

K. Naruse · M. Tanaka · H. Takeda *Editors*

Medaka

A Model for
Organogenesis,
Human Disease,
and Evolution



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Kiyoshi Naruse • Minoru Tanaka
Hiroyuki Takeda
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Front cover:

Lower: Medaka inbred line Hd-rRIII whose genome has been decoded (*upper*, male; *lower*, female).

Upper right: Fertilized eggs of medaka; oil droplet can be observed inside an egg.

Inset: Medaka *bis* mutant (*right*) displays a defect in the craniofacial skeleton (*arrows*), as described in Chapter 6.

Back cover:

Fluorescent images of three transgenic medaka just after hatching, with green and red fluorescence used for visualizing different types of tissues. The transgenic medaka were generated by Shuhei Nakamura in the Tanaka Lab. Photographic design by Hiroyo Kaneko.

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Preface

The medaka (*Oryzias latipes*), a small egg-laying freshwater teleost, has become an excellent model for studying developmental genetics and evolutionary biology. We have witnessed a vast explosion of zebrafish research during the past two decades. However, we believe we need to show that medaka research has a longer and outstanding history in basic biology (see Chap. 1), especially in the field of genetics and sex determination – for example, the first demonstration in any species of Y-linked inheritance (Aida 1921), the first successful sex reversal in vertebrates (Yamamoto 1953), and the identification of the male-determining gene, *DMY/Dmrt1bY*, the first nonmammalian equivalent of *SRY* (Matsuda et al. 2002; Nanda et al. 2002). Furthermore, the Mendelian law of inheritance was confirmed in medaka in 1910, one of the earliest and most prominent achievements in vertebrate genetics (Toyama 1916). In addition to sharing common features with zebrafish, medaka have several advantages: a smaller genome (about 800 Mb, half the size of the zebrafish genome), the existence of polymorphic and highly fertile inbred strains, and the fact that they are growth permissive at a wide range of temperatures in embryonic development. Recently, large-scale mutagenesis projects conducted by several groups in Japan delivered a vastly expanded pool of medaka mutant stocks (Furutani-Seiki et al. 2004). Probably owing to the long evolutionary distance between medaka and zebrafish (110–160 million years apart), some medaka mutations appear to have unique phenotypes and vice versa, demonstrating the advantage of using multiple teleost genetic models. A good example could be a recent report on duplicated *fgf-r1s*, *fgfr1a*, and *fgfr1b*. Because of the differential subfunctionalization between medaka and zebrafish, a mutation in *fgfr1a* results in distinct phenotypes: loss of the trunk-tail in medaka embryos but reduced scale formation in zebrafish adults (Rohner et al. 2009).

The medaka genome sequencing project started in late 2002 and was successfully completed in 2007, thanks to the available inbred lines (Kasahara et al. 2007). The high-quality draft genome and mutants have greatly facilitated ongoing medaka research and have begun to attract the attention of biologists who are currently working with other model organisms. However, although the community of medaka researchers is growing, it is still limited to Japan and a few other countries – Germany, for one. To boost this trend worldwide, a medaka protocol book was published in 2009 to help those newly establishing the medaka system in their laboratories

(Kinoshita et al. 2009). The present book was then planned to complement the protocol book, to provide researchers with frontline medaka research and to point to future directions. The reader will soon realize that medaka research encompasses a wide range of fields, e.g., genetics, embryology, disease, immunology, oncology, reproduction biology, evolutionary biology, and genome science, which have been implemented by the National BioResource Project (NBRP, MEXT) for medaka (<http://www.shigen.nig.ac.jp/medaka/>). Finally, although not included in this book, several lines of embryonic stem cells have been established by a group in Singapore (Yi et al. 2009), promising future genetic engineering of medaka, such as targeted gene knockout. We hope this book will be a valuable starting point for those who are new to the field and will help maintain collaboration and interaction among the growing fish research community worldwide.

We wish to thank all colleagues who contributed to the book for their time and effort. We also thank Misato Kochi, Aiko Hiraguchi, and Yuko Matsumoto at Springer Japan for their helpful editorial assistance.

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References

- Aida T (1921) On the inheritance of color in a fresh-water fish, *Aplocheilichthys latipes* Temmick and Schlegel, with special reference to sex-linked inheritance. *Genetics* 6:554–573
- Furutani-Seiki M, Sasado T, Morinaga C, Suwa H, Niwa K, Yoda H, Deguchi T, Hirose Y, Yasuoka A, Henrich T, Watanabe T, Iwanami N, Kitagawa D, Saito K, Asaka S, Osakada M, Kunimatsu S, Momoi A, Elmasri H, Winkler C, Ramialison M, Loosli F, Quiring R, Carl M, Grabher C, Winkler S, Del Bene F, Shinomiya A, Kota Y, Yamanaka T, Okamoto Y, Takahashi K, Todo T, Abe K, Takahama Y, Tanaka M, Mitani H, Katada T, Nishina H, Nakajima N, Wittbrodt J, Kondoh H (2004) A systematic genome-wide screen for mutations affecting organogenesis in medaka, *Oryzias latipes*. *Mech Dev* 121:647–658
- Kasahara M, Naruse K, Sasaki S, Nakatani Y, Qu W, Ahsan B, Yamada T, Nagayasu Y, Doi K, Kasai Y, Jindo T, Kobayashi D, Shimada A, Toyoda A, Kuroki Y, Fujiyama A, Sasaki T, Shimizu A, Asakawa S, Shimizu N, Hashimoto S, Yang J, Lee Y, Matsushima K, Sugano S, Sakaizumi M, Narita T, Ohishi K, Haga S, Ohta F, Nomoto H, Nogata K, Morishita T, Endo T, Shin IT, Takeda H, Morishita S, Kohara Y (2007) The medaka draft genome and insights into vertebrate genome evolution. *Nature (Lond)* 447:714–719
- Kinoshita M, Murata K, Naruse K, Tanaka M (2009) A laboratory manual for medaka biology. Wiley-Blackwell, Ames, IA
- Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Morrey CE, Shibata N, Asakawa S, Shimizu N, Hori H, Hamaguchi S, Sakaizumi M (2002) DMY is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature (Lond)* 417:559–563
- Nanda I, Kondo M, Hornung U, Asakawa S, Winkler C, Shimizu A, Shan Z, Haaf T, Shimizu N, Shima A, Schmid M, Scharl M (2002) A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. *Proc Natl Acad Sci USA* 99:11778–11783

- Rohner N, Bercsenyi M, Orban L, Kolanczyk ME, Linke D, Brand M, Nusslein-Volhard C, Harris MP (2009) Duplication of *fgfr1* permits Fgf signaling to serve as a target for selection during domestication. *Curr Biol* 19:1642–1647
- Toyama K (1916) Some examples of the Mendelian characters (in Japanese). *Nihon Ikushugaku Kaiho* 1:1–9
- Yamamoto T (1953) Artificially induced sex-reversal in genotypic males of the medaka (*Oryzias latipes*). *J Exp Zool* 123:571–594
- Yi M, Hong N, Hong Y (2009) Generation of medaka fish haploid embryonic stem cells. *Science* 326:430–433

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Chapter 1

A Glance at the Past of Medaka Fish Biology

Hiroshi Hori

Abstract The physiology, embryology, and genetics of medaka have been extensively studied for the past 100 years. Here we review the past of medaka fish biology as genetic model systems for early development, pigmentation, sex determination, and human diseases.

1.1 The Origin of Medaka Research

Medaka (*Oryzias latipes*) is a small fish native to East Asia, commonly confined to freshwater such as brooks, ponds, and paddy fields. Medaka literally means “with high eyes,” whereas *O. latipes* is a fish that is most frequently found in rice (*Oryza sativa*) fields, having a large leg-like anal fin.

Biological research with medaka was initiated in the mid-19th century by von Siebold (Fig. 1.1), a German physician and naturalist, who became famous for his study of Japanese flora and fauna. During his stay in Japan as a resident physician (1823–1829), he not only conducted research on Japanese natural history, but also contributed enormously to the modernization of the country, especially to the westernization of Japanese medicine. Using his fish specimens collected in Japan, Temminck and Schlegel (1846) described the taxonomic characters of medaka in Siebold’s *Fauna Japonica* (Fig. 1.2), assigned it to the genus *Poecilia*, and used the name *Poecilia latipes*. *Oryzias latipes* was called by this name in 1906 by Jordan and Snyder, famous American fish taxonomists, who considered medaka to belong to a monotypic genus, *Oryzias*. A good number of other relatives such as *Oryzias luzonensis*, *Oryzias curvinotus*, and others were named with some other variations after this genus (Takehana et al. 2005; see Uwa 1990; Fig. 1.3).

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Fig. 1.1 Philipp Franz von Siebold (1796–1866), illustrated by Keiga Kawahara. (With permission of Nagasaki Museum of History and Culture, and The University Museum, The University of Tokyo)

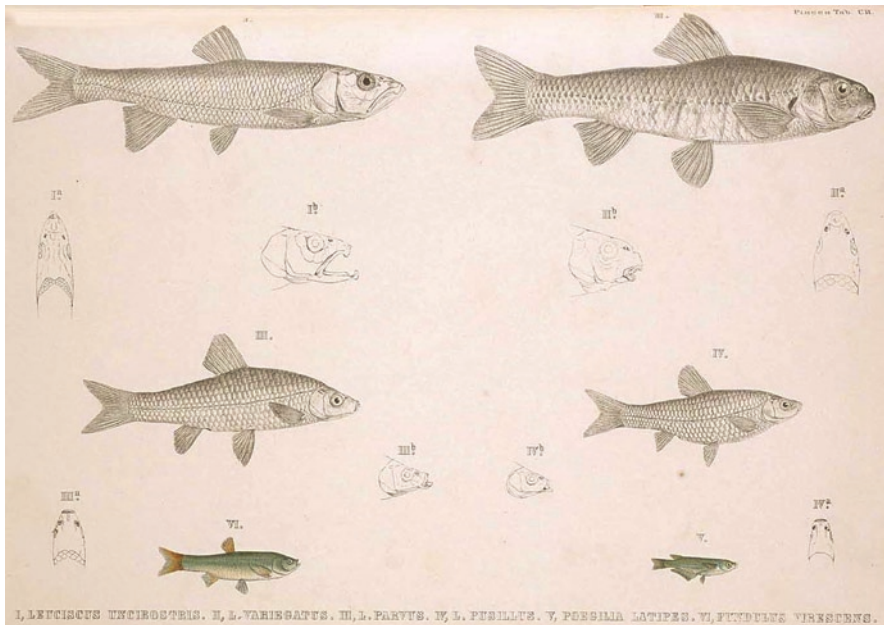


Fig. 1.2 Medaka and other freshwater fish in Japan. Medaka (*Poecilia latipes*) was introduced by Temminck and Schlegel in Siebold's *Fauna Japonica* in 1846. I., *Leuciscus uncirostris* (Hasu); II., *Leuciscus variegatus* (Higai); III., *Leuciscus parvus* (Motsugo); IV., *Leuciscus pusillus* (Motsugo); V., *P. latipes* (medaka; bottom right); VI.; *Fundulus virescens* (Motsugo? bottom left). (Copyright 2000, Kyoto University Library. With permission of Kyoto University Library, <http://edb.kulib.kyoto-u.ac.jp/exhibit/b05/b05cont.html>)



Fig. 1.3 Hiroshi Uwa (1942–1993). (Photograph by Kiyoshi Naruse at Sulawesi, Indonesia)

1.2 An Object of Genetic Research

Because the medaka has been commonly reared as an ornamental fish in Japan, several natural color mutants, such as orange-red type, white type, and albino type, have been isolated and maintained as pets by hobbyists for a long time. In the Edo Era (1838), a famous Japanese-style painter, Bai-en Mohri, illustrated these strains in his encyclopedia (Fig. 1.4). These strains lend themselves excellently to fish genetics. In fact, among vertebrates, it is the first animal in which the Mendelian laws were proven to be valid, using color mutants (Ishikawa 1916; Toyama 1916).

Aida (1921; Fig. 1.5) also used such mutants in his genetic study and found the sex-limited inheritance of the *R* (red) locus that controls the expression of orange-red color in epithelial pigment cells. Because the dominant *R* allele is on the Y chromosome and the recessive *r* allele is on the X chromosome, this allows for the determination of sex genotype just by looking at body color; males have an orange-red phenotype (X^rY^R), whereas females are white (X^rX^r). It should be noted that the first discovery of sex chromosomes was accomplished in insects, that is, the bug *Pyrrhocoris* and various grasshopper species, in the early 1900s (see Ault 1996). Later, of course, Morgan and his students discovered an ideal genetic model system in the fruit fly *Drosophila melanogaster*, which has an XY type of sex determination. Note that determination is by a balance of female determinants on the X chromosome in *Drosophila*, rather than being achieved by the Y chromosome



Fig. 1.4 (color online) Medaka illustrated by Bai-en Motohisa Mouri (1798–1851) in his encyclopedia, *Baien-gyofu*. Three medaka fish were drawn: from left to right, shiro-medaka (white, *bbrr*), hi-medaka (orange-red, *bb*), and wild-type (black-brown). (With permission of National Diet Library, Japan, <http://www.ndl.go.jp>)



Fig. 1.5 Tatsuo Aida (1872–1957). (Photograph by the late Toki-o Yamamoto. By courtesy of Tokihiko Yamamoto)

(Fig. 1.6). If there are two X chromosomes in a diploid cell ($2X:2A$), the fly is female (Bridges 1921), or sex is primarily determined by the ratio of the number of X chromosomes to the number of sets of autosomes (A) (see Cline and Meyer 1996); this means the Y of *Drosophila* is genetically recessive on male determination.

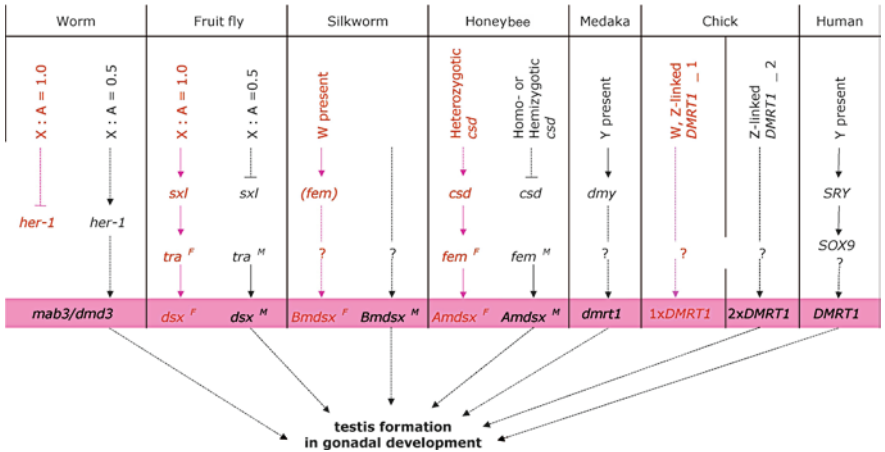


Fig. 1.6 (color online) Bottom-up evolution of sex-determining cascades in metazoans. Molecular pathways leading to the formation of the male gonad in the worm (*Caenorhabditis elegans*), fruit fly (*Drosophila melanogaster*), silkworm moth (*Bombyx mori*), honeybee (*Apis mellifera*), medaka fish (*Oryzias latipes*), chick (*Gallus gallus*), and human (*Homo sapiens*). Although the upstream regulatory factors can change rapidly (Marin and Baker 1998), some of the downstream regulatory genes, such as *DMRT* genes, have been conserved in different sexual differentiation pathways, supporting the bottom-up hypothesis of the sex determination pathway by Wilkins (1995). Conserved *dm* domain genes (see text) are indicated with pink box. Black and red gene names indicate male and female type genes, respectively. *csd*, complementary sex determiner; *dmd3*, doublesex/mab-3 domain family member 3; *dmrt1*, *dsx*- and *mab3*-related transcription factor 1; *dmy*, *dm* domain gene on Y chromosome; *dsx*, doublesex; *fem*, feminizer; *her-1*, hermaphroditization of *XO-1*; *mab3*, male abnormal 3; *SOX9*, *SRY*-box 9; *SRY*, sex-determining region Y; *sxl*, sex lethal; *tra*, transformer. A, autosome; W, X, and Y, W, X, and Y chromosome, respectively. [Figure 1 of Herpin and Schartl (2008) was revised with additional data from the human (Raymond et al. 2000), chick (Smith et al. 2009), and silkworm; see Fujii and Shimada 2007)]

On the other hand, the Y of medaka carries a dominant instead of a recessive male-determining gene. Thus, Aida (1921) in *Oryzias* has the credit for the first discovery of Y-linked dominant inheritance in organisms, and it was 40 years before the discovery of a dominant male determinant on the human Y chromosome (Jacobs and Strong 1959). By this seminal and two subsequent articles (Aida 1921, 1930, 1936), the “egg” of medaka research was laid down, yet it took another two decades for full “eclosion.”

1.3 Medaka as a Model Animal for Sex Determination and Sexual Differentiation

Since the work of Aida in 1921, many scientists have used medaka as a model animal, especially in the field of sex determination and sexual differentiation. Yamamoto (1953; Fig. 1.7), who was then famous for his research on the fertilization wave (see



Fig. 1.7 Toki-o Yamamoto (1906–1977). (With permission of Medakafish Homepage, Nagoya University, <http://www.bio.nagoya-u.ac.jp:8000/>)

Yamamoto 1975), established the *d-rR* strain (*d*omesticated *r* and *R*) using the sex-limited inheritance of the *R* locus, and demonstrated artificial induction of sex reversal with estrogen and androgen. He also published the key reference for medaka as a genetic model system, *Medaka (Killifish) Biology and Strains* (Yamamoto 1975).

Studies of medaka sex determination and differentiation finally resulted in identification of the primary sex-determination gene, *dmy* (*dm* domain gene on *Y* chromosome, also known as *dmrt1bY*; Matsuda et al. 2002; Nanda et al. 2002). The *dm* domain is a conserved zinc-finger-like DNA-binding motif named after *dsx* (*doublesex*) of the fly *D. melanogaster* and *mab3* (*male abnormal 3*) of the worm *Caenorhabditis elegans*. *dsx* and *mab3* are implicated in sex determination pathways, whereas the top sex regulatory genes of the fly and the worm are not conserved (Marin and Baker 1998; see Fig. 1.6). Interestingly, *dmy* is apparently homologous to medaka *dmrt1* (*dsx* and *mab3*-related transcription factor 1) and *DMRT1* of mammals, which plays a major role in sex determination. It is well known that the *Sry* gene in mammals is the first “primary sex determination gene” on the *Y* chromosome isolated in vertebrates (Sinclair et al. 1990). *dmy* is also known as the trigger of sex determination, and in a sense, “the functional equipment” of *Sry*. However, it should be noted that *Sry* is a variable top component of the testicular differentiation cascade (Raymond et al. 2000), but *dmy* homologs, including *dsx*, *mab3*, *Bmdsx*, and *dmrt1*, are conserved and are the most downstream components of the cascade (Raymond et al. 2000; Fujii and Shimada 2007; Herpin and Scharl 2008; Smith et al. 2009; see Fig. 1.6). Thus far, these results are consistent with the model proposed by Wilkins (1995) that sex determination pathways evolve from bottom to top in a retrograde fashion. *DMRT* appears to be a common terminal regulator in all hitherto analyzed pathways: this suggests a com-

mon evolutionary origin of sexual regulation in metazoans, and the discovery of *dmy* in medaka has sparked a renewed debate on the cascade of sex determination.

Another unique investigation in this field is on the role of germ cells in the sex differentiation of gonads in the medaka. Kurokawa et al. (2007) generated a germ cell-deficient strain by injection of morpholino antisense oligonucleotides against *cxcr4*. The results included female-to-male sex reversal of their secondary sex characteristics, accompanied by increased levels of androgen and reduced levels of estrogen, suggesting an importance of germ cells in sexual dimorphism of the gonads. Furthermore, by using transgenic methods and clonal analysis, Nakamura et al. (2010) identified germline stem cells in the ovary of the medaka, providing a model system for studying vertebrate stem cell niches. The germinal cradle (= interwoven thread-like cords of *sox9b*-expressing somatic cells in the ovarian cord of medaka) is reminiscent of the germarium from the *Drosophila* ovary, reflecting a fundamental process governing oogenesis across animal species.

1.4 Tomita Collection and Mutant Analysis

An early geneticist working on the medaka identified a broad variety of spontaneous mutants, and more than 80 spontaneous mutants that affect pigmentation, gross morphology, eye, fin, and skeletal development are currently known (Kelsh et al. 2004). All of these are natural mutants, and their documentation was achieved by Tomita at Nagoya University (Fig. 1.8). He screened these mutants by extensive but simple backcrossing. Today, they are being routinely maintained in a stock center (Tomita collection) and made available for a vast range of research at the cellular



Fig. 1.8 Hideo Tomita (1931–1998). (Photograph by Kiyoshi Naruse)

and molecular levels. Genes of interest include *Da* (*double anal fins*; Ohtsuka et al. 2004), *pl* (*pectoral fin-less*; Okamoto and Kuwada 1991), *el* (*eyeless*; Winkler et al. 2000; Loosli et al. 2001), *rs-3* (*reduced scales*; Kondo et al. 2001), and *pc* (*polycystic kidney*; Hashimoto et al. 2009), and there are several skin color mutants (see below). The transparent strain (named *see-through*), which is useful for studying internal processes in vivo, was generated by combining recessive alleles at four skin color loci (Wakamatsu et al. 2001).

As a genetic model system, medaka has played a key role in our understanding of vertebrate pigmentation. Tomita described independent five genetic loci responsible for melanophore pigmentation as exemplified by *b*, *dl*, *dl2*, *i-1*, and *i-3* (Kelsh et al. 2004). Among them, albino *i-1* has amelanotic skin and red eyes. Koga et al. (1995) revealed that the *tyrosinase* gene is responsible for the *i-1* mutation, as in the case of human patients with oculocutaneous albinism type 1 (*OCA1*). This was the first mutated gene identified in medaka, suggesting that medaka is a good model for studying human diseases at the molecular level. Another interesting finding from this work is that a transposable element (*Tol1* or *Tol2*) is inserted in three of the four *i*-locus alleles (*i1*, *i4*, *ib*) (Koga and Hori 1997). *Tol2* is the first active transposable element reported from vertebrates (Koga et al. 1996), and is now extensively used for transposon-mediated transgenesis in zebrafish (Asakawa and Kawakami 2009).

There is another “albino” mutant in medaka; it has amelanotic skin, but its eyes are melanized and black, and it is traditionally called an orange-red variant (see Hi-medaka in Fig. 1.4). The fish has the “*b*” allele at the *b* locus (Aida 1921; Shimada et al. 2002). Fukamachi et al. (2001) revealed this “*b*” allele to feature mutations in the *solute carrier family 45 member 2* (*slc45a2*) gene by means of positional cloning. After his work, it was demonstrated that the orthologous gene in human is also mutated in patients with *OCA4*, as well as in *underwhite* mice, “*cream*” horses, and silver chickens and quail. The phenotypes in these different species are quite similar to one another in that melanin deposition is severely suppressed in the skin, whereas the eyes become slightly pigmented during maturation. Fukamachi et al. (2004) also identified Tomita’s *i-3* mutant as *OCA2*, which has a mutation in the *pink-eyed dilution* (*p*) gene. Taken together, three of the four types of human *OCA* exist in medaka. It may therefore provide insights into the mechanisms of conservation of melanin synthesis among vertebrates, and medaka offer good models for investigating the human diseases at early stages of development (Packer 2001; Cheng 2008).

The cellular processes underlying organ formation in vertebrates are largely unknown. In the case of eye development, however, members of several of the known families of signaling molecules, such as sonic hedge hog (*shh*), fibroblast growth factor (*fgf*), and nodal, are clearly involved in retina formation in medaka. It has thus already proved to be a useful developmental biology model (Wittbrodt et al. 2002). Recently, Rembold et al. (2006) visualized early vertebrate eye morphogenesis at the single-cell level of resolution by in vivo imaging in medaka. Analysis of mutants demonstrated that the retina-specific transcription factor rx3 determines the convergence and migration behavior of retinal progenitor cells.

The authors claimed that the migration of individual cells mediates essential steps of organ morphogenesis, and that medaka could be good models for investigating organ formation at the molecular and cellular levels.

1.5 Genetic Polymorphism, Inbred Strains, and Linkage Map Construction

Morphological polymorphisms were initially found in wild populations of medaka with different geographic origins (Egami et al. 1990; Fig. 1.9). Previous allozymic analyses and recent DNA sequence comparisons of orthologous genes have consistently indicated that there are two widely separated populations of the fish in Japan: northern and southern. Today, we know that genome-wide single nucleotide polymorphisms (SNPs) differ at an average rate of 3.42% between the two populations; this is, to our knowledge, the highest SNP rate seen in any vertebrate species (Kasahara et al. 2007). These polymorphisms between northern and southern inbred strains (see following) have proven invaluable for genetic mapping, and using SNP markers, Naruse et al. (2000, 2004) established a genome-wide linkage map.

The establishment of several inbred lines from genetically different natural populations is unique to medaka. In 1974, Hyodo-Taguchi and Egami succeeded in generating the first inbred strains after at least 20 generations of successive brother–sister matings. So far, at least 13 inbred strains, 3 from the northern and 10 from the southern populations, have been described (Hyodo-Taguchi and Egami 1985; Ishikawa et al. 1997). After three decades, one of the inbred strains, *Hd-rR*, originally derived from Yamamoto's *d-rR*, was utilized for bacterial artificial chromosome (BAC) library



Fig. 1.9 Nobuo Egami (1925–1989). [Reprinted with permission from *Zoological Science* 6:1041–1044 (1989). Copyright 1989 by Zoological Society of Japan]

construction (Matsuda et al. 2001; Naruse et al. 2004) and a genome sequencing project (Kasahara et al. 2007).

Recently, the quantitative trait locus (QTL) of craniofacial traits of two inbred medaka strains (*HNI* and *Hd-rR* from northern and southern populations, respectively) was analyzed by Kimura et al. (2007). A phenotypic survey of F₂ progeny showed 304 of 444 quantified traits to be gender dependent and 379 traits to differ significantly between the two inbred strains. Thus, many craniofacial traits are highly heritable in medaka. Furthermore, 66 of 89 traits were localized in the medaka genome with interval mapping. This study was the first demonstration that the QTL of morphological traits in medaka could be identified by genetic analysis.

1.6 Ecotoxicology

During the past 20 years, medaka has also found application as a good test system for environmental research. Systematic testing for its susceptibility to physical or chemical mutagens was conducted, and in the course of these experiments conditions for systematic mutagenesis were established. Shima and Shimada (1991) developed a valuable approach for radiation-induced germ cell mutagenesis using medaka (Shima and Shimada 1994; Baroiller et al. 1999). As previously mentioned, Yamamoto (1953) early demonstrated artificial induction of sex reversal with estrogen and androgen in medaka. Clearly, the phenotypic sex of medaka is influenced not only by the genotype, but also by environmental factors such as endocrine disrupters. Indeed, evaluation of the toxicity of endocrine active chemicals has been described with use of sex-related genetic markers and alterations in gonadal development, including the induction of testis-ova formation in medaka (Urushitani et al. 2007). Hinton et al. (2005) reviewed these studies.

1.7 Genome Project

A genome project is much more than just whole genome sequencing. It is a coordinated generation of resources that contribute to our understanding of how a genome works. The medaka genome sequencing project commenced in 2002, and the draft genome sequence of the inbred *Hd-rR* strain was published by Kasahara et al. (2007). In the assembled medaka genome (700 megabases), which is less than half of the zebrafish genome, a total of 20,141 genes were predicted (Fig. 1.10). Of these, 16,414 (81.5%) have homologs in at least one of six other vertebrates, that is, human, mouse, chicken, *Tetraodon*, *Takifugu*, and zebrafish, and also with gene clusters of Aves, amphibians, ray-finned fish, and ascidians in the UniGene database. The remaining 3,727 (18.5%), with no homologs in the

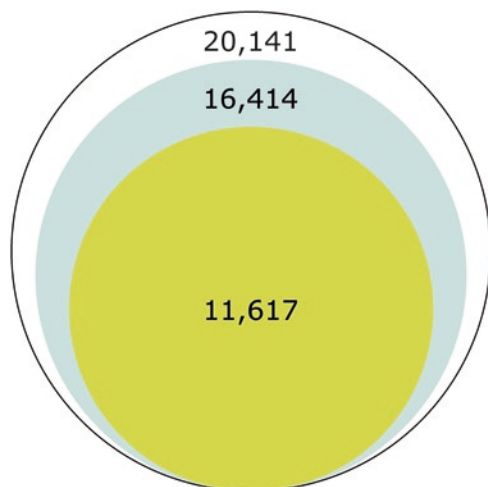


Fig. 1.10 (color online) Breakdown of medaka gene homologs in other species. *White circle*, 20,141 nonredundant candidate genes. *Blue circle*, 16,414 homologs, each found in at least one vertebrate species known to date (see text). *Green circle*, 11,617 homologs, each found in the human. [Simplified from Fig. 2 in Kasahara et al. (2007)]

databases, provide medaka-specific candidates. Note here that 11,617 (57.7%) feature human orthologs, and 925 of the 1,395 human disease genes known to date have strong orthologs among medaka genes. These homologs should provide strong insights into conserved mechanisms for body plans among vertebrates, and the medaka could be a good model for investigating human hereditary diseases of unknown origin.

Furthermore, whole genome comparisons among zebrafish, *Takifugu*, medaka, and mammalian draft genome sequences have revealed novel insights into the diversification of fish species and shed light on vertebrate genome evolution. During 450 million years (MY) of vertebrate evolution, eight major interchromosomal rearrangements took place in a remarkably short period of about 50 MY after the whole genome duplication event in the teleost ancestor, and afterward, intriguingly, the medaka genome preserved its ancestral karyotype for more than 300 MY (Kasahara et al. 2007).

1.8 Mutagenesis Screening

In addition to these genome resources, mutagenesis screening for the isolation of mutants with a specific phenotype has been established, and projects to identify the causal genes of mutants have been conducted in several laboratories (Furutani-Seiki et al. 2004; Yokoi et al. 2007). The generation of hundreds of medaka mutants and

the unique phenotypes observed have made this organism an attractive model system for vertebrate genetics and genomics that nicely complements the zebrafish. Obviously no other vertebrates would have permitted research obtaining such rich results at such a low cost (Wittbrodt et al. 2002). Furthermore, the draft genome sequence of the medaka involves the efficient determination of chromosome map positions by fine mapping using thousands of mapped markers [expressed sequence tags (ESTs) and SNPs], and finally should enable us to identify candidate genes using existing information from other vertebrate genomes. Genomic tools are already available, and there is no doubt that the draft genome sequence of the medaka has greatly accelerated the study of genes on the bases of their expression pattern and mutant phenotype alike.

One of the most significant studies using these isolated mutants and associated methods has focused on understanding the motile cilium in development and disease (Roy 2009). Motility defects in cilia and flagella often result in primary ciliary dyskinesia in humans. However, the mechanisms underlying cilia formation and function are only poorly understood. Omran et al. (2008) reported a new gene in a medaka mutant, *kintoun* (*ktu*), involved in the cilia formation process, in particular contributing to cytoplasmic assembly of dyneins that power ciliary motility. This gene was also found to be mutated in the *pf13* mutant of a unicellular alga, *Chlamydomonas*, as well as in primary ciliary dyskinesia patients from two affected families. In the absence of *ktu*/*PF13*, both outer and inner dynein arms are missing or defective in the axoneme, leading to loss of motility. Cilia and flagella that have diverse roles in cell motility and sensing extracellular signals are highly conserved eukaryote organelles from unicellular algae to mammals. Thus, medaka not only offer good models for investigating human diseases but also may provide strong insights into conserved mechanisms for cytoplasmic processes in eukaryotes.

Another unique report is that recently published by Hashimoto et al. (2009). They identified the medaka *pc* gene, an ortholog of human *GLIS3*, as the causative agent for cystic kidney and neonatal diabetes in human. Transcripts of *pc*/*glis3* were observed to be abundantly expressed in both the renal tubular epithelium and the beta cells of the pancreas in medaka. Medaka *pc* mutants exhibited clear cystic kidney disease in their renal organs; however, no mutant phenotypes were found in the pancreatic tissues. In this case, the mutant phenotypes are tissue specific, which indicates underlying differences in the genome and the degree of functional redundancy. Ultimately, differences in phenotype between mammals and the medaka positioned the *pc* mutant as a kidney-specific disease model of human *GLIS3* mutations.

1.9 Perspectives

Several years ago, the future of medaka as a research object was succinctly predicted by Wittbrodt et al. (2002): “Genome sequencing has yielded a plethora of new genes, the function of which can be unraveled through comparative genomic approaches. Increasingly, developmental biologists are turning to fish as model

genetic systems because they are amenable to studies of gene function. Zebrafish has already secured its place as a model vertebrate and now its Far Eastern cousin – medaka – is emerging as an important model fish, because of recent additions to the genetic toolkit available for this organism.”

Today, the first phase of deciphering the medaka genome has been completed with the important revelation that about 60% of the total of 20,141 genes have human orthologues (see Fig. 1.10). This fact clearly means that medaka could be a good model for investigating various human diseases, as well as providing strong insights into conserved biological processes in vertebrates. The zebrafish has already secured its place as a model vertebrate, but it should be noted here that, as in any model system, zebrafish has its limitations as to the types of questions that can be addressed. The most prominent example is sex determination, as discussed above, for which nothing is known in zebrafish. Furthermore, despite several large-scale mutagenesis screens in zebrafish, the number of mutations corresponding to examples in mouse and human is still limited. Mutants of medaka show a significantly different spectrum of phenotypes from those of zebrafish. In addition, medaka exhibits sophisticated social behavior, exemplified by the finding of mating dance and schooling behavior in swimming (Iwamatsu 2006): this highlights their potential as a model for analysis of behavior. Therefore, studies of medaka not only can complement those of zebrafish but should further provide important medaka-specific and original vertebrate phenotypes.

References

- Aida T (1921) On the inheritance of color in a fresh-water fish, *Aplocheilus latipes* Temminck and Schlegel, with special reference to sex-linked inheritance. *Genetics* 6:554–573
- Aida T (1930) Further genetical studies of *Aplocheilus latipes*. *Genetics* 15:1–16
- Aida T (1936) Sex reversal in *Aplocheilus latipes* and a new explanation of sex differentiation. *Genetics* 21:136–153
- Asakawa K, Kawakami K (2009) The *Tol2*-mediated Gal4-UAS method for gene and enhancer trapping in zebrafish. *Methods* 49:275–281
- Ault JG (1996) Contributions of insect systems to the chromosome theory of inheritance and to our understanding of chromosome behavior on the spindle. *Int J Insect Morphol Embryol* 25:93–114
- Baroiller JF, Guien Y, Fostier A (1999) Endocrine and environmental aspects of sex differentiation in fish. *Cell Mol Life Sci* 55:910–931
- Bridges CB (1921) Triploid intersexes in *Drosophila melanogaster*. *Science* 54:252–254
- Cheng KC (2008) Skin color in fish and humans: impacts on science and society. *Zebrafish* 5:237–242
- Cline TW, Meyer BJ (1996) Vive la difference: males vs. females in flies vs. worms. *Annu Rev Genet* 30:637–702
- Egami N, Yamagami K, Shima A (1990) Biology of the medaka (in Japanese). Tokyo University Press, Tokyo
- Fujii T, Shimada T (2007) Sex determination in the silkworm, *Bombyx mori*: a female determinant on the W chromosome and the sex-determining gene cascade. *Semin Cell Dev Biol* 18:379–388
- Fukamachi S, Shimada A, Shima A (2001) Mutations in the gene encoding B, a novel transporter protein, reduce melanin content in medaka. *Nat Genet* 28:381–385

- Fukamachi S, Asakawa S, Wakamatsu Y, Shimizu N, Mitani H, Shima A (2004) Conserved function of medaka pink-eyed dilution in melanin synthesis and its divergent transcriptional regulation in gonads among vertebrates. *Genetics* 168:1519–1527
- Furutani-Seiki M, Sasado T, Morinaga C, Suwa H, Niwa K, Yoda H, Deguchi T, Hirose Y, Yasuoka A, Henrich T, Watanabe T, Iwanami N, Kitagawa D, Saito K, Asaka S, Osakada M, Kunimatsu S, Momoi A, Elmasri H, Winkler C, Ramialison M, Loosli F, Quiring R, Carl M, Grabher C, Winkler S, Del Bene F, Shinomiya A, Kota Y, Yamanaka T, Okamoto Y, Takahashi K, Todo T, Abe K, Takahama Y, Tanaka M, Mitani H, Katada T, Nishina H, Nakajima N, Wittbrodt J, Kondoh H (2004) A systematic genome-wide screen for mutations affecting organogenesis in medaka, *Oryzias latipes*. *Mech Dev* 121:647–658
- Hashimoto H, Miyamoto R, Watanabe N, Shiba D, Ozato K, Inoue C, Kubo Y, Koga A, Jindo T, Narita T, Naruse K, Ohishi K, Nogata K, Shin-I T, Asakawa S, Shimizu N, Miyamoto T, Mochizuki T, Yokoyama T, Hori H, Takeda H, Kohara Y, Wakamatsu Y (2009) Polycystic kidney disease in the medaka (*Oryzias latipes*) *pc* mutant caused by a mutation in the *Gli-Similar3 (glis3)* gene. *PLoS One* 4:e6299
- Herpin A, Scharl M (2008) Regulatory putsches create new ways of determining sexual development. *EMBO Rep* 9:966–968
- Hinton DE, Kullman SW, Hardman RC, Volz DC, Chen PJ, Carney M, Bencic DC (2005) Resolving mechanisms of toxicity while pursuing ecotoxicological relevance? *Mar Pollut Bull* 51:635–648
- Hyodo-Taguchi Y, Egami N (1985) Establishment of inbred strains of the medaka *Oryzias latipes* and the usefulness of the strains for biomedical research. *Zool Sci* 2:305–316
- Ishikawa M (1916) Medaka no taishoku no iden ni tsuite (On the inheritance of body colors in the medaka, *Oryzias latipes*). *Fukuoka Ikadaigaku Zasshi (J Fukuoka Med Coll)* 9:259–267 (in Japanese)
- Ishikawa Y, Hyodo-Taguchi Y, Tatsumi K (1997) Medaka fish for mutant screens. *Nature (Lond)* 386:234
- Iwamatsu T (2006) The integrated book for the biology of the medaka, 2nd edn. Daigaku Kyoiku, Tokyo (in Japanese)
- Jacobs PA, Strong JA (1959) A case of human intersexuality having a possible XXY sex-determining mechanism. *Nature (Lond)* 183:302–303
- Jordan DS, Snyder JO (1906) A review of the Poeciliidae or killifishes of Japan. *Proc US Natl Mus* 31:287–290
- Kasahara M, Naruse K, Sasaki S, Nakatani Y, Qu W, Ahsan B, Yamada T, Nagayasu Y, Doi K, Kasai Y, Jindo T, Kobayashi D, Shimada A, Toyoda A, Kuroki Y, Fujiyama A, Sasaki T, Shimizu A, Asakawa S, Shimizu N, Hashimoto S, Yang J, Lee Y, Matsushima K, Sugano S, Sakaizumi M, Narita T, Ohishi K, Haga S, Ohta F, Nomoto H, Nogata K, Morishita T, Endo T, Shin-I T, Takeda H, Morishita S, Kohara Y (2007) The medaka draft genome and insights into vertebrate genome evolution. *Nature (Lond)* 447:714–719
- Kelsh RN, Inoue C, Momoi A, Kondoh H, Furutani-Seiki M, Ozato K, Wakamatsu Y (2004) The Tomita collection of medaka pigmentation mutants as a resource for understanding neural crest cell development. *Mech Dev* 121:841–859
- Kimura T, Shimada A, Sakai N, Mitani H, Naruse K, Takeda H, Inoko H, Tamiya G, Shinya M (2007) Genetic analysis of craniofacial traits in the medaka. *Genetics* 177:2379–2388
- Koga A, Hori H (1997) Albinism due to transposable element insertion in fish. *Pigment Cell Res* 10:377–381
- Koga A, Inagaki H, Bessho Y, Hori H (1995) Insertion of a novel transposable element in the tyrosinase gene is responsible for an albino mutation in the medaka fish, *Oryzias latipes*. *Mol Gen Genet* 249:400–405
- Koga A, Suzuki M, Inagaki H, Bessho Y, Hori H (1996) Transposable element in fish. *Nature (Lond)* 383:30
- Kondo S, Kuwahara Y, Kondo M, Naruse K, Mitani H, Wakamatsu Y, Ozato K, Asakawa S, Shimizu N, Shima A (2001) The medaka *rs-3* locus required for scale development encodes ectodysplasin-A receptor. *Curr Biol* 11:1202–1206

- Kurokawa H, Saito D, Nakamura S, Katoh-Fukui Y, Ohta K, Baba T, Morohashi K, Tanaka M (2007) Germ cells are essential for sexual dimorphism in the medaka gonad. *Proc Natl Acad Sci USA* 104:16958–16963
- Loosli F, Winkler S, Burgdorf C, Wurmbach E, Ansorge W, Henrich T, Grabher C, Arendt D, Carl M, Krone A, Grzebisz E, Wittbrodt J (2001) Medaka eyeless is the key factor linking retinal determination and eye growth. *Development (Camb)* 128:4035–4044
- Marin I, Baker BS (1998) The evolutionary dynamics of sex determination. *Science* 281:1990–1994
- Matsuda M, Kawato N, Asakawa S, Shimizu N, Nagahama Y, Hamaguchi S, Sakaizumi M, Hori H (2001) Construction of a BAC library derived from the inbred *Hd-rR* strain of the teleost fish, *Oryzias latipes*. *Genes Genet Syst* 76:61–63
- Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Morrey CE, Shibata N, Asakawa S, Shimizu N, Hori H, Hamaguchi S, Sakaizumi M (2002) *DMY* is a Y-specific *DM*-domain gene required for male development in the medaka fish. *Nature (Lond)* 417:559–563
- Nakamura S, Kobayashi K, Nishimura T, Higashijima S, Tanaka M (2010) Identification of germline stem cells in the ovary of the teleost medaka. *Science* 328:1561–1563
- Nanda I, Kondo M, Hornung U, Asakawa S, Winkler C, Shimizu A, Shan Z, Haaf T, Shimizu N, Shima A, Schmid M, Scharl M (2002) A duplicated copy of *DMRT1* in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. *Proc Natl Acad Sci USA* 99:11778–11783
- Naruse K, Fukamachi S, Mitani H, Kondo M, Matsuoka T, Kondo S, Hanamura N, Morita Y, Hasegawa K, Nishigaki R, Shimada A, Wada H, Kusakabe T, Suzuki N, Kinoshita M, Kanamori A, Terado T, Kimura H, Nonaka M, Shima A (2000) A detailed linkage map of medaka, *Oryzias latipes*: comparative genomics and genome evolution. *Genetics* 154:1773–1784
- Naruse K, Tanaka M, Mita K, Shima A, Postlethwait J, Mitani H (2004) Medaka gene map: the trace of ancestral vertebrate proto-chromosomes revealed by comparative gene mapping. *Genome Res* 14:820–828
- Ohtsuka M, Kikuchi N, Yokoi H, Kinoshita M, Wakamatsu Y, Ozato K, Takeda H, Inoko H, Kimura M (2004) Possible roles of *zic1* and *zic4*, identified within the medaka *Double anal fin* (*Da*) locus, in dorsoventral patterning of the trunk-tail region (related to phenotypes of the *Da* mutant). *Mech Dev* 121:873–882
- Okamoto H, Kuwada JY (1991) Outgrowth by fin motor axons in wild type and a finless mutant of the Japanese medaka fish. *Dev Biol* 146:49–61
- Omran H, Kobayashi D, Olbrich H, Tsukahara T, Loges NT, Hagiwara H, Zhang Q, Leblond G, O'Toole E, Hara C, Mizuno H, Kawano H, Fliegauf M, Yagi T, Koshida S, Miyawaki A, Zentgraf H, Seithe H, Reinhardt R, Watanabe Y, Kamiya R, Mitchell DR, Takeda H (2008) Ktu/PF13 is required for cytoplasmic pre-assembly of axonemal dyneins. *Nature (Lond)* 456:611–616
- Packer A (2001) Medaka on the move. *Nat Genet* 28:302
- Raymond CS, Murphy MW, O'Sullivan MG, Bardwell VJ, Zarkower D (2000) *Dmrt1*, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. *Genes Dev* 14:2587–2595
- Rembold M, Loosli F, Adams RJ, Wittbrodt J (2006) Individual cell migration serves as the driving force for optic vesicle evagination. *Science* 313:1130–1134
- Roy S (2009) The motile cilium in development and disease: emerging new insights. *BioEssays* 31:694–699
- Shima A, Shimada A (1991) Development of a possible nonmammalian test system for radiation-induced germ-cell mutagenesis using a fish, the Japanese medaka (*Oryzias latipes*). *Proc Natl Acad Sci USA* 88:2545–2549
- Shima A, Shimada A (1994) The Japanese medaka, *Oryzias latipes*, as a new model organism for studying environmental germ-cell mutagenesis. *Environ Health Perspect* 102(suppl 12):33–35
- Shimada A, Fukamachi S, Wakamatsu Y, Ozato K, Shima A (2002) Induction and characterization of mutations at the *b* locus of the medaka, *Oryzias latipes*. *Zool Sci* 19:411–417

- Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischau AM, Lovell-Badge R, Goodfellow PN (1990) A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature (Lond)* 346:240–244
- Smith CA, Roeszler KN, Ohnesorg T, Cummins DM, Farlie PG, Doran TJ, Sinclair AH (2009) The avian Z-linked gene *DMRT1* is required for male sex determination in the chicken. *Nature (Lond)* 461:267–271
- Takehana Y, Naruse K, Sakaizumi M (2005) Molecular phylogeny of the medaka fishes genus *Oryzias* (Belontiiformes: Adrianichthyidae) based on nuclear and mitochondrial DNA sequences. *Mol Phylogenet Evol* 36:417–428
- Temminck CJ, Schlegel H (1846) Siebold's Fauna Japonica, Pisces. Lyden Museum, Lyden
- Toyama K (1916) Ichinino Mendel seisitu ni tsuite (On some Mendelian characters). *Nippon Ikusyugakkai Hokoku (Rep Jpn Breed Soc)* 1:1–9
- Urushitani H, Katsu Y, Kato Y, Tooi O, Santo N, Kawashima Y, Ohta Y, Kisaka Y, Lange A, Tyler CR, Johnson RD, Iguchi T (2007) Medaka (*Oryzias latipes*) for use in evaluating developmental effects of endocrine active chemicals with special reference to gonadal intersex (testis–ova). *Environ Sci* 14:211–233
- Uwa H (1990) Karyotype and evolution. In: Egami N, Yamagami K, Shima A (eds) *Biology of the medaka*. Tokyo University Press, Tokyo, pp 162–182 (review in Japanese)
- Wakamatsu Y, Pristiyazhnyuk S, Kinoshita M, Tanaka M, Ozato K (2001) The see-through medaka: a fish model that is transparent throughout life. *Proc Natl Acad Sci USA* 98:10046–10050
- Wilkins AS (1995) Moving up the hierarchy: a hypothesis on the evolution of a genetic sex determination pathway. *BioEssays* 17:71–77
- Winkler S, Loosli F, Henrich T, Wakamatsu Y, Wittbrodt J (2000) The conditional medaka mutation eyeless uncouples patterning and morphogenesis of the eye. *Development (Camb)* 127:1911–1919
- Wittbrodt J, Shima A, Scharlt M (2002) Medaka—a model organism from the Far East. *Nat Rev Genet* 3:53–64
- Yamamoto T (1953) Artificially induced sex-reversal in genotypic males of the medaka (*Oryzias latipes*). *J Exp Zool* 123:603–616
- Yamamoto T (1975) Medaka (killifish) biology and strains. Keigaku, Tokyo
- Yokoi H, Shimada A, Carl M, Takashima S, Kobayashi D, Narita T, Jindo T, Kimura T, Kitagawa T, Kage T, Sawada A, Naruse K, Asakawa S, Shimizu N, Mitani H, Shima A, Tsutsumi M, Hori H, Wittbrodt J, Saga Y, Ishikawa Y, Araki K, Takeda H (2007) Mutant analyses reveal different functions of *fgfr1* in medaka and zebrafish despite conserved ligand–receptor relationships. *Dev Biol* 304:326–337

Part I
Genomics, Genetics, and Resources

Chapter 2

Genetics, Genomics, and Biological Resources in the Medaka, *Oryzias latipes*

Kiyoshi Naruse

Abstract *Oryzias latipes*, also known as the medaka or Japanese killifish, has long been considered one of the most powerful animal models in the field of genetics. The Japanese researchers Toyama and Ishikawa confirmed the Mendelian law of inheritance in medaka in 1910, one of the earliest and most prominent achievements in vertebrate genetics. Medaka Y-linked inheritance was subsequently reported in 1921 by Aida, representing the first report of this phenomenon in animals. Yamamoto established the first sex linkage map for medaka and described the differences in recombination frequency between sexes. He also reported for the first time, autosomal linkage between the *i* and *ci* loci in fish. Following the development of PCR technology, several attempts were made to construct a genome-wide genetic linkage map in medaka, zebrafish, pufferfish, and other fish species. In the initial stages of these experiments, fingerprint-type markers were used as they did not require any prior genome knowledge. In the later phases, single-locus type markers that amplify specific regions of the genome in the presence of sequence information were used. The map generated using the single-locus type markers enabled researchers to compare the linkage relationships between orthologous genes. The teleosts underwent specific whole genome duplication (third WGD). Finally, the medaka genome sequencing project, in addition to the *Tetraodon* genome project, provided a high-quality draft genome sequence for both medaka and *Tetraodon*. These data confirmed the third WGD, allowed the successful reconstruction of the preduplicated proto-chromosomes, and described a potential scenario that all led to the generation of the present medaka, *Tetraodon*, and zebrafish genomes. This analysis also identified the highly conserved synteny of the orthologous genes present in the teleost fishes.

Over the past 100 years of medaka research, numerous important biological resources have been developed. The first resource included the body color mutants such as the orange-red and white medaka. The *d-rR* strain was the first of these

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strains to be established and served the specific purpose of linking genetic sex with body color. To date, more than 600 mutants have been reported. In terms of genetic resources, more than 3,000 primer sequences that specifically amplify medaka genomic regions and medaka genome sequences are now widely available via genome browsers. The bacterial artificial chromosome (BAC) and Fosmid clones cover the vast majority of the medaka genome, and 355,000 full-length cDNA clones and expressed sequence tags (ESTs) are also now available. Importantly, most of these resources are publicly available through the National BioResource Project Medaka.

2.1 Mendelism and Medaka: A Brief History of Medaka Genetics in the Early 20th Century

Biology in the 20th century began with the rediscovery of Mendel's laws of inheritance by Hugo de Vries, Carl Correns, and Erich von Tschermak in 1900. Although most of the research undertaken at that time was conducted on plant species, including pea plants and evening primrose, arguments supporting a Mendelian law of inheritance in other organisms, especially animals, remained popular. In 1901, William Bateson and Edith R. Sanders demonstrated that the pea comb in the chicken was dominant to the single comb, and that the pea and single comb phenotypes were segregated at a ratio of 3:1 in the F_2 generation. Castle and Allen (1903) subsequently reported that the albino phenotype in the mouse, guinea pig, and rabbit was a recessive trait.

In Japan, Kametaro Toyama, who received fame as one of the founders of silk-worm genetics, reported on the heredity nature of body color variation in medaka in 1916. This study represents one of the most important studies in medaka genetics. Toyama previously described the black (wild-type), orange-red, and white body color variants in 1908, and reported that when the parents of each strain are pure, that is, they have no prior experience of crossing with medaka of differing body colors, the offspring from each strain demonstrated the identical body color to their parents. The term coined for the mechanism underlying this process was the pure-line theory. In addition, Toyama also identified that fish with a white body color were always female, and that this phenomenon was one example of the sex-limited inheritance previously described by Thomas Hunt Morgan in his study of *Drosophila* with white eyes (1910). In 1910, Toyama subsequently obtained 190 and 261 offspring with a black body color following the reciprocal crossing of black and orange-red medaka. These findings indicated that black body color was dominant and that orange-red was recessive, supporting Mendel's law of dominance. In 1910, Toyama obtained 10 black and 3 orange-red medaka by crossing F_1 fishes, and 187 black and 208 orange-red fish by backcrossing the F_1 females with orange-red males. In addition, he generated 25 black and 32 orange-red fish by backcrossing the F_1 males and orange-red females. In these experiments, Toyama was able to clearly demonstrate the law of segregation. In experiments undertaken

in 1909, Toyama crossed white females with black males and obtained 68 F₁ fishes, all with a black body color. Later, in 1910, he generated 100 F₂ fish. Of these fish, 60 were black, 20 were orange-red, 15 were blue, and 5 were white, corresponding to a ratio of 9:3:3:1. Thus, in these two sets of experiments, Toyama was able to further demonstrate the law of independent assortment. Thus he clearly demonstrated the Mendelian laws of inheritance including the laws of dominance, segregation, and independent assortment. Similar results were also obtained by Chiyomatsu Ishikawa around the same time (1912).

In 1915, Makoto Ishihara crossed black females and white “males” and obtained 81 F₁ generation offspring that were all black in body color. In the F₂ generation, he generated 61 black, 22 orange-red, 16 blue, and 6 white offspring, corresponding to the 9:3:3:1 ratio; interestingly, the white males were present in his study (Ishihara 1916). If the white medaka reported in Ishihara’s experiment and that reported in Toyama’s experiment represent the same mutant, white males may be generated following crossing-over of the X and Y chromosomes. Following these studies, Tatsuo Aida (1921) demonstrated that the gene controlling carotenoid deposition in the yellow pigment cells, the *r* locus, was located on the Y chromosome. He also demonstrated that the wild-type allele of the *r* locus was located on the Y chromosome and that the recessive or mutant allele was located on the X chromosome. Thus, the white medaka appeared to be homozygous for the recessive allele, suggesting that females are homogametic. This evidence indicated that the medaka sex determination system is heterogametic for males and homogametic for females (XX–XY). In addition to Aida, Schmidt (1920) and Winge (1922), who investigated the guppy, shared the credit for the first discovery of Y-linked inheritance in animals. Medaka is the one of the earliest vertebrates, and at the very least the first fish species in which Mendelian laws of inheritance were confirmed.

2.2 Linkage and Genome-Wide Genetic Mapping

2.2.1 Early Studies on Linkage Analysis in Medaka

Following the study by Aida, Toki-o Yamamoto further investigated sex determination and differentiation in medaka (1951). Yamamoto was the first to establish the *d-rR* strain, in which generated females are white and males are orange-red because of the presence of the wild-type allele at the *r* locus on the Y chromosome. As a result of this linkage, researchers were able to determine the sex of the fish based on body color. In addition, Yamamoto was also able to successfully induce the XY sex-reversal female by providing an estrogen-containing diet (1953) and the XX sex-reversal male by feeding with an androgen-containing diet (1958). Yamamoto reported the linkage map of the medaka sex chromosome, which is represented as LG1 in the most current linkage map (Naruse et al. 2000, 2004b). As XX represent females and XY represent males under normal conditions (Aida 1921), it is possible to generate females with an XY chromosome composition by feeding the fish

estrogen in their diet. Yamamoto described the two sex chromosome linkage maps containing one chromosome from normal males and another from an induced heterogametic female (Yamamoto 1964). The map distance between the *r* locus and the sex determination gene *y*, which was identified as *DMY/DMRT1-Yb* (Matsuda et al. 2002; Nanda et al. 2002), has been measured as 0.2 ± 0.01 centimorgans (cM) in normal males and 1.0 ± 0.4 cM in sex-reversal females (Yamamoto 1964). Interestingly, the recombination frequency during female meiosis was five times higher than that during male meiosis in this region. This report represented the first description of differences in recombination frequency between male and female meiosis. On the autosomal linkage map, the *ci* and *i* loci were found to be linked and exhibited a map distance of 4.6 ± 1.2 cM (Yamamoto and Oikawa 1973; this was the first reported example of autosomal linkage in teleosts. The genes encoded by the *i* and *ci* loci were identified using position-based cloning and were found to encode tyroninase (Koga et al. 1995) and somatolactin (Fukamachi et al. 2004b), respectively. Hideo Tomita also identified two autosomal linkages between the diluted xanthophore-2 (*dx-2*) and the concentrated xanthophore-2 (*co*) loci (12.2 ± 1.1 in female meiosis and 12.9 ± 0.6 in male meiosis), and between the dispersed xanthophore (*di*) and white leucophore (*wl*) loci (1985). Although more than 60 visible mutants were identified, it was almost impossible to assign all these loci using ordinary three-point linkage analysis.

2.2.2 Toward a Genome-Wide Genetic Map

The development of polymerase chain reaction (PCR)-based technology has allowed the identification of polymorphisms in genomic DNA and permitted the establishment of a genome-wide genetic map. The initial linkage analysis aimed at constructing a genome-wide map using PCR-based mapping methods, which was published by Wada et al. in 1995, involved the use of random amplified polymorphic DNA (RAPD) technology (Welsh and McClelland 1990). In this method, prior genomic information was not required for the detection of polymorphisms, as arbitrary primers and a low PCR annealing temperature (37°C) were used. No requirement of prior genomic information represented one of the most significant advantages over other alternative methods including traditional restriction fragment length polymorphism (RFLP) analysis using Southern blotting. The map generated via this method consisted of 28 linkage groups with 170 RAPD markers, three pigment patterns, and five enzyme-coding loci that spanned 2,480 cM. Ohtsuka et al. (1999) also published a RAPD-based linkage map to assign the double anal fin (*Da*) mutant locus and constructed a genetic map consisting of 26 linkage groups. As the medaka contains a set of 48 diploid chromosomes (Ojima and Hitotsumachi 1969), gaps in these maps still remained. A linkage map comprising 24 linkage groups was subsequently published by Naruse et al. in 2000. This map represented the first skeletal-level map and consisted of 633 markers including 488 amplified fragment length polymorphisms

(AFLP) (Vos et al. 1995), 28 RAPD, 34 inter-sequences between two short interspersed repeated elements (IRS), 75 expressed sequence tags (ESTs), four sequence tagged site markers, and four phenotypic markers, and covered 1,354.5 cM in male meiosis. Two inbred lines, AA2 and HNI, were used for the construction of backcross typing panels wherein AA2 females were crossed with F₁ males. AFLP markers were mainly used for loci in the construction of this skeletal map; as with the RAPD markers, there was no need for prior genomic knowledge regarding the organisms used for the analysis. In addition, the AFLP markers generally provided more reproducible PCR amplification of DNA fragments than the RAPD markers.

Other novel markers used in the generation of this map were the markers termed PCR-RFLP markers (Naruse et al. 2000). In contrast to the AFLP and RAPD marker methods, this method used specific primers that amplified the corresponding gene regions in the genomic DNA. Following amplification of the specific genomic regions, polymorphisms between parents were detected using simple restriction enzyme digestion. One of the most interesting results from this linkage map was the number of *Hox* gene clusters present; 22 *Hox* genes mapped to seven different linkage groups: LG7, LG8, LG111, LG15, LG16, LG19, and LG21. In zebrafish, seven *Hox* gene clusters were also identified (Amores et al. 1998). However, the precise composition of the *Hox* clusters differed between medaka and zebrafish. Medaka contained two *Hox A*, two *Hox B*, one *Hox C*, and two *Hox D* (Naruse et al. 2000), whereas the zebrafish contained two *Hox A*, two *Hox B*, two *Hox C*, and one *Hox D* (Amores et al. 1998). The common ancestor of zebrafish and medaka has been hypothesized to be the common ancestor of all euteleosts (Miya et al. 2003; Yamanoue et al. 2007). Thus, the combined results from zebrafish and medaka strongly suggested that the common ancestor contained eight *Hox* clusters, indicating an extra whole genome duplication (third WGD) in the teleost lineage.

2.2.3 Medaka Gene Catalogs and Gene-Based Linkage Maps

The genetic map published in 2000 mainly consisted of anonymous DNA markers including RAPD, AFLP, and microsatellites (Naruse et al. 2000). For the precise genome-wide comparison of the organization of the genes between different organisms, an accurate gene-based map is crucial. One possible method to generate the genome-wide gene map for medaka is the use of PCR-RFLP markers. To apply this method, knowledge of the medaka genome sequence, in particular the protein-coding regions (cDNA), was essential. At this time, the medaka cDNA information listed on the DDBJ/Genbank/EMBL database presented only a few hundred cDNAs. Thus, we decided to establish a medaka gene catalog using EST analysis. EST analysis is a simple method used for the cataloging of expressed genes. The first step in the process involves the construction of a cDNA library containing various mRNA sources including adult, specific embryonic developmental stage tissue, and cultured cells. The cDNA libraries are then plated onto agar media and the clones are isolated one by one using a colony-picking apparatus. Each colony is then

inoculated into 384- or 96-well plates. Finally, the sequences at 5'- and 3'-ends of the clones are determined. In our experiments, we first constructed a full-length cDNA library from adult HNI males using the oligo-capping method (Maruyama and Sugano 1994), and then constructed five additional cDNA libraries from cultured medaka cell lines, OLHNI with and without photo-reactivation (20 J/m² UV), OLHNI with gamma irradiation, ovarian cells, and a mixture of male and female liver cells. From these libraries we obtained 18,527 sequences and identified 8,676 independent clusters using clustering analysis of the 5'-end sequences. These EST data were obtained from the M base website (http://mbase.bioweb.ne.jp/~dclust/medaka_top.html).

A further EST project was conducted at the National Institute of Genetics (Kimura et al. 2004). In this project, five different cDNA libraries were constructed from three different developmental embryonic stages including stage 23 (segmentation stage), stage 35–36 (5 days), and stage 40 (fry stage). From these libraries, 132,082 sequences from both ends of the clones were characterized, and 12,429 clusters of 3'-end sequences were identified using clustering analysis. These sequence data were adequate for primer design and the subsequent amplification of specific medaka gene regions. Figure 2.1 demonstrates an example of the amplification of specific regions using primers based on EST sequences, and the detection of polymorphisms between AA2 and HNI using the PCR-RFLP method. Four of eight enzymes including *DdeI*, *FokI*, *HaeIII*, *HinfI*, *MnII*, *MspI*, *RsaI*, and *MseI* represent RFLP in this example.

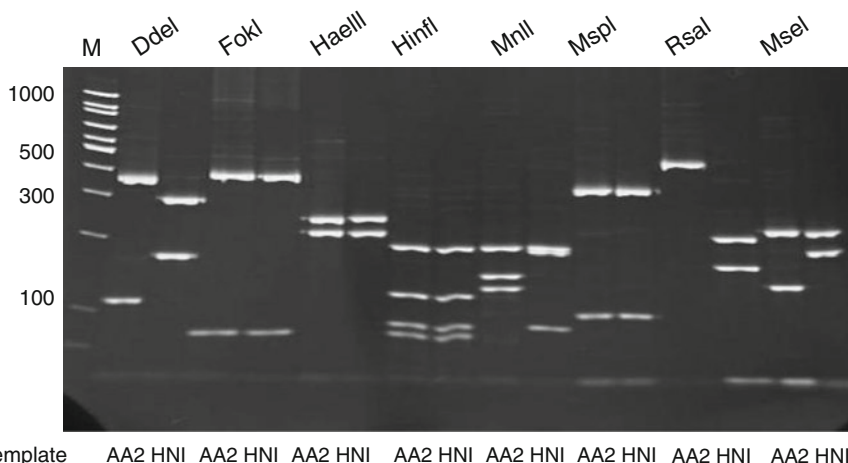


Fig. 2.1 Representative polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) data. RFLP were observed for four of the eight enzymes. *M*, 100-bp ladder marker. AA2 and HNI genomic DNA were used as the template for PCR amplification

On average, 30% of the primers that were based on only EST sequences could be used for mapping in this experiment. Using this method, we constructed a medaka gene map consisting of 818 genes and EST on the single meiotic backcross panel in the AA2 and HNI inbred lines (Naruse et al. 2004a). All primer sequences and enzymes showing RFLP are shown on the M base website. The greatest advantage for using specific primers to construct genetic maps is the reproducibility of amplification of specific genomic regions. Although DNA fingerprint-type DNA markers including RAPD and AFLP are applicable for the construction of genetic maps in the absence of prior genomic information, the markers are highly strain specific, and it is therefore impossible to identify and compare orthologous regions between different organisms. In contrast, PCR primers based on region-specific markers are applicable to mapping within a species or even within related species and may be used to identify orthologous and homologous regions in organisms exhibiting sequence similarity.

Using these features, we analyzed the conserved synteny between medaka and zebrafish, using the human as an outgroup. Figure 2.2 shows the oxford grids for medaka and human and for medaka and zebrafish. Medaka and human were found to share 104 conserved syntenic segments, including at least three orthologous pairs in the data set. Medaka and zebrafish also showed conserved synteny. We hypothesized that the number of hypothetical proto-chromosomes using conserved synteny data among medaka, zebrafish, and human and the number of preduplicated proto-chromosomes is 12 (Naruse et al. 2004a). Whole genome

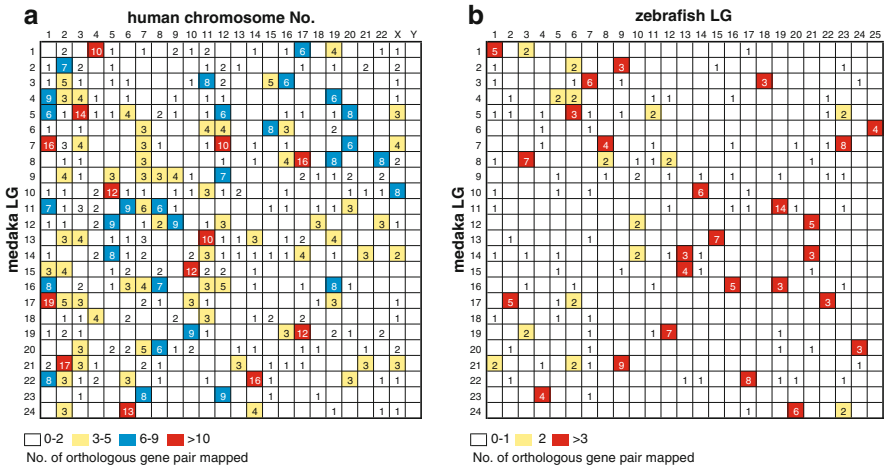


Fig. 2.2 Oxford grid display of the conserved synteny between medaka and human (a) and medaka and zebrafish (b). Numbers in the grids represent the number of orthologous gene pairs between the two species. Clusters of orthologous gene pairs were observed in both the medaka–human and medaka–zebrafish comparisons, indicating that conserved synteny was demonstrated among these species

sequence data in medaka and other teleosts were essential for the description of the preduplicated proto-chromosomes and the scenario leading to the evolution of the present fish genome.

2.2.4 Genetic Features of Medaka

The establishment of the genome-wide linkage map for medaka has allowed the definition of many of the features of the medaka genome. As described in the previous section, Yamamoto defined the differences in recombination frequency of sex chromosomes between male and female meiosis using normal males and sex-reversal females. The recombination frequency between the sex-determining locus *y* and the *r* locus in normal males is 0.2%, whereas that of sex-reversal females is 1% (Yamamoto 1964). Detailed analysis of recombination frequency in sex chromosomes has also been undertaken using 21 sequence-tagged site (STS) markers (Kondo et al. 2001a). This study was able to demonstrate that the genetic length of the sex chromosome was shorter during male meiosis than in female meiosis, and that the region containing male recombination suppression was located within close proximity to the sex-determining gene *y*. In the telomeric regions, the recombination frequency in males was higher than that in females. Differences in recombination frequencies between male and female meiosis have been reported in other fish species including the tiger pufferfish (Kai et al. 2005), turbot (Bouza et al. 2007), and rainbow trout (Rexroad et al. 2008). In general, recombination in males is suppressed compared with females. Even though the cumulative length of the linkage group is similar, chromosome-specific variations in recombination rate between sexes were observed (Rexroad et al. 2008). The recombination rates in telomeric regions are higher during male meiosis than during female meiosis. Recombination in females is relatively even along the chromosome, with the exception of the telomeric regions. This trend is not only apparent for the sex chromosomes but has also been observed for autosomes in fish (Rexroad et al. 2008; Kimura et al. 2005; Kai et al. 2005).

Another important observation was the reported suppression of recombination in LG11 in the Hd-rR/HNI-typing panel. Eight of 17 markers were clustered in a single genetic position in LG11 (Kimura et al. 2005). Clustering of the markers was also observed in the F₂ panel of the Hd-rR and Kaga strains. In contrast, this cluster was not observed in the Hd-rR and Kunming backcross panel. The Kaga and HNI strains belong to the Northern Japanese population whereas the Kunming belongs to the China–West Korean population. This phenomenon is explained by an inversion in the Northern Japanese population (Kimura et al. 2005). Thus, careful attention must be paid when determining position-based cloning of the mutants located on LG11. It is impossible to narrow down the responsible region using genetic mapping for reasons of the lack of recombination in this genomic region. In this case it would seem appropriate to select fish from the China–West Korean population or the East Korea population for the construction of a high-resolution typing panel.

2.3 Medaka Genome Sequencing and Genomic Features

2.3.1 Medaka Genome Sequencing

The medaka genome sequence project was launched in the autumn of 2002, and the genome assembly database was opened to the public in 2004 through the medaka genome browser UTGB (<http://medaka.utgenome.org/>). The draft-level genome sequencing was completed in 2006 (Kasahara et al. 2007). As part of this project, we sequenced the genome of the Hd-rR inbred strain, a descendant of the d-rR strain. The fact that we were able to achieve this was one of the most important factors for the success of the medaka genome sequencing within a relatively short time. With the exception of the sex-determining region, there were no sequence differences between the parental alleles, so we were able to assemble the medaka genome as a haploid genome. There were two other important factors in the design strategy for the medaka genome sequence project. One factor involved the whole genome shotgun sequencing process that was undertaken to assemble the medaka genome, and the second factor involved the construction of high-density linkage maps using single nucleotide polymorphic (SNP) markers. Using this strategy, the generated scaffolds were aligned on the high-density linkage map and the chromosomal position of each scaffold was determined. The accuracy of assembly of the relatively long scaffolds was then verified, as the single scaffolds must be assigned to the same linkage group. If this was not found to be the case, we carefully investigated the assembly again and corrected the mis-assembly. Via this method, we were able to map 2,544 SNP markers in two backcross panels with 93 individual female and male meiosis events, and a F₂ panel with 368 individuals. A female backcross panel was used for the main typing panel, and the other two were used for genotyping of the genomic regions with relatively low recombination frequency in female meiosis. The resolution of the typing panel was 1.1 cM, which corresponded to an average of 500 kbp. N50 scaffold size was 1.4 Mbp in this assembly, so the resolution was high enough for the alignment of the scaffolds on this linkage map. Table 2.1 shows the assembly and mapping statistics of the medaka genome sequencing. The raw output of the RAMEN assembler was 764 Mbp, including singleton shotgun reads and short scaffolds less than 2 kbp. In general, singleton shotgun reads and short scaffolds were from repetitive regions in the genome and on occasion from contaminants. After removal of these data, the cumulative length of the generated scaffolds was 700.4 Mbp. We were then able to assign the map position of scaffolds whose cumulative length was 627 Mbp (89.7%) without any ambiguity.

In addition to the Hd-rR genome, we also sequenced the HNI inbred line genome. The sequence coverage was found to be 2.8-fold genome equivalents with a 2.3-kbp-insert plasmid library. The cumulative length of the generated scaffolds was 648 Mbp in the HNI assembly. Assembly data of the Hd-rR strain are publicly available though UTGB (<http://medaka.utgenome.org/>), Ensembl (<http://medaka.utgenome.org/>), the UCSC genome browser (<http://genome.ucsc.edu/>), and the

Table 2.1 Summary of the medaka genome sequencing

<i>Assembly statistics</i>	
Number of reads	13.8 million
Sequence coverage	10.6 fold
Clone coverage	53.7 fold
N50 contig size	9.8 kbp
N50 scaffold (supercontig) size	1.41 Mbp
N50 ultracontig size	5.1 Mbp
<i>Mapping statistics</i>	
Number of mapped loci	2544
Number of unmapped loci	4
Resolution of typing panel	1.1 cM (92 individuals)
Anchored	
oriented	582.1 M (83.1%),
unoriented	16.1 M (2.3%),
unordered	29.8 M (4.3%)
Unanchored	72.4 M (10.3%)
Total	700.4 Mbp

medaka map in NBRP Medaka (<http://medakagb.lab.nig.ac.jp/index.html>). Information regarding the HNI strain can also be obtained from the medaka genome project website (<http://dolphin.lab.nig.ac.jp/medaka/>). Blat alignment between Hd-rR and HNI genome is provided at the UTGB website.

2.3.2 Genomic Features of Medaka

2.3.2.1 Polymorphisms in the Medaka Genome

As described, we have sequenced the Hd-rR and HNI genomes. These two inbred lines are representative strains from the Southern and Northern Japanese populations, respectively, and the lineage separation of these two populations has been shown to date back more than 4 million years (Takehana et al. 2003). The comparison of these two genomes enables detection of polymorphisms on a genome-wide scale. We found that the substitution rate in the whole genome was 34.246/kbp (3.42%), 18.077/kbp (1.80%) in the exonic region and 33.984/kbp (3.39%) in the intronic region. For the detailed analysis of genomic polymorphisms among medaka natural populations, we analyzed SNP detected in the 47 PCR-amplified fragments from the Hd-rR, d-rR, and Nago strains from the Southern Japanese population; the HNI, Niigata, and Kaga strains from the Northern Japanese population; the HSOK strain from the East Korean population; and Taiwan and the Kunming strain from the China–West Korea population. Following close observation of the validity of SNP sites, we focused on the 475 SNP located between the Hd-rR and HNI strains. Among the 475 SNP, 130 (27%) and 28 (5.8%) were

found to be polymorphic within the Southern and Northern populations, respectively. One of these mutations also happened to be shared in both populations. The remaining 318 SNP were a common SNP (fixed mutation) for each population. In other words, 66.9% (318/475) of SNPs between HNI and Hd-rR stains were conserved in any combination of respective individuals or strains from the Southern and Northern Japanese populations. These data demonstrate a recent expansion of the Northern Japanese population and strict genetic separation of these two populations after divergence without any obvious sexual separation or speciation. This information may lead to the further enhancement of genetic mapping of mutants and the identification of strain-specific phenotype traits.

2.3.2.2 Repeat Elements

Repeat elements detected using Repeat Masker and Repbase data (version 9.11) revealed that 8.3% of the medaka genome sequence occupies known repeats including SINE, LINE DNA elements, simple repeats, and low complexity. The de novo repeat detection results suggest that 9.2% of the medaka genome occupies novel repeats. Thus, a total 17.5% of the medaka genome sequences consisted of repeat elements.

2.3.2.3 Construction of the Medaka Gene Model

In 2006, species-specific gene/cDNA information including full-length cDNA sequences was rare for medaka. Only 189 full-length cDNA sequences in total were available on the medaka UniGene database. Thus, we used the ab initio gene prediction method combined with transcription start site (TSS) information with 5'-end serial analysis of gene expression (5'-SAGE) (Hashimoto et al. 2004). 5'-SAGE libraries were constructed from a mixture of 0- to 7-day-old embryos and a mixture of adult tissues. We first collected more than 1 million 5'-SAGE tags and identified the TSS sites. We then searched the genes using newly developed gene prediction methods based on genescan. As a result of this evidence-based gene prediction method, we identified 20,141 medaka genes. We then compared the medaka gene model with the genes of six other vertebrates including human, mouse, chicken, *Tetraodon nigroviridis*, *Takifugu rubripes*, and zebrafish in the RefSeq database, and with the gene clusters for Aves, amphibians, ray-finned fish, and ascidians in the Unigene database using the tblastx function. We identified that 3,727 medaka genes exhibited no significant homology with any other species examined with loose criteria (e-value, $<10^{-4}$). This relatively large number of medaka-specific genes served as an interesting resource for analysis of the evolution of protein-coding and noncoding genes in medaka. Others genes were also found to demonstrate significant homology with other species. A total of 11,809 genes were found in all six species including human, mouse, chicken, *T. nigroviridis*, *T. rubripes*, and zebrafish; 12,821 were found in all three fish species *T. nigroviridis*, *T. rubripes*,

and zebrafish; and 15,565 were found in one of the three fish species. A total of 16,414 homologs were found in at least one of the six species including Unigene clusters of Aves, amphibians, ray-finned fish, ascidians, and *Takifugu* genomes.

2.4 Conserved Synteny and Reconstruction of Ancestral Fish Chromosomes

2.4.1 Orthologous Chromosomes in Medaka, Tetraodon, and Zebrafish

The medaka gene models described in the previous section enabled us to undertake a comprehensive analysis of the conserved synteny among vertebrates. Figure 2.3 demonstrates the oxford display for the numbers of orthologous genes shared between medaka, *Tetraodon*, and zebrafish. In the medaka and *Tetraodon* display, a single medaka chromosome corresponded to the single *Tetraodon* chromosome, with the exception of *Tetraodon* chromosomes (Tni) 1, 2, and 3. These chromosomes corresponded with 2 medaka chromosomes: Tni1 corresponded to medaka chromosome (Ola) 4 and 10, Tni 2 to Ola 19 and Ola 21, and Tni3 to Ola2 and Ola 8. This information likely arose from chromosomal fusions in the *Tetraodon* lineage, as Ola 4, Ola 10, Ola 19, Ola 21, Ola 2, and Ola 8 corresponded to 1 or more zebrafish chromosomes. In the medaka–zebrafish display, 11 zebrafish chromosomes corresponded one-to-one with medaka chromosomes; additional chromosomes corresponded one-to-two or many-to-many. Zebrafish represents one of the most basally diverged species in the teleost phylogeny. Its lineage separation from the common ancestor of medaka–*Tetraodon*–zebrafish (MTZ ancestor) extends back to more than 300 million years ago. Even for medaka and *Tetraodon*, their lineage separation extends back to 150 million years ago. These results suggest that inter-chromosomal rearrangements in the teleost lineage are less frequent compared to that of mammals, whose radiation time is approximately 103 million years (Murphy et al. 2001).

2.4.2 Reconstruction of the Ancestral Proto-Chromosomes

One of the most prominent pieces of genomic evidence in the teleost lineage is the extra third WGD. Extra Hox clusters and extra genes observed in several other fish species provide strong evidence for the occurrence of a third WGD. Analysis of paralogous gene copy distribution in the medaka genome showed that particular combinations of duplicated gene sets tend to colocate to two particular chromosomes (Kasahara et al. 2007). Similar analysis in the *Tetraodon* genome has also shown this pattern of distribution for duplicated genes (Jaillon et al. 2004). Figure 2.4 depicts a model of whole genome duplication and the subsequent genome arrangement in the teleost lineage. Following divergence of the tetrapod lineage that leads to the generation of mammals, the third WGD was expected to

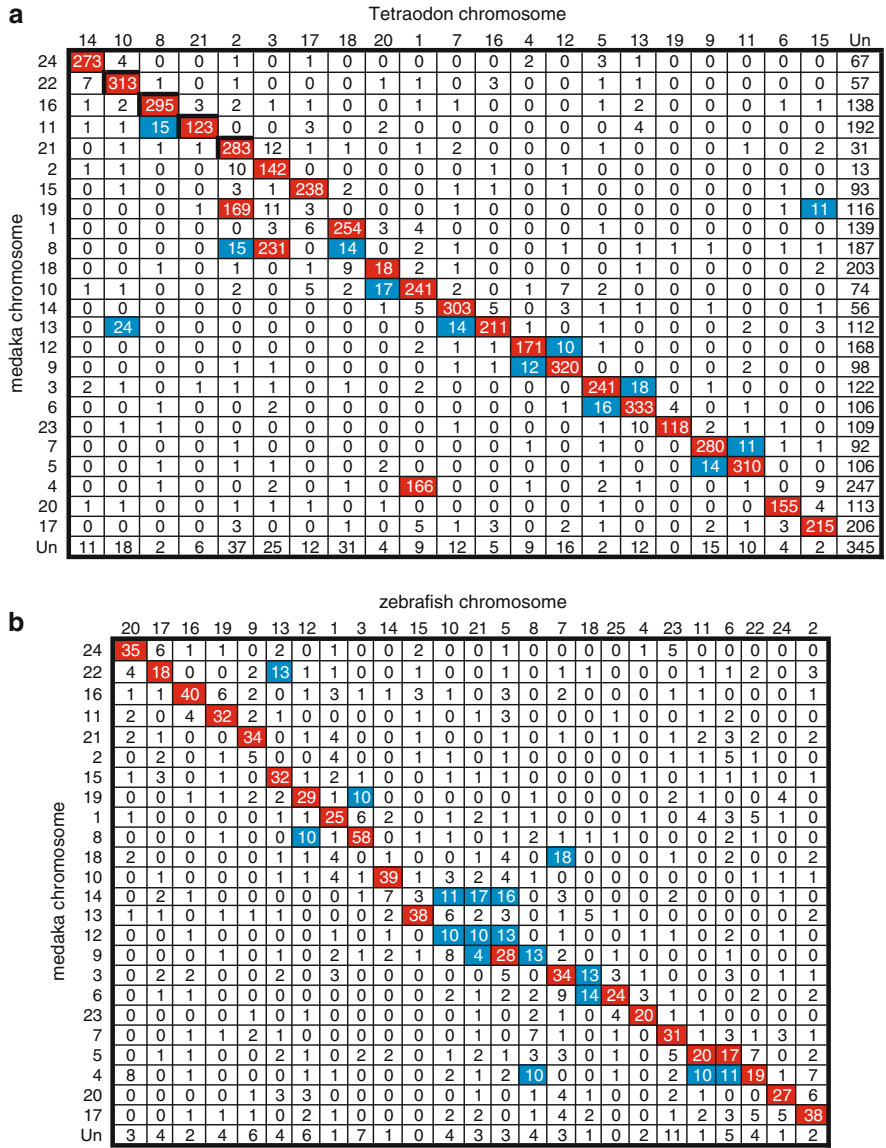


Fig. 2.3 Oxford grid display of the conserved synteny between medaka and *Tetraodon* (a) and medaka and zebrafish (b). Numbers in each grid represent the number of orthologous gene pairs between the two species. With the exception of *Tetraodon* chromosomes 1, 2, and 3, the medaka–*Tetraodon* matrix shows one-to-one correspondence with each chromosome. The medaka–zebrafish matrix shows a relatively complex correspondence for each chromosome when comparing the medaka and *Tetraodon* matrix. Eleven chromosomes in the zebrafish showed a one-to-one correspondence with the medaka chromosomes. The remaining zebrafish chromosomes displayed a correspondence of “one to two” to “many to many”

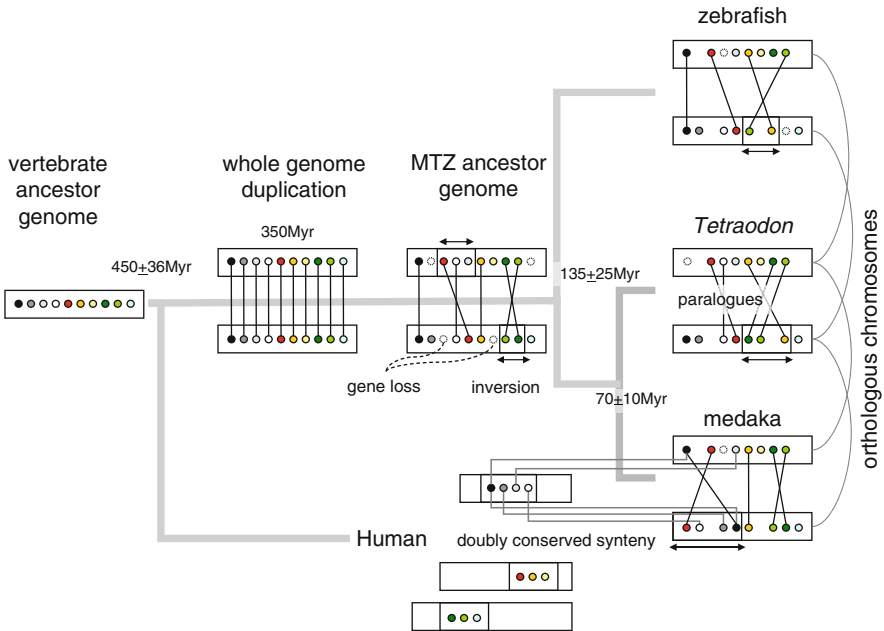


Fig. 2.4 A model of chromosomal evolution in vertebrates. After divergence of the tetrapod lineage from its vertebrate ancestor, whole genome duplication followed in the ray-finned fish. After rearrangements including gene loss and inversion, medaka, *Tetraodon*, and zebrafish (MTZ) ancestors were generated and differentiated into the present medaka, *Tetraodon*, and zebrafish genomes in the absence of major inter-chromosomal rearrangements. In the tetrapod lineage leading to the generation of humans, a single ancestral chromosome was divided into several segments by fusion and fission events in the mammalian lineage. Myr, million years

have happened in the medaka, *Tetraodon*, and zebrafish (MTZ) ancestor, so that duplicated genes in the MTZ ancestor evolved in a lineage-specific manner without large inter-chromosomal rearrangements. In the mammalian lineage, an ancestral chromosome was thought to have divided into several chromosomal segments with relatively frequent inter-chromosomal rearrangements. Thus, the particular human genomic segments are likely to exhibit conserved synteny with two particular fish chromosomes (DCS), as a consequence of a third WGD in the teleost lineage. Conversely, the human chromosome segments with DCS in two particular fish chromosomes should be consistent with a single ancestral chromosome. Given this expectation, we reconstructed the preduplicated proto-chromosomes in ray-finned fish and defined the sequence of events that most likely leads to the present genome structures of medaka, *Tetraodon*, and zebrafish. Figure 2.5 outlines the results of this analysis: there were 13 preduplicated proto-chromosomes in the haploid. Following the third WGD and eight major rearrangements, the MTZ ancestor should have contained 24 chromosomes. The present genomes of the medaka, *Tetraodon*, and zebrafish were formed with lineage-specific rearrangements, that is, three chromosomal fusions in the *Tetraodon* lineage, several chromosomal fusions and fissions in the zebrafish lineage, and no obvious rearrangements in medaka.

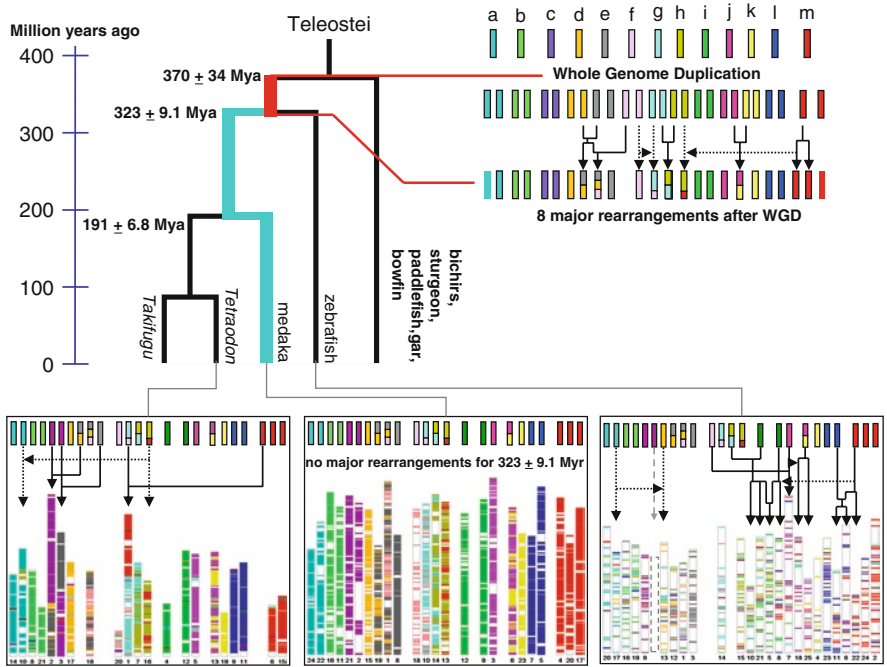


Fig. 2.5 Reconstruction of the preduplicated proto-chromosomes in ray-finned fish and the potential scenario that led to the formation of the present medaka, *Tetraodon*, and zebrafish genomes. The chromosomes *a* to *m* represent preduplicated proto-chromosomes. After whole genome duplication (WGD), eight major rearrangements occurred to form the medaka, *Tetraodon*, and zebrafish ancestors that contained 24 chromosomes. In the *Tetraodon* lineage, three chromosomal fusions occurred to generate the present *Tetraodon* genome that contains 21 chromosomes. In the zebrafish lineage, several fusion, fission, and translocation events occurred to form the present zebrafish genome. The numbers under each chromosome represent the chromosome number in each species. The presented divergence time of each lineage is in accordance with Yamanoue et al. (2007). Mya, million years ago

2.5 Future Perspectives

During the past 100 years, important information has been generated that has permitted the precise definition of the medaka genome. Initially, body color mutants such as the orange-red variety (the *b* locus mutant) and the white (double mutant at the *b* and *r* loci) strain served as the only experimental tools for these studies. These spontaneous body color mutants provided crucial information on medaka genetics early in the 20th century. Extensive efforts to identify the mutant from natural populations continued into 1990, and enabled identification of more than 60 viable mutants exhibiting specific phenotypes (Tomita 1982, 1993). Causative genes for the *b*, *rs3*, *i*, and *i-3* loci were identified using position-based cloning (Fukamachi et al. 2001, 2004a,b; Kondo et al. 2001b; Koga et al. 1995, 1996). These genes were

also mutated in the human, where they were found to cause a number of important hereditary diseases (see Chaps.6–13). Thus, the numerous spontaneous viable medaka mutants serve as reliable models for hereditary human diseases. In addition to these viable mutants, more than 400 *N*-ethyl-*N*-nitrosourea (ENU)-induced mutants are also available (see Chap.5). The high-quality draft genome sequences of medaka are also one of the most important resources (Kasahara et al. 2007). These data have enabled rapid identification of genes underlying the mutations using positional cloning and are indispensable for genomic information using comparative genomic analysis. The bacterial artificial chromosome (BAC)/fosmid clones developed during the genome sequencing project were rich sources for the construction of transgenic fish containing fluorescence protein tags such as green fluorescent protein and Ds-Red2. We are now able to modify BAC/fosmid clones using in vivo recombination techniques (Nakamura et al. 2008), eliminating the need for tedious cut-and-paste procedures in the generation of transgenic constructs. The generation of EST and cDNA including full-length cDNA clones are also important resources. More than 68,000 unique sequences have been deposited in the DDBJ/GenBank/EMBL databases to date. These sequences can be used for probe design to detect gene expression via in situ hybridization. More recently, we have developed full-length cDNA clones, so that 355,000 clones with 499,944 sequences from eleven full-length cDNA libraries are now available from NBRP Medaka (<http://www.shigen.nig.ac.jp/medaka/top/top.jsp>). NBRP Medaka provides medaka strains as live resources and BAC/Fosmid/cDNA as genomic resources. Additional resources including phylogenetic information of medaka and their relatives, an atlas, and a medaka-specific genome browser are also provided. Medaka now represents a valid model system for the study of both vertebrate morphogenesis and disease ontogeny.

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References

- Aida T (1921) On the inheritance of color in a freshwater fish, *Aplocheilichthys latipes* Temminck and Schlegel, with special reference to sex-linked inheritance. *Genetics* 6:554–573
- Amores A, Force A, Yan YL, Joly L, Amemiya C, Fritz A, Ho RK, Langeland J, Prince V, Wang YL, Westerfield M, Ekker M, Postlethwait JH (1998) Zebrafish *hox* clusters and vertebrate genome evolution. *Science* 282:1711–1714
- Bateson W, Saunders ER (1901) Experimental studies in the physiology of heredity. Report to The Evolution Committee of the Royal Society of London, London

- Bouza C, Hermida M, Pardo BG, Fernandez C, Fortes GG, Castro J, Sanchez L, Presa P, Perez M, Sanjuan A, de Carlos A, Alvarez-Dios JA, Ezcurra S, Cal RM, Piferrer F, Martinez P (2007) A microsatellite genetic map of the turbot (*Scophthalmus maximus*). *Genetics* 177:2457–2467
- Castle WE, Allen GM (1903) The heredity of albinism. *Proc Am Acad Arts Sci* 38:603–622
- Fukumachi S, Shimada A, Shima A (2001) Mutations in the gene encoding B, a novel transporter protein, reduce melanin content in medaka. *Nat Genet* 28:381–385
- Fukumachi S, Asakawa S, Wakamatsu Y, Shimizu N, Mitani H, Shima A (2004a) Conserved function of medaka pink-eyed dilution in melanin synthesis and its divergent transcriptional regulation in gonads among vertebrates. *Genetics* 168:1519–1527
- Fukumachi S, Sugimoto M, Mitani H, Shima A (2004b) Somatolactin selectively regulates proliferation and morphogenesis of neural-crest derived pigment cells in medaka. *Proc Natl Acad Sci USA* 101:10661–10666
- Hashimoto S, Suzuki Y, Kasai Y, Morohoshi K, Yamada T, Sese J, Morishita S, Sugano S, Matsushima K (2004) 5'-end SAGE for the analysis of transcriptional start sites. *Nat Biotechnol* 22:1146–1149
- Ishihara M (1916) On the inheritance of body color of medaka (Medaka no taishoku no iden ni tsuite). *Fukuoka Ikadaigaku Zashi* 9:259–267
- Ishikawa C (1912) *Genshu Kairyō Ron*. Suisan Koshujo Publication
- Jaillon O, Aury JM, Brunet F, Petit JL, Stange-Thomann N, Mauceli E, Bouneau L, Fischer C, Ozouf-Costaz C, Bernot A, Nicaud S, Jaffe D, Fisher S, Lutfalla G, Dossat C, Segurens B, Dasilva C, Salanoubat M, Levy M, Boudet N, Castellano S, Anthouard R, Jubin C, Castelli V, Katinka M, Vacherie B, Biemont C, Skalli Z, Cattolico L, Poulain J, de Berardinis V, Cruaud C, Duprat S, Brottier P, Coutanceau JP, Gouzy J, Parra G, Lardier G, Chapple C, McKernan KJ, McEwan P, Bosak S, Kellis M, Volff JN, Guigo R, Zody MC, Mesirov J, Lindblad-Toh K, Birren B, Nusbaum C, Kahn D, Robinson-Rechavi M, Laudet V, Schachter V, Quetier F, Saurin W, Scarpelli C, Wincker P, Lander ES, Weissenbach J, Crollius HR (2004) Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature (Lond)* 431:946–957
- Kai W, Kikuchi K, Fujita M, Suetake H, Fujiwara A, Yoshiura Y, Ototake M, Venkatesh B, Miyaki K, Suzuki Y (2005) A genetic linkage map for the tiger pufferfish, *Takifugu rubripes*. *Genetics* 171:227–238
- Kasahara M, Naruse K, Sasaki S, Nakatani Y, Qu W, Ahsan B, Yamada T, Nagayasu Y, Doi K, Kasai Y, Jindo T, Kobayashi D, Shimada A, Toyoda A, Kuroki Y, Fujiyama A, Sasaki T, Shimizu A, Asakawa S, Shimizu N, Hashimoto SI, Yang J, Lee Y, Matsushima K, Sugano S, Sakaizumi M, Narita T, Ohishi K, Haga S, Ohta F, Nomoto H, Nogata K, Morishita T, Endo T, Shin-I T, Takeda H, Morishita S, Kohara Y (2007) The medaka draft genome and insights into vertebrate genome evolution. *Nature (Lond)* 447:714–719
- Kimura T, Jindo T, Narita T, Naruse K, Kobayashi D, Shin-I T, Kitagawa T, Sakaguchi T, Mitani H, Shima A, Kohara Y, Takeda H (2004) Large-scale isolation of ESTs from medaka embryos and its application to medaka developmental genetics. *Mech Dev* 121:915–932
- Kimura T, Yoshida K, Shimada A, Jindo T, Sakaizumi M, Mitani H, Naruse K, Takeda H, Inoko H, Tamiya G, Shinya M (2005) Genetic linkage map of medaka with polymerase chain reaction length polymorphisms. *Gene (Amst)* 363:24–31
- Koga A, Inagaki H, Bessho Y, Hori H (1995) Insertion of a novel transposable element in the tyrosinase gene is responsible for an albino mutation in the medaka fish, *Oryzias latipes*. *Mol Gen Genet* 249:400–405
- Koga A, Suzuki M, Inagaki H, Bessho Y, Hori H (1996) Transposable element in fish. *Nature (Lond)* 383:30
- Kondo M, Nagao E, Mitani H, Shima A (2001a) Differences in recombination frequencies during female and male meioses of the sex chromosomes of the medaka, *Oryzias latipes*. *Genet Res* 78:23–30
- Kondo S, Kuwahara Y, Kondo M, Naruse K, Mitani H, Wakamatsu Y, Ozato K, Asakawa S, Shimizu N, Shima A (2001b) The medaka rs-3 locus required for scale development encodes ectodysplasin-A receptor. *Curr Biol* 11:1202–1206

- Maruyama K, Sugano S (1994) Oligo-capping: a simple method to replace the cap structure of eukaryotic messenger-RNAs with oligoribonucleotides. *Gene (Amst)* 138:171–174
- Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Morrey CE, Shibata N, Asakawa S, Shimizu N, Hori H, Hamaguchi S, Sakaizumi M (2002) DMY is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature (Lond)* 417:559–563
- Miya M, Takeshima H, Endo H, Ishiguro NB, Inoue JG, Mukai T, Satoh TP, Yamaguchi M, Kawaguchi A, Mabuchi K, Shirai SM, Nishida M (2003) Major patterns of higher teleostean phylogenies: a new perspective based on 100 complete mitochondrial DNA sequences. *Mol Phylogenet Evol* 26:121–138
- Morgan TH (1910) Sex limited inheritance in *Drosophila*. *Science* 22:120–122
- Murphy WJ, Eizirik E, O'Brien SJ, Madsen O, Scally M, Douady CJ, Teeling E, Ryder OA, Stanhope MJ, de Jong WW, Springer MS (2001) Resolution of the early placental mammal radiation using Bayesian phylogenetics. *Science* 294:2348–2351
- Nakamura S, Saito D, Tanaka M (2008) Generation of transgenic medaka using modified bacterial artificial chromosome. *Dev Growth Differ* 50:415–419
- Nanda I, Kondo M, Hornung U, Asakawa S, Winkler C, Shimizu A, Shan Z, Haaf T, Shimizu N, Shima A, Schmid M, Scharl M (2002) A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. *Proc Natl Acad Sci USA* 99:11778–11783
- Naruse K, Fukamachi S, Mitani H, Kondo M, Matsuoka T, Kondo S, Hanamura N, Morita Y, Hasegawa K, Nishigaki R, Shimada A, Wada H, Kusakabe T, Suzuki N, Kinoshita M, Kanamori A, Terado T, Kimura H, Nonaka M, Shima A (2000) A detailed linkage map of medaka, *Oryzias latipes*: comparative genomics and genome evolution. *Genetics* 154:1773–1784
- Naruse K, Hori H, Shimizu N, Kohara Y, Takeda H (2004a) Medaka genomics: a bridge between mutant phenotype and gene function. *Mech Dev* 121:619–628
- Naruse K, Tanaka M, Mita K, Shima A, Postlethwait J, Mitani H (2004b) A medaka gene map: the trace of ancestral vertebrate proto-chromosomes revealed by comparative gene mapping. *Genome Res* 14:820–828
- Ohtsuka M, Makino S, Yoda K, Wada H, Naruse K, Mitani H, Shima A, Ozato K, Kimura M, Inoko H (1999) Construction of a linkage map of the medaka (*Oryzias latipes*) and mapping of the Da mutant locus defective in dorsoventral patterning. *Genome Res* 9:1277–1287
- Ojima Y, Hitotsumachi S (1969) The karyotype of the medaka, *Oryzias latipes*. *Chrom Inf Serv* 10:15–16
- Rexroad CE, Palti Y, Gahr SA, Vallejo RL (2008) A second generation genetic map for rainbow trout (*Oncorhynchus mykiss*). *BMC Genet* 9:74
- Schmidt J (1920) Racial investigations. IV. The genetic behavior of a secondary sexual character. *C R Lab Trav Carlsberg* 14:1–12
- Takehana Y, Nagai N, Matsuda M, Tsuchiya K, Sakaizumi M (2003) Geographic variation and diversity of the cytochrome b gene in Japanese wild populations of medaka, *Oryzias latipes*. *Zool Sci* 20:1279–1291
- Tomita H (1982) Gene analysis in the medaka (*Oryzias latipes*). *Fish Biol J Medaka* 1:7–9
- Tomita H (1985) Study on the mutant of the medaka *co* and *di*. *Fish Biol J Medaka* 3:5–16
- Tomita H (1993) A study on the mutant pectoral-finless, *pl*, of medaka *Oryzias latipes*. *Fish Biol J Medaka* 5:31–32
- Toyama K (1916) Some examples of the Mendelian characters. *Nihon Ikushugaku Kaiho* 1:1–9
- Vos P, Hogers R, Bleeker M, Reijans M, Vandelee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA-fingerprinting. *Nucleic Acids Res* 23:4407–4414
- Wada H, Naruse K, Shimada A, Shima A (1995) Genetic-linkage map of a fish, the Japanese medaka *Oryzias latipes*. *Mol Mar Biol Biotechnol* 4:269–274
- Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res* 18:7213–7218

- Winge O (1922) One-sided masculine and sex-linked inheritance in *Lebistes reticulatus*. J Genet 12:145–162
- Yamamoto T (1951) Artificial sex-reversal in the genotypic males of the medaka, *Oryzias latipes*. Jpn J Genet 26:245
- Yamamoto T (1953) Artificially induced sex-reversal in genotypic males of the medaka (*Oryzias latipes*). J Exp Zool 123:571–594
- Yamamoto T (1958) Artificial induction of functional sex-reversal in genotypic females of the medaka (*Oryzias latipes*). J Exp Zool 137:227–264
- Yamamoto T (1964) Linkage map of sex chromosomes in the medaka, *Oryzias latipes*. Genetics 50:59–64
- Yamamoto T, Oikawa T (1973) Linkage between albino (i) and color interfere (ci) in the medaka *Oryzias latipes*. Jpn J Genet 48:315–329
- Yamanoue Y, Miya M, Matsuura K, Yagishita N, Mabuchi K, Sakai H, Katoh M, Nishida M (2007) Phylogenetic position of tetraodontiform fishes within the higher teleosts: Bayesian inferences based on 44 whole mitochondrial genome sequences. Mol Phylogenet Evol 45:89–101

Chapter 3

Chromatin-Associated Periodicity in Genetic Variation Downstream of Transcriptional Start Sites*

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Abstract Might DNA sequence variation reflect germline genetic activity and underlying chromatin structure? We investigated this question using the medaka (Japanese killifish, *Oryzias latipes*) by comparing the genomic sequences of two strains (Hd-rR and HNI) and by mapping approximately 37.3 million nucleosome

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cores from Hd-rR blastulae and 11,654 representative transcription start sites from six embryonic stages. We observed a distinctive ~200-base pair (bp) periodic pattern of genetic variation downstream of transcription start sites; the rate of insertions and deletions longer than 1 bp peaked at positions of approximately +200, +400, and +600 bp, whereas the point mutation rate showed corresponding valleys. This ~200-bp periodicity was correlated with the chromatin structure, with nucleosome occupancy minimized at positions 0, +200, +400, and +600 bp. These data exemplify the potential for genetic activity (transcription) and chromatin structure to contribute to molding the DNA sequence on an evolutionary time scale.

Mutation and repair characteristics of DNA sequence in experimental systems have been shown in a number of cases to reflect structures in chromatin. For one well-studied experimental system, ultraviolet-irradiated yeast (*Saccharomyces cerevisiae*), repair rates for a set of DNA nucleosome core regions are lower than in the surrounding linker regions (Tijsterman et al. 1999; Svejstrup 2002; Wellinger and Thoma 1997; Suter et al. 1997). Correlations between chromatin structure and mutation rates have also been suggested in analysis of human and yeast genomes (Higasa and Hayashi 2006; Prendergast et al. 2007; Washietl et al. 2008). The draft genome sequences of two inbred medaka strains, Hd-rR and HNI (Kasahara et al. 2007), provide a remarkable opportunity for extensive comparison between genomic variation and structural features in the genome. The two strains are cross-fertile, yet their genomes are substantially different [~3.42% single nucleotide polymorphisms (SNP)] (Kasahara et al. 2007). For analysis of chromatin and transcriptional effects on genetic variation, tissue samples including totipotent (germline tissue) would be most relevant, as mutational events in the germline would uniquely contribute to shaping the genome over evolutionary time (Ozsolak et al. 2007; Whitehouse et al. 2007; Schones et al. 2008).

To characterize transcriptional activity patterns from the medaka genome at embryonic stages, we collected 25-nucleotide (nt) 5'-end mRNA tags for 1-, 2-, 3-, 5-, 10-, and 14-day-old Hd-rR medaka embryos. Among a total of ~38.5 million 5'-end tags collected, ~26.2 million (68.14%) were successfully aligned to unique positions in the medaka genome. Starting with a rough assumption that one cell contains ~300,000 mRNA molecules (Velculescu et al. 1999), single-copy-per-cell RNAs would be represented by ~100 of the ~26.2 million tags. To define a set of active transcription start sites (TSSs), we used a clustering algorithm yielding 11,654 clusters of ≥ 100 tags. More than 98.4% of neighboring clusters were separated by >100 base pairs (bp) from their nearest neighbor. A reference TSS for each cluster was defined as the position with the most 5'-end tags.

The substitution and indel rates within 1,000 bp of the reference TSSs in the 11,654 TSS clusters tend to reach a valley at the TSSs (Fig. 3.1a), suggesting relative selective constraint within promoters. This finding is consistent with reports of high conservation around TSS regions in mammals (Taylor et al. 2006). Our analysis in medaka uncovers an additional pattern: the substitution rate (see blue line) showed peaks at +100 and +300 bp and valleys at +200 and +400 bp around the

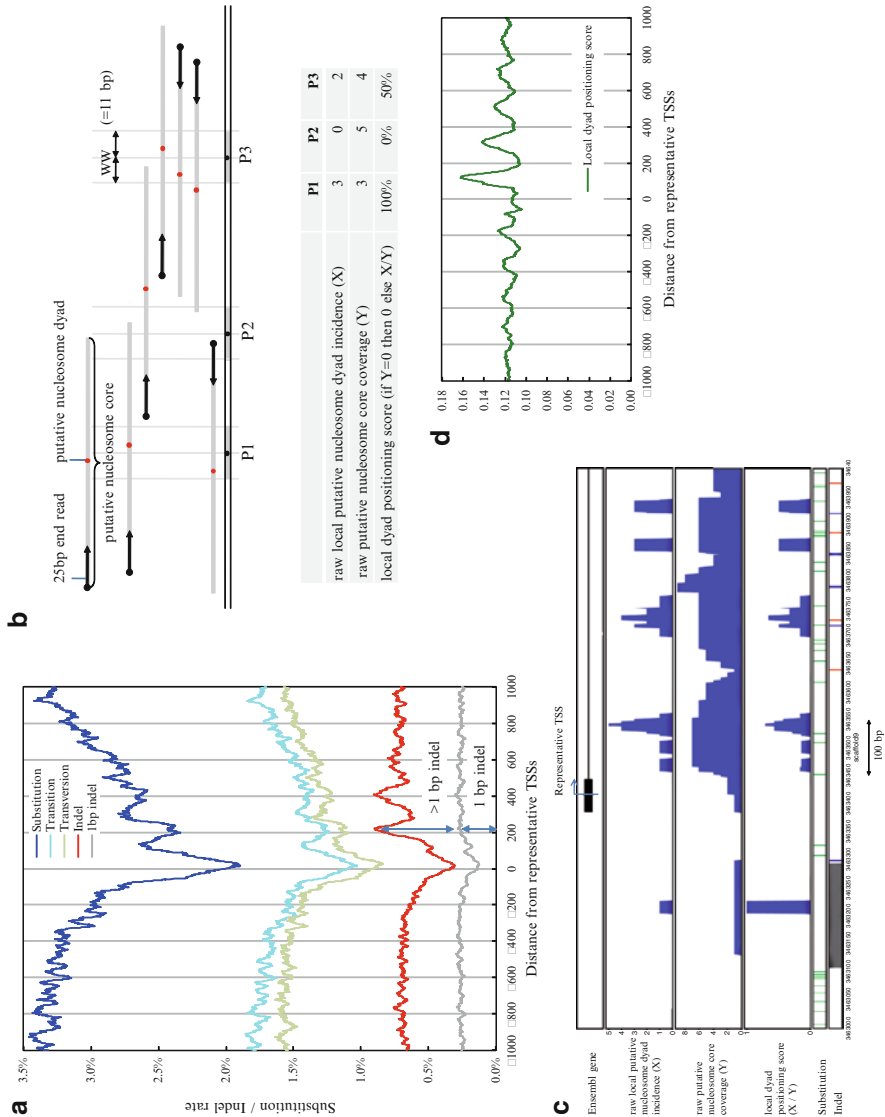
TSSs (the same pattern was also seen in the transition and transversion rates). The indel rate (Fig. 3.1a) was minimal at the TSSs and maximal at +200 bp; additional peaks were evident at +400 and +600 bp. These peaks define regions where indel mutation rates were significantly greater than the average rate (0.59%) for the entire genome, with the signal weakening with increasing distance from TSSs. The indel dataset was then split into a “1-bp” category (37.46%) and the remaining “>1-bp” category of indels. The peaks at +200, +400, and +600 bp are generated by the increase in the >1-bp category, whereas the 1-bp indel rate does not yield an evident periodicity (see Fig. 3.1a). Comparisons of genetic variation to TSSs were possible in human to chimpanzee or mouse to rat, although not limited to germline or embryo TSSs (Sasaki et al. 2009). A limited periodicity in substitution rates may be present for these genomes, albeit much smaller in magnitude than that observed with the early transcriptome TSS data from medaka.

The ~200-bp periodicity of the substitution and indel rates in medaka suggested the involvement of nucleosome structure. We isolated mononucleosome core DNAs from micrococcal nuclease-digested chromatin from blastulae [0.5-day-old embryos that maintain germline character in some (or all) cells (Hong et al. 1998)] and sequenced 67 million DNA ends to 36 bp (Valouev et al. 2008). The first 25 bp were sufficient to map 37.3 million ends (55.7% of sequenced reads) to unique locations in the medaka genome.

The distribution of distances between nucleosome start and end reads presents a significant peak at ~147 bp, coincident with the size of nucleosome cores and indicative of some degree of constraint in nucleosome positioning (Sasaki et al. 2009). To assess nucleosome spacing intervals, we analyzed the distribution of distances between start positions of mapped nucleosome ends (Johnson et al. 2006) (Valouev et al. 2008). We observed a small peak at 165 bp, which indicated that adjacent nucleosomes in regions with conserved positioning are likely to be located at approximately 165-bp intervals (~18-bp linker), while a distinctive ~200-bp spacing (~50-bp linker) was seen downstream of TSSs (see below).

Our metric for nucleosome position at individual sites in the genome (see Fig. 3.1b) counts the number of putative nucleosome dyads in a 23-bp “sliding window” and divides this by the total number of nucleosomes impinging on this window to obtain a localized dyad positioning score (Fig. 3.1b). The 23-bp window (± 1 helical turn) is used to accommodate observed variability in nuclease cleavage around nucleosome termini (Sasaki et al. 2009; Valouev et al. 2008).

The distribution of nucleosome dyad indicators, substitutions, and indels around several TSS sites is shown in Fig. 3.1c. For global analysis, positioning scores (X/Y) were taken into account only in areas covered by multiple nucleosome reads (87.1% of genomic positions; the remaining 12.9% correspond in part to repetitive sequences that occupy 17.5% of the medaka genome) (Kasahara et al. 2007). In unique regions supported with multiple nucleosome core coverage, putative nucleosome dyads that occur reproducibly in a defined neighborhood allow us to define positioned nucleosomes. The average local dyad positioning score has local minima at positions +200, +400, +600, and +800 bp from the TSSs (see Fig. 3.1d), which suggests the presence of phased arrays of nucleosomes every



~200 bp downstream of the TSS (Ozsolak et al. 2007; Whitehouse et al. 2007; Schones et al. 2008; Ioshikhes et al. 2006; Albert et al. 2007; Lee et al. 2007; Mavrich et al. 2008).

By contrast to the decreased substitution rate in nucleosome linker regions, the indel rate for medaka had peaks at positions +200, +400, and +600 bp from the TSSs, which implied that indels of length >1 bp are more likely to occur at DNA linker regions. One possible explanation is that DNA linker regions have more indel mutations than the rest of the genome; this idea is supported by the higher indel rate on a genome-wide scale (not limited to TSS regions) in the DNA linkers in regions occupied by positioned nucleosomes (Fig. 3.2). One might wonder if the substitution rate increases toward the positioned dyads in nonpromoter regions; however, this tendency was not observed (Fig. 3.2a). These observations suggest an interplay of transcription and nucleosome positioning in determining susceptibility to substitutions and indel mutations.

Transcription-coupled DNA repair (TCR), a mechanism that protects transcribed regions from mutations, may contribute to the observed sequence effects (Svejstrup 2002; Francino et al. 1996; Green et al. 2003; Polak and Arndt 2008). TCR is thought to work simultaneously with mRNA transcription involving RNA polymerases I and II, resulting in an asymmetrical effect with an overabundance of G+T over A+C downstream from the TSSs (through an excess of C-to-T mutations over G-to-A mutations) (Francino et al. 1996; Green et al. 2003). A significant asymmetry of the base composition is found in examining natural variation in the medaka genome at TSSs (Fig. 3.3a). Examining reciprocity in frequencies of the 12 possible base substitutions in 319 transcribed loci (121.1 kbp, in total; regions where ancestry could be inferred by comparison with sequence data from an out-group species), only the C-to-T versus G-to-A in the transcribed regions downstream of TSSs showed a significant strand bias (Fig. 3.3b) ($P=0.044$). This result is consistent with TCR as one of the factors contributing to the character of natural sequence variation in these regions.

Several possible causal and structural relationships may link sequence composition to mutagenesis rates and nucleosome positioning around TSSs. One rather simple explanation for the remarkable periodicity in mutation rates might have been an underlying bias in sequence composition in nucleosome core regions that favored certain types of mutations, whereas distinct sequence composition in linkers would favor other types of mutations. We addressed this possibility by examining sequence composition in general and around sites of genetic variation as a function of positioning relative to nucleosomes and TSSs (Sasaki et al. 2009). This analysis

Fig. 3.1 Diversity rates and nucleosome positions around transcriptional start sites (TSSs). **a** The *x*-axis shows the distance from the representative TSSs in the medaka (Hd-rR) genome. For smoothing of lines, a running average over a 23-bp window (one full turn of the helix in each direction) is depicted. **b Top:** Putative nucleosome dyads (73 bp from start of sequence read) and cores (147 bp). **Bottom:** The distinct meanings of the three nucleosome indicators. **c** Distribution of nucleosomes, substitutions, and indels surrounding a TSS. **d** The average local dyad positioning score

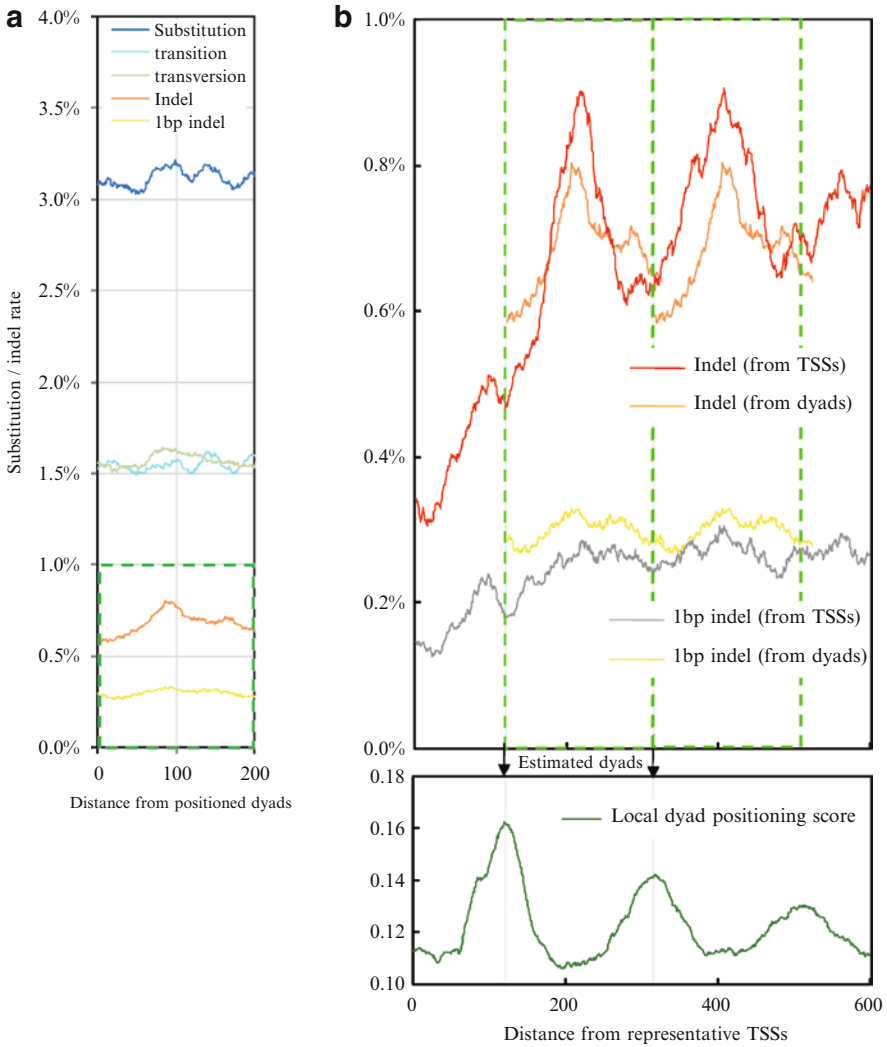


Fig. 3.2 Mutational spectra at positions around 8,181 positioned dyads that are isolated from their neighboring dyads by >165 bp and are covered by an average of 5.44 putative nucleosome cores on a genome-wide scale (excluding TSSs and coding regions). **a** In nonpromoter regions, where transcription does not occur, the two locations in the distinct strands are positionally equivalent in a nucleosome core if they are the same distance from the dyad. The *x*-axis presents the distance. **b** An expanded view of the indel rates enclosed in the *square* in **a** is duplicated in tandem, and the two copies are overlaid for comparison with equivalent measurements relative to TSSs in Fig. 3.1a. *Bottom:* The estimated dyads (arrows) aligned with dyad positioning score near TSSs (expanded from Fig. 3.1d)

gave no indication that differential mutagenesis could be accounted for by an initial sequence bias. A second intriguing possibility is that mutagenesis rates are influenced toward periodicity, not by the structural constraints of the chromatin template, but by functional constraints related to overall organismal fitness. Thus, for

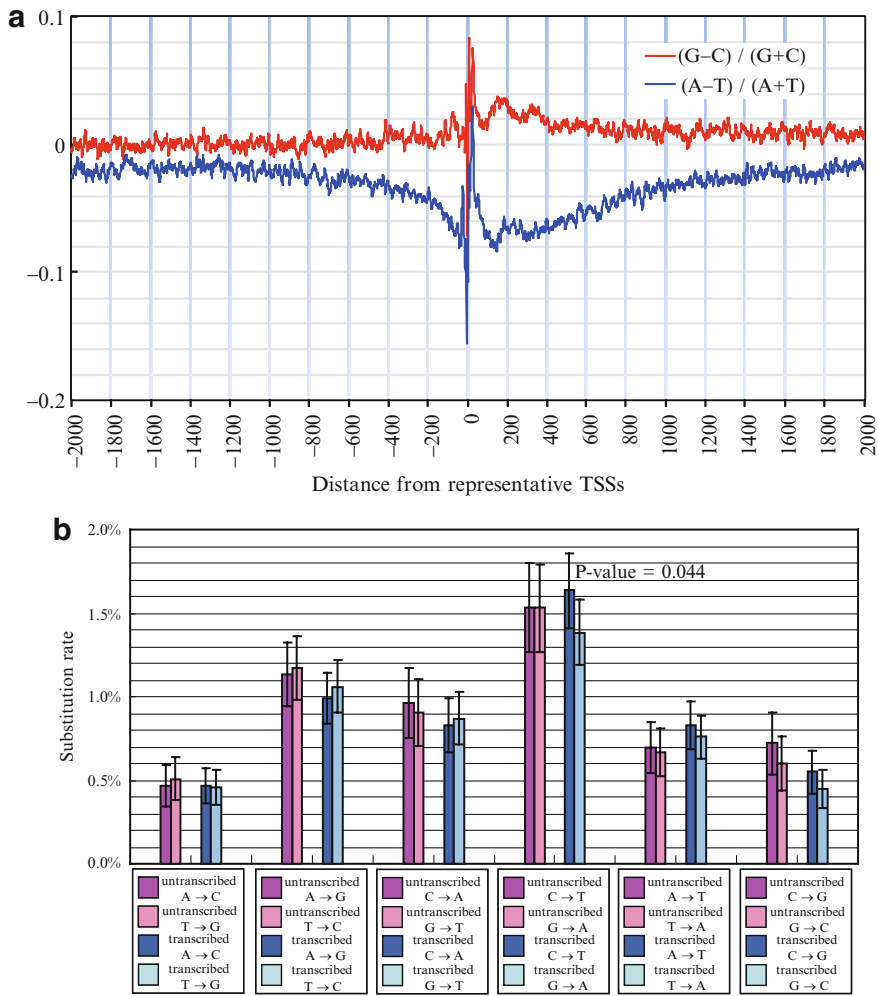


Fig. 3.3 **a** Base composition surrounding TSSs. **b** Substitution rates around TSSs. Rates for each substitution and its complement and their 95% confidence intervals are indicated side by side for untranscribed and transcribed regions that are upstream and downstream of TSSs, respectively

example, it would be conceivable that substitutions might be underrepresented in a critical set of linker sequences that are essential in maintaining specific transcription complexes and nucleosome-based structures downstream of TSSs. We do not favor this explanation for the medaka data, given that indel mutations show an opposite distribution, occurring more frequently in the linker regions. Instead, the biases in genetic variation seem most likely to represent structural constraints of the chromatin template during the mutagenic processes that medaka has encountered during evolutionary time. The mechanistic points at which nucleosomes may have influenced mutagenesis and/or repair processes in medaka evolution are (by definition) not known. The ability of nucleosomes in model assay systems to block repair of

certain DNA lesions (Wellinger and Thoma 1997) certainly provides a precedent for the observed higher substitution rates in core regions. The complementary pattern of indels in medaka could reflect any of several conceivable linker and/or core differences (e.g., higher susceptibility of cores to breakage or less precise break repair in linkers).

For any species, the balance of specific mutagenic and repair processes occurring over history would have shaped the genome in potentially unique ways; thus, not all genomes would be expected to show a qualitatively or quantitatively equivalent “shadow” of germline chromatin structure. Our working model for the basis of structural variation between the genomes of these two inbred medaka strains is that chromatin structure influences mutagenesis, which in turn influences genetic variation, to provide the observed periodic pattern near the 5'-ends of germline-transcribed genomic segments. We expect the influence of chromatin structure to be a general feature of sequence evolution throughout the genome and the biosphere.

References

- Albert I, Mavrich T et al (2007) Translational and rotational settings of H2A.Z nucleosomes across the *Saccharomyces cerevisiae* genome. *Nature (Lond)* 446(7135):572–576
- Francino MP, Chao L et al (1996) Asymmetries generated by transcription-coupled repair in enterobacterial genes. *Science* 272(5258):107–109
- Green P, Ewing B et al (2003) Transcription-associated mutational asymmetry in mammalian evolution. *Nat Genet* 33(4):514–517
- Higasa K, Hayashi K (2006) Periodicity of SNP distribution around transcription start sites. *BMC Genomics* 7:66
- Hong Y, Winkler C et al (1998) Production of medakafish chimeras from a stable embryonic stem cell line. *Proc Natl Acad Sci USA* 95(7):3679–3684
- Ioshikhes I, Albert I et al (2006) Nucleosome positions predicted through comparative genomics. *Nat Genet* 38(10):1210–1215
- Johnson S, Tan F et al (2006) Flexibility and constraint in the nucleosome core landscape of *Caenorhabditis elegans* chromatin. *Genome Res* 16(12):1505–1516
- Kasahara M, Naruse K et al (2007) The medaka draft genome and insights into vertebrate genome evolution. *Nature (Lond)* 447(7145):714–719
- Lee W, Tillo D et al (2007) A high-resolution atlas of nucleosome occupancy in yeast. *Nat Genet* 39(10):1235–1244
- Mavrich T, Jiang C et al (2008) Nucleosome organization in the *Drosophila* genome. *Nature (Lond)* 453(7193):358–362
- Ozsolak F, Song J et al (2007) High-throughput mapping of the chromatin structure of human promoters. *Nat Biotechnol* 25(2):244–248
- Polak P, Arndt P (2008) Transcription induces strand-specific mutations at the 5'-end of human genes. *Genome Res* 18(8):1216–1223
- Prendergast J, Campbell H et al (2007) Chromatin structure and evolution in the human genome. *BMC Evol Biol* 7:72
- Sasaki S, Mello CC et al (2009) Chromatin-associated periodicity in genetic variation downstream of transcriptional start sites. *Science* 323(5912):401–404
- Schones D, Cui K et al (2008) Dynamic regulation of nucleosome positioning in the human genome. *Cell* 132(5):887–898
- Suter B, Livingstone-Zatchej M et al (1997) Chromatin structure modulates DNA repair by photolyase in vivo. *EMBO J* 16(8):2150–2160

- Svejstrup J (2002) Mechanisms of transcription-coupled DNA repair. *Nat Rev Mol Cell Biol* 3(1):21–29
- Taylor M, Kai C et al (2006) Heterotachy in mammalian promoter evolution. *PLoS Genet* 2(4):e30
- Tijsterman M, de Pril R et al (1999) RNA polymerase II transcription suppresses nucleosomal modulation of UV-induced (6–4) photoproduct and cyclobutane pyrimidine dimer repair in yeast. *Mol Cell Biol* 19(1):934–940
- Valouev A, Ichikawa J et al (2008) A high-resolution, nucleosome position map of *C. elegans* reveals a lack of universal sequence-dictated positioning. *Genome Res* 18(7):1051–1063
- Velculescu V, Madden S et al (1999) Analysis of human transcriptomes. *Nat Genet* 23(4):387–388
- Washietl S, Machne R et al (2008) Evolutionary footprints of nucleosome positions in yeast. *Trends Genet* 24(12):583–587
- Wellinger R, Thoma F (1997) Nucleosome structure and positioning modulate nucleotide excision repair in the non-transcribed strand of an active gene. *EMBO J* 16(16):5046–5056
- Whitehouse I, Rando O et al (2007) Chromatin remodelling at promoters suppresses antisense transcription. *Nature (Lond)* 450(7172):1031–1035

Chapter 4

Transposable Elements *Tol1* and *Tol2*

Akihiko Koga

Abstract Medaka harbors active DNA-based transposable elements in its genome. This feature is unique among vertebrates and has enabled a wide range of studies on DNA-based elements, specifically on such topics as their transposition mechanisms, their population dynamics, and their contribution to genome evolution as natural mutagens. Findings of particular significance include the rapid expansion of an element in the medaka genome, the elevation of the mutation rate of host genes, and the disappearance of elements after they have functioned as mutagens. In addition to these contributions to basic biology, active DNA-based elements from medaka have also contributed to the development of tools for genetic manipulations, such as gene transfer, mutagenesis, gene tagging, and promoter/enhancer trapping. These tools are now used in a wide range of vertebrates, including humans, mice, and zebrafish.

4.1 Transposable Elements

Transposable elements are repetitive sequences that are, or were at some time in the past, capable of moving from one chromosomal location to another. DNA-based transposable elements form one major group of transposable elements. They are transposed directly from DNA to DNA mostly in a cut-and-paste fashion, in contrast to RNA-mediated elements, which are copied into RNA before their reverse transcription products are inserted into the chromosomes. DNA-based elements are present in the genomes of plants, animals, and fungi as well as in prokaryote genomes, but the DNA-based elements in vertebrates are exceptional in that they are inactivated. The transposition reaction requires some molecular factors, in some or all of which the vertebrate DNA-based elements are deficient. An exception to

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this exception is medaka. *Tol1* and *Tol2* are the two known active elements residing in the medaka genome; each has all factors necessary for transposition. “*Tol*” stands for “transposable element of *Oryzias latipes*,” and the numbers indicate the order of identification.

4.2 Identification

Before the advent of genome sequence databases, new transposable elements were most frequently encountered as extra sequences present in mutant genes of host organisms, especially genes controlling body color. The *Activator* element of maize, for example, was identified as an insertion sequence in the *waxy* gene of a mutant strain exhibiting mosaic kernel color (Fedoroff et al. 1983). The *P* element of *Drosophila* was an insertion in the *white* locus of a white-eyed mutant, wild-type flies having red eyes (Rubin et al. 1982). The identification of *Tol1* and *Tol2* occurred in a similar manner. Among Hideo Tomita’s collection of spontaneous mutant medaka, there were several mutants with albino phenotypes resulting from absence of or reduction in the black pigment melanin (Tomita 1975). It was known that albinism in humans and mice is often caused by deficiency in the tyrosinase protein (Yokoyama et al. 1990). This enzyme mediates the initial steps of melanin synthesis by oxidizing the amino acid tyrosine. Hiroshi Hori and his research group at Nagoya University first cloned the wild-type tyrosinase gene of medaka (Inagaki et al. 1994) and then analyzed the structure of this gene in the albino mutants. In the process, they found *Tol1* as an extra DNA fragment in the tyrosinase gene of a complete albino mutant (Koga et al. 1995), and *Tol2* in another mutant of a quasi-albino phenotype (Koga et al. 1996). *Tol2* was also found in the same gene of a third mutant exhibiting a weak albino phenotype (Iida et al. 2004). Figure 4.1 shows the locations of these insertions.

4.3 Structure

A DNA-based transposable element can be regarded as an enzyme–substrate system. The enzyme is called a transposase, and a gene coding for the enzyme is carried by the element itself. The substrate for the transposase enzyme is again the element itself. The transposase recognizes the element, excises it from the DNA molecule in which it resides, cuts DNA at another location, and then inserts the element there.

The nucleotide sequence recognized by the transposase is not that of the entire element but only those of the terminal regions. Thus, even if the transposase gene of an element decays or is deleted, the element can be transposed as long as it retains its terminal regions and the transposase is supplied by another element present in the same cell. Such a deficient copy of the element is called a nonautonomous copy, whereas one carrying an intact transposase gene is called an autonomous copy.

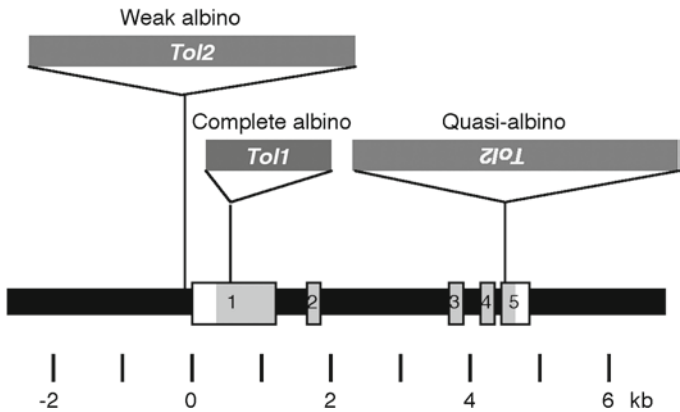


Fig. 4.1 Copies of *Tol1* and *Tol2* identified as insertion sequences in the tyrosinase gene. The scale is in kilobase pairs, 0 being assigned to the major transcription initiation site of the tyrosinase gene. Exons are shown as boxes with numerals, in which the coding regions are *lightly shaded*. The *heavily shaded boxes* toward the top of the figure are transposable elements. The names of the elements are shown *inside*, their orientation being that of the transcription of their transposase gene. The phenotypes of mutants caused by the respective insertions are described *above* the elements. For the actual colors of these mutant fish, see Koga and Hori (1997, 2001) and Iida et al. (2005)

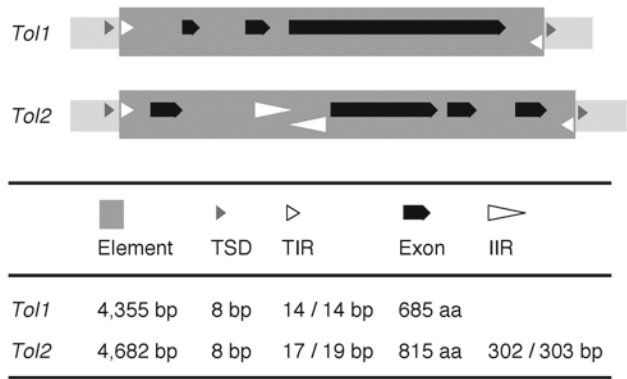


Fig. 4.2 Structure of autonomous copies of *Tol1* and *Tol2*. Locations of their components are illustrated and sizes are tabulated. *TSD*, target site duplication; *TIR*, terminal inverted repeat; *IIR*, internal inverted repeat

Figure 4.2 illustrates the structure of autonomous copies of *Tol1* and *Tol2*. Both elements belong to the *hAT* transposable element family (Koga et al. 1999, 2007a), which is one major group of DNA-based elements (Calvi et al. 1991). The name of this family is an acronym standing for three elements found in the early period of transposable element research: *hobo* of *Drosophila*, *Activator* of maize, and *Tam3* of snapdragon.

4.4 Independent Systems

The two elements are independent of each other as transposase–substrate systems, which was demonstrated by assaying transposition in four combinations: (1) *Tol1* transposase and nonautonomous *Tol1* copy; (2) *Tol1* transposase and nonautonomous *Tol2* copy; (3) *Tol2* transposase and nonautonomous *Tol1* copy; and (4) *Tol2* transposase and nonautonomous *Tol2* copy. Transposition was observed in cases (1) and (4) but not in cases (2) and (3). Thus, the respective transposases have specificity for the nucleotide sequences of their own elements (Koga et al. 2008).

4.5 Contribution to Genetic Variation

Genetic variation is a prerequisite for evolution, and DNA-based elements are thought to be a major factor supplying variation to the genomes of their host organisms. Actually, three new mutations have been caused by insertions of *Tol1* and *Tol2*, as shown in Fig. 4.1. An important fact is that these were spontaneous mutations: Tomita (1975) identified the mutants, but the mutations had occurred earlier under natural conditions.

Genetic variation is also provided by the excision of *Tol1* and *Tol2*. As is often the case with DNA-based elements, excision of *Tol1* and *Tol2* is not always precise. Figure 4.3 shows examples of nucleotide sequences at and around excision sites of *Tol1*, some of which indicate precise excisions whereas others indicate imprecise excisions. Most imprecise excisions result from parts of the target site duplications being left over. It was also observed that nucleotides at a terminus of the element were sometimes left over, and that the chromosome regions were sometimes truncated beyond the target site duplication. It is noteworthy that various new mutations leading to different phenotypes were generated from a single insertion mutation (Koga et al. 2006).

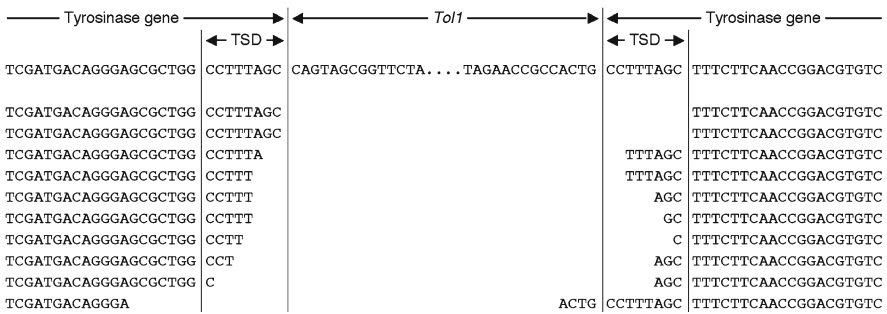


Fig. 4.3 Nucleotide sequences of the region around the *Tol1* insertion point. The *top line* is the sequence of the *Tol1*-carrying tyrosinase gene. *Other lines* are sequences of excision products from individual animals. *Blank spaces* indicate the absence of corresponding nucleotides. *TSD*, target site duplication

4.6 Transposition Burst

In some DNA-based elements of insects and plants, there are reports of sudden increases in transposition frequency. Such a phenomenon is called a transposition burst, and it is often accompanied by an elevation of the mutation rate of host genes. A well-known example is the transposition burst of the *Drosophila P* element that was associated with extraordinarily high mutation rates of various genes (Bingham et al. 1982). In some cases, the effects were so strong as to cause sterility or lethality in *P*-element-carrying animals. Genetic analyses revealed that a transposition burst of the *P* element occurs when an autonomous *P* copy is delivered by a sperm into the cytoplasm of an egg that does not have a repressor protein.

A transposition burst involving the medaka *Tol2* element has been observed, but its underlying mechanisms are not yet clear (Koga et al. 2006). In this case, the phenotypic mutation rate at the tyrosinase gene represented a 1,000-fold increase over mutation rates not associated with transposable elements. Numerous simultaneous insertions were observed throughout the genome.

4.7 Genomic Organization

Tol1 is present as hundreds of copies per haploid medaka genome; most of these, probably more than 99%, are nonautonomous copies (Koga et al. 1995, 2007a). Internal deletion is the primary cause of the changes from autonomy to nonautonomy, as is the case for the maize *Activator* element (Fedoroff et al. 1983) and the *Drosophila P* element (Nitasaka and Yamazaki 1994). The deleted regions differ from copy to copy, causing heterogeneity in the element length (Koga et al. 2007a).

The copy number of *Tol2* is as small as can be accurately estimated using a genomic Southern blot analysis. In an assay with 12 samples collected from natural populations, the minimum number of bands in a lane was 11 and the maximum was 30 (Koga and Hori 1999). Another assay with 12 different samples showed a minimum of 10 and a maximum of 25 (Koga et al. 2000). These data, along with some other unpublished data, yielded an average copy number of 18.2. Because most copies in natural populations can be thought to be in a hemizygous state (Koga and Hori 1999), an average medaka fish contains about 9 copies of *Tol2* per haploid genome. Some laboratory strains were also examined for copy number: the HNI, Hd-rR, and HO5 strains have 11, 13, and 13 copies per haploid genome, respectively (unpublished results).

In contrast to that of *Tol1*, the structure of *Tol2* is highly homogeneous among copies. More than 400 copies were examined for length using Southern blot and polymerase chain reaction (PCR) analyses, and all were revealed to be 4.7 kb in length. Eight copies were cloned and sequenced, and no sequence variation was found except for a variation in the number of consecutive T residues present in the first exon (Koga and Hori 1999; Koga et al. 2000). These data suggest that virtually all copies of *Tol2* are autonomous.

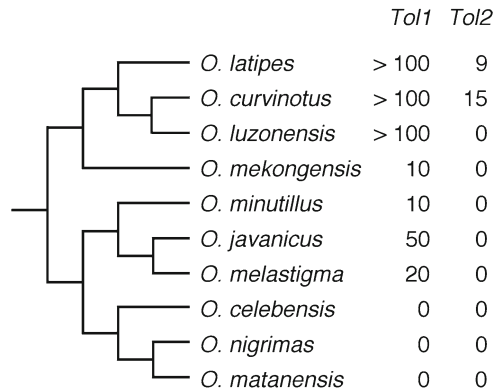


Fig. 4.4 Copy numbers of *Tol1* and *Tol2* in ten species in the genus *Oryzias* (*O.*). The phylogenetic tree was redrawn with data from Naruse (1996) and Takehana et al. (2005). The copy numbers of *Tol1* and *Tol2* are the roughly estimated numbers per haploid genome; they were obtained by counting hybridization bands on autodiagrams of genomic Southern blots (Koga et al. 2000, 2002b; unpublished data are also included)

4.8 Distribution Among Species

The genus *Oryzias* contains about 20 known species (Naruse 1996; Takehana et al. 2005). Ten of these species were examined by Southern blot analysis for the presence of *Tol1* and *Tol2* (Koga et al. 2000, 2002b). As Fig. 4.4 shows, *Tol1* is present in seven of the ten species. The three species that did not exhibit hybridization signals (*Oryzias celebensis*, *Oryzias matanensis*, and *Oryzias nigrimas*) all inhabit the island of Sulawesi (Celebes). *Tol1* might have been lost in the common ancestor of these fishes. *Tol2* is present in two of the ten species (*O. latipes* and *Oryzias curvinotus*), which are relatively closely related even within the genus.

4.9 Possibility of Horizontal Transfer

Medaka inhabits a large area of East Asia, including Japan, Korea, and China, and exhibits geographic variation (Sakaizumi et al. 1987). Even between individuals from Northern Japan and Southern Japan, there is nucleotide sequence variation throughout the genome. The average percent difference in nucleotides has been estimated at 3.42% (Kasahara et al. 2007). Among *Tol2* copies, however, there is virtually no sequence variation between fish from Northern Japan and those from Southern Japan (Koga et al. 2000). A plausible explanation for this fact is that *Tol2* is a newcomer to the medaka genome and that the time elapsed since its arrival has not been sufficiently long for variation to have accumulated. The presence of *Tol2* not only in *O. latipes* (medaka) but also in *O. curvinotus* allowed this explanation to be tested in an experiment measuring how much *O. curvinotus* *Tol2* differs from *O. latipes* *Tol2*.

A *Tol2* copy was cloned from *O. curvinotus* and sequenced. The result was surprising: except for the number of consecutive T's, its sequence was identical to that of *O. latipes Tol2* over the entire 4.7-kb-long element. Thus, *Tol2* was shown to be highly homogeneous, not only among individuals within species but also between different species in the genus (Koga et al. 2000). For comparison, a 2.0-kb-long region of the tyrosinase gene from each of the two species was also sequenced, revealing that more than 100 base substitutions have been accumulated in this region.

The most likely explanation for the near-total lack of sequence divergence between the two species is a recent *Tol2* invasion into one or both species. This explanation could involve either a horizontal transfer of *Tol2* from one species to the other or a transfer into the two species from a common source.

Recently, evidence for a simultaneous invasion of a DNA-based element into various tetrapod animals was reported (Pace et al. 2008). The element was named *Space Invader*, and the authors discuss the possibility that a virus with the ability to infect a wide range of species served as a vector for the element. Such an infection event might have occurred in the case of *Tol2*, as this could explain why *Tol2* is present in medaka throughout their entire geographic range with virtually no sequence variation.

4.10 Genetic Tools

DNA-based elements can be used as powerful tools for genetic manipulations. The simplest form of application is as transgenesis vectors. Two components should be prepared for this application, one being a donor and the other a helper. A donor is a transposable element copy that serves as a vehicle for a gene or other DNA fragment to be delivered into the chromosome. The most frequently used type of donor is plasmid DNA extracted from bacteria, but other sorts of DNAs, such as bacterial artificial chromosome (BAC)- or PCR-generated DNA, are also used. A helper is either a DNA clone of the transposase gene placed downstream of an appropriate promoter, or in vitro synthesized RNA with nucleotide sequence information that serves as an mRNA for the transposase.

Mutagenesis is a similar application that also uses a donor and a helper. When the element from a donor is inserted in or around a gene, and the inserted element causes a phenotype alteration, the resulting mutant can be identified by phenotype screening. A big advantage of mutagenesis performed with transposable elements over that performed with chemicals or radiation is that the responsible gene can be readily cloned because the inserted element serves as a tag for cloning. The nucleotide sequence of the inserted element is known, so its flanking chromosomal regions can be easily cloned by inverse PCR, thermal asymmetrical interlaced (TAIL)-PCR, hybridization, or other similar methods. Thus, the entire process, from inducing mutations to cloning genes, is called gene tagging.

This system can be used for promoter trapping and/or enhancer trapping by mounting a reporter gene that either lacks a promoter or contains a weak promoter into the element of the donor. A signal for a splicing acceptor site is often added to the reporter gene for the purpose of increasing the chance of successful expression of the inserted reporter gene.

DNA-based elements and RNA-mediated elements each have features that make them useful genetic manipulation tools. A point unique to the former is the ease of removing an integrated element from the chromosome. DNA-based elements are transposed mostly in a cut-and-paste manner, the “cut” aspect of the process being excision. Excision can be expected to occur when a helper is supplied to the transformants obtained by transgenesis and the mutants obtained by mutagenesis. A precise excision may indicate that the phenotypic change is caused solely by the insertion. Imprecise excisions, meanwhile, may be useful for generating different mutations of the same gene.

DNA-based elements widely used in vertebrates for these purposes include *piggyBac*, identified from the cabbage looper moth (Fraser et al. 1996), *Sleeping Beauty*, molecularly reconstructed and resurrected from salmonid fishes (Ivics et al. 1997), and *Tol1* and *Tol2*, naturally occurring as active elements in medaka. These are members of different transposable element families that have different characters in structure, dependences on species and cell types, insertion site preferences, etc. A big advantage of *Tol1* and *Tol2* is their large cargo capacity (Koga et al. 2007b). Another point to be mentioned is that these are similar elements but independent of each other. The availability of these two elements would make it possible to transfer two different genes sequentially or simultaneously into a single recipient. Subsequent removal of either gene would be achievable by using the appropriate transposase.

Many sophisticated systems have already been developed with *Tol1* and *Tol2*. Directions for use and results obtained are described in several papers (Kwan et al. 2007; Koga et al. 2007b, 2008; Villefranc et al. 2007; Korzh 2007; Kawakami 2007; Asakawa et al. 2008; Urasaki et al. 2008). The last article describes a simple method used in zebrafish, in which delivery of the donor and helper into eggs is not necessary, as these have already been integrated into the chromosomes. The transposase gene is silent under regular laboratory conditions, but can be switched on by a heat-shock treatment.

Tol1 and *Tol2* originate from medaka; for this reason, these elements are not efficient tools for use in medaka itself, unfortunately for medaka researchers. An average medaka fish carries hundreds of *Tol1* copies and nine *Tol2* copies per haploid genome. Transposase that is artificially supplied may mediate transposition of these endogenous copies, and this is likely to cause extra deleterious mutations in transgenesis experiments and to interfere with identification of mutant genes in mutagenesis experiments. One possible way to solve this problem is to establish a fish line that is free of these elements. This approach appears to be achievable, at least for *Tol2*, because its copy number is low and it is subject to spontaneous excision (unpublished data). A simple way to attain a medaka line without *Tol2* is to repeat, over generations, the selection of fish with lower copy numbers. An artificial supply of transposase will be useful for saving time in this process.

In contrast, the other species in the genus *Oryzias* appear to present no obstacles to the use of *Tol2* as a genetic tool, with the exception of *O. curvinotus*. In fact, the mobility of *Tol2* has already been demonstrated in *Oryzias luzonensis* (Koga and Hori 2000) and *O. melastigma* (Koga et al. 2002a).

References

- Asakawa K, Suster ML, Mizusawa K, Nagayoshi S, Kotani T, Urasaki A, Kishimoto Y, Hibi M, Kawakami K (2008) Genetic dissection of neural circuits by *Tol2* transposon-mediated Gal4 gene and enhancer trapping in zebrafish. *Proc Natl Acad Sci USA* 105:1255–1260
- Bingham PM, Kidwell MG, Rubin GM (1982) The molecular basis of P-M hybrid dysgenesis: the role of the P element, a P-strain-specific transposon family. *Cell* 29:995–1004
- Calvi BR, Hong TJ, Findley SD, Gelbart WM (1991) Evidence for a common evolutionary origin of inverted repeat transposons in *Drosophila* and plants: *hobo*, *activator*, and *Tam3*. *Cell* 66:465–471
- Fedoroff N, Wessler S, Shure M (1983) Isolation of the transposable maize controlling elements Ac and Ds. *Cell* 35:235–242
- Fraser MJ, Ciszczon T, Elick T, Bauser C (1996) Precise excision of TTAA-specific lepidopteran transposons *piggyBac* (IFP2) and *tagalong* (TFP3) from the baculovirus genome in cell lines from two species of Lepidoptera. *Insect Mol Biol* 5:141–151
- Iida A, Inagaki H, Suzuki M, Wakamatsu Y, Hori H, Koga A (2004) The tyrosinase gene of the *i^b* albino mutant of the medaka fish carries a transposable element insertion in the promoter region. *Pigment Cell Res* 17:158–164
- Iida A, Takamatsu N, Hori H, Wakamatsu Y, Shimada A, Shima A, Koga A (2005) Reversion mutation of *i^b* oculocutaneous albinism to wild-type pigmentation in medaka fish. *Pigment Cell Res* 18:382–384
- Inagaki H, Bessho Y, Koga A, Hori H (1994) Expression of the tyrosinase-encoding gene in a colorless melanophore mutant of the medaka fish, *Oryzias latipes*. *Gene (Amst)* 150:319–324
- Ivics Z, Hackett PB, Plasterk RH, Izsvak Z (1997) Molecular reconstruction of *Sleeping Beauty*, a *Tc1*-like transposon from fish, and its transposition in human cells. *Cell* 91:501–510
- Kasahara M, Naruse K, Sasaki S et al (2007) The medaka draft genome and insights into vertebrate genome evolution. *Nature (Lond)* 447:714–719
- Kawakami K (2007) *Tol2*: a versatile gene transfer vector in vertebrates. *Genome Biol* 8 suppl 1:S7
- Koga A, Hori H (1997) Albinism due to transposable element insertion in fish. *Pigment Cell Res* 10:377–381
- Koga A, Hori H (1999) Homogeneity in the structure of the medaka fish transposable element *Tol2*. *Genet Res* 73:7–14
- Koga A, Hori H (2000) Detection of *de novo* insertion of the medaka fish transposable element *Tol2*. *Genetics* 156:1243–1247
- Koga A, Hori H (2001) The *Tol2* transposable element of the medaka fish: an active DNA-based element naturally occurring in a vertebrate genome. *Genes Genet Syst* 76:1–8
- Koga A, Inagaki H, Bessho Y, Hori H (1995) Insertion of a novel transposable element in the tyrosinase gene is responsible for an albino mutation in the medaka fish, *Oryzias latipes*. *Mol Gen Genet* 249:400–405
- Koga A, Suzuki M, Inagaki H, Bessho Y, Hori H (1996) Transposable element in fish. *Nature (Lond)* 383:30
- Koga A, Suzuki M, Maruyama Y, Tsutsumi M, Hori H (1999) Amino acid sequence of a putative transposase protein of the medaka fish transposable element *Tol2* deduced from mRNA nucleotide sequences. *FEBS Lett* 461:295–298

- Koga A, Shimada A, Shima A, Sakaizumi M, Tachida H, Hori H (2000) Evidence for recent invasion of the medaka fish genome by the *Tol2* transposable element. *Genetics* 155:273–281
- Koga A, Hori H, Sakaizumi M (2002a) Gene transfer and cloning of flanking chromosomal regions using the medaka fish *Tol2* transposable element. *Mar Biotechnol* 4:6–11
- Koga A, Sakaizumi M, Hori H (2002b) Transposable elements in medaka fish. *Zool Sci* 19:1–6
- Koga A, Iida A, Hori H, Shimada A, Shima A (2006) Vertebrate DNA transposon as a natural mutator: the medaka fish *Tol2* element contributes to genetic variation without recognizable traces. *Mol Biol Evol* 23:1414–1419
- Koga A, Shimada A, Kuroki T, Hori H, Kusumi J, Kyono-Hamaguchi Y, Hamaguchi S (2007a) The *Toll* transposable element of the medaka fish moves in human and mouse cells. *J Hum Genet* 52:628–635
- Koga A, Higashide I, Hori H, Wakamatsu Y, Kyono-Hamaguchi Y, Hamaguchi S (2007b) The *Toll* element of medaka fish is transposed with only terminal regions and can deliver large DNA fragments into the chromosomes. *J Hum Genet* 52:1026–1030
- Koga A, Cheah FS, Hamaguchi S, Yeo GH, Chong SS (2008) Germline transgenesis of zebrafish using the medaka *Toll* transposon system. *Dev Dyn* 237:2466–2474
- Korzh V (2007) Transposons as tools for enhancer trap screens in vertebrates. *Genome Biol* 8 suppl 1:S8
- Kwan KM, Fujimoto E, Grabher C, Mangum BD, Hardy ME, Campbell DS, Parant JM, Yost HJ, Kanki JP, Chien CB (2007) The Tol2kit: a multisite gateway-based construction kit for *Tol2* transposon transgenesis constructs. *Dev Dyn* 236:3088–3099
- Naruse K (1996) Classification and phylogeny of fishes of the genus *Oryzias*. *Fish Biol J Medaka* 8:1–10
- Nitasaka E, Yamazaki T (1994) The relationship between DNA structural variation and activities of *P* elements in *P* and *Q* strains of *Drosophila melanogaster*. *Heredity* 73:608–615
- Pace JK 2nd, Gilbert C, Clark MS (2008) Repeated horizontal transfer of a DNA transposon in mammals and other tetrapods. *Proc Natl Acad Sci USA* 105:17023–17028
- Rubin GM, Kidwell MG, Bingham PM (1982) The molecular basis of P-M hybrid dysgenesis: the nature of induced mutations. *Cell* 29:987–994
- Sakaizumi M, Uwa H, Jeon S-R (1987) Genetic diversity of the East Asian populations of the freshwater fish, *Oryzias*. *Zool Sci* 4:1003
- Takehana Y, Naruse K, Sakaizumi M (2005) Molecular phylogeny of the medaka fishes genus *Oryzias* (Belontiiformes: Adrianichthyidae) based on nuclear and mitochondrial DNA sequences. *Mol Phylogenet Evol* 36:417–428
- Tomita H (1975) Mutant genes in the medaka. In: Yamamoto T (ed) *Medaka (killifish): biology and strains*. Yugenkusha, Tokyo, pp 251–272
- Urasaki A, Asakawa K, Kawakami K (2008) Efficient transposition of the *Tol2* transposable element from a single-copy donor in zebrafish. *Proc Natl Acad Sci USA* 105:19827–19832
- Villefranc JA, Amigo J, Lawson ND (2007) Gateway compatible vectors for analysis of gene function in the zebrafish. *Dev Dyn* 236:3077–3087
- Yokoyama T, Silversides DW, Waymire KG, Kwon BS, Takeuchi T, Overbeek PA (1990) Conserved cysteine to serine mutation in tyrosinase is responsible for the classical albino mutation in laboratory mice. *Nucleic Acids Res* 18:7293–7298

Chapter 5

A Systematic Screen for Mutations Affecting Organogenesis in Medaka

Makoto Furutani-Seiki

Abstract The use of a single species for mutagenesis screening will not be sufficient to uncover all gene functions because of functional overlap of related genes in vertebrates. Medaka fish with accumulated knowledge and expertise in genetics and biology were successfully adopted for a systematic screen for mutations affecting organogenesis. Despite exhaustive screens in zebrafish, a large number of mutants with new distinct phenotypes were identified in the large-scale medaka screen, indicating that by carrying out screens in both species more mutants and/or phenotypes can be found and that screens in medaka and zebrafish are complementary. Straightforward comparison of phenotypes, ease of side-by-side analysis using the same techniques, and ease of raising the mutants of medaka and zebrafish in an aquarium, together with their draft genome sequences, facilitate further genetic dissection. Thus, the two fish models will provide essential supplements to finding the most difficult targets in mammalian mutant screens, mutants that become lethal in the uterus during development. Analyses of mutants of these three vertebrate models neatly complement each other and will lead to elucidation of the global functions of the vertebrate genome.

Abbreviations

ENU *N*-Ethyl *N*-nitrosourea
TILLING Targeting induced local lesions in genomes

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5.1 Introduction

Mutants of model organisms are the most important tools for obtaining insights into the function of the genes at the level of the whole organism. Systematic phenotype-driven mutant screening involves generation of mutants by random mutagenesis of the genome and screening for the phenotype affecting the process of interest. Thus, the phenotype-driven screen is an unbiased approach in contrast to the gene-driven approach to generate gene knockout animals. Mutants are classified according to the phenotypes, and genes required for each class of mutants are defined by genetic complementation analysis. The value of phenotype-oriented mutant screening is that it systematically dissects the process into genetically defined steps, and the genes required for each step can be identified. This approach has been applied to invertebrate animals and plants, such as *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Arabidopsis thaliana* (Brenner 1974; Nüsslein-Volhard and Wieschaus 1980; Mayer et al. 1991). In vertebrates, systematic screens have been carried out in two teleost fish species, zebrafish (Driever et al. 1996; Haffter et al. 1996a) and medaka fish (Furutani-Seiki et al. 2004), and in the mouse (Hrabe de Angelis et al. 2000; Nolan et al. 2000a,b).

The medaka, *Oryzias latipes*, has been established as an experimental animal since the 1920s with accumulated knowledge of genetics and biology of sex determination (Aida 1921), radiation response (Egami 1969) and tumorigenesis (Hyodo-Taguchi and Matsudaira 1984; Masahito et al. 1989), and evolution and development (Wittbrodt et al. 2002). Genetic tools have been generated including inbred strains (Hyodo-Taguchi and Egami 1985) and a fine genetic map with polymorphic markers (Naruse et al. 2000). A draft genome sequence became available owing to the inbred strain and its small genome size (one-half of that of zebrafish and only twice that of fugu) (Kasahara et al. 2007). The substantial phylogenetic distance between medaka and zebrafish allows the identification of different phenotypes and conserved and divergent gene functions by comparison of the phenotypes (Furutani-Seiki and Wittbrodt 2004). Resources equivalent to those of zebrafish are available in medaka: expressed sequence tags (ESTs) (Kimura et al. 2004; Lo et al. 2008), bacterial artificial chromosome (BAC) contigs (Khorasani et al. 2004), and the TILLING (targeting-induced local lesions in genomes) mutant library for identifying mutants of genes of interest (Taniguchi et al. 2006). Medaka not only complements zebrafish for further genetic dissection of vertebrate genome function but also has unique features that facilitate genetic studies: (1) endogenous transposons for transgenesis (Koga et al. 1995); (2) development at low temperatures for identification of temperature-sensitive alleles; (3) inbred strains that give low phenotypic variation and allow cell transplantation analysis in adults, for example, to validate carcinoma cells; and (4) reliable storage of frozen sperm for maintaining mutant strains.

Based on these useful features of medaka, a systematic phenotype-driven screen was carried out (Furutani-Seiki et al. 2004). Taking the collection of medaka mutants affecting organogenesis from our systematic screen as examples, I discuss here the value of medaka for the genetic study of organogenesis in vertebrates.

5.2 Why Are Fish Models Useful for Identifying Mutants Affecting Organogenesis?

Among the vertebrate model animals, the small teleost fish zebrafish and medaka complement the mouse in the genetic dissection of vertebrate organogenesis.

Close observation of organogenesis at multiple time points facilitates isolation of mutants because organogenesis is a dynamic process. Embryonic development outside the mother's body and the translucent body during embryogenesis are important attributes of the two fish species. In situ hybridization and antibody staining also assist in the isolation of mutants, while the use of transgenic strains with green fluorescent protein (GFP) reporters has revolutionized mutant screens by allowing observation of organogenesis in live embryos in unprecedented detail.

Survival of mutant fish embryos greatly contributes to identification of new phenotypes. The genetic analysis of genes that are used multiple times in different tissues and at different times during development is not straightforward, because early lethality of mutants often prevents finding phenotypes at later stages. This problem can be alleviated by use of the fish models. (1) Fish mutant embryos suffer much less from defects in blood circulation compared to the mouse, as exemplified by the isolation of large numbers of mutants affecting heart formation in zebrafish (Chen et al. 1996; Stainier et al. 1996). (2) Use of alleles of mutants that survive for a longer time often allows the detection of later phenotypes. Multiple alleles of one gene with different lethality can be isolated by a systematic screen of mutations induced by the point mutagen *N*-ethyl *N*-nitrosourea (ENU). (3) The presence of maternal RNA in fish protects embryos from early lethality during gastrulation and allows manifestation of phenotypes during organogenesis. Although mouse embryos have maternal RNA only during the early cell division stages, fish embryos have maternal RNA or proteins that persist longer, with zygotic transcription starting after the 1,000-cell stage. Hence, zygotic mutants of fish survive longer toward organogenesis. (4) Interspecies difference of lethality between medaka and zebrafish (see sub-functionalization, as discussed below) is also useful for discovering later phenotypes.

The spectrum of mutants isolated by the phenotype-driven screens in vertebrates reflects these advantageous features of the fish models. The majority of the fish mutations are recessive and embryonic lethal, whereas the majority of mouse mutations identified by the phenotype-driven screens are dominant and embryonic viable mutations. In the mouse, the F_1 animals are screened for the dominant phenotypes after they are born, because an F_3 screen is labor-, space-, and time intensive, and direct observation during organogenesis is difficult during large-scale screening in the mouse. Most recessive embryonic lethal mutations in the mouse have been generated by the gene-driven approach. Taken together, the two fish models complement the mouse.

Large-scale screens are feasible in medaka because they are easily reared in a relatively compact space and have a reasonably short generation time (3 months) and low maintenance costs. Because a relatively small-scale focused medaka

mutant screen can also be carried out in a laboratory, these screens also identified many interesting mutants (Loosli et al. 2000; Ishikawa 2000; Hibiya et al. 2009; Miyake et al. 2008; Omran et al. 2008; Sakaguchi et al. 2006; Sakamoto et al. 2004; Shimada et al. 2008; Tanaka et al. 2004).

5.3 Why Do Medaka Complement Zebrafish for the Genetic Study of Organogenesis?

The use of a single species for mutagenesis screening will not be sufficient to uncover all gene functions in vertebrates for several reasons. (1) Functional overlap occurs among related genes. (2) Repeated usage of the same gene during development has the consequence that lethality caused by the early requirement of a gene makes it difficult to detect its late phenotypes. (3) Species-specific features, for example, differences in the signaling cascade network, cause difficulties. There is accumulating evidence that these issues can be alleviated by the use of two fish species, medaka and zebrafish, where usage of genes varies between them.

5.3.1 *Further Genetic Dissection of Vertebrate Genome Function*

5.3.1.1 Divergent Functional Overlap Between Medaka and Zebrafish

A duplication of the genome occurred in the ancestor of teleosts 350 million years ago. Medaka and zebrafish were separated from their last common ancestor between 115 and 200 million years ago, a distance that roughly corresponds to the distance between human and chicken (Fig. 5.1) (Wittbrodt et al. 2002). As a consequence of a genome duplication and rediploidization that generates paralogous groups of (duplicated) genes, one of the paralogs is free either to take over a partial function (complementing the other paralog; sub-functionalization), or to acquire a new function (neo-functionalization), or to vanish (dis-functionalization) (Ohno 1970; Amores et al. 1998). The analysis of the two fish species thus allows functions unidentifiable in one species to be uncovered in the other: (1) Phenotypes masked by the two redundant genes can be visible in another species in the case of dis-functionalization. (2) Mutations in the individual fish paralogs can represent a subset of the features of the complex mouse phenotype, resulting from sub-functionalization as a consequence of the genome duplication. Phenotypes of later functions masked by lethality of the embryo as a result of early function can be seen if early and late functions have been divided between the paralogs by sub-functionalization. This sub-functionalization is likely to be different in different teleost species. (3) New phenotypes can be detected for the gene generated by neo-functionalization.

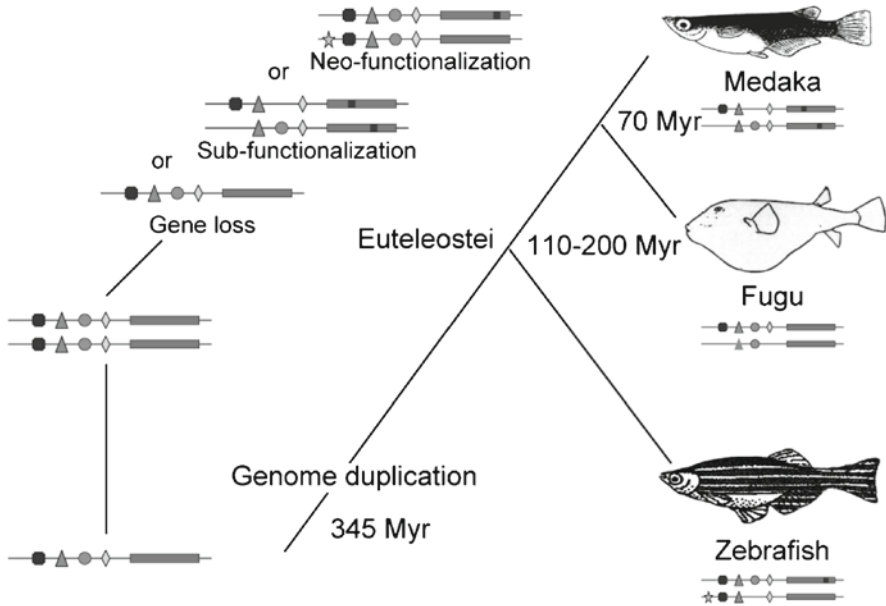


Fig. 5.1 Evolutionary relationships of teleost model systems and potential consequences of a genome duplication at the base of their radiation. As schematized, the duplication of a gene or genome can lead to subsequent gene loss, to sub-functionalization or neo-functionalization of the paralogs generated in the duplication. Sub- and neo-functionalisation are not limited to the noncoding region (*square, triangle, circle, and rhombus*), but can occur as well in the coding region (*oblong*; differences in *solid black*). (Adopted from Furutani-Seiki and Wittbrodt 2004)

Because the medaka and zebrafish embryos develop in a similar manner, the direct comparison of a phenotype and its functional and evolutionary interpretation is relatively straightforward: similar phenotypes indicate a conservation of functional units employed to control embryonic development, but do not necessarily imply that orthologous genes are affected by the respective mutations. Only the molecular identification of the mutated gene will allow the evaluation of whether its function is conserved or species specific. When the disruption of an orthologous gene results in the same phenotype in both species, we can assume a conserved gene function. Conversely, when the disruption of an orthologous gene results in a different phenotype in the two species, this shows that its function has diverged in evolution. As the overall development is very similar, this would consequently imply that the respective developmental modules are different at the molecular level. To unravel such similarities and differences of developmental modules is one of the big potential benefits of this comparative analysis of medaka and zebrafish. To detect similarities and differences, the evolutionary distance between the species compared must be just right. If it is too small (e.g., mouse–man), there will be very few informative differences; if it is too large (e.g., mouse–fish), there will be too many. The evolutionary distance between medaka and zebrafish seems ideal for such comparisons (Furutani-Seiki and Wittbrodt 2004).

5.3.1.2 Different Mutability of the Genome

The combination of mutagenesis approaches in medaka and zebrafish will in part also overcome differential mutabilities of genes in the genome caused by the genomic environment.

5.3.1.3 Different Visibility of Target Tissue

Identification of mutants is strongly dependent on the robustness of the assay used, such as the visibility of the tissue in a morphology-based screen. Consequently, the number of mutants affecting conspicuous tissues, such as the notochord and the melanophores in zebrafish, which have been identified are higher than those affecting less conspicuous tissues such as liver (Haffter et al. 1996b). In medaka, the liver is more conspicuous than in zebrafish whereas the notochord is less conspicuous. Thus, the number of mutations per mutagenized genome in the medaka screen is higher for the mutations affecting the liver and lower for those affecting the notochord. Thus, performing a mutagenesis screen in the species where the target tissue is more conspicuous, or the assay is more robust, will significantly strengthen an equivalent mutagenesis screen in the other species (Table 5.1).

5.3.2 Study of Species-Specific Features

Medaka and zebrafish have several species-specific features that are amenable to the genetic studies discussed here. Two particularly good examples representing these features are sex determination and adult pigment patterning, which are best studied in medaka and zebrafish, respectively (Furutani-Seiki and Wittbrodt 2004).

Medaka has an XX, XY sex-determination system similar to mammals, with the male-determining locus on the Y chromosome, whereas in zebrafish none of the many markers on the genetic map is sex linked and little is known about sex determination.

Table 5.1 Summary of identified genes according to the affected tissues

Tissues affected by the mutations	Genomes screened	Genes (mutations)
Forebrain	1,588	21 (28)
Primordial germ cells	450	10 (12)
Gonad	428	13 (16)
Lateral line	432	4 (4)
Liver	162	19 (22)
Thymus	502	13 (15)
Eye	529	22 (25)
Retino-tectal pathfinding	184	5 (7)
Somite	1,588	9 (12)
γ -Ray sensitivity	780	3 (3)

Sex determination has been the target of intensive research since the late 1950s (Yamamoto 1965; reviewed by Scharl 2004), and recently a member of the *dmrt* gene family was found to be a sex-determining gene in medaka, representing the first nonmammalian sex determination gene in vertebrates (Matsuda et al. 2002; Nanda et al. 2002). The success of an intensive research effort is further manifested in a relatively high number of mutants that affect gonad formation and sex determination in medaka (see Sect. 5.4.3.3).

The beautiful stripes of the adult zebrafish are an excellent system to gain insights into autonomous pattern formation of pigment cells in the skin (Haffter et al. 1996b). Mutations in nine loci affect formation of the stripes in zebrafish (Parichy 2006). Medaka does not exhibit a particularly ordered body pigmentation in adults, but shows a simpler pattern of relatively homogeneously dispersed pigment cells.

5.4 The Kyoto Medaka Mutant Screen

5.4.1 Design of the Systematic Screen

To identify zygotic recessive mutations, a classical three-generation in-crossing scheme was used to generate F_3 embryos homozygous for mutations induced in founder males (Fig. 5.2). The wild-type strains with low background mutations and high fecundity suitable for a mutagenesis screen (Kyoto-Cab), and the strain that is polymorphic for genetic mapping (Kyoto-Kaga), were established (Furutani-Seiki et al. 2004). To introduce mutations into the germ line, male founder adult fish were treated with ENU (Shima and Shimada 1988), which has been used for zebrafish (Mullins and Nüsslein-Volhard 1993; Solnica-Krezel et al. 1994) and mice (Russell and Montgomery 1982). ENU is known to introduce point mutations very efficiently and relatively randomly in spermatogonia.

The scale of the Kyoto medaka screen was approximately one half but its mutagenesis rate was about double that of the initial Tübingen zebrafish screen. Approximately 1,137 F_2 families were used to produce homozygous F_3 embryos bearing mutations: 6,088 intercross pairs successfully produced progeny, and 24,887 clutches (a clutch is a set of eggs produced per mating) were used for mutant screening. Nearly 260,000 F_3 embryos were inspected during the screen. In total, 1,588 mutagenized haploid genomes were screened for recessive mutations.

5.4.2 Detection of Mutant Phenotypes

Morphological abnormalities in live embryos were screened at three stages of development: stage (st.) 19–21 (27–34 hpf, hours post fertilization), st. 25–27 (50–58 hpf), and st. 32–35 (4 dpf, days post fertilization). Screening at the earliest stage identified defects in early embryonic patterning, including gastrulation,

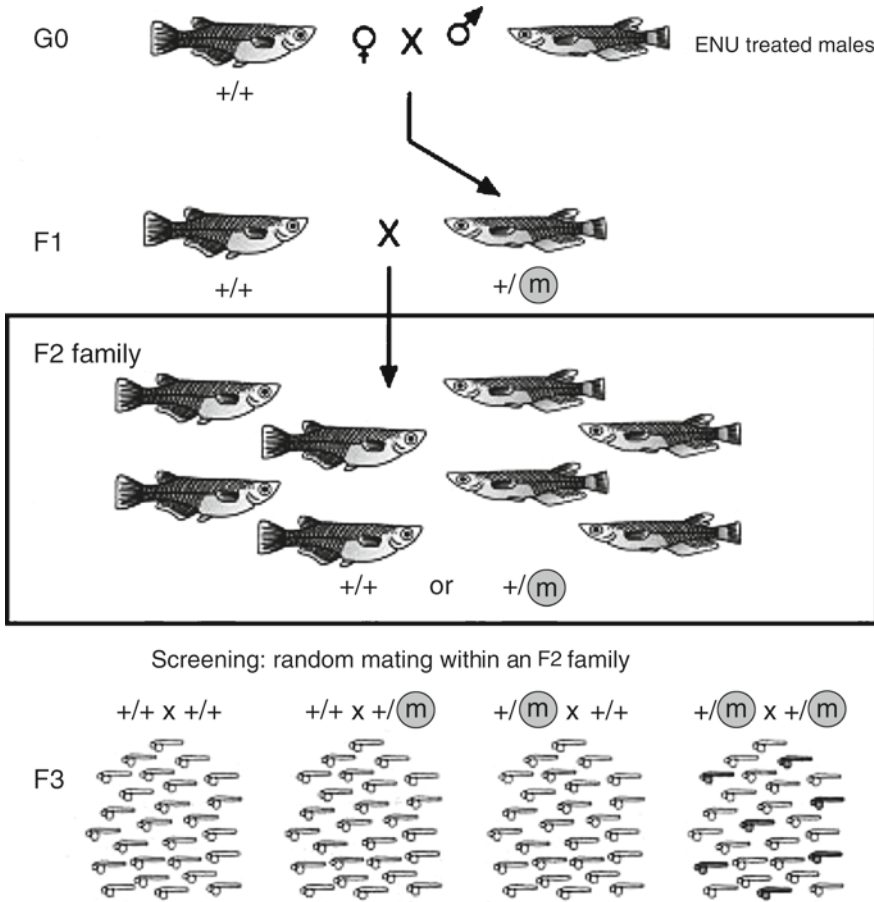


Fig. 5.2 A scheme of breeding of mutagenized fish population to identify recessive mutations (adopted from Haffter et al. 1996a,b, with modification). Males 4 weeks after treatment with *N*-ethyl *N*-nitrosourea (ENU) were mated with wild-type females to produce F_1 progeny, which are heterozygous carriers of mutations derived from the mutagenized paternal genome. Many pairs of F_1 fish were mated to generate F_2 families, each consisting of about 60 fish derived from a single pair. A mutation designated as *m* harbored in an F_1 parent is transmitted to one-half of the F_2 families. Random brother–sister mating within an F_2 family is expected to produce homozygotes of the mutation at a frequency of 1:4, assuming that the Mendelian ratio applies: When both F_2 parents are heterozygous for mutation *m*, one-fourth of their F_3 progeny will be homozygous for *m* and exhibit the mutant phenotype (shown on the right side). (Adopted from Furutani-Seiki et al. 2004)

dorsoventral polarity, body axis formation, and early tissue degeneration. Screening at the second time point focused on the rudiments of organs, such as the eye vesicles, brain, heart primordium, otic vesicles, somites, and notochord. At the third time point, abnormalities in organ morphogenesis were detected by screening the olfactory bulbs, brain ventricles, otic vesicles, liver, heart, vasculature, and pectoral fin buds.

Special attention was paid to morphological abnormalities accompanied by cell degeneration because specific patterning defects can produce localized cell degeneration at later stages (Furutani-Seiki et al. 1996).

To confirm that abnormalities were genetic rather than the result of compromised egg quality, we made simultaneous observations of embryos at different stages but derived from the same parents. Although zebrafish lay about 100 eggs once in 1–2 weeks, medaka spawns 10–40 eggs daily. We collected embryos from each pair for 5 consecutive days per week over a 2-week period, and used these different clutches of embryos in multiple assays, allowing pairs to be readily rescreened to confirm the reproducibility of the phenotypes and to look for Mendelian ratios as an indicator of genetic nature.

Various screening procedures were used to detect more subtle abnormalities in specific structures or functional processes in addition to morphological criteria (Fig. 5.3). Germline cells, thymocytes, optic, cranial, and lateral line nerves, and optic tectal projections of retinal ganglion cell (RGC) axons were visualized by in situ hybridization, immunostaining, or fluorescent dye injection in fixed embryos. In addition, the potential for DNA repair and bilirubin and lipid metabolism associated with liver function were examined (Fig. 5.3).

In situ hybridization with a *vasa* probe allowed the visualization of primordial germ cells (PGCs) (Fig. 5.3a, a') and differentiated germ cells (Fig. 5.3b–b") at st. 26 (59 hpf) and 15 dpf, respectively (Shinomiya et al. 2000). The *rag1* probe was used to detect thymocytes in st. 32 embryos (Fig. 5.3c, c'). Immunostaining using a mixture of antiacetylated tubulin and HNK1 antibodies was performed to detect axons forming cranial and lateral line nerves at st. 32 (Fig. 5.3d, d'). The topographic projection of axons from RGCs to the optic tectum was visualized by injecting the fluorescent carbocyanine dyes DiI and DiO at the ventrotemporal and dorsonasal positions, respectively, of retinas, as previously described in a screen for zebrafish retinotectal mutants (Fig. 5.3e, e') (Baier et al. 1996). Liver-associated physiological activity involving bile and lipid metabolism was scored by bile color (Fig. 5.3g) and the accumulation of a fluorescent metabolite of PED6, a phospholipase A2 substrate, (Farber et al. 2001) in the gallbladder in living embryos (Fig. 3f, f'). DNA repair activity during embryogenesis was tested by examining recovery after exposure of developing embryos to a sublethal dose of γ -ray radiation (Fig. 5.3h, h').

Together, using all the screening methods, a total of 2,031 embryonic lethal mutations were identified (see Table 5.1). As medaka embryos hatch at 9 dpf at 28°C, mutant embryos that failed to hatch by this time were defined as carrying embryonic lethal mutations. Among these mutations, we selected 372 for further analyses on the basis of their specific phenotypes. F_2 parents carrying the mutations of interest were outcrossed to wild-type fish, and the F_3 progeny were raised and used for reidentification and recovery of mutations. From the F_3 generation, a total of 312 mutations were recovered and subjected to more detailed characterization.

To dissect organogenesis into genetically defined steps and identify the loci required for each step, mutations were grouped into several classes according to their phenotypes. Complementation testing was completed for 142 mutations defining 113 distinct genetic loci.

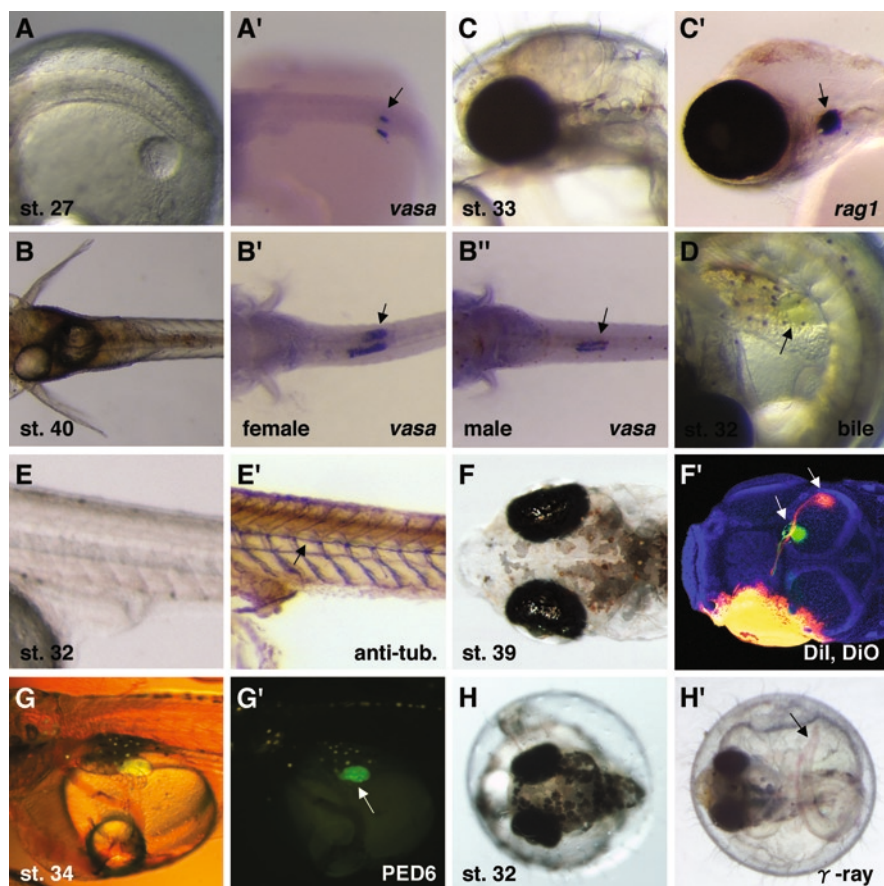


Fig. 5.3 Various assays employed in screening and analysis of medaka mutants (see text). (Adopted from Furutani-Seiki et al. 2004)

5.4.3 Mutant Phenotypes

5.4.3.1 Forebrain Mutants

Thirty-three mutations in 25 loci required for the formation of the forebrain were identified (Kitagawa et al. 2004). The mutations were grouped into two major phenotypic classes: group 1 included mutations in 11 genes that resulted in a reduced telencephalon and group 2 mutations in 14 genes produced abnormal morphology of the telencephalon without significantly affecting its size. In zebrafish, the development of the telencephalon is affected in *knollnase* (*kas*), *masterblind* (*mbf*), and *silberblick* (*slb*) embryos (Heisenberg et al. 1996), and these mutants are associated with midline defects resulting in cyclopia or a curly tail-down phenotype (Brand et al. 1996).

Mutations in medaka affecting forebrain formation appear distinct from those in zebrafish as judged by their morphology (Fig. 5.4), although more detailed comparison of medaka and zebrafish mutant phenotypes using specific markers is necessary. Localized focal cell degeneration is often caused by earlier patterning defects. The *baltan* mutant that showed severe degeneration in the forebrain is associated with early brain patterning defects. To date, 17 loci have been mapped and 4 cloned.

5.4.3.2 Lateral Line Mutants

The lateral line system is a sensory organ found in fish and amphibians. The posterior lateral line nerve (PLLn) shows stereotyped projections from the hindbrain to the neuromasts, groups of cells receiving sensory stimuli in the trunk. Mutations in four

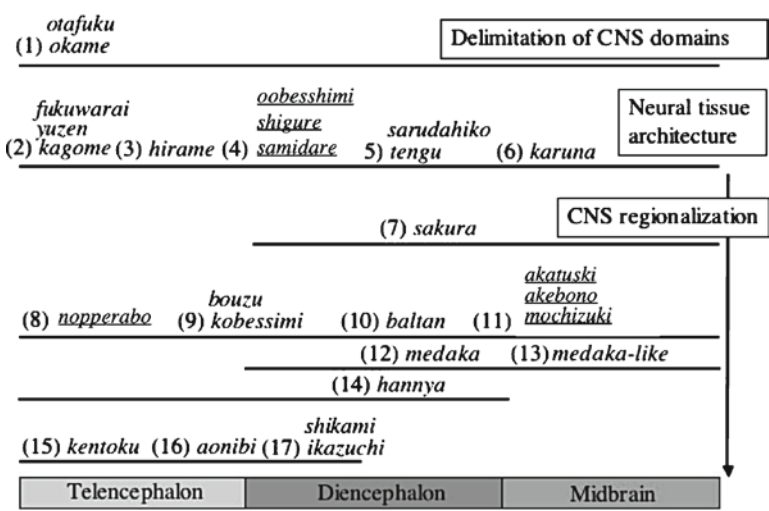


Fig. 5.4 Schematic presentation of medaka mutations affecting the anterior central nervous system (CNS) and possible correspondence with the phenotypes of zebrafish mutants. Medaka mutants are roughly classified into 17 groups according to their phenotype, and the regions of CNS affected by the group of mutations are indicated by *horizontal lines*. Possible underlying defects at cellular and tissue levels responsible for the mutant phenotype are indicated in the *squares*. Medaka mutants displaying phenotypes resembling those of zebrafish mutations are *underlined*. Group 4 mutants *oobesshimi*, *shigure*, and *samidare* exhibit a phenotype similar to that of the *parachute* zebrafish mutant. Group 8 mutant *nopperabo* has an analogous phenotype to that of *masterblind* and *headless* mutants. Group 11 mutants *akatsuki*, *akebono*, and *mochizuki* display the phenotype resembling those of *one-eyed-pinhead* zebrafish mutants. Remaining mutations, possibly representing unique phenotypes of medaka mutations, are indicated in *blue*. The correspondence to the zebrafish mutant phenotype is only the authors' interpretation and does not imply a genetic correspondence. This scheme is drawn merely to provide the readers with an overview of mutant phenotypes, and the details are not necessarily precise. (Adopted from Furutani-Seiki et al. 2004)

genes were isolated that affected the PLLn trajectory (Yasuoka et al. 2004). Among the mutations identified, *kazura* (*kaz*) and *yanagi* (*yan*) mutations displayed specific defects in projection of the posterior lateral line (PLL) nerve. The *yan* and *kaz* mutants also exhibited defects in the migration of PGCs. *yan* was identified to be a mutation in *cxc7* that remains unidentified in zebrafish (Sasado et al. 2008).

5.4.3.3 Primordial Germ Cell Mutants

Alterations in PGC number and distribution were screened by in situ hybridization at st. 27 using the *vasa* probe. Identified mutations were classified into two major groups, those affecting distribution of PGCs and those causing a decrease in the number of PGCs (Sasado et al. 2004). In the first group, mutations in nine genes caused an altered distribution of PGCs, affecting PGC migration, accumulation, and redistribution. The majority of mutant phenotypes in this class were associated with other morphological abnormalities, suggesting that the mutated gene is involved in several different aspects of organogenesis. The *yanagi* (*yan*) and *kazura* (*kaz*) mutation had PGC distribution and lateral line defects. *kaz* and *yan* were identified to be a mutation in chemokine receptors *cxc4b* and *cxc7* that were expressed in PGC and somites respectively (Sasado et al. 2008). CXCR7 and CXCR4 mediated regulation of PGC routing is conserved in medaka and zebrafish (refs). All of the mutations that reduced the number of PGCs exerted their effects only when the mother was a heterozygous carrier, indicating the contribution of maternal factors to the determination of PGC abundance.

5.4.3.4 Germ Cell Mutants

Mutations affecting germ cell and gonad formation were screened at the larval stage when the sex of an animal can be distinguished by the abundance of *vasa*-positive germ cells. The female gonad is large and asymmetrical and the male gonad is smaller and symmetrical (see Fig. 5.3).

The mutations in 13 genes affecting proliferation and/or distribution of germ cells during gonadal development were classified into four groups (Morinaga et al. 2004). In the first group that had an increased number of germ cells, the *hotei* mutation caused the tumorous growth of immature germ cells in homozygous juvenile adults and male-to-female sex reversal. Positional cloning identified *hotei* to be a mutation in the *anti-Müllerian hormone receptor type II* (*amhrII*) gene (Morinaga et al. 2007). Anti-Müllerian hormone (Amh) induces regression of the Müllerian duct, which forms female genital organs in amniotes, but Amh is expressed in lower vertebrates lacking the Müllerian duct. Analysis of the *hotei* mutant revealed the conserved function of *amhrII* for the sex-dependent regulation of germ cell proliferation and follicular development. The second group of mutants with a decreased number of germ cells included the *zenzai* mutant (Saito et al. 2007). The third and fourth group of mutants had abnormal distribution of germ

cell clusters accompanied by an irregular or by a fragmented gonad shape, respectively, suggesting that an interaction between germ cells and the non-germline gonadal anlage might be affected in these mutants.

5.4.3.5 Eye Mutants

The Kyoto screen together with previous morphological screens identified 60 mutations that affect retinal development (Loosli et al. 2004). The mutants were grouped into five classes: 11 mutants had defective neural plate and optic vesicle formation, 15 mutants showed impaired growth of optic vesicles, 18 mutants were defective in optic cup development, 13 mutants showed abnormal retinal differentiation, 12 of which had small eyes and 1 had enlarged eyes, and 3 mutants had abnormal retinal pigmentation.

5.4.3.6 Retino-Tectal Mutants

In medaka, as in other lower vertebrates, the axons of RGCs project to the visual center of the brain, the optic tectum. To project the image on the retina precisely, all the RGC axons connect to the opposite side (contralateral) of the tectum, preserving the topological relationship with different points on the retina. Mutations in five genes affecting the projections of RGC axons to the retina were identified (Yoda et al. 2004). The misrouting of RGC axons occurred either between the retina and chiasm (group 1 mutants) or between the chiasm and the tectum (group 2 mutants). Although misrouted axons reached the tectum on the same side (ipsilateral) of the retina in group 1 mutants, misrouted axons did not project to the tectum in group 2 mutants. In contrast to previously described zebrafish mutants (Karlstrom et al. 1996), defects appear to be restricted to RGC axons as other tissues appear unaffected in the medaka mutants.

5.4.3.7 Thymus Mutants

Cells derived from three germ layers contribute to thymus organogenesis: neural crest cells from the ectoderm, lymphocytes from the mesoderm, and cells of the thymic anlage from the endodermal pharyngeal pouch. Twenty-four mutations defining at least 13 genes were isolated by the screen using *rag1* (*recombination activating gene 1*) expression in thymocytes as a marker of thymus development (Iwanami et al. 2004). These mutants were classified into three groups based on the associated defects in pharyngeal arch formation. The group 2 and 3 mutants had mild or severe defects in the pharyngeal from which the thymus anlagen develops. In these mutants, the primary defect may reside in the thymus anlage, leading to the failure of thymus formation. The group 1 mutants with defective *rag1* expression showed normal pharyngeal arch development, suggesting that the mutations disrupt

colonizing lymphocytes. Nine of these mutations were mapped and four of them were cloned. The *hokecha* (*hkc*) mutation with mild pharyngeal arch defects was found in the *WDR55* gene carrying the tryptophan-aspartate-repeat motif (Iwanami et al. 2008). *WDR55* is a novel nucleolar protein involved in the production of ribosomal RNA (rRNA). Defects in *WDR55* cause aberrant accumulation of rRNA intermediates and cell-cycle arrest, indicating that *WDR55* is a nuclear modulator of rRNA synthesis and cell-cycle progression. Although *WDR55*-null mice are lethal before implantation and therefore the effects on thymus development are not known, a mutation in *WDR55* in zebrafish also leads to analogous defects in thymus development, suggesting its function is conserved between the two species (Amsterdam et al. 2004).

5.4.3.8 Mutants Affecting Liver Development and Function

Liver mutant screening was carried out using multiple parameters, morphological inspection, in situ hybridization staining of the gut tube, bile color, and PED6 live staining of lipid metabolism (Watanabe et al. 2004). Mutations in 19 genes were classified into five phenotypic groups affecting morphogenesis, laterality, bile color, lipid metabolism, and endoderm formation. Mutations in the first group, *kakurenbo*, *hiogi*, and *origami*, affect the size of the liver as well as the morphology of the gall bladder and gut, suggesting that these genes might be involved in gut tube morphogenesis. Five genes have been mapped so far and two of them cloned.

5.4.3.9 Somitogenesis Mutants

Mutations in nine genes affected somite formation and mutants were classified into two groups (Elmasri et al. 2004). Group 1 mutations caused phenotypes characterized by the complete or partial absence of somites or somite boundaries, and group 2 mutations resulted in fused somites or somites of irregular size and shape. The majority of the mutants exhibited somitic phenotypes, such as individually fused somites and irregular somite sizes, that were distinct from those found in zebrafish (van Eeden et al. 1996). Three mutations were also isolated that produced characteristic phenotypes similar to those of zebrafish mutations affecting the Delta/Notch signaling pathway.

5.4.3.10 γ -Ray Sensitivity Mutants

To identify mutants that exhibit increased sensitivity to γ -ray radiation during development, a new type of screen was designed (Aizawa et al. 2004). After irradiation of F_3 embryos using a dose at which wild-type embryos readily recover from damage, mutants with lower whole embryo viability were screened. Three genes, termed *ric1-3* (radiation-induced curly tailed), were identified in this screen.

The *ric1* mutant has a defect in the repair mechanism of DNA double-strand breaks induced by induced by γ -rays, suggesting a DNA surveillance function for the *ric1* gene product during early embryogenesis. Furthermore, it was found that *ric1* is required for DNA repair in germ cells by the analysis using the *Olvas-GFP* transgene that allowed survival of PGC after irradiation (Aizawa et al. 2007).

5.5 Conclusion and Perspective

The molecular bases of phenotypic divergence between medaka and zebrafish await cloning of the mutations and further analysis of the phenotypes in the two species. These analyses have been greatly facilitated because comparison of the phenotypes is straightforward between medaka and zebrafish, most of the standard experimental procedures can be applied to both species, both fish species can be raised in the same aquarium, and zebrafish-equivalent resources of EST, BAC, TILLING mutant library, and the draft genome sequence are available in medaka. Therefore, a large proportion of the medaka mutant collection will complement and extend analyses on zebrafish and mouse mutants and expand our understanding of general mechanisms underlying organogenesis in vertebrates.

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References

- Aida T (1921) On the inheritance of color in a fresh-water fish, *Aplocheilurus latipes* Temminck and Schlegel, with special reference to sex-linked inheritance. *Genetics* 6:554–573
- Aizawa K, Mitani H, Kogure N, Shimada A, Hirose Y, Sasado T, Morinaga C, Yasuoka A, Yoda H, Watanabe T, Iwanami N, Kunimatsu S, Osakada M, Suwa H, Niwa K, Deguchi T, Hennrich T, Todo T, Shima A, Kondoh H, Furutani-Seiki M (2004) Identification of radiation-sensitive mutants in the medaka, *Oryzias latipes*. *Mech Dev* 121:895–902
- Aizawa K, Yori K, Kaminaga C, Yamashita T, Kinoshita M, Oda S, Mitani H (2007) Responses of embryonic germ cells of the radiation-sensitive medaka mutant to gamma-irradiation. *J Radiat Res (Tokyo)* 48:121–128
- Amores A, Force A, Yan YL, Joly L, Amemiya C, Fritz A, Ho RK, Langeland J, Prince V, Wang YL, Westerfield M, Ekker M, Postlethwait JH (1998) Zebrafish hox clusters and vertebrate genome evolution. *Science* 282:1711–1714
- Amsterdam A, Nissen RM, Sun Z, Swindell EC, Farrington S, Hopkins N (2004) Identification of 315 genes essential for early zebrafish development. *Proc Natl Acad Sci USA* 101:12792–12797
- Baier H, Klostermann S, Trowe T, Karlstrom RO, Nusslein-Volhard C, Bonhoeffer F (1996) Genetic dissection of the retinotectal projection. *Development (Camb)* 123:415–425
- Brand M, Heisenberg CP, Warga RM, Pelegri F, Karlstrom RO, Beuchle D, Picker A, Jiang YJ, Furutani-Seiki M, van Eeden FJ, Granato M, Haffter P, Hammerschmidt M, Kane DA, Kelsh RN, Mullins MC, Odenthal J, Nusslein-Volhard C (1996) Mutations affecting development of

- the midline and general body shape during zebrafish embryogenesis. *Development (Camb)* 123:129–142
- Brenner S (1974) The genetics of *Caenorhabditis elegans*. *Genetics* 77:71–94
- Chen JN, Haffter P, Odenthal J, Vogelsang E, Brand M, van Eeden FJ, Furutani-Seiki M, Granato M, Hammerschmidt M, Heisenberg CP, Jiang YJ, Kane DA, Kelsh RN, Mullins MC, Nusslein-Volhard C (1996) Mutations affecting the cardiovascular system and other internal organs in zebrafish. *Development (Camb)* 123:293–302
- Driever W, Solnica-Krezel L, Schier AF, Neuhauss SC, Malicki J, Stemple DL, Stainier DY, Zwartkruis F, Abdelilah S, Rangini Z, Belak J, Boggs C (1996) A genetic screen for mutations affecting embryogenesis in zebrafish. *Development (Camb)* 123:37–46
- Egami N (1969) Kinetics of recovery from injury after whole-body x-irradiation of the fish *Oryzias latipes* at different temperatures. *Radiat Res* 37:192–201
- Elmasri H, Winkler C, Liedtke D, Sasado T, Morinaga C, Suwa H, Niwa K, Henrich T, Hirose Y, Yasuoka A, Yoda H, Watanabe T, Deguchi T, Iwanami N, Kunitatsu S, Osakada M, Loosli F, Quiring R, Carl M, Grabher C, Winkler S, Del Bene F, Wittbrodt J, Abe K, Takahama Y, Takahashi K, Katada T, Nishina H, Kondoh H, Furutani-Seiki M (2004) Mutations affecting somite formation in the medaka (*Oryzias latipes*). *Mech Dev* 121:659–671
- Farber SA, Pack M, Ho SY, Johnson ID, Wagner DS, Dosch R, Mullins MC, Hendrickson HS, Hendrickson EK, Halpern ME (2001) Genetic analysis of digestive physiology using fluorescent phospholipid reporters. *Science* 292:1385–1388
- Furutani-Seiki M, Wittbrodt J (2004) Medaka and zebrafish, an evolutionary twin study. *Mech Dev* 121:629–637
- Furutani-Seiki M, Jiang YJ, Brand M, Heisenberg CP, Houart C, Beuchle D, van Eeden FJ, Granato M, Haffter P, Hammerschmidt M, Kane DA, Kelsh RN, Mullins MC, Odenthal J, Nusslein-Volhard C (1996) Neural degeneration mutants in the zebrafish, *Danio rerio*. *Development (Camb)* 123:229–239
- Furutani-Seiki M, Sasado T, Morinaga C, Suwa H, Niwa K, Yoda H, Deguchi T, Hirose Y, Yasuoka A, Henrich T, Watanabe T, Iwanami N, Kitagawa D, Saito K, Asaka S, Osakada M, Kunitatsu S, Momoi A, Elmasri H, Winkler C, Ramialison M, Loosli F, Quiring R, Carl M, Grabher C, Winkler S, Del Bene F, Shinomiya A, Kota Y, Yamanaka T, Okamoto Y, Takahashi K, Todo T, Abe K, Takahama Y, Tanaka M, Mitani H, Katada T, Nishina H, Nakajima N, Wittbrodt J, Kondoh H (2004) A systematic genome-wide screen for mutations affecting organogenesis in medaka, *Oryzias latipes*. *Mech Dev* 121:647–658
- Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, Kane DA, Odenthal J, van Eeden FJ, Jiang YJ, Heisenberg CP, Kelsh RN, Furutani-Seiki M, Vogelsang E, Beuchle D, Schach U, Fabian C, Nusslein-Volhard C (1996a) The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development (Camb)* 123:1–36
- Haffter P, Odenthal J, Mullins MC, Lin S, Farrell MJ, Vogelsang E, Haas F, Brand M, Vaneeden FJM, Furutani-Seiki M, Granato M, Hammerschmidt M, Heisenberg CP, Jiang YJ, Kane DA, Kelsh RN, Hopkins N, Nusslein-Volhard C (1996b) Mutations affecting pigmentation and shape of the adult zebrafish. *Dev Genes Evol* 206:260–276
- Heisenberg CP, Brand M, Jiang YJ, Wargha RM, Beuchle D, van Eeden FJ, Furutani-Seiki M, Granato M, Haffter P, Hammerschmidt M, Kane DA, Kelsh RN, Mullins MC, Odenthal J, Nusslein-Volhard C (1996) Genes involved in forebrain development in the zebrafish, *Danio rerio*. *Development (Camb)* 123:191–203
- Hibiya K, Katsumoto T, Kondo T, Kitabayashi I, Kudo A (2009) Brpf1, a subunit of the MOZ histone acetyl transferase complex, maintains expression of anterior and posterior Hox genes for proper patterning of craniofacial and caudal skeletons. *Dev Biol* 329:176–190
- Hrabe de Angelis MH, Flawinkel H, Fuchs H, Rathkolb B, Soewarto D, Marschall S, Heffner S, Pargent W, Wuensch K, Jung M, Reis A, Richter T, Alessandrini F, Jakob T, Fuchs E, Kolb H, Kremmer E, Schaeble K, Rollinski B, Roscher A, Peters C, Meitinger T, Strom T, Steckler T, Holsboer F, Klopstock T, Gekeler F, Schindewolf C, Jung T, Avraham K, Behrendt H, Ring J, Zimmer A, Schughart K, Pfeffer K, Wolf E, Balling R (2000) Genome-wide, large-scale production of mutant mice by ENU mutagenesis. *Nat Genet* 25:444–447

- Hyodo-Taguchi Y, Egami N (1985) Establishment of inbred strains of the medaka *Oryzias latipes* and the usefulness of the strains for biomedical research. *Zool Sci* 2:305–316
- Hyodo-Taguchi Y, Matsudaira H (1984) Induction of transplantable melanoma by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in an inbred strain of the teleost *Oryzias latipes*. *J Natl Cancer Inst* 73:1219–1227
- Ishikawa Y (2000) Medakafish as a model system for vertebrate developmental genetics. *Bioessays* 22:487–495
- Iwanami N, Takahama Y, Kunimatsu S, Li J, Takei R, Ishikura Y, Suwa H, Niwa K, Sasado T, Morinaga C, Yasuoka A, Deguchi T, Hirose Y, Yoda H, Henrich T, Ohara O, Kondoh H, Furutani-Seiki M (2004) Mutations affecting thymus organogenesis in medaka, *Oryzias latipes*. *Mech Dev* 121:779–789
- Iwanami N, Higuchi T, Sasano Y, Fujiwara T, Hoa VQ, Okada M, Talukder SR, Kunimatsu S, Li J, Saito F, Bhattacharya C, Matin A, Sasaki T, Shimizu N, Mitani H, Himmelbauer H, Momoi A, Kondoh H, Furutani-Seiki M, Takahama Y (2008) WDR55 is a nucleolar modulator of ribosomal RNA synthesis, cell cycle progression, and teleost organ development. *PLoS Genet* 4:e1000171
- Karlstrom RO, Trowe T, Klostermann S, Baier H, Brand M, Crawford AD, Grunewald B, Haffter P, Hoffmann H, Meyer SU, Muller BK, Richter S, van Eeden FJ, Nusslein-Volhard C, Bonhoeffer F (1996) Zebrafish mutations affecting retinotectal axon pathfinding. *Development (Camb)* 123:427–438
- Kasahara M, Naruse K, Sasaki S, Nakatani Y, Qu W, Ahsan B, Yamada T, Nagayasu Y, Doi K, Kasai Y, Jindo T, Kobayashi D, Shimada A, Toyoda A, Kuroki Y, Fujiyama A, Sasaki T, Shimizu A, Asakawa S, Shimizu N, Hashimoto S, Yang J, Lee Y, Matsushima K, Sugano S, Sakaizumi M, Narita T, Ohishi K, Haga S, Ohta F, Nomoto H, Nogata K, Morishita T, Endo T, Shin IT, Takeda H, Morishita S, Kohara Y (2007) The medaka draft genome and insights into vertebrate genome evolution. *Nature (Lond)* 447:714–719
- Khorasani MZ, Hennig S, Imre G, Asakawa S, Palczewski S, Berger A, Hori H, Naruse K, Mitani H, Shima A, Lehrach H, Wittbrodt J, Kondoh H, Shimizu N, Himmelbauer H (2004) A first generation physical map of the medaka genome in BACs essential for positional cloning and clone-by-clone based genomic sequencing. *Mech Dev* 121:903–913
- Kimura T, Jindo T, Narita T, Naruse K, Kobayashi D, Shin IT, Kitagawa T, Sakaguchi T, Mitani H, Shima A, Kohara Y, Takeda H (2004) Large-scale isolation of ESTs from medaka embryos and its application to medaka developmental genetics. *Mech Dev* 121:915–932
- Kitagawa D, Watanabe T, Saito K, Asaka S, Sasado T, Morinaga C, Suwa H, Niwa K, Yasuoka A, Deguchi T, Yoda H, Hirose Y, Henrich T, Iwanami N, Kunimatsu S, Osakada M, Winkler C, Elmasri H, Wittbrodt J, Loosli F, Quiring R, Carl M, Grabher C, Winkler S, Del Bene F, Momoi A, Katada T, Nishina H, Kondoh H, Furutani-Seiki M (2004) Genetic dissection of the formation of the forebrain in medaka, *Oryzias latipes*. *Mech Dev* 121:673–685
- Koga A, Inagaki H, Bessho Y, Hori H (1995) Insertion of a novel transposable element in the tyrosinase gene is responsible for an albino mutation in the medaka fish, *Oryzias latipes*. *Mol Gen Genet* 249:400–405
- Lo L, Zhang Z, Hong N, Peng J, Hong Y (2008) 3640 Unique EST clusters from the medaka testis and their potential use for identifying conserved testicular gene expression in fish and mammals. *PLoS One* 3:e3915
- Loosli F, Koster RW, Carl M, Kuhnlein R, Henrich T, Mucke M, Krone A, Wittbrodt J (2000) A genetic screen for mutations affecting embryonic development in medaka fish (*Oryzias latipes*). *Mech Dev* 97:133–139
- Loosli F, Bene FD, Quiring R, Rembold M, Martinez-Morales JR, Carl M, Grabher C, Iquel C, Krone A, Wittbrodt B, Winkler S, Sasado T, Morinaga C, Suwa H, Niwa K, Henrich T, Deguchi T, Hirose Y, Iwanami N, Kunimatsu S, Osakada M, Watanabe T, Yasuoka A, Yoda H, Winkler C, Elmasri H, Kondoh H, Furutani-Seiki M, Wittbrodt J (2004) Mutations affecting retina development in medaka. *Mech Dev* 121:703–714
- Masahito P, Aoki K, Egami N, Ishikawa T, Sugano H (1989) Life-span studies on spontaneous tumor development in the medaka (*Oryzias latipes*). *Jpn J Cancer Res* 80:1058–1065
- Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Morrey CE, Shibata N, Asakawa S, Shimizu N, Hori H, Hamaguchi S, Sakaizumi M (2002) DMY is a Y-specific

- DM-domain gene required for male development in the medaka fish. *Nature (Lond)* 417:559–563
- Mayer U, Ruiz RAT, Berleth T, Misera S, Jürgens G (1991) Mutations affecting body organization in the *Arabidopsis* embryo. *Nature (Lond)* 353:402–407
- Miyake A, Higashijima S, Kobayashi D, Narita T, Jindo T, Setiamarga DH, Ohisa S, Orihara N, Hibiya K, Konno S, Sakaguchi S, Horie K, Imai Y, Naruse K, Kudo A, Takeda H (2008) Mutation in the *abcb7* gene causes abnormal iron and fatty acid metabolism in developing medaka fish. *Dev Growth Differ* 50:703–716
- Morinaga C, Tomonaga T, Sasado T, Suwa H, Niwa K, Yasuoka A, Henrich T, Watanabe T, Deguchi T, Yoda H, Hirose Y, Iwanami N, Kunimatsu S, Okamoto Y, Yamanaka T, Shinomiya A, Tanaka M, Kondoh H, Furutani-Seiki M (2004) Mutations affecting gonadal development in medaka, *Oryzias latipes*. *Mech Dev* 121:829–839
- Morinaga C, Saito D, Nakamura S, Sasaki T, Asakawa S, Shimizu N, Mitani H, Furutani-Seiki M, Tanaka M, Kondoh H (2007) The *hotei* mutation of medaka in the anti-Müllerian hormone receptor causes the dysregulation of germ cell and sexual development. *Proc Natl Acad Sci USA* 104:9691–9696
- Mullins MC, Nüsslein-Volhard C (1993) Mutational approaches to studying embryonic pattern formation in the zebrafish. *Curr Opin Genet Dev* 3:648–654
- Nanda I, Kondo M, Hornung U, Asakawa S, Winkler C, Shimizu A, Shan ZH, Haaf T, Shimizu N, Shima A, Schmid M, Scharlt M (2002) A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. *Proc Natl Acad Sci USA* 99:11778–11783
- Naruse K, Fukamachi S, Mitani H, Kondo M, Matsuoka T, Kondo S, Hanamura N, Morita Y, Hasegawa K, Nishigaki R, Shimada A, Wada H, Kusakabe T, Suzuki N, Kinoshita M, Kanamori A, Terado T, Kimura H, Nonaka M, Shima A (2000) A detailed linkage map of medaka, *Oryzias latipes*: comparative genomics and genome evolution. *Genetics* 154:1773–1784
- Nolan PM, Peters J, Strivens M, Rogers D, Hagan J, Spurr N, Gray IC, Vizor L, Brooker D, Whitehill E, Washbourne R, Hough T, Greenaway S, Hewitt M, Liu X, McCormack S, Pickford K, Selley R, Wells C, Tymowska-Lalanne Z, Roby P, Glenister P, Thornton C, Thang C, Stevenson JA, Arkell R, Mburu P, Hardisty R, Kiernan A, Erven A, Steel KP, Voegelings S, Guenet JL, Nickols C, Sadri R, Nasse M, Isaacs A, Davies K, Browne M, Fisher EM, Martin J, Rastan S, Brown SD, Hunter J (2000a) A systematic, genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse. *Nat Genet* 25:440–443
- Nolan PM, Peters J, Vizor L, Strivens M, Washbourne R, Hough T, Wells C, Glenister P, Thornton C, Martin J, Fisher E, Rogers D, Hagan J, Reavill C, Gray I, Wood J, Spurr N, Browne M, Rastan S, Hunter J, Brown SD (2000b) Implementation of a large-scale ENU mutagenesis program: towards increasing the mouse mutant resource. *Mamm Genome* 11:500–506
- Nüsslein-Volhard C, Wieschaus E (1980) Mutations affecting segment number and polarity in *Drosophila*. *Nature (Lond)* 287:795–801
- Ohno S (1970) *Evolution by gene duplication*. Springer, Heidelberg
- Omran H, Kobayashi D, Olbrich H, Tsukahara T, Loges NT, Hagiwara H, Zhang Q, Leblond G, O'Toole E, Hara C, Mizuno H, Kawano H, Fliegauf M, Yagi T, Koshida S, Miyawaki A, Zentgraf H, Seithe H, Reinhardt R, Watanabe Y, Kamiya R, Mitchell DR, Takeda H (2008) Ktu/PF13 is required for cytoplasmic pre-assembly of axonemal dyneins. *Nature (Lond)* 456:611–616
- Parichy DM (2006) Evolution of *Danio* pigment pattern development. *Heredity* 97:200–210
- Russell LB, Montgomery CS (1982) Supermutagenicity of ethylnitrosourea in the mouse spot test: comparisons with methyl nitrosourea and ethylnitrosourea. *Mutat Res* 92:193–204
- Saito D, Morinaga C, Aoki Y, Nakamura S, Mitani H, Furutani-Seiki M, Kondoh H, Tanaka M (2007) Proliferation of germ cells during gonadal sex differentiation in medaka: insights from germ cell-depleted mutant *zenzai*. *Dev Biol* 310:280–290
- Sakaguchi S, Nakatani Y, Takamatsu N, Hori H, Kawakami A, Inohara K, Kudo A (2006) Medaka unextended-fin mutants suggest a role for Hoxb8a in cell migration and osteoblast differentiation during appendage formation. *Dev Biol* 293:426–438

- Sakamoto D, Kudo H, Inohaya K, Yokoi H, Narita T, Naruse K, Mitani H, Araki K, Shima A, Ishikawa Y, Imai Y, Kudo A (2004) A mutation in the gene for delta-aminolevulinic acid dehydratase (ALAD) causes hypochromic anemia in the medaka, *Oryzias latipes*. *Mech Dev* 121:747–752
- Sasado T, Morinaga C, Niwa K, Shinomiya A, Yasuoka A, Suwa H, Hirose Y, Yoda H, Henrich T, Deguchi T, Iwanami N, Watanabe T, Kunimatsu S, Osakada M, Okamoto Y, Kota Y, Yamanaka T, Tanaka M, Kondoh H, Furutani-Seiki M (2004) Mutations affecting early distribution of primordial germ cells in medaka (*Oryzias latipes*) embryo. *Mech Dev* 121:817–828
- Sasado T, Yasuoka A, Abe K, Mitani H, Furutani-Seiki M, Tanaka M, Kondoh H (2008) Distinct contributions of CXCR4b and CXCR7/RDC1 receptor systems in regulation of PGC migration revealed by medaka mutants kazura and yanagi. *Dev Biol* 320:328–339
- Schartl M (2004) A comparative view on sex determination in medaka. *Mech Dev* 121:639–645
- Shima A, Shimada A (1988) Induction of mutations in males of the fish *Oryzias latipes* at a specific locus after gamma-irradiation. *Mutat Res* 198:93–98
- Shimada A, Yabusaki M, Niwa H, Yokoi H, Hatta K, Kobayashi D, Takeda H (2008) Maternal-zygotic medaka mutants for fgfr1 reveal its essential role in the migration of the axial mesoderm but not the lateral mesoderm. *Development (Camb)* 135:281–290
- Shinomiya A, Tanaka M, Kobayashi T, Nagahama Y, Hamaguchi S (2000) The vasa-like gene, olvas, identifies the migration path of primordial germ cells during embryonic body formation stage in the medaka, *Oryzias latipes*. *Dev Growth Differ* 42:317–326
- Solnica-Krezel L, Schier AF, Driever W (1994) Efficient recovery of ENU-induced mutations from the zebrafish germline. *Genetics* 136:1401–1420
- Stainier DY, Fouquet B, Chen JN, Warren KS, Weinstein BM, Meiler SE, Mohideen MA, Neuhauss SC, Solnica-Krezel L, Schier AF, Zwartkruis F, Stemple DL, Malicki J, Driever W, Fishman MC (1996) Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. *Development (Camb)* 123:285–292
- Tanaka K, Ohisa S, Orihara N, Sakaguchi S, Horie K, Hibiya K, Konno S, Miyake A, Setiamarga D, Takeda H, Imai Y, Kudo A (2004) Characterization of mutations affecting embryonic hematopoiesis in the medaka, *Oryzias latipes*. *Mech Dev* 121:739–746
- Taniguchi Y, Takeda S, Furutani-Seiki M, Kamei Y, Todo T, Sasado T, Deguchi T, Kondoh H, Mudde J, Yamazoe M, Hidaka M, Mitani H, Toyoda A, Sakaki Y, Plasterk RH, Cuppen E (2006) Generation of medaka gene knockout models by target-selected mutagenesis. *Genome Biol* 7:R116
- van Eeden FJ, Granato M, Schach U, Brand M, Furutani-Seiki M, Haffter P, Hammerschmidt M, Heisenberg CP, Jiang YJ, Kane DA, Kelsh RN, Mullins MC, Odenthal J, Warga RM, Allende ML, Weinberg ES, Nüsslein-Volhard C (1996) Mutations affecting somite formation and patterning in the zebrafish, *Danio rerio*. *Development* 123:153–164
- Watanabe T, Asaka S, Kitagawa D, Saito K, Kurashige R, Sasado T, Morinaga C, Suwa H, Niwa K, Henrich T, Hirose Y, Yasuoka A, Yoda H, Deguchi T, Iwanami N, Kunimatsu S, Osakada M, Loosli F, Quiring R, Carl M, Grabher C, Winkler S, Del Bene F, Wittbrodt J, Abe K, Takahama Y, Takahashi K, Katada T, Nishina H, Kondoh H, Furutani-Seiki M (2004) Mutations affecting liver development and function in Medaka, *Oryzias latipes*, screened by multiple criteria. *Mech Dev* 121:791–802
- Wittbrodt J, Shima A, Schartl M (2002) Medaka: a model organism from the Far East. *Nat Rev Genet* 3:53–64
- Yamamoto TO (1965) Estriol-induced XY females of the medaka (*Oryzias latipes*) and their progenies. *Gen Comp Endocrinol* 5:527–533
- Yasuoka A, Hirose Y, Yoda H, Aihara Y, Suwa H, Niwa K, Sasado T, Morinaga C, Deguchi T, Henrich T, Iwanami N, Kunimatsu S, Abe K, Kondoh H, Furutani-Seiki M (2004) Mutations affecting the formation of posterior lateral line system in medaka, *Oryzias latipes*. *Mech Dev* 121:729–738
- Yoda H, Hirose Y, Yasuoka A, Sasado T, Morinaga C, Deguchi T, Henrich T, Iwanami N, Watanabe T, Osakada M, Kunimatsu S, Wittbrodt J, Suwa H, Niwa K, Okamoto Y, Yamanaka T, Kondoh H, Furutani-Seiki M (2004) Mutations affecting retinotectal axonal pathfinding in medaka, *Oryzias latipes*. *Mech Dev* 121:715–728

Part II

Organogenesis and Disease Models

Chapter 6

Medaka Bone Development

Akira Kudo

Abstract Recent advances of medaka bone development are described, which include osteoblast- and osteoclast-specific cells and marker genes, by using new techniques: screening of medaka bone and fin ray mutants followed by positional cloning, and transgenic lines employing osteoblast and osteoclast specific promoters linked with a fluorescent protein, in addition to electron microscopy for histology. Because live imaging becomes the most powerful tool for characterization of cell development and organ formation, studies of bone development in medaka as an animal model bring new molecular and cellular insights of bone by visual data from the medaka line specifically derived from coupling the transgenic line with a mutant.

6.1 Preface

Bone is the highly specialized connective tissue serving as the internal support system in all vertebrates. The extracellular matrix of bone is mineralized, which gives rigidity and strength to the skeleton and maintains calcium homeostasis. Bone is composed of mainly type I collagen and hydroxyapatite that is deposited in the organic matrix in bone. In mammals, bone structure consists of two forms, cortical and cancellous bones. The two forms differ in their primary function; that is, cortical bone provides mechanical and protective functions whereas cancellous bone provides metabolic function. Bone is derived from four different cell types in mammals: osteoblasts, osteoclasts, and bone lining cells located along the bone surface, and osteocytes within the bone (Marks and Hermey 1996). In bone development, neural crest cells contribute to the craniofacial bone, the sclerotome compartment of the somites generates the axial skeleton, and the lateral plate

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mesoderm forms the limb mesenchyme for the limb skeletons. Ossification is an important process in bone development, which is controlled by two mechanisms: that for intramembranous (for cortical bone) and that for endochondral (for cancellous bone) ossification (Yang 2009).

Recent advances in the study of medaka bone biology have demonstrated that the structure of bone and cartilage and bone-related genes expressed in osteoblasts, chondrocytes, and osteoclasts are well conserved between medaka and mammals; however, endochondral bone ossification and osteocytes are not present in medaka or in other fishes. In addition, in fish osteoclastogenesis, osteoclast progenitors are derived from the kidneys; that is, because the medaka has no bone marrow cavity, the hematopoietic stem cells exist in the kidney.

6.2 Bone in Medaka

Small-sized freshwater teleost fishes such as the zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) show suitable properties for the study of embryogenesis and organogenesis owing to their compact size, short generation time, and, in particular, transparency during their embryonic stages. Moreover, their embryos develop outside the mother's body, hence allowing visual live inspection and direct manipulation of cells and tissues (Ishikawa 2000).

In accord with recent progress in genomic analyses, medaka has gained much attention as a model animal of human diseases whereby the genetic background of various human diseases may be precisely deduced. Consequently, large-scale screening has provided hundreds of mutant lines that will help to identify the genes which are essential for a wide range of embryonic processes and are candidates for the cause of various human diseases (Furutani-Seiki et al. 2004; Sakamoto et al. 2004; Tanaka et al. 2004; Sakaguchi et al. 2006). In organogenesis, fish and mammals utilize similar genes; for example, fish have a bicameral heart (Sehnert and Stainier 2002; North and Zon 2003), which is equivalent to the early developmental stage of the human heart, and the fin is the homologous organ of the tetrapod limb (Garritty et al. 2002). The basic bone structure of medaka is completed at an early stage, just after hatching (Hayashida et al. 2004). Therefore, this organism is very useful for mutant screening. In fact, by recently conducting large-scale mutagenesis experiments, we obtained bone mutants having phenotypes not yet observed in zebrafish.

It is important to note that the medaka has an acellular bony skeleton lacking osteocytes (Ekanayake and Hall 1988; Inohaya et al. 2007). Moreover, cells comparable to the highly active mature osteoclasts seen in mammals and birds, which are multinucleate cells bearing a ruffled border (Lakkakorpi and Vaananen 1996; Lerner 2000; Takeshita et al. 2000), have been recently found in medaka. This fact implies a unique process of bone growth and development in this small teleost fish that is distinct from that in mammalian species. Superficially, it may not appear appropriate to utilize medaka as a model animal to clarify the mechanisms of bone formation and bone diseases of humans. However, the medaka homologs of human

bone-inducing and/or bone-regulating factors such as twist, core binding factor $\alpha 1$ (Cbfa1/Runx2), and osteoprotegerin (OPG) have been found in recent studies; and expression analysis of these genes has shown that their functions are well conserved among the vertebrates (Yasutake et al. 2004; Inohaya and Kudo 2000; Wagner et al. 2003). Despite the accumulation of large amounts of data on bone-related molecular profiles in medaka, no precise histological analysis of osteogenesis, including the presence or absence of osteoclasts in the acellular bony skeleton, has been performed except for the electron microscopic study by Ekanayake and Hall (1988), which was limited to the process forming the vertebral centrum. Here, I describe the recent progress in modern medaka bone research employing transgenic and mutant techniques and show that medaka is a good animal model for human diseases.

6.3 Mutant Screening

The medaka bone mutant screening was carried out by us. To induce mutations in the spermatogonial cells, we treated wild-type males of the Cab strain of medaka with ethylnitrosourea (ENU). Medaka fish were earlier shown to be successfully mutagenized by a single treatment with 0.1–2 mM ENU for 2 h (Ishikawa et al. 1999). A single-locus test using three pigmentation loci revealed that the average mutation rate was 3.4×10^{-4} when the fish were exposed to 1 mM ENU for 2 h. This rate was significantly lower than the average mutation rate ($1\text{--}3 \times 10^{-3}$) obtained in studies performed on zebrafish, in which the fish were exposed to 3 mM ENU for 1 h, with the treatment repeated up to six times (Mullins et al. 1994). We first applied this zebrafish protocol to the medaka. However, most of the fish were killed in such experiments. Thereafter, we treated the fish with 2.5 or 3 mM ENU for 2 h and repeated the treatment only once. Three weeks after the ENU treatment, the fish were crossed with the wild type to obtain the F_1 progeny. After maturation, a pair of the F_1 fish was crossed with each other to generate an F_2 family, and F_2 siblings were intercrossed to obtain homozygous mutants.

At 3, 5–6, and 9–10 days post fertilization (dpf), embryos and larvae were scored for abnormalities in the morphology of the developing blood cells (Sakamoto et al. 2004; Tanaka et al. 2004; Moriyama et al. 2010) and organs, including heart (Taneda et al. 2010), blood vessels, ears, and fins. At 13 dpf, larvae with normal morphology were fixed and stained with alizarin red to score the bone patterning.

By staining with alizarin red, the patterning of the calcified bones was scored and eight mutations were found to affect the vertebral patterning (Ohisa et al. 2010); a few mutations caused fusion of the vertebrae, whereas the others caused asymmetrical fusion of the vertebrae, such that the vertebrae displayed a zigzag shape. When we examined somitogenesis at the early segmentation stages, we found defective somite formation in the latter group of mutants, but no obvious abnormalities in the former group.

The patterning of the fin rays, the bony structures supporting the fins, was affected in nine mutants. In the wild-type larvae, the fin rays extended ventrally

away from the body axis. However, in the *bis* mutant, the fin rays extended both dorsally and ventrally (Sakaguchi et al. 2006). The other eight mutants had no or very small fin rays (Hibiya et al. 2009).

6.4 Vertebral Bone

The vertebral structure varies among species, but most vertebrates basically have vertebrae consisting of neural arches, hemal arches, and vertebral body (centrum). In higher vertebrates such as birds and mammals, the adjacent vertebral bodies join with an intervertebral disk, which is composed of an outer annulus fibrosus and inner nucleus pulposus. Cells derived from the sclerotome, a part of the somite, migrate toward the notochord, differentiate into cartilage, and form the model for the vertebrae and intervertebral disks. These cartilaginous anlagen of the vertebral column are subsequently replaced by bone through endochondral ossification. In teleosts, the formation of the chordal centrum is defined by the direct mineralization of the notochordal sheath by a direct intersegmental deposition of bone. Yasutake et al. (2004) demonstrated sclerotome development during medaka embryogenesis by using various sclerotomal marker genes: that is, *twist*, *pax1*, *pax9*, and *bapx1*. The medaka sclerotome appears at the ventral-most region of the somite, and the sclerotome-derived cells migrate dorsally and distribute around the notochord, neural tube, and axial blood vessels. From the results of knockdown analysis using morpholinos (MO) for the *twist* gene, which knockdown inhibits neural arch formation, *twist* was shown to function in the differentiation process of the sclerotome-derived osteoblasts. Subsequently, this MO knockdown was found to be effective within 3 days, which was the first experimental result to show the effectiveness of MO in medaka embryos. Moreover, *pax1* and *pax9* are indispensable for the development of the vertebral body and neural arch (Mise et al. 2008).

In the analysis of vertebral column formation (Inohaya et al. 2007), medaka embryos of various developmental stages are stained with alizarin red to reveal areas of calcification (Fig. 6.1). In the day 3 embryos (stage 30), only the otoliths are stained with alizarin red. In the day 4 embryos (stage 35), the cleithrum, parasphenoid, opercular bone, and a part of the first vertebra are stained with the dye. The mineralization of the vertebral column starts from the anterior part of the notochord in the day 5 embryo (stage 37). The mineralization of each centrum is initiated bilaterally at the dorsal part of the notochord and then expands to the ventral region. Thereafter, in the just-hatched larvae (day 6, stage 39), the mineralization of the vertebral column progresses to the posterior part of the notochord; and the formation of the neural arches starts from the anterior part of the vertebral column. The formation of the hemal arches is observed in the day 3 larvae. The basic structure of the medaka vertebral column is established within 9 days after fertilization.

Histological analysis and osteoblast lineage-specific promoter-driven transgenic medaka demonstrate bone development at the cellular level by tracing cell movement. Inohaya et al. (2007) developed two transgenic lines, the medaka *twist*

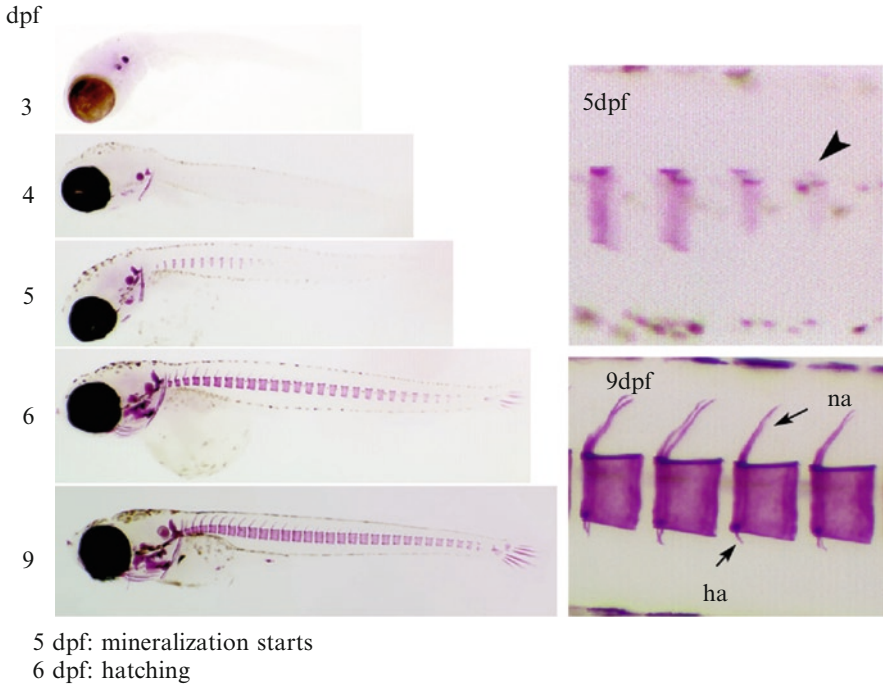


Fig. 6.1 Development of vertebral column lateral views of alizarin red-stained embryos of various developmental stages. At 5 days post fertilization (dpf), the mineralization of each centrum starts at the dorsal part of the notochord (arrowhead). *na*, neural arch (arrow); *ha*, hemal arch (arrow); *dpf*, days post fertilization. [Figure reproduced from Inohaya et al. (2007)]

promoter line and the medaka *osteocalcin* promoter transgenic line, which visualize the osteoblast progenitors and mature osteoblasts, respectively. As shown in Fig. 6.2, the deposition of the bone matrix proceeds in the direction external to the notochord, and the growth of the centrum proceeds remarkably at the anterior and posterior ends of each centrum. Also, the newly formed bone matrix at the anterior and posterior ends of each centrum is much thicker than that in its middle part, indicating that bone matrix is secreted more actively at the edges adjacent to the intervertebral ligament. In summary, the intervertebral region plays an important role in centrum growth, and the sclerotomal cells participate in the vertebral column formation in medaka. Renn and Winkler (2009) established the osterix promoter-driven transgenic medaka, which provides insight into the appearance of early osteoblasts in live imaging. These transgenic lines having the *twist*, *osterix*, and *osteocalcin* promoter characterize osteoblast-lineage cells in this order from the early to late stage of osteoblast differentiation. Although there are only a few reports on zebrafish vertebral formation, a recent paper (Laue et al. 2008) describes interesting phenotypes of the retinoic acid-oxidizing enzyme *cyp26b1* mutants, which display a reduction in midline cartilage and hyperossification of facial and axial bones. As a representative

Chordal centrum and perichordal centrum

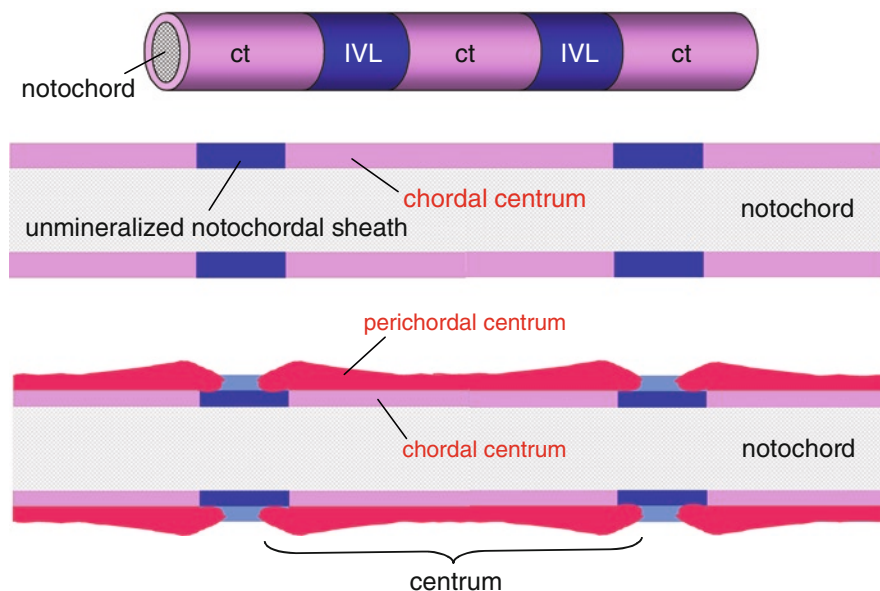


Fig. 6.2 Scheme of vertebral column formation *IVL* indicates an intervertebral region including an intervertebral ligament and bone edges of two adjoining centra; *ct*, centrum. The vertebra comprises two kinds of centrum, namely, the chordal centrum and the perichordal centrum. Mineralization occurs within the notochordal sheath, and then the chordal centrum is first formed within the notochordal sheath before the formation of the perichordal centrum. Next, the dynamic growth of the perichordal centrum occurs because the osteoblast progenitors, originating from the sclerotome, are first located in the intervertebral region to participate in bone matrix formation; and then they become located in the centrum region and differentiate into mature osteoblasts. The middle part of the perichordal bone layer grows more slowly than the anterior or posterior end of each centrum

medaka mutant, the *vbi* mutant was shown by Ohisa et al. (2010) to display defects in notochord sheath formation and dwarfism. The *vbi* gene encodes *sec24d* a subunit of coatamer complex, which is critical for protein transportation from the endoplasmic reticulum to the Golgi apparatus. The data show that the defect of *sec24d* causes specific accumulation of type II collagen inside of cells. To reveal the molecular mechanisms of vertebral bone development, further analyses of mutant medaka are required. Recently, we found that *wnt4b* is essential for the segment patterning of the vertebral column in medaka (Inohaya et al. 2010).

6.5 Osteoclasts

In mammals, osteoclasts differentiate from osteoclast progenitors, which are a type of macrophage, into mononuclear tartrate-resistant acid phosphatase (TRAP)-positive, cathepsin K-positive osteoclasts that subsequently become mature

multinucleate osteoclasts. Both mononuclear and multinucleate osteoclasts resorb bone after forming a ruffled border (Lerner 2000; Takeshita et al. 2000). Witten et al. (1997) reported mononuclear osteoclasts in the acellular bony skeleton of a teleost. In the bony skeleton of the zebrafish, which comprises cellular bones (bones with osteocytes), both mono- and multinucleate TRAP-positive cells lack a ruffled border (Witten et al. 2001).

We performed a thorough examination of the expression patterns of certain osteoclast-related genes, and also carried out histochemical and ultrastructural analyses to identify the putative sites of bone remodeling in the acellular bony skeleton of the medaka. Nemoto et al. (2007) isolated three osteoclast-related genes, *cathepsin K*, *TRAP*, and *V-ATPase*, from medaka, performed RNA in situ hybridization of these genes, and thereby obtained molecular evidence for the presence of osteoclasts in this small teleost. Some of the TRAP-positive cells display all the morphological characteristics equivalent to those of mammalian osteoclasts. These cells are associated primarily with the shedding teeth and their supporting bones (pedicles), where alkaline phosphatase (ALPase)-positive osteoblasts are also observed, implying progressive bone remodeling associated with tooth replacement in this region. In contrast, the inner aspects of the neural and hemal arches of the vertebral column, which are the only sites of bone resorption other than the tooth-bearing bones, show rather flat TRAP-positive osteoclasts, which are essential for bone modeling of neural and hemal arches; and this specific bone resorption provides the space for the neural tube and blood vessel. The results of histological analysis have provided ample evidence that allows us to conclude the existence of dynamic bone remodeling by the coupled actions of osteoblasts and osteoclasts in the skeletal system of the medaka, particularly in the tooth-bearing pharyngeal bones. These data indicate this small fish to be a good experimental model for the study of bone remodeling and modeling, especially for in vivo analysis of osteoclast differentiation. Furthermore, recent advances in the development of osteoclast-specific transgenic medaka, obtained by using TRAP and cathepsin K promoters, demonstrate osteoclast lifespan, differentiation, fusion, resorption, and death by in vivo imaging (Chatani et al. 2008).

6.6 Fin Ray

The fin rays consist of segmented calcified bones and exhibit a periodic actiniform pattern. Vertebrate limb development begins with the specification of limb position and polarities. Members of the fibroblast growth factor (FGF) family of proteins are critical for the induction, maintenance, and function of the apical ectodermal ridge (AER), an ectodermal region of limbs, which is necessary for the outgrowth of the limb bud. Following the specification and formation of the limb buds, three-dimensional axes need to be constructed in the developing limbs. It has been revealed that a molecular cascade involving the key molecule Sonic hedgehog establishes the anterior–posterior (AP) axis of the limb. As for proximal–distal (PD) patterning of the limbs, it is specialized by several Hox-family molecules.

Two positional clonings from medaka fin ray mutants revealed the molecular mechanisms of fin ray formation, which are related to Hox regulation. Sakaguchi et al. (2006) identified the *hoxb8a* mutant, *unextended-fin (ufi)*, in which all the fin tissues are malformed (Fig. 6.3). As the abnormal phenotype is observed in the caudal fin, the *ufi* phenotype suggests that the medaka Hoxb8a has a fundamental role in the formation of appendages protruding from the trunk. Moreover, the numbers of proliferating cells and osteoblastic cells in the mutant are increased, suggesting that the medaka Hoxb8a functions in the outgrowth of appendages through the regulation of cell migration and osteoblast differentiation. Figure 6.4

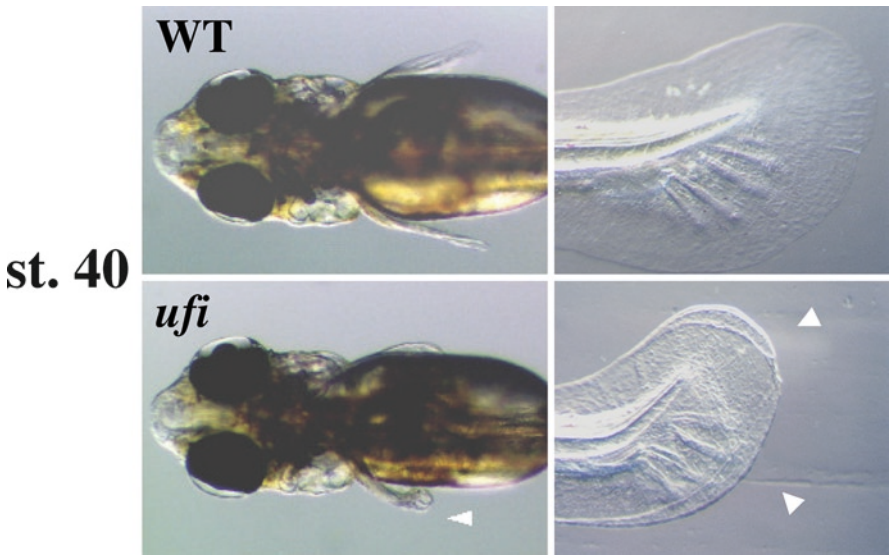


Fig. 6.3 The medaka fin mutant *unextended-fin (ufi)*. All the fin tissues of *ufi* are malformed. Arrowheads indicate malformations. WT, wild type; *st.*, stage

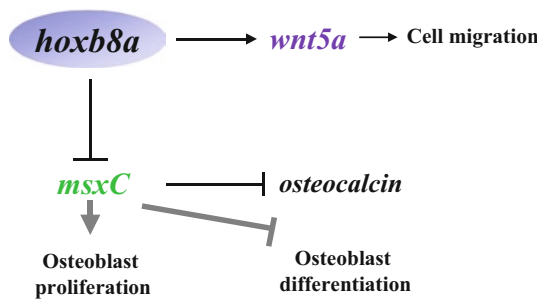


Fig. 6.4 Schematic showing possible functions of Hoxb8a in the formation of appendage tissues

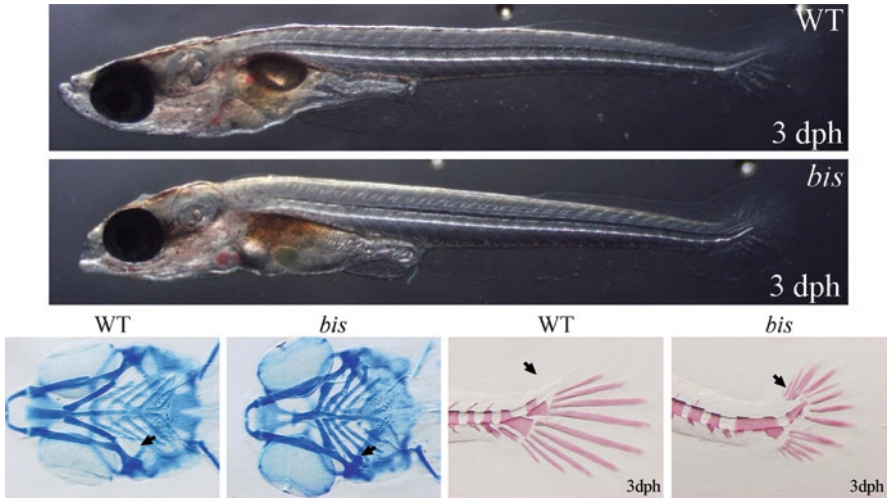


Fig. 6.5 The medaka mutant *biaxial symmetries* (*bis*) shows defects in both the anterior–posterior axis of the craniofacial skeleton and the dorsal–ventral axis of the caudal skeleton. Arrows point to the ectopic proliferating cells at the dorsal side of the caudal region in the *bis* mutant, compared with the wild type (*WT*)

shows a schematic of the possible functions of Hoxb8a. Hoxb8a activates cell migration via *wnt5a*, the noncanonical Wnt pathway, during fin growth. Hoxb8a suppresses the osteoblast proliferation by repressing *msxC*, and promotes the osteoblast differentiation.

Hibiya et al. (2009) characterized a medaka mutant named *biaxial symmetries* (*bis*), in which *brpf1*, a subunit of the MOZ histone acetyl transferase (HAT) complex, is mutated (Fig. 6.5). The *bis* mutant displays patterning defects in both the anterior–posterior axis of the craniofacial skeleton and the dorsal–ventral axis of the caudal skeleton. *Brpf1* is required for the maintenance of expression of 5' and 3' *hox* genes (Fig. 6.6). This regulation commonly occurs in mice, as detected by the analysis of *MOZ*-deficient mice, demonstrating the common role of the MOZ HAT complex in vertebrates, a complex that is required for the proper patterning for skeletal development.

6.7 Fin Regeneration

The medaka fin regenerates about 2 weeks after amputation. Fin regeneration proceeds by formation of a thickened wound epidermis and blastema at the end of the stump. The blastema is a mass of undifferentiated mesenchymal cells that reconstitute the missing part of the fin, suggesting that blastema formation is essential for the regeneration process (Nakatani et al. 2007). For identification of genes that play

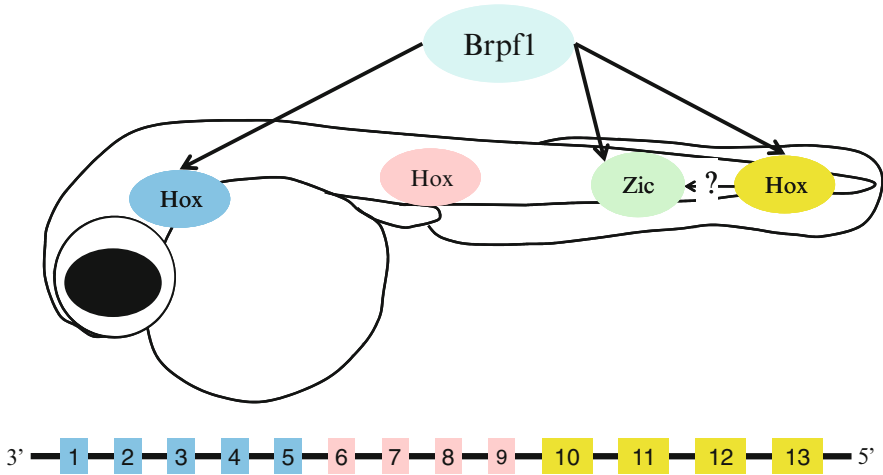


Fig. 6.6 The promising function of Brpf1 in the Hox gene transcription. Brpf1 maintains the transcription of 3' and 5' Hox family genes but not that of central Hox genes. Zic genes are involved in caudal skeleton formation; however, their molecular relation with Hox genes is uncertain

important roles in the blastema formation and differentiation leading to reconstitution of fin structures, expressed sequence tag (EST) analysis was performed by using two cDNA libraries, one from the generating fin at 3 days post amputation (dpa) and the other from that at 10 dpa to characterize genes associated with blastema formation and regenerative outgrowth, respectively (Katogi et al. 2004). Collagens such as type I, II, V, VIII, X, XI, and XII are found in the ESTs, and of these collagens, type X collagen is specifically expressed in the regenerating fin ray at 10 dpa. Observations of transverse sections indicate strong expression in the prospective mineralizing region of the fin ray, which gradually decreases in the proximal portion where the fin ray becomes completely mineralized. Furthermore, osteonectin expression is found in the regenerating fin ray at 10 dpa. Because osteonectin is a 32-kDa glycoprotein that forms a complex with collagen fibers and hydroxyapatite, it is thought to initiate active mineralization in skeletal tissues. The expression patterns of *type X collagen* and *osteonectin* suggest that bone deposition in the regenerating fin ray is caused by the accumulation of collagens and their subsequent mineralization induced by osteonectin. Regarding blastema formation, microarray screening of transcripts upregulated during regeneration showed that 12 transcripts were detected either in differentiating cartilage, basal wound epidermis, or blastema (Nishidate et al. 2007). In addition, to find the detailed cellular behaviors and their molecular basis, a chemical inhibitor was used, specifically to reveal the role of phosphoinositide 3-kinase (PI3K) signaling (Nakatani et al. 2008). The PI3K inhibitor impaired fin regeneration by suppressing the migration of mesenchymal cells fated to the blastema and the induction of blastema-specific gene expression, suggesting that mesenchymal migration and the

resulting induction of blastema-specific gene expression are necessary steps for blastema formation. In this analysis, the vascular anatomy of the developing medaka is very useful information strongly connected with regeneration (Fujita et al. 2006).

6.8 Otolith

The mechanisms of inner ear formation and otolith calcification remain unknown. In the analysis of mRNA expression of biomineralization-related genes in the otic vesicles of medaka, as otolith formation is based on matrix vesicle mineralization, *osteonectin* and *type II collagen (col2a)* show spatiotemporal expression patterns during otic development in medaka (Nemoto et al. 2008). Particularly, a chondrocyte-modulating and angiogenesis-inhibiting factor, *chondromodulin-1*, is expressed exclusively in the medial wall of the otic vesicle.

6.9 Postscript

Medaka bone development is faster than that of zebrafish, indicating that mutant screening and morpholinos knockdown experiments are more suitable for the medaka than for the zebrafish. These beneficial properties resulted in generation of vertebral bone mutants that are available only in the medaka. Although the studies on medaka bone development have just started, we expect much progress in this field by efficiently performing the positional cloning of mutants and by employing new technologies including inducible gene expression-transgenic medaka.

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References

- Chatani M, Inohaya K, Kudo A (2008) In-vivo imaging for osteoclasts in medaka, showing the evidence of bone remodeling (2008). In: Abstracts, International Conference on Zebrafish Development and Genetics, p 314
- Ekanayake S, Hall BK (1988) Ultrastructure of the osteogenesis of acellular vertebral bone in the Japanese medaka, *Oryzias latipes* (Teleostei, Cyprinodontidae). *Am J Anat* 182:241–249
- Fujita M, Isogai S, Kudo A (2006) The vascular anatomy of the developing medaka, *Oryzias latipes*: a complementary fish model for the cardiovascular research of vertebrate. *Dev Dyn* 235:734–746

- Furutani-Seiki M, Sasado T, Morinaga C et al (2004) A systematic genome-wide screen for mutations affecting organogenesis in medaka, *Oryzias latipes*. *Mech Dev* 121:647–658
- Garrity DM, Childs S, Fishman MC (2002) The heartstrings mutation in zebrafish causes heart/fin Tbx5 deficiency syndrome. *Development (Camb)* 129:4635–4645
- Hayashida Y, Kawamura T, Hori-e R, Yamashita I (2004) Retinoic acid and its receptors are required for expression of aryl hydrocarbon receptor mRNA and embryonic development of blood vessel and bone in the medaka fish, *Oryzias latipes*. *Zool Sci* 21:541–551
- Hibiya K, Katsumoto T, Kondo T, Kitabayashi I, Kudo A (2009) Brpf1, a subunit of the MOZ histone acetyl transferase complex, maintains expression of anterior and posterior Hox genes for proper patterning of craniofacial and caudal skeletons. *Dev Biol* 329:176–190
- Inohaya K, Kudo A (2000) Temporal and spatial patterns of cbfal expression during embryonic development in the teleost, *Oryzias latipes*. *Dev Genes Evol* 210:570–574
- Inohaya K, Takano Y, Kudo A (2007) The teleost intervertebral region acts as a growth center of the centrum: in vivo visualization of osteoblasts and their progenitors in transgenic fish. *Dev Dyn* 236:3031–3046
- Inohaya K, Takano Y, Kudo A (2010) Production of Wnt4b by floor plate cells is essential for the segmental patterning of the vertebral column in medaka. *Development* 137:1807–1813
- Ishikawa Y (2000) Medakafish as a model system for vertebrate developmental genetics. *BioEssays* 22:487–495
- Ishikawa Y, Hyodo-Taguchi Y, Aoki K, Yasuda T, Matsumoto A, Sasanuma M (1999) Induction of mutations by ENU in the medaka germline. *Fish Biol J Medaka* 10:27–29
- Katogi R, Nakatani Y, Shin-I T, Kohara Y, Inohaya K, Kudo A (2004) Large-scale analysis of the genes involved in fin regeneration and blastema formation in the medaka, *Oryzias latipes*. *Mech Dev* 121:861–872
- Lakkakorpi PT, Vaananen HK (1996) Cytoskeletal changes in osteoclasts during the resorption cycle. *Microsc Res Tech* 33:171–181
- Laue K, Janicke M, Plaster N, Sonntag C, Hammerschmidt M (2008) Restriction of retinoic acid activity by Cyp26b1 is required for proper timing and patterning of osteogenesis during zebrafish development. *Development (Camb)* 135:3775–3787
- Lerner UH (2000) Osteoclast formation and resorption. *Matrix Biol* 19:107–120
- Marks SC, Hermey DC (1996) The structure and development of bone. In: Bilezikian JP et al (eds) *Principles of bone biology*. Academic Press, London
- Mise T, Iijima M, Inohaya K, Kudo A, Wada H (2008) Function of Pax1 and Pax9 in the sclerotome of medaka fish. *Genesis* 46:185–192
- Moriyama A, Inohaya K, Maruyama K, Kudo A (2010) Bcf medaka mutant reveals the essential role of c-myb in both primitive and definitive hematopoiesis. *Dev Biol* 345:133–143
- Mullins MC, Hammerschmidt M, Haffter P, Nusslein-Volhard C (1994) Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. *Curr Biol* 4:189–202
- Nakatani Y, Kawakami A, Kudo A (2007) Cellular and molecular processes of regeneration, with special emphasis on fish fins. *Dev Growth Differ* 49:145–154
- Nakatani Y, Nishidate M, Fujita M, Kawakami A, Kudo A (2008) Migration of mesenchymal cell fated to blastema is necessary for fish fin regeneration. *Dev Growth Differ* 50:71–83
- Nemoto Y, Higuchi K, Baba O, Kudo A, Takano Y (2007) Multinucleate osteoclasts in medaka as evidence of active bone remodeling. *Bone (NY)* 40:399–408
- Nemoto Y, Chatani M, Inohaya K, Hiraki Y, Kudo A (2008) Expression of marker genes during otolith development in medaka. *Gene Expr Patterns* 8:92–95
- Nishidate M, Nakatani Y, Kudo A, Kawakami A (2007) Identification of novel markers expressed during fin regeneration by microarray analysis in medaka fin. *Dev Dyn* 236:2685–2693
- North TE, Zon LI (2003) Modeling human hematopoietic and cardiovascular diseases in zebrafish. *Dev Dyn* 228:568–583
- Ohisa S, Inohaya K, Takano Y, Kudo A (2010) sec24d encoding a component of COPII is essential for vertebra formation, revealed by the analysis of the medaka mutant, vbi. *Dev Biol* 342:85–95

- Renn J, Winkler C (2009) Osterix-mCherry transgenic medaka for in vivo imaging of bone formation. *Dev Dyn* 238:241–248
- Sakaguchi S, Nakatani Y, Takamatsu N, Hori H, Kawakami A, Inohaya K, Kudo A (2006) Medaka unextended-fin mutants suggest a role for Hoxb8a in cell migration and osteoblast differentiation during appendage formation. *Dev Biol* 293:426–438
- Sakamoto D, Kudo H, Inohaya K, Yokoi H, Narita T, Naruse K, Mitani H, Shima A, Ishikawa Y, Imai Y, Kudo A (2004) A mutation in the gene for δ -aminolevulinic acid dehydratase (ALAD) causes hypochromic anemia in the medaka *Oryzias latipes*. *Mech Dev* 121:747–752
- Sehnert AJ, Stainier DY (2002) A window to the heart: can zebrafish mutants help us understand heart disease in humans? *Trends Genet* 18:491–494
- Takeshita S, Kaji K, Kudo A (2000) Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts. *J Bone Miner Res* 15:1477–1488
- Tanaka K, Ohisa S, Orihara N, Sakaguchi S, Horie K, Hibiya K, Konno S, Miyake A, Setiamarga D, Takeda H, Imai Y, Kudo A (2004) Characterization of mutations affecting embryonic hematopoiesis in the medaka, *Oryzias latipes*. *Mech Dev* 121:739–746
- Taneda Y, Konno S, Makino S, Morioka M, Fukuda K, Imai Y, Kudo A, Kawakami A (2010) Epigenetic control of cardiomyocyte production in response to a stress during the medaka heart development. *Dev Biol* 340:30–40
- Wagner TU, Renn J, Riemensperger T et al (2003) The teleost fish medaka (*Oryzias latipes*) as genetic model to study gravity dependent bone homeostasis in vivo. *Adv Space Res* 32:1459–1465
- Witten PE, Bendahmane M, Abou-Haila A (1997) Enzyme histochemical characteristics of osteoblasts and mononucleated osteoclasts in a teleost fish with acellular bone (*Oreochromis niloticus*, Cichlidae). *Cell Tissue Res* 287:591–599
- Witten PE, Hansen A, Hall BK (2001) Features of mono- and multinucleated bone resorbing cells of the zebrafish *Danio rerio* and their contribution to skeletal development, remodeling, and growth. *J Morphol* 250:197–207
- Yang Y (2009) Skeletal morphogenesis and embryonic development. In: *Primer on the metabolic bone diseases and disorders of mineral metabolism*, 7th edn. American Society for Bone and Mineral Research (ASBMR), Washington, DC
- Yasutake J, Inohaya K, Kudo A (2004) Twist functions in vertebral column formation in the medaka, *Oryzias latipes*. *Mech Dev* 121:883–894

Chapter 7

Anatomical Atlas of Blood Vascular System of Medaka

Sumio Isogai and Misato Fujita

Abstract The zebrafish and the medaka provide a number of advantageous features for the analysis of cardiovascular development. To fully exploit the advantages of the teleost fish, there must be detailed knowledge of the normal pattern of the vascular system from the embryo through to the adult, which enables us to detect the mutants, interpret the results of experimental or genetic perturbations, and conduct cross-species comparisons. We visualized the gill, intestinal, renal, and parietal (including brain and spinal cord) blood vascular systems in adult medaka using intravascular resin casting and dye injection methods. Here, we provide an anatomical atlas of blood vascular systems of adult fish with brief descriptions to give a morphological foundation for future cardiovascular research using the medaka.

7.1 Introduction

Zebrafish and the medaka provide a number of advantageous features as model organisms for morphological, experimental, and genetic analysis, including their small size, fecundity, development external to the mother, and the optical clarity of the embryo. The optical clarity and accessibility of the embryo permit efficient application of experimental embryological methods for in vivo analysis of cardiovascular development. Because of their small size, the embryos receive enough

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oxygen, by passive diffusion, to survive and continue to develop in reasonably normal fashion for several days even in the complete absence of blood circulation, facilitating phenotypic analysis of animals with circulatory defects. This combination of features has made it possible to perform large-scale forward genetic screens to isolate embryonic and early larval lethal mutations in the zebrafish and the medaka that specifically affect the circulatory system. The screens have yielded some mutants with phenotypes that closely resemble human genetic diseases and therefore can serve as models for them. To fully exploit the advantages of the teleost fish for studying cardiovascular development, there must be detailed knowledge of the normal pattern of the system from the embryo through to the adult to enable us to detect mutants, interpret the results of experimental or genetic perturbations, and perform cross-species comparisons. In the zebrafish, the rapid progress of various infrastructure initiatives is facilitating study of the developing heart, blood cells, and blood vessels. We previously characterized the vascular anatomy of the developing zebrafish embryo and early larva using microangiography (<http://eclipse.nichd.nih.gov/nichd/lmg/redirect.html>). These studies revealed that the developing vasculature of the zebrafish differs in certain respects from the primary circulatory system conserved in vertebrates. The medaka is a complementary fish model that holds promise to provide essential information on the development of the primary vertebrate vascular system that is lacking in the zebrafish (<http://www.shigen.nig.ac.jp/medaka/atlas/>). To establish a firm morphological foundation for future cardiovascular research using medaka, we provide here an anatomical atlas of the blood vascular system of the adult medaka fish, visualized using intravascular resin casting and dye injection methods.

7.2 Branchial Blood Vascular System

The sinus venosus, which receives venous blood from the hepatic veins and from the right and left common cardinal veins, opens into the atrium of the medaka heart. The ventricle and atrium are partitioned off by atrioventricular valves. The tapering rostral end of the ventricle, provided also with valves, passes into the bulbus arteriosus, and from there continues into the ventral aorta (Fig. 7.1a–d). There are four pairs of fully developed *holobranches* carrying double gill filaments in medaka (Fig. 7.2a, b, d). The first three pairs of afferent branchial vessels branch off independently from the tapering ventral aorta whereas the common root of the fourth afferent branchial vessel and the pharyngeal artery arises recurrently from the ventral aorta near the heart (Figs. 7.1a and 7.2a). In each gill arch, the single afferent vessel passes underneath the efferent vessel, which bifurcates at its ventralmost side, and passes dorsally up the arch along the lateral side of the efferent vessel (Fig. 7.2c–e). The afferent filament vessel stems from the afferent branchial vessel brings venous (less oxygenated) blood into the gill filament, and then the afferent lamella vessel into the gill lamella (Fig. 7.2c–e, g). Richly oxygenated blood from the gill vascular plexus flows out of the gill lamella and filaments via the efferent lamella and filament vessels, respectively (Fig. 7.2f), and then drains into the

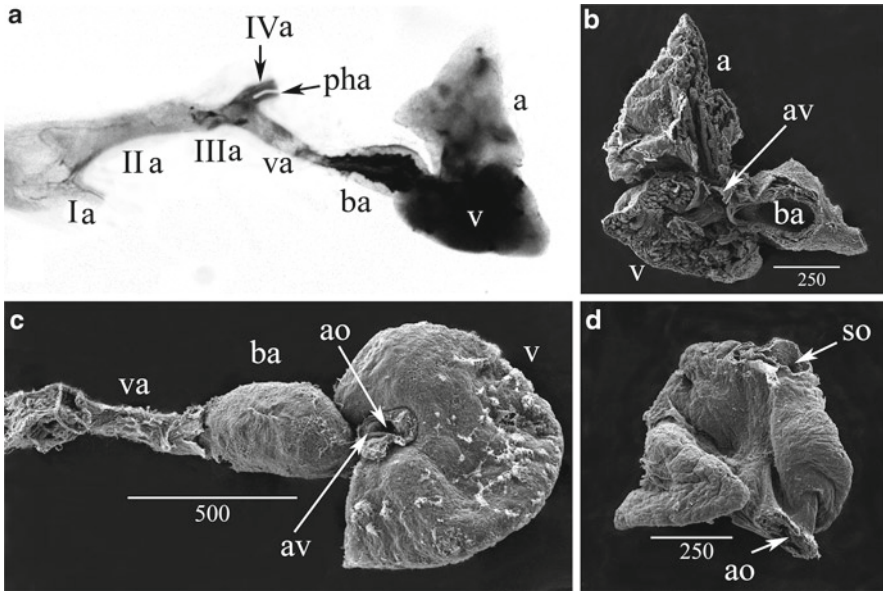


Fig. 7.1 Heart. **a** Left lateral view of dye-injected heart. **b** Scanning electron microscopic (SEM) image of atrium, ventricle, and bulbus arteriosus viewed from medial side. To show the inside of the heart, the right wall was removed. **c** SEM image of ventricle, bulbus arteriosus, and ventral aorta viewed from dorsal side. To show the ventricle, the atrium was removed. **d** SEM image of atrium. Dorsal is to the *top* in **a**, **b**, and **d**; rostral is to the *left* in **a**, **c**. *Ia–IVa*, first to fourth afferent branchial vessels; *a*, atrium; *ao*, atrioventricle ostium; *av*, atrioventricle valve; *ba*, bulbus arteriosus; *pha*, pharyngeal artery; *so*, sinuatrial ostium; *va*, ventral aorta; *v*, ventricle. Bars **a–d**, micrometers (μm)

efferent branchial vessel (Fig. 7.2b–e). As shown in Fig. 7.2b, e, the single efferent vessel bifurcates at its dorsalmost end. The first and second efferent branchial vessels merge into the lateral dorsal aorta on each side and then unite into the single dorsal aorta beneath the vertebrae. The merged third and fourth efferent branchial vessels join separately with the single aorta at a more caudal point. In contrast to the other gills, the pseudobranch receives oxygen-rich blood by the orbital artery (designated also as the afferent vessel of pseudobranch in medaka) directly from the internal carotid artery and redrains into the orbital artery by the efferent vessel (Fig. 7.2h; and see Fig. 7.8a, d).

7.3 Intestinal Blood Vascular System

7.3.1 Coeliaco-Mesenteric Artery

The coeliaco-mesenteric artery branches off ventrolaterally to the right side from the dorsal aorta, just caudal to the junction of the aortic radix and junction of the third and fourth efferent branchial vessels with the aorta (Fig. 7.3b–1). As a unique

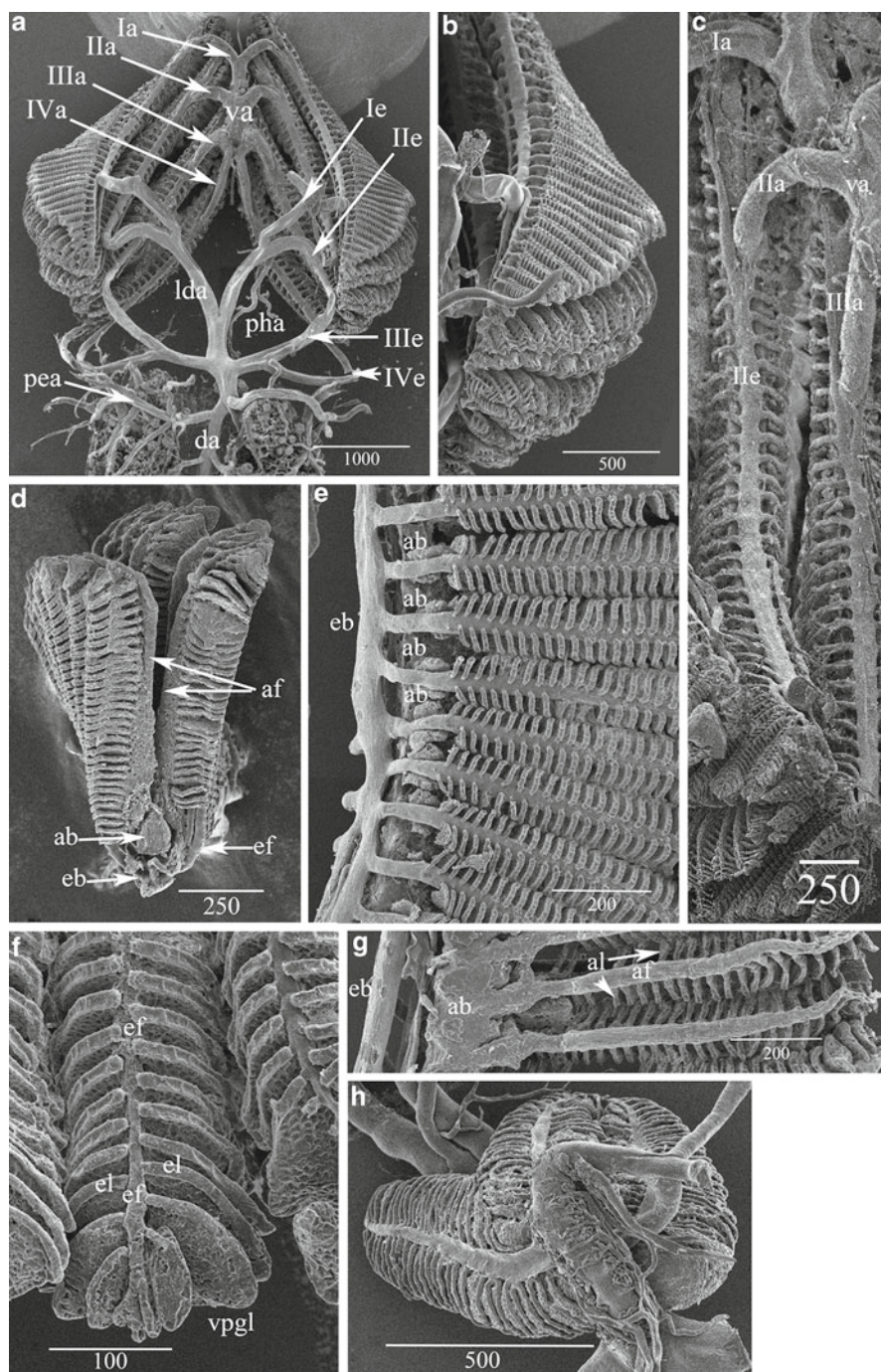


Fig. 7.2 Branchial blood vascular system visualized by corrosive resin casts (SEM images). **a** Branchial vascular system viewed from dorsal side. **b** Closer view of first to fourth holobranch on right side. **c** Afferent branchial vessel passes underneath the efferent branchial vessel at the pharyngeal base. **d** Holobranch carrying double gill filaments. **e** Outer view of gill filaments.

arterial trunk to the alimentary canal, it runs caudally through to the rectum, giving branches into all the visceral organs and gonads (Fig. 7.3c) except the caudal end of the rectum, anus, and cloaca, which are supplied by the ventral intersegmental artery (Fig. 7.3g-1, g-2). Following the pharynx (Fig. 7.3b-1, b-2), the alimentary canal of the medaka is anatomically divided into four parts – esophagus, cranial intestine, caudal intestine, and rectum – although it is difficult to distinguish histologically the gastric part from the intestine (Iwamatu). The bulged cranial intestine (*pseudogaster*), adjacent to the liver and spleen, runs ventral-caudally within the left ventral side of the body cavity. The following thinner caudal intestine makes a right-angle turn in a horizontal plane, extends convolutedly craniodorsally along the right lateral body wall, and then makes another sharp U-shaped turn caudally to the rectum (Fig. 7.3a1–a4). The swim bladder is a median dorsal diverticulum of the alimentary canal lying in a position retroperitoneal to the gonads. The gonadal artery stems from the proximal part of the coeliaco-mesenteric artery, and the red body of the swim bladder (Fig. 7.3d) seems to share its branch with the gonadal artery. Caudal to the gonadal artery, the pseudogaster-lienal artery, which supplies the wall of the pseudogaster and the spleen, also emerges from the coeliaco-mesenteric artery (Fig. 7.3c). We could not recognize any proper hepatic arteries. Giving small branches to the intestine, the coeliaco-mesenteric artery runs along the dorsal wall of the rectum. Each part of the gut shows characteristic features in its vascular patterning (Fig. 7.3e1–e3, f1–f3, g1–g3; esophagus is not shown).

7.3.2 Intestinal Veins and Hepatic Portal System

The hepatic portal system is composed of veins that collect blood from the intestine and transport it to the sinusoids of the liver. Beyond the liver sinusoids, the blood is recollected into the hepatic veins to empty into the common cardinal veins. In medaka, the cranial (superior) mesenteric vein collects blood from the caudal half of the pseudo-gastric intestine and the anterior part of the caudal intestine, while the caudal (inferior) mesenteric vein runs cranially collecting blood from the caudal end of the caudal intestine and the rectum. The caudal mesenteric vein merges with the lienal vein first, and then with the cranial mesenteric vein, forming the hepatic portal vein, which runs along the bile duct to transport the venous blood from the intestine to the hepatic sinusoids (Fig. 7.3c). Blood from the cranial part of the pseudogastric intestine drains directly into the hepatic sinusoids without traveling through the portal venous system (Fig. 7.3e-1).

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Fig. 7.2 (continued) **f** Regularly arranged gill lamellae at the tip of gill filament. **g** Inner view of gill filaments. To show the medial side of the holobranch, the hemibranch was removed. **h** Pseudobranch. Rostral is to the top in **a**, **b**, and **c**. *Ia–IVa* (*ab*), first to fourth afferent branchial vessel; *Ie–IVe* (*eb*), first to fourth efferent branchial vessel; *af*, afferent filament vessel; *al*, afferent lamella vessel; *da*, dorsal aorta; *ef*, efferent filament vessel; *el*, efferent lamella vessel; *lda*, lateral dorsal aorta; *pea*, pectoral (subclavian) artery; *pha*, pharyngeal artery; *va*, ventral aorta; *vpgl*, vascular plexus of gill lamella. Bars **a–h**, μm

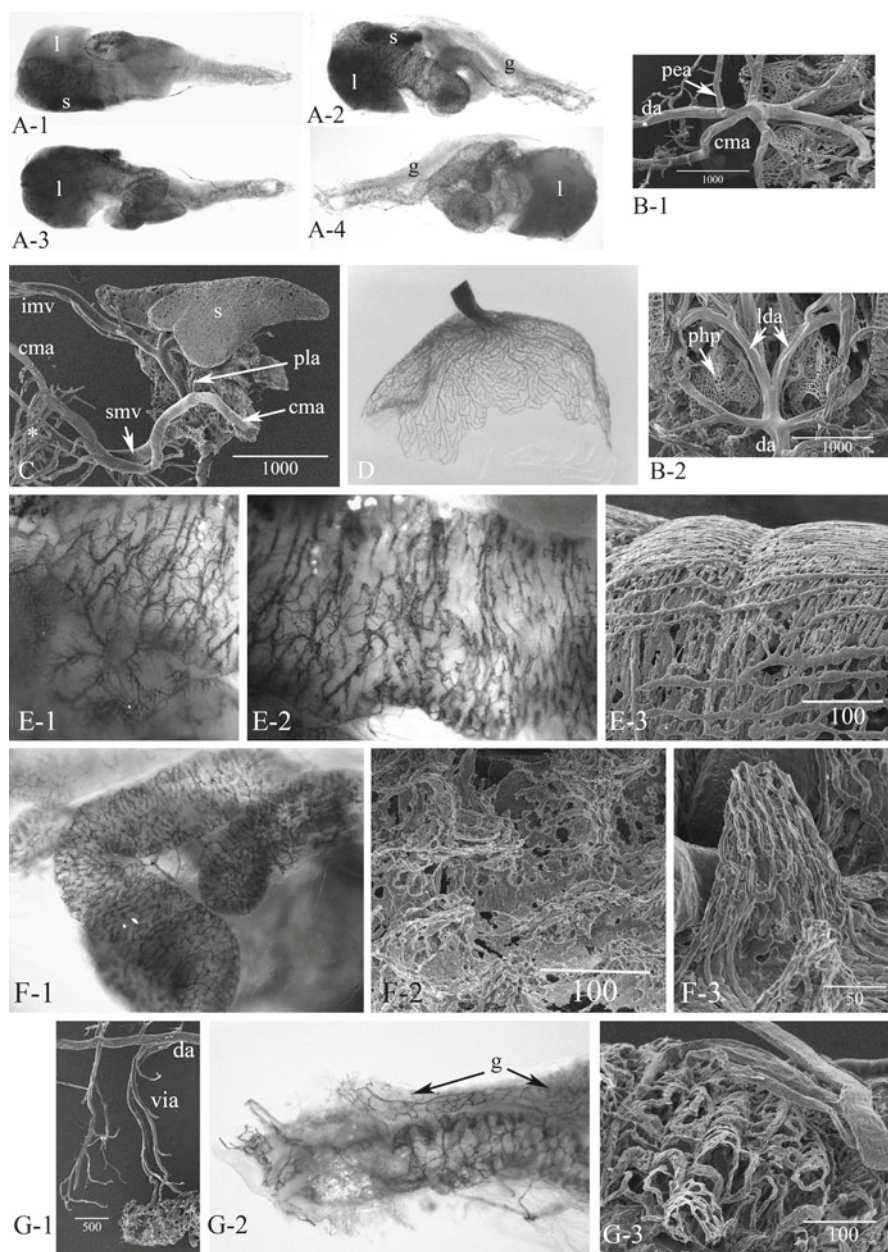


Fig. 7.3 Intestinal blood vascular system visualized by dye injection and corrosive resin casts (SEM images). **a** Contour of intestinal loop: 1, dorsal view; 2, left lateral view; 3, ventral view; 4, right lateral view. **b** Root of coeliaco-mesenteric artery and pharyngeal vascular plexus (with bone): 1, viewed from right dorsolateral side; 2, viewed from dorsal side. **c** Branching pattern of coeliaco-mesenteric artery and hepatic portal vein viewed from right dorsolateral side. **d** Rete mirabile in the red body of swim bladder. **e** 1, Direct drainage from pseudogaster vascular plexus

7.4 Renal Blood Vascular System

7.4.1 Renal Arteries

The teleostean definitive kidney (mesonephros) has generally been divided into the head kidney, consisting of lymphomyeloid tissue, and the trunk kidney, composed of numerous nephrons and interstitial lymphomyeloid tissue. Although the medaka kidney does appear to be divided into a flat cranial and a cylindrical caudal part, this distinction is not clear at the histological level because the entire kidney is composed of both nephrons and interstitial lymphoid tissue. The cranial part (head kidney) is supplied with arterial blood by the pectoral (subclavian) artery cranially and by intersegmental arteries more caudally. Both arteries branch off several sprigs into the renal tissue as they pass along it (Figs. 7.4a and 7.5a). These renal arterioles further branch, distributing to glomeruli in a manner that resembles a bunch of grapes (Fig. 7.6b). In the caudal part (trunk kidney), renal tissue receives its arterial supply from sprigs branching directly from the dorsal aorta or from the intersegmental arteries (Fig. 7.6d). Each segmentally appearing caudal renal arteriole serves as the afferent artery for a single glomerulus (or often, double glomeruli) (Figs. 7.5e–h and 7.6d). The efferent arteries from each glomerulus, in either the cranial or caudal part, drain into the mesonephric sinusoid plexus described below (Fig. 7.6c). It should be noted that the pronephric glomerulus persists into adulthood in medaka (Fig. 7.6a).

7.4.2 Renal Veins and Renal Portal System

The venous blood from the trunk and the tail does not go directly cranial ward through the original posterior cardinal veins, but is instead shunted through a capillary network around the mesonephric tubules and ducts (mesonephric sinusoid), from whence it returns cranially through the posterior and common cardinal veins. The single posterior cardinal vein (the continuation of the caudal vein) passes cranially between the right and left mesonephric ducts, bifurcating along each nephric duct in the cranial trunk region. In medaka, the right posterior cardinal vein mostly

←
Fig. 7.3 (continued) into hepatic sinusoid; 2, 3, vascular plexus of pseudogaster. **f** 1, Vascular plexus of intestine; 2, vascular plexus of intestinal villi arranged in lines; 3, vascular plexus in an intestinal villus. **g** 1, Ventral intersegmental artery distributing to the caudal end of rectum, anus, and cloaca; 2, vascular plexuses in anus, rectum, and gonad; 3, closer view of rectum. Dorsal is to the *top* and rostral is to the *right* in **b-1**, **c**, **f-1**, **g-1**, **g-2**; dorsal is to the *top* and rostral is to the left in **e-1**, **e-2**. *cma*, coeliaco-mesenteric artery; *da*, dorsal aorta; *g*, gonad; *imv*, caudal (inferior) mesenteric vein; *l*, liver; *lda*, lateral dorsal aorta; *pea*, pectoral (subclavian) artery; *php*, pharyngeal vascular plexus; *pla*, pseudogaster-lienal artery; *s*, spleen; *smv*, cranial (superior) mesenteric vein; *via*, ventral intersegmental artery; *, small branches to the intestine. Bars **b**, **c**, **e**–g, μm

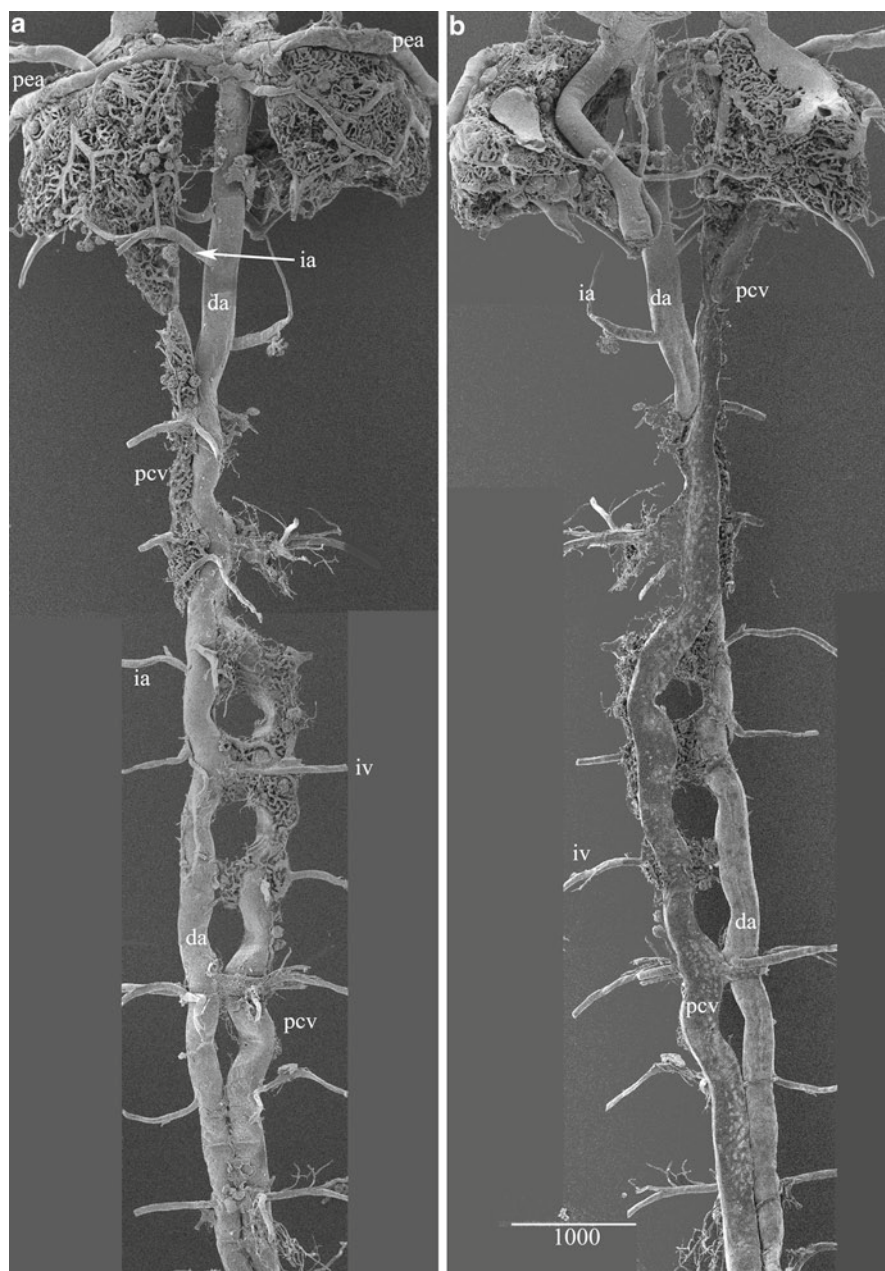


Fig. 7.4 Renal portal system visualized by corrosive resin casts (SEM images): **a** dorsal view; **b** ventral view. Cranial is to the *top* in **a**, **b**. *da*, dorsal aorta; *ia*, intersegmental artery; *iv*, intersegmental vein; *pcv*, posterior cardinal vein; *pea*, pectoral (subclavian) artery. Bar **b** 1,000 μm

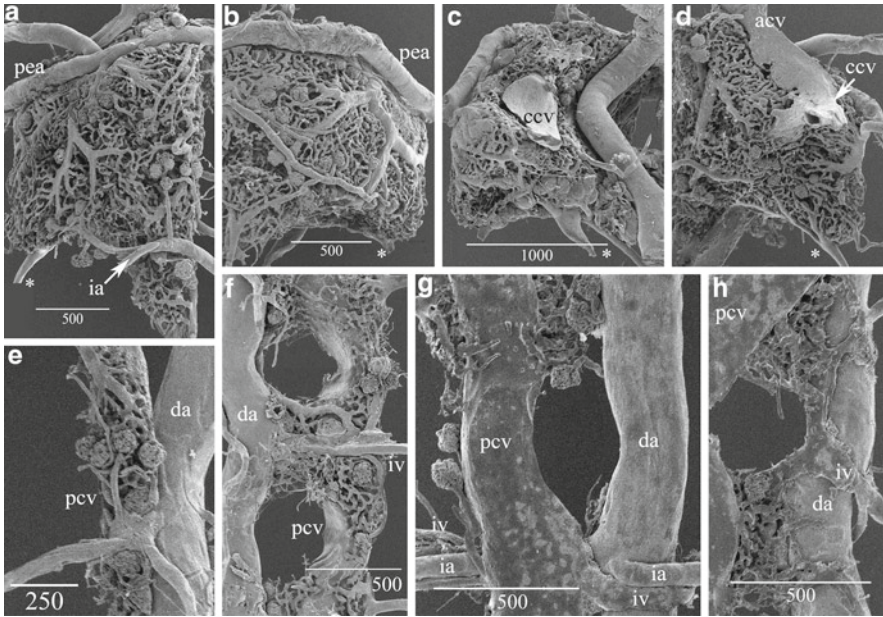


Fig. 7.5 Cranial (head) and caudal (trunk) mesonephric sinusoids. **a** Left cranial mesonephric sinusoid viewed from dorsal side. **b** Right cranial mesonephric sinusoid viewed from dorsal side. **c** Right cranial mesonephric sinusoid viewed from ventral side. **d** Left cranial mesonephric sinusoid viewed from ventral side. **e** Posterior cardinal vein breaks up into sinusoidal plexus in caudal mesonephron viewed from dorsal side. **f** Caudal mesonephric sinusoid viewed from dorsal side. **g, h** Caudal mesonephric sinusoid viewed from ventral side. Cranial is to the *top*. *acv*, anterior cardinal vein; *ccv*, common cardinal vein; *da*, dorsal aorta; *ia*, intersegmental artery; *iv*, intersegmental vein; *pcv*, posterior cardinal vein; *pea*, pectoral (subclavian) artery; *, veins from lateral body wall. *Bars a–c, e–h*

develops as a major renal portal vein, bringing venous blood from the tail and the most of the trunk into the mesonephric sinusoid, whereas the left posterior cardinal vein remains as a remnant route only from the left cranial trunk region. (Note that the posterior cardinal vein shifts its course from the right to the left in this specimen; see Fig. 7.4a, b.) The posterior cardinal vein breaks up into a sinusoidal plexus (Fig. 7.5e) tightly tangled with the tubules and ducts of the cranial part of the renal system (Figs. 7.4a, b and 7.5a–d). The blood is recollected into renal veins to drain into the anterior cardinal vein, and from there into the common cardinal veins (Fig. 7.5c, d). The intersegmental veins and the veins from the lateral body wall bringing venous blood into the mesonephric sinusoid also work as renal portal veins (Fig. 7.5a–d). In the cylindrical caudal part of the kidney, every intersegmental vein breaks up into a miniature sinusoidal plexus that intermingles with the adjacent renal tubule and duct, serving as a renal portal vein before draining into the posterior cardinal vein (Fig. 7.5e–h).

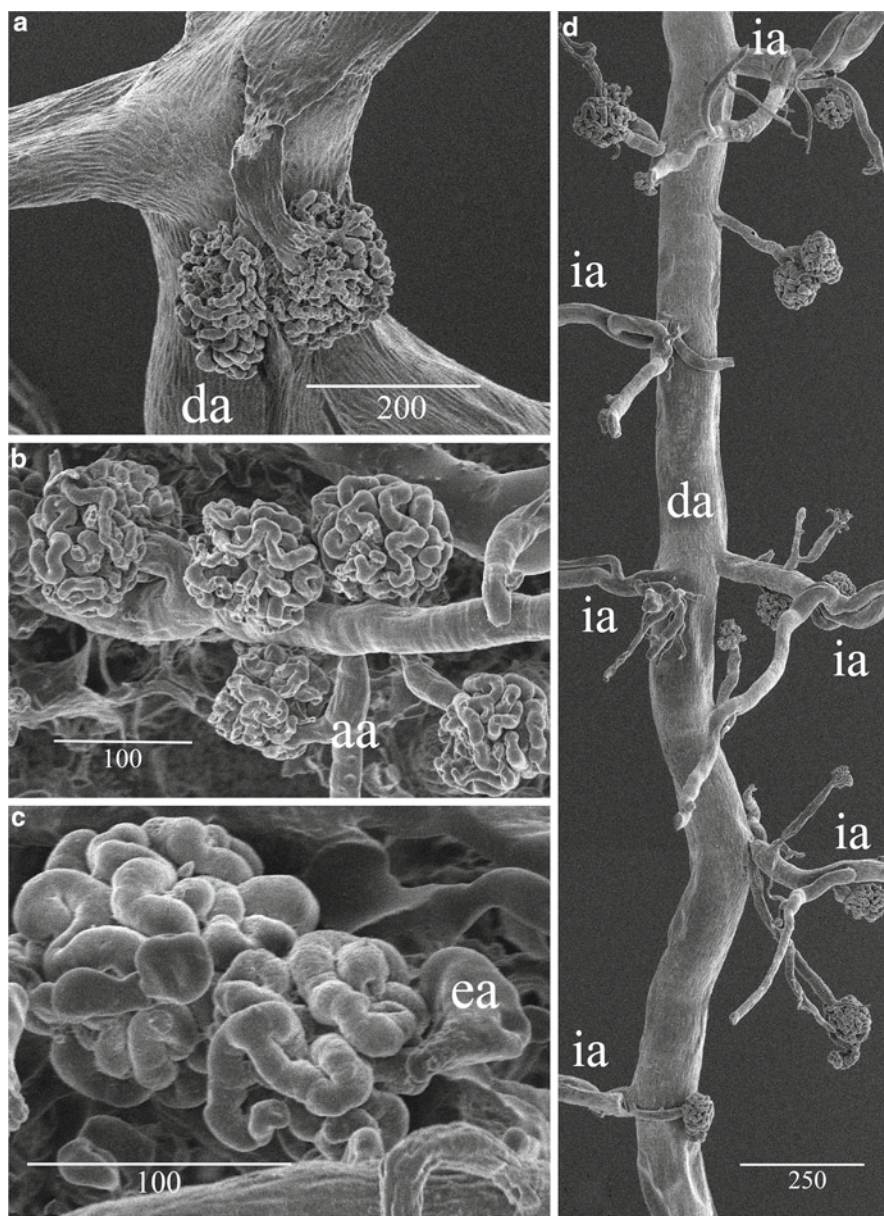


Fig. 7.6 Pronephric glomerulus and mesonephric glomeruli. **a** Remnant pronephric glomerulus. **b** Glomeruli in cranial (head) mesonephron. **c** Doubled glomeruli in caudal (trunk) mesonephron. **d** In the caudal (trunk) mesonephron, renal afferent arteries branch off directly from the dorsal aorta or from the intersegmental arteries. Cranial is to the *top* in **a** and **d**. *aa*, afferent artery; *da*, dorsal aorta; *ea*, efferent artery; *ia*, intersegmental artery. Bars **a–d**, μm

7.5 Parietal Blood Vascular System

7.5.1 *Blood Vascular System for the Trunk and Tail*

In the trunk, the single dorsal aorta runs caudally beneath the vertebrae, continuing as the caudal artery in the tail, where it enters into the closed hemal arches of the vertebral column (Fig. 7.7a,c). In the myo-septa, segmentally arranged arteries branch off from the dorsal aorta or caudal artery to penetrate the muscle segments on each side of the body and extremities (Fig. 7.7a). The dorsal intersegmental arteries and their sprigs supply the individual myomeres of the epaxial musculature, vertebrae, and spinal cord in the trunk and tail (Fig. 7.7c–g); they also supply small branches to the dorsal fin and the musculature at its dorsal end (Fig. 7.7k). In the trunk region, the intercostal arteries adjacent to each rib branch off dorsally from the intersegmental vessels and then run ventral-caudally directly beneath the peritoneum in every myoseptum, supplying the hypaxial musculature of the belly on each side (Fig. 7.7d, e). Some of these (iliac arteries) distribute to the pelvic fin and its musculature in the caudal part of the trunk (Fig. 7.7i). In the tail, instead of intercostals, ventral intersegmental arteries branch off ventrally from the caudal artery to supply the hypaxial musculature and the anal fin (Fig. 7.7c, f, j). The lateral intersegmental artery, originating from the dorsal intersegmental, runs laterally within the horizontal myoseptum, and splits dorsally and ventrally beneath the skin. The lateral intersegmentals supply the superficial muscle, the skin, and the lateral portions of the epaxial and hypaxial musculature of the trunk and tail (Fig. 7.7d, e). The pectoral (subclavian) artery branches off from the cranialmost part of the dorsal aorta, running along the pectoral girdle to distribute blood to the pectoral fin and its musculature (Figs. 7.2a, 7.4a, b, 7.5a, b and 7.7d, e). The caudal artery runs caudally and then splits dorsally and ventrally at the base of the tail. The dorsal and ventral branches divide into a large number of smaller vessels in the fin (Fig. 7.7g, k). The blood is collected via collateral veins, draining into the caudal vein. It has been believed that the intersegmental veins alternate more or less regularly with the arteries so that in each segment an intersegmental vein is located on one side of the body and an intersegmental artery on the other side, but this is not the case. An irregular alternative arrangement of intersegmental arteries and veins generally occurs, with no consistent pattern. Each intersegmental vein has a dorsal and ventral branch; the ventral branches are also designated as the intercostal veins when they are located in the trunk region. These branches drain from the lateral muscles of the trunk and tail, subdermal muscles, and the fin musculature either into the caudal vein, posterior cardinal vein, or the renal portal system, depending upon the portion of the body in which they are located (Figs. 7.4, 7.5, and 7.7c–g). The dorsal and lateral intersegmental veins penetrate into the dorsal side of the kidney, and the intercostal veins penetrate into the ventral edge of the kidney as the miniature renal portal veins mentioned earlier (Figs. 7.4 and 7.5). The ventral intersegmental veins from the cloacal region carry venous blood either to the caudal vein or to the mesonephric sinusoids. The veins for the extremities generally run parallel to the corresponding extremity

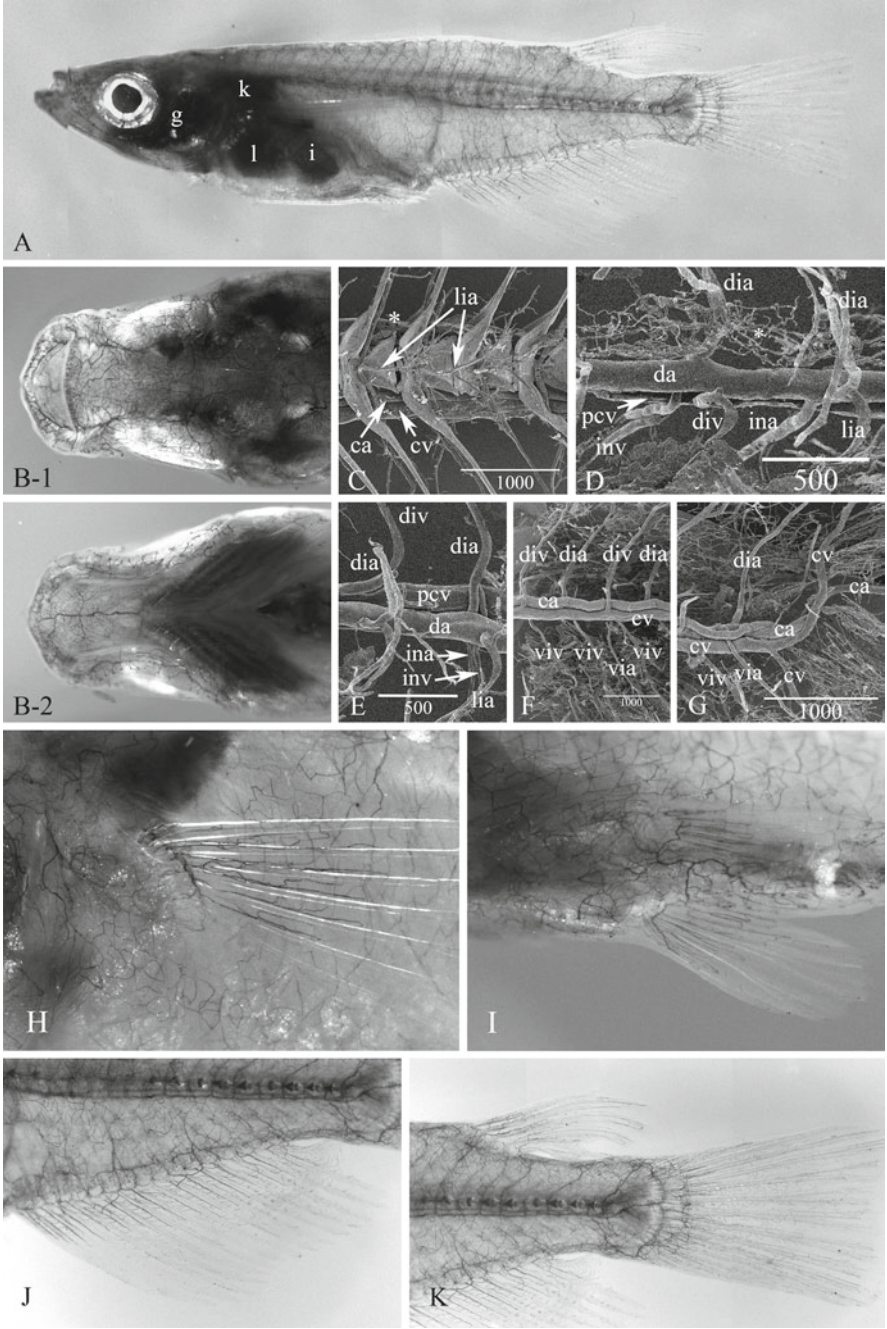


Fig. 7.7 Parietal blood vascular system visualized by dye injection and corrosive resin casts (SEM images). **a** Left lateral view of dye-injected see-through medaka. **b-1** Vascular network in nasal sac and upper jaw viewed from dorsal side. **b-2** Vascular network in lower jaw viewed from

arteries. The pectoral (subclavian) vein opens into the common cardinal vein. The iliac veins lead the blood from the pelvic fin to the posterior cardinal vein or mesonephric sinusoids.

7.5.2 Blood Vascular System for the Head

An internal carotid artery extends rostrally from the junction of the aortic radix of first and second efferent branchialis with the lateral dorsal aorta (Fig. 7.8a). Just rostral to this junction, the relatively thick orbital artery (designated also as the afferent pseudobranchial vessel in medaka) branches off from the internal carotid and, receiving the efferent pseudobranchial vessel, takes its main course to the orbital region. It distributes to the external eye muscles, choroidal vascular plexus (Fig. 7.8g), and choroidal body (bipolar rete mirabile, which is a complex of arterial and venous capillaries) (Fig. 7.8e, f). Further rostrally, a nasal artery stems from the internal carotid to supply the nasal sac and upper jaw (Figs. 7.7b-1, b-2, and 7.8a).

7.5.3 Blood Vascular System for the Brain and Spinal Cord

At their rostral end the internal carotids come closer to one another, then turn dorsal ward, then split rostrally and caudally. The rostral ophthalmic arteries reach to the eyes and penetrate into the cavity of the spherical sclera as the retinal arteries (Fig. 7.8a,g). The caudal branches of the internal carotid (posterior communicating artery of circle of Willis) run caudolaterally and then come closer medially each other to merge into the single basilar artery (Fig. 7.8a,c,d). As already mentioned, the dorsal intersegmental arteries branch smaller vessels off to the vascular plexus



Fig. 7.7 (continued) ventral side. **c** Vascular network viewed from left side. Spinal cord, caudal artery, and caudal vein enter into the closed neural and hemal arches of the vertebral column in the tail. **d** Branching pattern of dorsal intersegmental, lateral intersegmental, and intercostal arteries and veins with vascular plexus of spinal cord in the trunk viewed from right dorsolateral side. **e** Branching pattern of dorsal intersegmental, lateral intersegmental, and intercostal arteries and veins in the cranial trunk region viewed from right dorsolateral side. **f** Dorsal and ventral intersegmental arteries and veins in the tail viewed from left side. Vascular system of tail fin (**g**), pectoral fin (**h**), pelvic fin (**i**), anal fin (**j**), and dorsal and tail fins (**k**) viewed from left side. Dorsal is to the top in **a**, **c-k**; rostral is to the left in **a**, **b**, **c**, and **f-k** but to the right in **d** and **e**. *ca*, caudal artery; *cv*, caudal vein; *da*, dorsal aorta; *dia*, dorsal intersegmental artery; *div*, dorsal intersegmental vein; *g*, gill; *i*, intestine; *ila*, iliac artery; *ina*, intercostal artery; *inv*, intercostal vein; *k*, kidney; *l*, liver; *lia*, lateral intersegmental artery; *liv*, lateral intersegmental vein; *pcv*, posterior cardinal vein; *via*, ventral intersegmental artery; *viv*, ventral intersegmental vein; *, vascular plexus of spinal cord. Bars **c-g**, μm

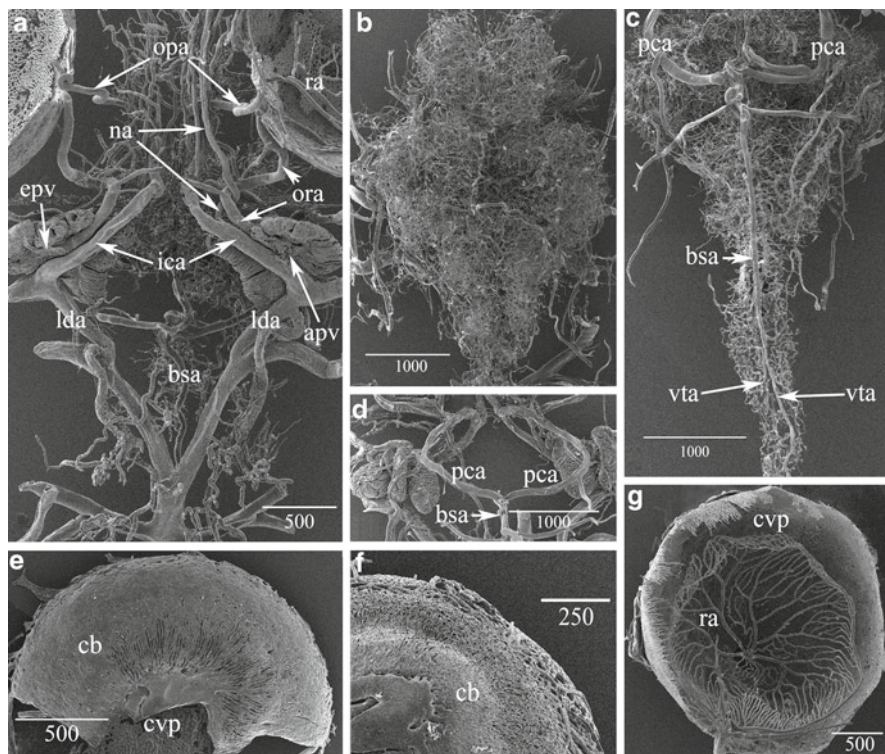


Fig. 7.8 Blood vascular system of brain and eye visualized by corrosive resin casts (SEM images). **a** Branching pattern of internal carotid artery viewed from ventral side. **b** Vascular network of brain viewed from dorsal side. **c** Vascular system of brain and spinal cord viewed from ventral side. **d** Posterior communicating artery of circle of Willis and single basilar artery viewed from dorsal side. Vascular network of brain was removed. **e** Rete mirabile of choroidal body. **f** Closer view of outer choroidal and inner retinal vascular plexus (**e**, **g**). Rostral is to the *top* in **a–d**. *apv*, afferent pseudobranchial vessel; *bsa*, basilar artery; *cb*, choroidal body (rete mirabile); *cvp*, choroidal vascular plexus; *epv*, efferent pseudobranchial vessel; *ica*, internal carotid artery; *lda*, lateral dorsal aorta; *na*, nasal artery; *opa*, ophthalmic arteries; *ora*, orbital artery; *pca*, posterior communicating artery (of circle of Willis); *ra*, retinal artery; *vta*, vertebral artery. Bars **a–g**, μm

of the spinal cord in the trunk and tail (Fig. 7.7d). At the cranialmost part of the plexus, a paired longitudinal vertebral artery appears to join the single basilar artery. This arterial system, consisting of the ophthalmic, caudal branch of internal carotids, basilar, and vertebral arteries, supplies blood to the brain from its ventral side (Fig. 7.8b–d). The blood from the rostral region of the head, the mouth, olfactory organ, eyeballs, and muscles, runs caudally, crossing over the eye on its dorsal side to drain into the anterior cardinal vein. The middle and posterior cerebral veins receive blood from the brain and drain to the anterior cardinal vein just caudal to the otic capsule.

7.5.4 *Perspective*

To make the medaka an excellent model organism for future cardiovascular research, we are compiling a complete series of developmental anatomical atlases from the embryo through to the adult. We previously characterized the vascular anatomy of the developing medaka embryo, and here we provide that of the adult fish. The detailed morphological analysis should be continued, however. We will present the remaining part from the early larva to the young fish in the near future.

Acknowledgments The authors thank Y. Wakamatsu and H. Hashimoto (Nagoya University) for providing See-through Medaka; and Y. Takahashi (Department of Anatomy, Iwate Medical University) and the members of the Bio-imaging Center of IMU for technical assistance. We are grateful to B.M. Weinstein (National Institutes of Health, USA) for critical reading of this manuscript.

References

- Goodrich ES (1958) Study on the structure and development of vertebrates. Dover, New York
Harder W (1975) Anatomy of fishes. E. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart
Iwamatsu T (1997) The integrated book for the biology of the medaka (in Japanese). Daigaku
Kyouiku Shuppan, Okayama
Romer AS (1971) The vertebrate body, 4th edn. Saunders, Philadelphia

Chapter 8

Kidney Development, Regeneration, and Polycystic Kidney Disease in Medaka

Hisashi Hashimoto

Abstract Medaka has a pronephros at early larval stages, and thereafter the mesonephros develops in the tissues around the pronephric tubule and duct. A marked increase in mesonephric nephrons continues until 2 to 3 months after hatching, and consequently the mesonephros consists of 200–300 nephrons on each side. The nephrogenic processes can be histologically featured in the developing mesonephros as three distinguishable stages: mesenchymal condensation, formation of a nephrogenic body, and maturation of the nephron. The appearance of mesenchymal condensates and nephrogenic bodies in the interstitial tissue indicates that the de novo nephrogenesis takes place actively. As these nephron precursors are positive for *wt1* expression, *wt1* could be a good marker of de novo nephrogenesis.

The program for nephron development can be reactivated in medaka during adulthood by artificial injury with chemicals. Intraperitoneal administration of gentamicin, damaging tubules, ducts, and the glomeruli, leads to a significant increase of the mesenchymal condensates and nephrogenic bodies in the injured kidney, which can be also recognized as *wt1*-positive cell masses. Thus, in contrast to mammals, medaka is capable of regenerating the kidney through de novo nephrogenesis, possibly by recruiting stem cells retained in the interstitial tissue of the adult kidney.

The medaka *pc* mutant shows lesions quite similar to those of the human genetic disease polycystic kidney disease (PKD): it develops numerous fluid-filled renal cysts and suffers from enlargement of the abdomen. Genetic linkage analysis identified the causative gene to be the medaka ortholog of *glis3*. In humans, the mutations in *GLIS3* have been reported to be involved in pathogenesis of pleiotropic genetic diseases including PKD and diabetes. Consistent with the medaka mutant phenotype, *glis3* mRNA is expressed in the epithelia of the renal tubule and duct. The cilia in the pronephric tubule are significantly shortened in the *pc* mutant. Glis3 protein is preferentially located in the cilium of renal epithelial cell. Similar to the other PKD genes reported previously, *glis3* may also play a crucial role in the ciliary structure or function.

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All the findings suggest that medaka serves as a good model for understanding the process of kidney development and regeneration as well as the pathogenesis of human genetic kidney diseases.

8.1 Introduction

The kidney plays a pivotal role in controlling humoral conditions, such as electrolytes, osmolarity, and blood pressure. Abnormalities of renal function sometimes result in serious conditions, such as acute and chronic renal failure, for which dialysis treatment is required. Millions of people throughout the world suffer from end-stage renal failure, with 280,000 in Japan. Kidney disease is associated with various systemic diseases, including diabetes and autoimmune diseases. The number of kidney disease patients who need dialysis treatment is increasing, and this has become one of the major issues for the cost of medical care in the world. Therefore, development of treatments for kidney disease is one of the most urgent issues to be resolved.

Kidney regeneration in mammals is one of the major subjects involved in development of new therapeutic strategies in renal medicine (Ricardo and Deane 2005; Verghese et al. 2008). A well-established characteristic of the adult mammalian kidney is the ability to recover from acute renal failure. This repair response is thought to occur through repopulation of the existing nephron cells. The formation of nephrons is terminated at embryonic or neonate stages, and it does not take place in the human adult kidney. It is thought that stem cells for nephrogenesis are not present or are dormant in the human adult kidney. In contrast to the mammalian kidney, the teleost fish kidney retains stem cells in adulthood and can regenerate the nephrons (Reimschuessel 2001). Studies using fish as a model organism have pioneered a new research field on kidney regeneration. However, analyses of the normal development and the regeneration process of kidney in fish are required for exploiting the fish system to develop a regenerative medical approach for human kidney diseases.

Polycystic kidney disease (PKD) is one of the major genetic diseases in the kidney and is the most common genetic cause of renal failure (Igarashi and Somlo 2007). It is characterized by the appearance of fluid-filled cysts in the renal tubules and collecting ducts of the kidney. Recent studies have proposed that renal cilia, which are immotile organelles projecting from the renal epithelium into the lumen of the tubule or duct, play a crucial role in cyst formation (Watnick and Germino 2003; Yoder 2007). Although many genes have been identified by analyzing PKD patients and animal models (Bisgrove and Yost 2006), the mechanisms underlying PKD pathogenesis are not fully elucidated.

Mammalian kidneys form from three successive structures – the pronephros, the mesonephros, and the metanephros (Gilbert 2003) – whereas medaka has only the first two forms (Fedorova et al. 2008). Despite evolutionary variation, the structure and formation of their functional unit, the nephron, are well conserved among vertebrate species. In this review, I first introduce the development and regeneration

process of the medaka kidney. I also explain the medaka PKD mutant *polycystic* (*pc*) and the potential use of the medaka mutant for understanding the pathogenesis of human PKD and for developing medical strategies for PKD.

8.2 Kidney Development in Medaka

8.2.1 Gross Morphology of Medaka Kidney

Medaka has a pair of kidneys that are located retroperitoneally, extending from the bases of the pectoral fins to the caudal reaches of the abdominal cavity (Fig. 8.1a). Their large anterior portions containing most of the nephrons are much larger than those of zebrafish whereas the caudal portions are smaller (Fig. 8.1b). Medaka hatchlings have functional pronephros consisting of a single pair of nephrons (Fig. 8.1c). The kidney size of the fry increases as mesonephric nephrons develops. Adult medaka kidneys contain both pronephros and mesonephros, in contrast to the kidneys of some fish species, which lose the pronephros after the mesonephros starts functioning (Harder 1975; Hickman and Trump 1969; Lagler et al. 1977; Reimschuessel 2001). The bean-shaped cranial portion is larger than the caudal portion, and this proportion persists throughout life.

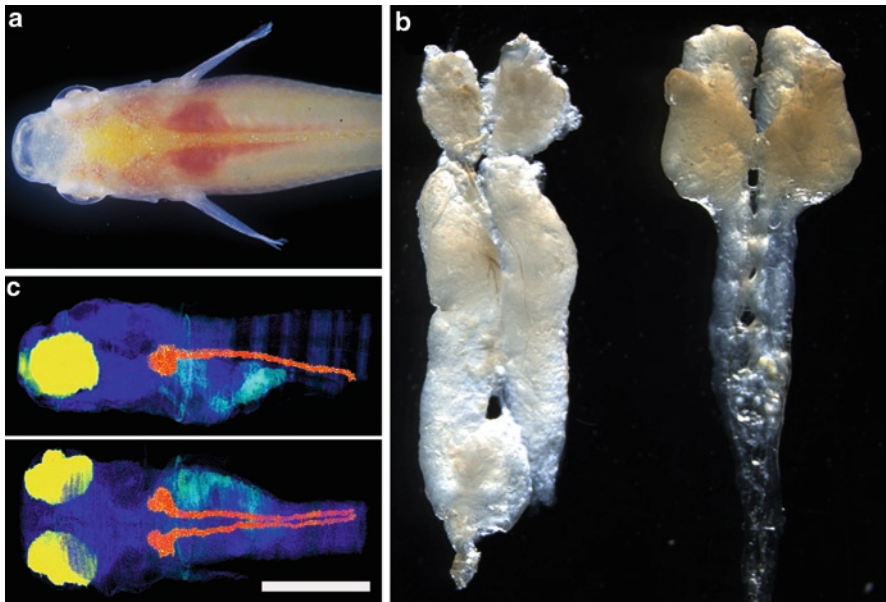


Fig. 8.1 Gross morphology of medaka kidney. **a** See-through medaka STII. Reddish kidneys from dorsal view. **b** Comparison of adult kidney morphology between medaka (*right*) and zebrafish (*left*). **c** Three-dimensional image of medaka hatchling. Bar **c** 300 μ m

8.2.2 Histological Anatomy of the Kidney and Nephron Development

A medaka hatchling has only the pronephros (Fig. 8.2a), which consists of a single pair of nephrons having the butterfly-shaped glomus external to the kidney capsule and the tubule connecting the glomus with the duct extending to the urinary bladder. Adult medaka fish have 200–300 nephrons in each of the kidneys, distributing with no particular distinction between medulla and cortex (Fig. 8.2b). The entire kidney

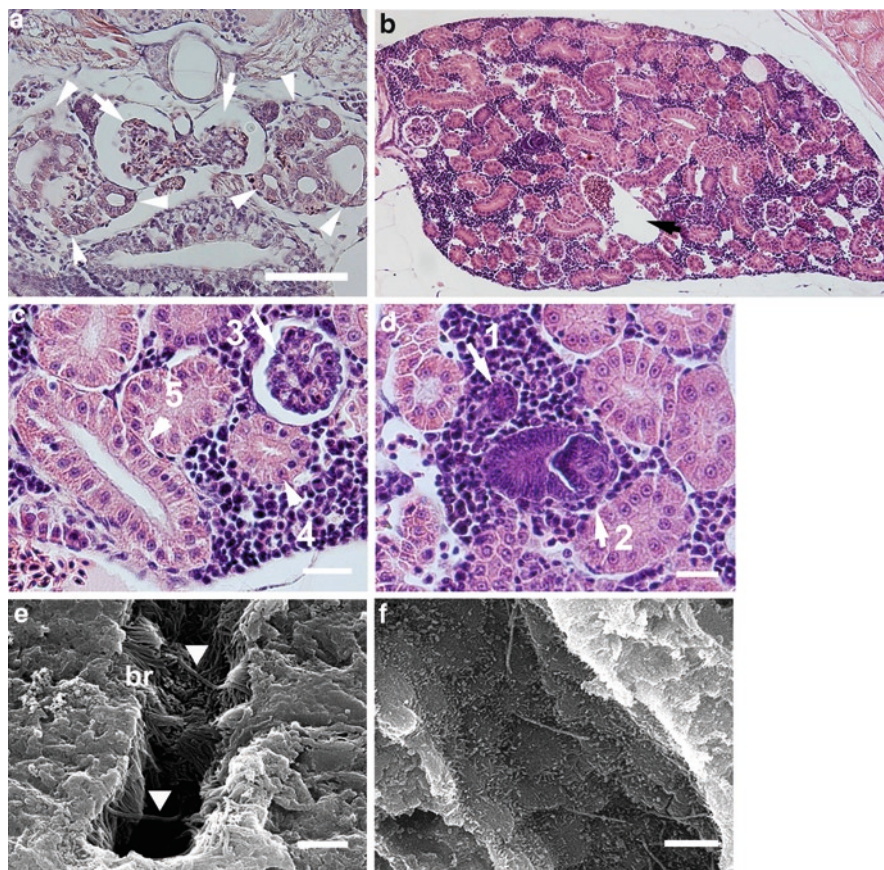


Fig. 8.2 Histology of medaka kidney. **a** The pronephros of the hatchling. *Arrows*, external glomeruli; *three arrowheads*, each of the bilateral borders of the renal tissue. **b** A section of the anterior portion of medaka 2 months post hatching (mph). *Black arrow*, subcardinal vein. **c**, **d** Magnified images of a section from 2 mph medaka. *Arrow-1*, mesenchymal condensate; *arrow-2*, nephrogenic body; *arrow-3*, mature glomerulus; *arrowhead-4*, proximal tubule; *arrowhead-5*, distal tubule. **e** Scanning electron microscope (SEM) image of the proximal tubule. *Arrowheads*, microvilli; *br*, brush border of microvilli. **f** SEM image of the distal tubule. *Bars* **a** 50 μm ; **b** 100 μm ; **c**, **d** 20 μm ; **e**, **f** 10 μm

is composed of both nephrons and interstitial lymphoid tissue (Fig. 8.2b). Three segmental structures of nephron are recognized by hematoxylin and eosin staining of the kidney section. The glomerulus is typically a round-shaped cell mass covered by the flat epithelium of Bowman's capsule. The proximal segment of the tubule is lined by tall columnar epithelial cells possessing a brush border on its apical surface (Fig. 8.2c, d, e), which is positive for *Locus tetragonolobus* lectin staining (see Fig. 8.5f). The distal tubular segment has a wide lumen lined by low columnar epithelial cells having a monocilium (Fig. 8.2c, f). In the developing kidney of medaka [10 days post hatching (dph) to 2 months post hatching (mph)], three different stages of nephrogenesis can be distinguished histologically (Fig. 8.2c, d). (1) Mesenchymal condensation: a cell mass of high cell density strongly stained by hematoxylin, appears in the mesenchyme. (2) Formation of a nephrogenic body: a condensed mesenchyme changes its shape to a tadpole-shaped nephrogenic body; its tail region corresponds to a presumptive tubule. The nephrogenic body may be equivalent to the comma- or S-shaped nephrogenic body in the mammalian kidney (Vainio and Lin 2002). (3) Maturation of nephron: the glomerulus becomes surrounded by a spacious Bowman's capsule and the tubular segments become eosinophilic, possibly because of completion of the mesenchymal–epithelial transition.

8.2.3 Mesonephros Development

Mesonephric nephrons start to develop at 4 dph as the condensates form in the mesenchyme in the dorsomedial region of the anterior pronephros. The first mesonephric nephron becomes mature within 10 dph. Thereafter, mesonephric nephrons continuously develop along the pronephric tubule and duct, rapidly increasing in number until 2 mph. Notably, after the number of nephrons reaches to a plateau, approximately 7% of total nephrons are still immature.

Wilms' tumor suppressor gene 1 (*wt1*) is reported to be a good marker of developing pronephric and mesonephric nephrons in zebrafish (Drummond et al. 1998; Kreidberg et al. 1993; Serluca and Fishman 2001). In medaka, *wt1* is detected in the precursor cells and later in the immature glomerulus of pronephric nephrons. During mesonephric development, *wt1*-positive cells masses are detected where the first mesonephric nephrons form (Fig. 8.3a, b), and they increase and appear along the pronephric tubule and duct (Fig. 8.3c, e). *wt1* is expressed strongly in the mesenchymal condensate (Fig. 8.3b, d, f, g) and later in the presumptive glomeruli (Fig. 8.3d). Expression of *wt1* disappears when the glomeruli become mature (Fig. 8.3f), indicating that *wt1* is a hallmark for developing nephrons in medaka. The *wt1*-expressing mesonephric nephrons appear in the posterior region in zebrafish, whereas these are located mainly in the anterior region of the medaka kidney (Fig. 8.3h, i). The difference in gross morphology of the kidney between medaka and zebrafish may reflect the distinct distribution of mesonephric nephrons.

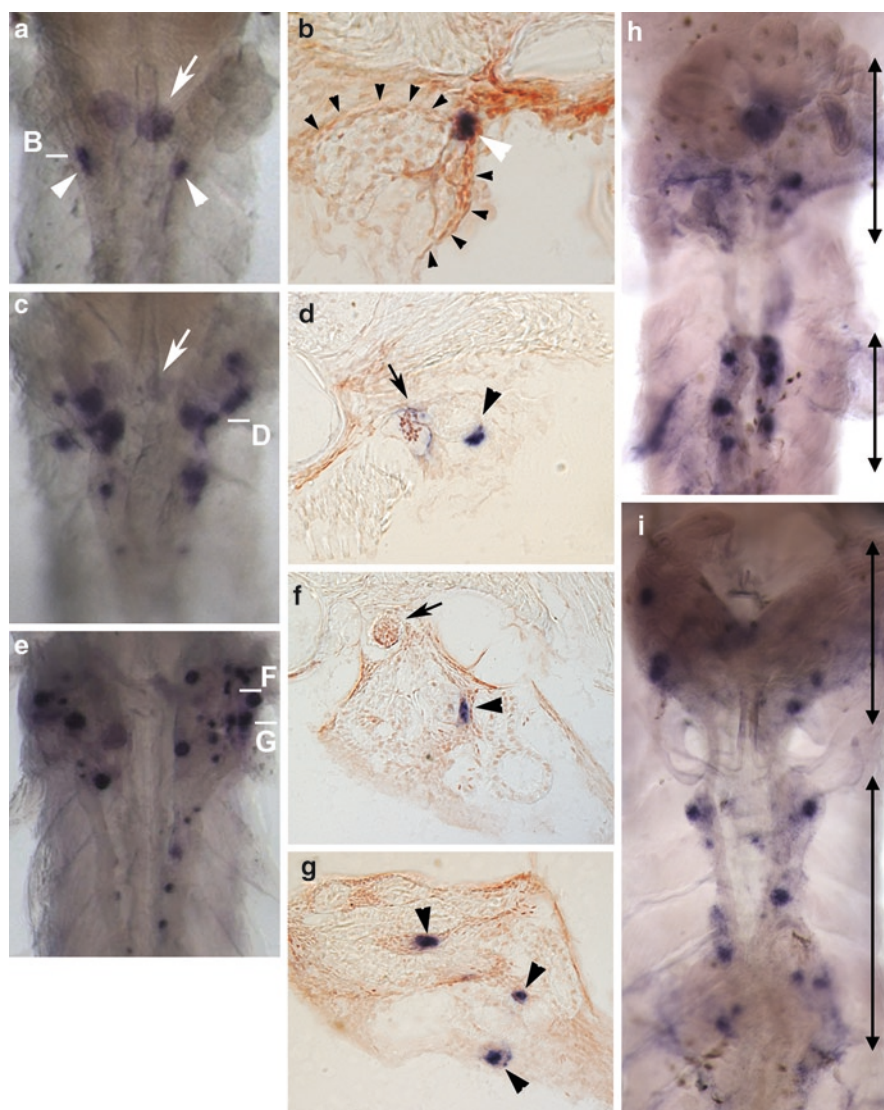


Fig. 8.3 *wt1* expression in the developing mesonephros of medaka and zebrafish. **a, b** Medaka 5 days post hatching (dph). *White arrowheads*, the first mesonephric nephron precursors positive for *wt1*; *white arrow*, left pronephric glomus; *black arrowheads*, border of renal tissue. **c, d** 10 dph medaka. *White arrow*, left pronephric glomus; *black arrow*, the first mesonephric nephron with weaker *wt1* expression; *arrowhead*, newly formed nephron precursor. **e, f, g** 20 dph medaka. *Arrowheads*, newly formed nephron precursors; *arrow*, a fully developed mesonephric nephron with *wt1* expression almost diminished. **b, d, f, g** Cross sections were made at the levels indicated by *bars* in **a, c**, and **e**. **h** 20 dph zebrafish. **i** 24 dph zebrafish. More *wt1*-positive signals are observed in the posterior portion of the kidney. *Double-headed arrows*, two separate nephrogenic zones

8.3 Kidney Regeneration in Medaka

8.3.1 *Repair of Nephrons in Mammals*

Renal recovery from sublethal injury is considered to recruit two different cell sources for repairing the damaged nephrons. One source is surviving tubular epithelial cells, characteristic for flattened and squamous shape (Cantley 2005). They migrate to and cover the denuded basement membrane and subsequently develop to (repopulate) a cuboidal and columnar epithelium. Another source may be bone marrow stem cells. The plasticity of bone marrow stem cells has been traditionally known as they can give rise to blood cells and connective and adipose tissue cells (Ricardo and Deane 2005). Recent studies suggest that bone marrow-derived cells can also contribute to adult renal cells in humans and other animal models (Lin et al. 2005; Rookmaaker et al. 2003). When human patients or experimental animals receive a kidney or bone marrow transplant, bone marrow-derived cells repopulate primarily in the renal proximal tubule as well as in the renal vasculature, interstitium, the renal tubules, and the glomeruli. However, these two cell sources are incapable of nephron neogenesis.

8.3.2 *Renal Damage and Repair in Medaka and Other Fish*

Administration of nephrotoxicants such as gentamicin also leads to renal damage in fish. Reimschuessel et al. reported a pioneering work using goldfish (Reimschuessel et al. 1990). As has been shown in goldfish, and later in other fish (Cormier et al. 1995; Reimschuessel et al. 1990, 1993; Reimschuessel and Biggs 1996; Salice et al. 2001), in medaka after intraperitoneal injection of gentamicin, damaged nephrons characteristic of a sloughing columnar epithelium and a denuded intact basement membrane are found (Fig. 8.4a, b). The damage in the glomerulus is visible under a transmission electron microscope (Fig. 8.4c–f). Repair of these damaged nephrons seems to be the first renal response to acute injury. Similar to the case in mammals, repopulation of the tubular epithelium occurs in goldfish, catfish, trout, zebrafish, and tilapia (Cormier et al. 1995; Reimschuessel et al. 1990, 1993; Reimschuessel and Biggs 1996; Salice et al. 2001).

8.3.3 *Nephron Neogenesis in Medaka and Other Fish*

In contrast to the mammalian kidneys, fish are capable of regenerating the kidney through de novo generation of nephrons, or neogenesis. Neogenesis recapitulates the normal development process of pronephros and mesonephros from the mesenchymal

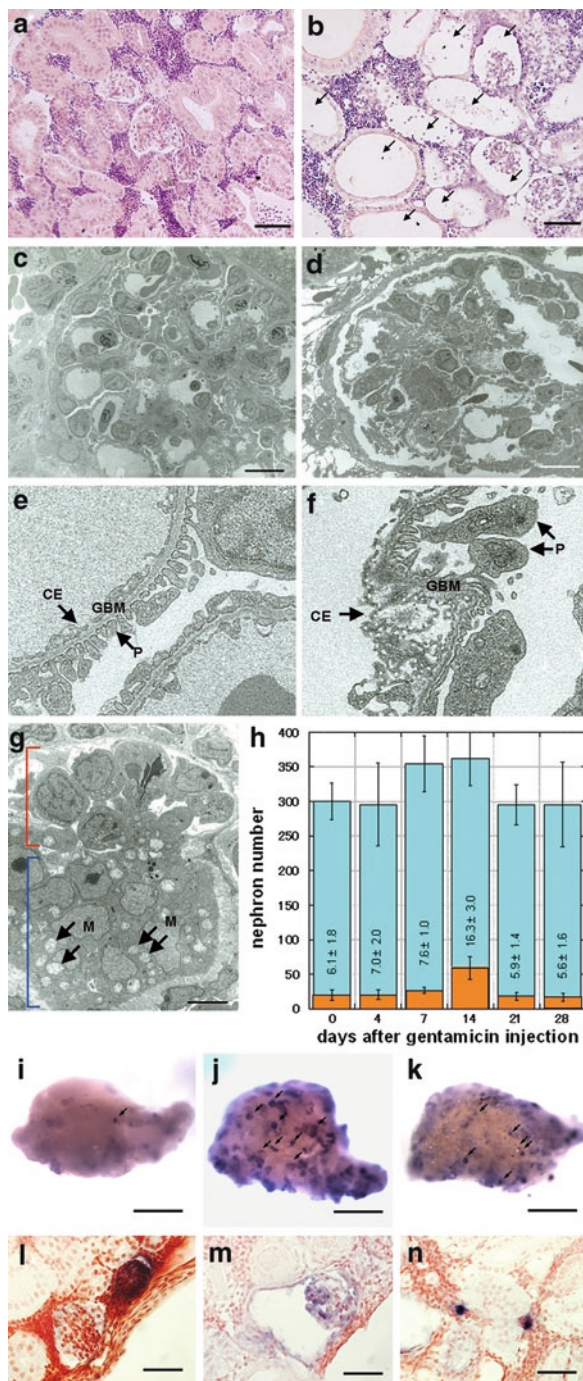


Fig. 8.4 (color online) Kidney regeneration in medaka. **a, b** Histology of normal kidney [**a**; 7 days after phosphate-buffered saline (PBS) injection] and damaged kidney (**b**; 3 days after gentamicin injection). **c, d** SEM image of normal (**c**) and damaged (**d**; 3 days after gentamicin injection) mature glomeruli. **e, f** High magnification of SEM image around the basement membrane. Compared with a normal glomerulus

tissue. In medaka, we have found significant increase in the number of de novo developing nephrons 2 weeks after toxicant administration (Fig. 8.4g, h). More direct evidence of de novo nephrogenesis in adult fish was provided by Elger et al., who reported that partial nephrectomy in the skate fish (the elasmobranch *Leucoraja erinacea*) induces proliferation of renal progenitor cells and leads to formation of a nephrogenic zone (Elger et al. 2003). Intriguingly, unilateral nephrectomy of mammalian adult kidneys also results in compensatory renal hypertrophy as a consequence of cellular hypertrophy but not as a result of nephron neogenesis (Drummond 2003).

8.3.4 Recapitulation of *wt1* Expression in Repair and Neogenesis of Medaka Kidney

The appearance of basophilic cell clusters, referred to above as mesenchymal condensates and nephrogenic bodies, is a hallmark for de novo nephrogenesis in fish, including medaka (Reimschuessel 2001; Watanabe et al. 2009). Similarly, in normal nephron development, increase in the number of *wt1*-positive cell masses is also a hallmark for the de novo nephrogenesis and repair response (Fig. 8.4i–n). All the regenerative phenomena just described suggest that the adult fish kidney retains stem cells, which can produce new nephrons in response to injury. These characteristics of the fish kidney may be relevant to the fact that the fish (e.g., medaka) kidneys display slight but continuous growth of mesonephros in adulthood (Fedorova et al. 2008), whereas mammalian kidneys do not generate new nephrons after a specific time point of development (in rat, up to 3 days after birth) (Karp et al. 1971).

Fig. 8.4 (continued) (e), a damaged glomerulus (f) displays a capillary endothelium that is abnormally arranged and detached from the glomerular basement membrane, and the podocytes are hypertrophic. CE, capillary endothelium; GBM, glomerular basement membrane; P, podocyte. g SEM image of nephrogenic body. Blue bar, portion of mitochondria-rich tubular segment precursor; red bar, portion of a developing glomerulus. h Number of developing nephrons and total nephrons (means + standard deviation). Orange bars, number of developing nephrons; blue bars, total number of nephrons. The ratios of developing nephrons to total nephrons are indicated above the orange bars. i–n *wt1* expression in gentamicin-treated kidneys. i, j, k External views of the whole kidney stained by *wt1* in situ hybridization. l, m, n Sections of *wt1*-stained kidney. i, l 3.5 mph. j, m Three days after gentamicin injection. k, n Fourteen days after gentamicin injection

8.4 Medaka *pc* Mutant as a PKD Model

8.4.1 What Is PKD?

Polycystic kidney disease (PKD) can be inherited in an autosomal dominant manner (ADPKD) or an autosomal recessive manner (ARPKD) (Igarashi and Somlo 2002). ADPKD occurs in 1:700 to 1:1,000 individuals, where 80% of the patients have a mutation in PKD1 and 20% a mutation in PKD2. ARPKD, caused by loss of fibrocystin, is rather rare (1:6,000–40,000). The PKD proteins – polycystin-1, polycystin-2, and fibrocystin – have all been found in the primary cilia of the renal tubules and the collecting ducts (Yoder 2007). Furthermore, many of the products of the genes that are responsible for renal cystic diseases, including nephronophthisis and Bardet–Biedl syndrome, localize to the cilium and/or to the basal body of the cilium (Chauvet et al. 2004; Taulman et al. 2001; Tobin and Beales 2007; Wang et al. 2004; Yoder 2007). These findings suggest that PKD is closely associated with abnormalities in the cilium. Actually, structural defects and/or dysfunction of the cilium link with PKD pathogenesis.

An interesting model has been proposed for the ciliary function in the mammalian kidney. In the kidney, a cilium projects from the apical surface of the renal tubular cell or from the ductal epithelial cell into the lumen (Nauli et al. 2003; Nauli and Zhou 2004). The cilium acts as a mechanosensor for urine flow and controls the size of the lumen in response to flow speed. Consistent with this, the cilium in mammalian kidney exhibits a 9+0 microtubule structure, which is characteristic of immotile cilia (stereocilia). In contrast, the cilium of medaka and zebrafish kidney has a 9+2 organization and is shown to move (Kramer-Zucker et al. 2005; Omran et al. 2008). The beating motion of the cilium in the lumen of the renal tubules is required for generation of urinary flow in the pronephric tubules and ducts. Although the role of the renal cilium may be slightly different between mammals and fish, our studies on the medaka mutant *pc* reveal a role of the renal cilium in the maintenance of kidney functions and pathogenesis of PKD in both mammals and fish.

8.4.2 Phenotype of Medaka *pc* Mutant

The medaka *pc* is a spontaneous mutant collected by Tomita in 1975. The *pc* mutant develops bilateral enlargement of the abdomen, which is inherited in an autosomal and recessive manner; it is externally apparent in early adulthood (approximately 1.5 mhp) and slowly progresses (Fig. 8.5a,b). The affected kidneys occupy most of the body cavity, displacing and compressing other internal organs (Fig. 8.5c,d). The ratios of the kidney weight to the body weight were about 30 times larger in the *pc* mutant than in the wild type. The *pc* mutant becomes sexually mature at approximately

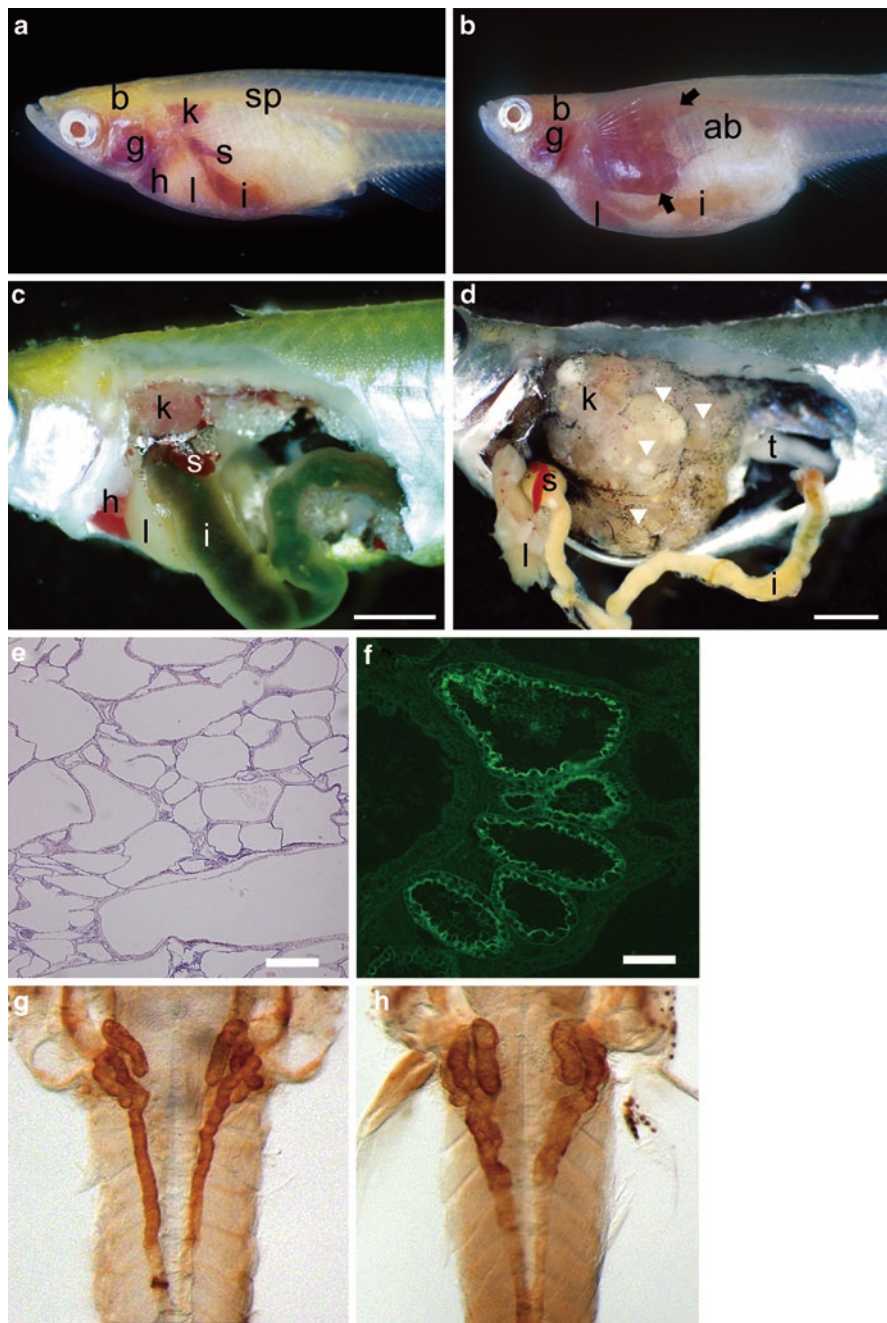


Fig. 8.5 Medaka *pc* mutant showing polycystic kidney disease (PKD). **a, b** External appearance of normal see-through (**a**) and *pc* see-through (**b**) medaka. **c, d** Morphology of kidney in orange-red variety (**c**) and *pc* mutant (**d**). **e** Histology of PKD in *pc* mutant (6 mph). **f** Cysts positive for lectin (*Locus tetragonolobus*) staining. **g, h** Whole mount immunostaining of Na⁺/K⁺ ATPase in wild-type (**g**) and *pc* mutant (**h**) pronephros (5 dph). *ab*, airbladder; *b*, brain; *g*, gill; *h*, heart; *i*, intestine; *k*, kidney; *l*, liver; *s*, spleen; *sp*, spinal cord; *t*, testis. Bars **c, d** 5 mm; **e** 200 μm; **f** 100 μm

2 mph, as does the wild-type fish, and dies at approximately 6 mph, earlier than the wild-type fish, which has a lifespan of 12–18 months under laboratory conditions (Mochizuki et al.).

8.4.3 Histology of PKD in the *pc* Mutant

In the *pc* mutant at the adult stage, the kidney is occupied by numerous fluid-filled cysts, lined by highly attenuated epithelial cells that form a single layer (Fig. 8.5e). Some fractions of the tubular epithelium lining the cysts are positive for lectin *Lotus tetragonolobus* (LTL) staining, suggesting that the cysts, although not all of them, are formed in the proximal segment of the renal tubule (Fig. 8.5f). The interstitial lymphoid cells of the renal tissue become lost as the kidney is occupied by the cysts.

8.4.4 Early Phase of the PKD Phenotype

Although abdominal enlargement can be externally recognized in the *pc* mutant within 1.5 mph at the earliest, tubular distension of the *pc* mutant kidney is detectable at 4–5 dph, when medaka fry has only the pronephros; this indicates that the renal cysts develop in the pronephros as well as in the mesonephros in the *pc* mutant. The early phase of renal distension can be viewed by immunostaining with anti-Na/K ATPase antibody (Fig. 8.5g,h).

8.4.5 The *pc* Locus Encodes *glis3*

By positional cloning, the causal gene of the medaka *pc* mutant has been identified to be *glis3*. GLIS3 was first identified as a homolog of Gli, a component of the Hedgehog (Hh) signaling pathway (Kim et al. 2003). Glis3 contains five C2H2-type zinc fingers that are closely related to the Gli and Sox families. Medaka Glis3 protein has 53% and 51% sequence similarities to human and mouse GLIS3, respectively. The medaka *glis3* gene consists of ten exons, the third exon of which is alternatively spliced to produce two different mRNAs (Fig. 8.6a,c). In the *pc*

Fig. 8.6 (continued) (CCCTTGTGCTGTCTTAGG) inverted repeat. **c** Structures of *glis3* mRNA (upper, wt) and predicted protein (lower, *pc*). Replacement of normal exons 5–10 by aberrant exons *p5–p9* leads to loss of four of five zinc fingers (green boxes) and nuclear localization signal (diamond shape). **d, e** *glis3* mRNA expression at 5 dph in wild type (**d**) and *pc* mutant (**e**). **f** *glis3* mRNA expression in the 20 dph wild-type (WT) kidney. **g, h, i** Cyst formation in *glis3* knockdown individuals. Gene-specific knockdown with antisense oligonucleotides resulted in dilation of the renal tubules (**h**), ducts (not seen in this figure), and glomera (**i**). In **g**, a cross section of a hatching fry with no notable dilation is shown. **j** *glis3* mRNA expression in the 5 pdh wild-type pancreas. Arrow, pronephros. **k, i** Insulin mRNA expression at 5 dph in wild type (**k**) and *pc* mutant (**i**). *gl*, glomus; *gu*, gut; *nc*, notochord; *nt*, neural tube; *rt*, renal tubule; *yo*, yolk; *stars*, typical dilation of the tubule

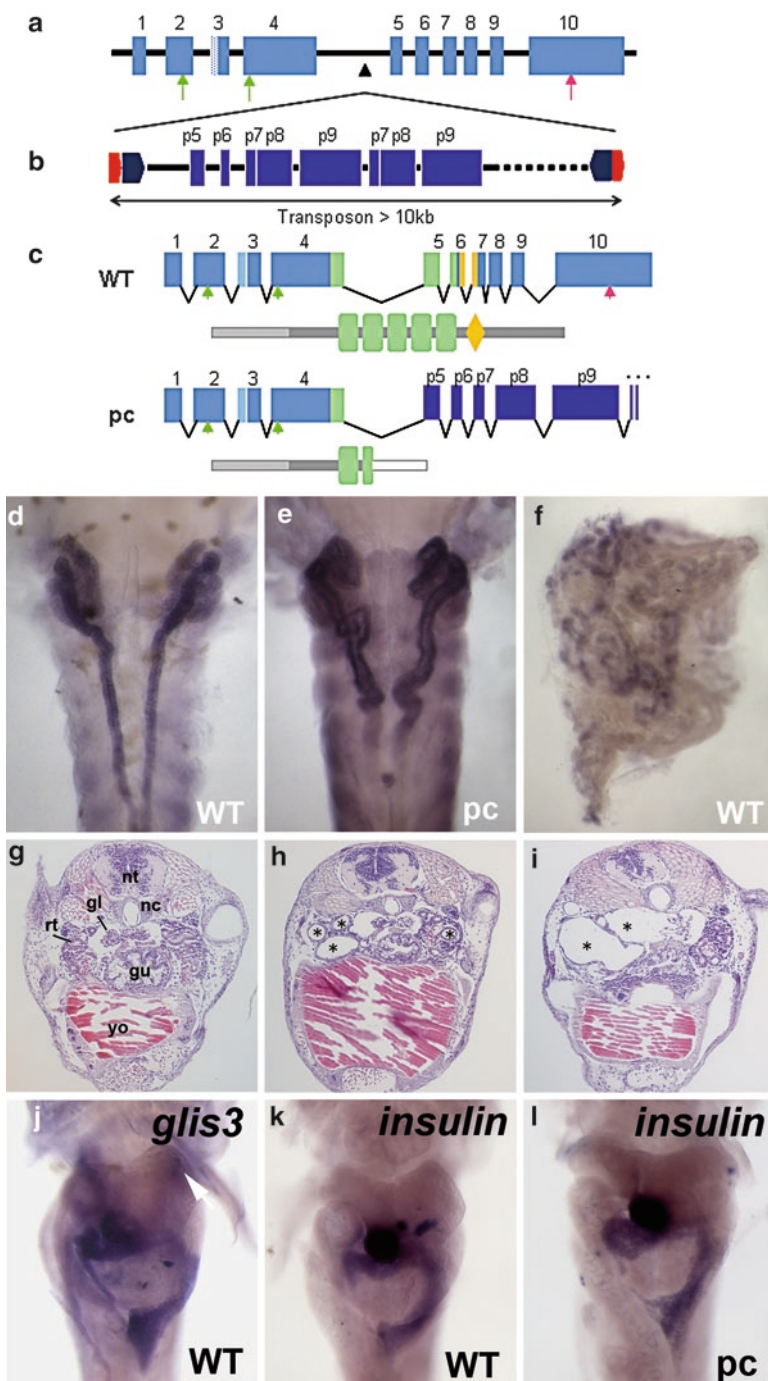


Fig. 8.6 (color online) *glis3* gene, causative for *pc* mutation. **a** Structure of medaka *glis3* gene. Alternative splicing of the 5'-region in exon 3 produces two different mRNAs with two different presumptive start codons. Arrows, positions of the start and the stop codons; arrowhead, site of the insertion of transposon in **b**. **b** Transposon insertion (>10 kb). The regions behaving as exons of the *pc* mutant mRNA are indicated by purple boxes (p5–p9). Red pentagon, 4 bp (TTAA) end sequence; navy pentagon, 18 bp

mutant, the 3'-region of the *glis3* transcript is replaced by an aberrant sequence that is not similar to that of any known gene. Because the *glis3* mRNA in the *pc* mutants lacks a normal 3'-region, the resulting defective Glis3 product has a normal N-terminal domain and one intact zinc finger but lacks the normal C-terminal domain including the four zinc fingers (Fig. 8.6c). Detailed analysis of the *pc* mutant genome has revealed that intron 4 in the *pc* mutant contains an insertion of a transposon-like element (Fig. 8.6b). *glis3* mRNA is detected in the tubular and ductal epithelium of pronephros and mesonephros (Fig. 8.6d,f). Thus, the *glis3* expression domain is consistent with manifestation of the *pc* phenotype. Despite the abnormal 3'-region, *glis3* mRNA is expressed in the affected tubules and ducts of the *pc* mutant (Fig. 8.6e). Furthermore, ablation of *glis3* function by knockdown with anti-sense oligonucleotide phenocopies the cystic kidney of the *pc* mutant (Fig. 8.6g–i). The *glis3* knockdown hatchlings show severe dilation of the pronephric tubule and duct and sometimes of Bowman's capsule (glomerular cyst). The diameter of the tubular lumen in the knockdown fry is three- to fourfold larger than in wild-type fry. *glis3* mRNA is also expressed in the pancreas (Fig. 8.6j), overlapping the expression domain of *insulin* mRNA (Fig. 8.6k). Nevertheless, the pancreatic cells positive for *insulin* mRNA do not seem to be affected in the *pc* mutant (Fig. 8.6l).

8.4.6 *pc/glis3* Is a Ciliary Gene

Expression analyses reveal that *glis3* expression is maintained in the ciliated epithelium of the definitive tubules and ducts of pronephros and mesonephros. In PKD models, pathological features are attributed to the absence or structural defects of cilia and/or ciliary dysfunction (Igarashi and Somlo 2007). Some of the ciliary defects in some zebrafish and medaka PKD models are manifested as impaired urine flow rate (Kramer-Zucker et al. 2005; Omran et al. 2008), which may be related to the active function of their motile renal cilia driving the urine to the urinary bladder. Dye secretion experiments (Fig. 8.7a–c) have suggested the

Fig. 8.7 (continued) Fluorescent images at 2 min (**b**) and 5 min (**c**) after dye injection. *Arrowhead*, dye excretion into the bladder. **d** The time after injection until first entry of fluorescence into the bladder was 153.0 ± 14.7 s (sec) in wild-type fry ($n=7$) and 348.0 ± 164.9 s in *pc* mutants ($n=9$). Time to excretion for each individual is indicated by a *triangle* (s). *Horizontal bars* show mean values. **e, f** Confocal fluorescence images of the cilia in tubular segments immunostained by anti-acetylated alpha-tubulin antibody (*arrowheads*): **e** wild type; **f** *pc* mutant. **g** Measurement results of ciliary length. The cilia were significantly shorter in *pc* mutants (10.73 ± 0.44 mm, $n=3$, at 0 dph and 10.43 ± 0.21 mm, $n=3$, at 5 dph; *blue triangles*) than in the wild type (14.97 ± 0.51 mm, $n=3$, at 0 dph and 15.28 ± 0.74 mm, $n=3$, at 5 dph; *orange triangles*) ($P < 0.005$). *Horizontal bars* show mean values. **h** Subcellular localization of *glis3* protein in Dai1 cells (mouse cultured renal epithelial cells). Enhanced green fluorescent protein (EGFP)-tagged *glis3* protein fluorescence was detected along the entire length of the cilium (**h1, h2**). Left, EGFP fluorescence; *middle*, acetylated alpha-tubulin, used as a cilium marker (Alexa 555); right, merged pictures. The *glis3* fluorescent signal was also detected in the nucleus (**h1**). *Bars e, f, h* 10 mm

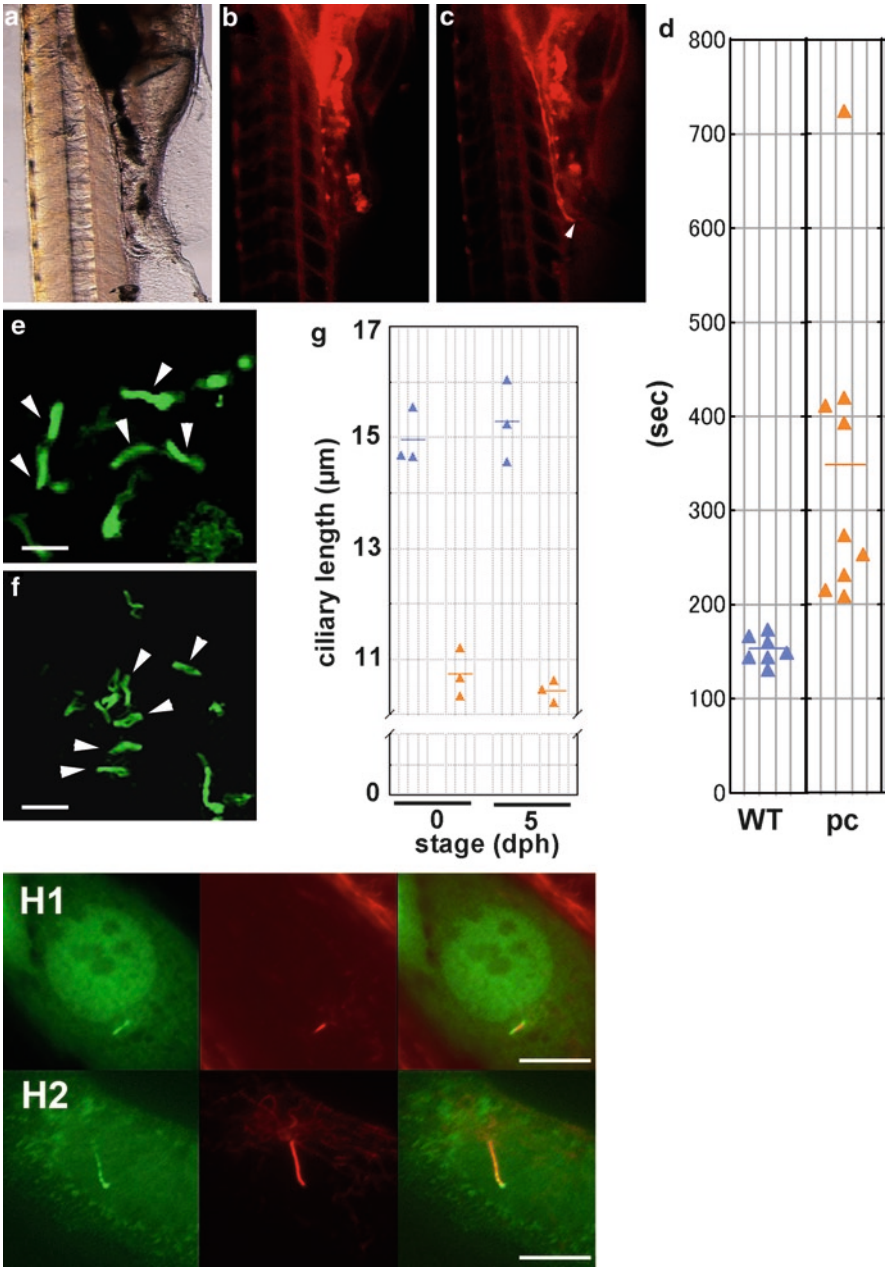


Fig. 8.7 (color online) Ciliary defects in *pc* mutant. **a, b, c** Dye excretion assay. The fluorescent dye (5% rhodamine-conjugated dextran) was injected into the common cardinal vein of 10 dph fry. The dye was filtered in the pronephric glomera, passed through the pronephric tubules and ducts, and subsequently excreted into the bladder. **a** Transmitted light image of the wild-type fry. **b, c**

reduction of urine flow rate in the *pc* mutant fry (1 day old), not caused by dilation of the tubules or ducts (Fig. 8.7d). Examination of the motility of renal cilia has further implied that in kidneys of the *pc* mutant the ciliary beat is indistinguishable from that observed in the wild type. However, measurement of ciliary length has shown that the cilia in the renal tubule are significantly shorter in *pc* mutants than in wild-type fish (Fig. 8.7e–g). These results imply that the abnormally short cilia in *pc* mutants are responsible for the relatively decreased rates of urine flow, and that this leads to the accumulation of urine and distension of the tubules and ducts in the pronephros. Further evidence implying a link between the cilia and PKD pathogenesis in the medaka *pc* mutant is derived from the analysis of the subcellular localization of the Glis3 protein (Fig. 8.7h). In the mouse renal epithelia cell line (Dai1 cell), the fluorescence of exogenously expressed Glis3-green fluorescent protein (Glis3-GFP) can be found in a punctuate fashion throughout the entire ciliated region, overlapping with the immunohistochemical signal from acetylated alpha-tubulin. In a large fraction of the cells, Glis3-GFP fluorescence is also detected in the nucleus, indicating that Glis3 protein is preferentially delivered to both the nucleus and the cilia in the Dai1 cells. All the results together suggest that Glis3 function is related to the cilia of renal epithelium in medaka. The localization of Glis3 protein to the cilia strongly implies its involvement in ciliary signaling to maintain the proper size of the tubular and ductal lumen. It is not clear if or how the reduction of the ciliary length is caused by the Glis3 dysfunction in the ciliated cells or is merely a consequence of the altered characteristics of the ciliated cells.

8.4.7 *Is the pc Mutant a Counterpart of the Human Patient with glis3 Mutation?*

In the human and mouse genome, there are three *GLIS* genes: *GLIS1*, *GLIS2*, and *GLIS3*. In the medaka genome database, four *glis* genes can be identified, including two *glis1* orthologs, one *glis2*, and one *glis3*. *rfx3* is located adjacent to the flanking region of medaka *glis3*, while the other *glis* genes are not adjacent to *rfx3* or other *rfx* isoform genes. This synteny is conserved in all vertebrate species examined (humans, mice, zebrafish, and pufferfish), indicating that medaka *glis3* is equivalent to human and mouse *GLIS3/Glis3*.

Senec et al. reported that mutations in *GLIS3* are responsible for a rare syndrome with pleiotropic phenotype that includes neonatal diabetes mellitus, congenital hypothyroidism, and in some cases cystic kidney (Senec et al. 2006). Three distinct alleles of *GLIS3* are involved in this syndrome. One allele, NDH1, which harbors a single base-pair insertion that results in a frameshift mutation and a truncated protein, causes cystic kidney, whereas the other two alleles, NDH2 and NDH3, which have large deletions in the *GLIS3*-flanking region, do not. The mutation found in *glis3* of the medaka *pc* mutant was caused by a large transposon inserted at a position corresponding to the second zinc finger, resulting in a defective zinc finger motif and the absence of the C-terminal region.

Truncation of the C-terminal region of GLIS3 has also been shown to inhibit its transcriptional promoting activity *in vitro*. These lines of evidence suggest that the C-terminal domain of Glis3 is crucial for optimal function of this protein in the kidney. Despite the allele-specific manifestation of cystic formation in human patients, the findings in medaka have clearly shown that loss of Glis3 function can lead to renal cyst formation across species. The *glis3*-knockdown but not the *pc* mutation leads, in some cases, to formation of glomerular cysts or to more severe and earlier onset dilation of the renal tubules (Fig. 8.6). The *Glis3*-deficient mice also develop glomerular cysts in nephrons of developing metanephros (Kang et al. 2009). These results imply that the *pc* mutation of *glis3* may be a hypomorphic allele.

The results from human patients indicate that *GLIS3* mutations cause neonatal diabetes despite alleles (Senee et al. 2006). The *Glis3*-deficient mice show significant increases in basal blood sugar level during the first few days after birth (Watanabe et al. 2009). The high levels of blood sugar are attributed to a decrease in the *Insulin* mRNA level in the pancreas, which is caused by impaired islet development and the subsequent impairment of insulin-producing cell formation. In the *Glis3*-deficient mice, other pancreatic endocrine cells including glucagon- and somatostatin-producing cells are also impaired. These observations suggest that Glis3 is required for the normal pancreatic endocrine cell formation in mice (Watanabe et al. 2009). By contrast, in the medaka *pc* mutant, no prominent phenotypes are found in the pancreatic tissues, although transcripts of *glis3* are abundantly expressed in the beta cells of medaka (see Fig. 8.6). Differing from the results in *Glis3*-deficient mice, no marked decrease in *insulin*-positive cells is observed in the *pc* mutants. It is not clear at present if endocrinological features of insulin-producing cells in medaka are equivalent to those in mammals. Notably, however, there are fundamental differences in the nutritional requirements of humans and fish. For example, amino acids rather than glucose in the blood are the most important insulinotropins in fish. The pathogenesis of diabetes may differ between mammals and fish. However, if it is assumed that some members of the *glis* family have similar activities (Kim et al. 2002, 2003, 2005), the overlapping expression of the different *glis* members in the pancreas but not in the kidney might preclude the occurrence of a pancreatic phenotype in the *pc* mutant. Ultimately, this difference in phenotype between mammals and medaka would position the *pc* mutant as a kidney-specific disease model of human *GLIS3* mutations.

8.5 Conclusions

Although the medaka mesonephros is evolutionarily different from the human metanephros, most of its fundamental features are well conserved, as is seen in the histological appearance and cellular nature of nephron development. As in mammalian kidneys, nephron stem cells, which derive from the mesenchymal cell aggregation, change their shape and are differentiated to a tadpole-shaped nephrogenic body and

finally form mature nephrons. When damaged by a nephrotoxicant, the medaka kidney (mesonephros) re-boosts the process of nephrogenesis, even in adulthood. Thus, medaka has the ability to regenerate the nephrons through repopulation of the existing cells and neogenesis from the renal stem cells in response to injury. *wt1* expression marks developing immature nephrons in the development and regeneration processes. Transgenic techniques to label the *wt1*-positive cells with GFP could be utilized to isolate the nephron progenitors and further to identify gene cascade(s) to control differentiation of nephrons. It is reported that the *Tg(wt1b:GFP)* transgenic zebrafish shows pronephros-specific GFP expression (Perner et al. 2007). As this transgenic line expresses GFP in all the nephron segments in the developing pronephros and also in other organs, identification of the enhancer element that drives expression in the developing nephrons would be needed for the regenerative studies described here. I wish to emphasize one great advantage of using the medaka as a model: a medaka fish has only 300 nephrons in the definitive kidney. Medaka also serves as a good source for identifying the genes of human genetic diseases, the phenotypes of which are relevant to medaka mutants. Completion of a high-quality draft genome will accelerate the identification of mutated genes from a large stock of medaka mutants including human disease models. Our findings on the *glis3/pc* mutants reveal mutations in the same gene in human and medaka results in the same or similar symptoms (phenotypes). Medaka mutants could be good models of human diseases for drug screening and therapeutic development.

References

- Bisgrove BW, Yost HJ (2006) The roles of cilia in developmental disorders and disease. *Development (Camb)* 133:4131–4143
- Cantley LG (2005) Adult stem cells in the repair of the injured renal tubule. *Nat Clin Pract Nephrol* 1:22–32
- Chauvet V, Tian X, Husson H, Grimm DH, Wang T, Hiesberger T, Igarashi P, Bennett AM, Ibraghimov-Beskrovnaya O, Somlo S, Caplan MJ (2004) Mechanical stimuli induce cleavage and nuclear translocation of the polycystin-1 C terminus. *J Clin Invest* 114:1433–1443
- Cormier SM, Neiheisel TW, Racine RN, Reimschuessel R (1995) New nephron development in fish from polluted waters: a possible biomarker. *Ecotoxicology* 4:157–168
- Drummond I (2003) The skate weighs in on kidney regeneration. *J Am Soc Nephrol* 14:1704–1705
- Drummond IA, Majumdar A, Hentschel H, Elger M, Solnica-Krezel L, Schier AF, Neuhauss SC, Stemple DL, Zwartkruis F, Rangini Z, Driever W, Fishman MC (1998) Early development of the zebrafish pronephros and analysis of mutations affecting pronephric function. *Development (Camb)* 125:4655–4667
- Elger M, Hentschel H, Litteral J, Wellner M, Kirsch T, Luft FC, Haller H (2003) Nephrogenesis is induced by partial nephrectomy in the elasmobranch *Leucoraja erinacea*. *J Am Soc Nephrol* 14:1506–1518
- Fedorova S, Miyamoto R, Harada T, Isogai S, Hashimoto H, Ozato K, Wakamatsu Y (2008) Renal glomerulogenesis in medaka fish, *Oryzias latipes*. *Dev Dyn* 237:2342–2352
- Gilbert SF (2003) *Developmental biology*. Sinauer, Sunderland
- Harder W (1975) *Anatomy of fishes*. Schweizerbart, Stuttgart
- Hickman CPJ, Trump BF (1969) The kidney. In: Hoar WS, Randall DJ (eds) *Fish physiology*. Academic Press, New York, pp 91–239

- Igarashi P, Somlo S (2002) Genetics and pathogenesis of polycystic kidney disease. *J Am Soc Nephrol* 13:2384–2398
- Igarashi P, Somlo S (2007) Polycystic kidney disease. *J Am Soc Nephrol* 18:1371–1373
- Kang HS, Beak JY, Kim YS, Herbert R, Jetten AM (2009) Glis3 is associated with primary cilia and Wwtr1/TAZ and implicated in polycystic kidney disease. *Mol Cell Biol* 29:2556–2569
- Karp R, Brasel JA, Winick M (1971) Compensatory kidney growth after uninephrectomy in adult and infant rats. *Am J Dis Child* 121:186–188
- Kim YS, Lewandoski M, Perantoni AO, Kurebayashi S, Nakanishi G, Jetten AM (2002) Identification of Glis1, a novel Gli-related, Kruppel-like zinc finger protein containing trans-activation and repressor functions. *J Biol Chem* 277:30901–30913
- Kim YS, Nakanishi G, Lewandoski M, Jetten AM (2003) GLIS3, a novel member of the GLIS subfamily of Kruppel-like zinc finger proteins with repressor and activation functions. *Nucleic Acids Res* 31:5513–5525
- Kim SC, Kim YS, Jetten AM (2005) Kruppel-like zinc finger protein Gli-similar 2 (Glis2) represses transcription through interaction with C-terminal binding protein 1 (CtBP1). *Nucleic Acids Res* 33:6805–6815
- Kramer-Zucker AG, Olale F, Haycraft CJ, Yoder BK, Schier AF, Drummond IA (2005) Cilia-driven fluid flow in the zebrafish pronephros, brain and Kupffer's vesicle is required for normal organogenesis. *Development (Camb)* 132:1907–1921
- Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, Jaenisch R (1993) WT-1 is required for early kidney development. *Cell* 74:679–691
- Lagler KF, Bardach JE, Miller RR, May Passino DR (1977) *Ichthyology*. Wiley, New York
- Lin F, Moran A, Igarashi P (2005) Intrarenal cells, not bone marrow-derived cells, are the major source for regeneration in postischemic kidney. *J Clin Invest* 115:1756–1764
- Mochizuki E, Fukuta K, Tada T, Harada T, Watanabe N, Matsuo S, Hashimoto H, Ozato K, Wakamatsu Y (2005) Fish mesonephric model of polycystic kidney disease in medaka (*Oryzias latipes*) pc mutant. *Kidney Int* 68:23–34
- Nauli SM, Zhou J (2004) Polycystins and mechanosensation in renal and nodal cilia. *BioEssays* 26:844–856
- Nauli SM, Alenghat FJ, Luo Y, Williams E, Vassilev P, Li X, Elia AE, Lu W, Brown EM, Quinn SJ, Ingber DE, Zhou J (2003) Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells. *Nat Genet* 33:129–137
- Omran H, Kobayashi D, Olbrich H, Tsukahara T, Loges NT, Hagiwara H, Zhang Q, Leblond G, O'Toole E, Hara C, Mizuno H, Kawano H, Fliegauf M, Yagi T, Koshida S, Miyawaki A, Zentgraf H, Seithe H, Reinhardt R, Watanabe Y, Kamiya R, Mitchell DR, Takeda H (2008) Ktu/PF13 is required for cytoplasmic pre-assembly of axonemal dyneins. *Nature (Lond)* 456:611–616
- Perner B, Englert C, Bollig F (2007) The Wilms tumor genes *wt1a* and *wt1b* control different steps during formation of the zebrafish pronephros. *Dev Biol* 309:87–96
- Reimschuessel R (2001) A fish model of renal regeneration and development. *ILAR J* 42:285–291
- Reimschuessel R, Biggs K (1996) Zebrafish model for nephron regeneration following injury. Cold Spring Harbor Symposium on Zebrafish Development and Genetics. Cold Spring Harbor, New York
- Reimschuessel R, Bennett RO, May EB, Lipsky MM (1990) Development of newly formed nephrons in the goldfish kidney following hexachlorobutadiene-induced nephrotoxicity. *Toxicol Pathol* 18:32–38
- Reimschuessel R, Bennett RO, May EA, Lipsky MM (1993) Pathological alterations and new nephron development in rainbow trout *Oncorhynchus mykiss* following tetrachloroethylene contamination. *J Zoo Anim Med* 24:503–507
- Ricardo SD, Deane JA (2005) Adult stem cells in renal injury and repair. *Nephrology (Carlton)* 10:276–282
- Rookmaaker MB, Smits AM, Tolboom H, Van't Wout K, Martens AC, Goldschmeding R, Joles JA, Van Zonneveld AJ, Grone HJ, Rabelink TJ, Verhaar MC (2003) Bone-marrow-derived cells contribute to glomerular endothelial repair in experimental glomerulonephritis. *Am J Pathol* 163:553–562

- Salice CJ, Rokous JS, Kane AS, Reimschuessel R (2001) New nephron development in goldfish (*Carassius auratus*) kidneys following repeated gentamicin-induced nephrotoxicosis. *Comp Med* 51:56–59
- Senec V, Chelala C, Duchatelet S, Feng D, Blanc H, Cossec JC, Charon C, Nicolino M, Boileau P, Cavener DR, Bougneres P, Taha D, Julier C (2006) Mutations in GLIS3 are responsible for a rare syndrome with neonatal diabetes mellitus and congenital hypothyroidism. *Nat Genet* 38:682–687
- Serluca FC, Fishman MC (2001) Pre-pattern in the pronephric kidney field of zebrafish. *Development (Camb)* 128:2233–2241
- Taulman PD, Haycraft CJ, Balkovetz DF, Yoder BK (2001) Polaris, a protein involved in left-right axis patterning, localizes to basal bodies and cilia. *Mol Biol Cell* 12:589–599
- Tobin JL, Beales PL (2007) Bardet–Biedl syndrome: beyond the cilium. *Pediatr Nephrol* 22:926–936
- Vainio S, Lin Y (2002) Coordinating early kidney development: lessons from gene targeting. *Nat Rev Genet* 3:533–543
- Verghese E, Weidenfeld R, Bertram JF, Ricardo SD, Deane JA (2008) Renal cilia display length alterations following tubular injury and are present early in epithelial repair. *Nephrol Dial Transplant* 23:834–841
- Wang S, Luo Y, Wilson PD, Witman GB, Zhou J (2004) The autosomal recessive polycystic kidney disease protein is localized to primary cilia, with concentration in the basal body area. *J Am Soc Nephrol* 15:592–602
- Watanabe N, Kato M, Suzuki N, Inoue C, Fedorova S, Hashimoto H, Maruyama S, Matsuo S, Wakamatsu Y (2009) Kidney regeneration through nephron neogenesis in medaka. *Dev Growth Differ* 51:135–143
- Watnick T, Germino G (2003) From cilia to cyst. *Nat Genet* 34:355–356
- Yoder BK (2007) Role of primary cilia in the pathogenesis of polycystic kidney disease. *J Am Soc Nephrol* 18:1381–1388

Chapter 9

Primary Ciliary Dyskinesia in Fish: The Analysis of a Novel Medaka Mutant *Kintoun*

Daisuke Kobayashi and Hiroyuki Takeda

Abstract Primary ciliary dyskinesia (PCD) is a phenotypically and genetically heterogeneous disorder manifested as dysfunction of motile cilia. Recent investigations using medaka and zebrafish as disease model systems have contributed to the current understanding of the formation and function of motile cilia. This chapter summarizes these data.

We also present our recent investigations of a novel PCD gene, *kintoun* (*ktu*), which was discovered in a medaka mutant. This gene was found to be mutated in patients with PCD from two affected families, as well as in the *Chlamydomonas pf13* mutant. In the absence of Ktu/PF13, both outer and inner dynein arms are missing or are defective in the axoneme, leading to a loss of motility. Biochemical and immunohistochemical studies show that Ktu/PF13 is one of the long-sought proteins involved in cytoplasmic preassembly of dynein arm complexes before intraflagellar transport loading into the ciliary compartment.

Abbreviations

DRC	Dynein regulatory complex
IDA	Inner dynein arm
IFT	Intraflagellar transport
KTU/Ktu	Kintoun
KV	Kupffer's vesicle
MT	Microtubule

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ODA	Outer dynein arm
OMIM	Online Mendelian inheritance in man
PCD	Primary ciliary dyskinesia
TEM	Transmission electron microscopy

9.1 Introduction

Cilia and flagella are hair-like organelles projecting from the cell surface that are important for a diversity of cell functions. Defects in the structure and function of sensory and motile cilia result in pleiotropic disorders, or ciliopathies.

Motility defects involving cilia and flagella often result in primary ciliary dyskinesia (PCD). We recently identified a novel PCD gene, *kintoun* (*ktu*). *ktu* was initially identified as a causative gene for a medaka ciliary mutant, and the homozygous loss-of-function mutations of its human ortholog were identified in two consanguineous families. In addition, we discovered that the *ktu* homolog in *Chlamydomonas* is PF13, a gene locus required for the formation of the outer dynein arm (ODA). In this chapter, we introduce this novel protein, which is critical for the proper function of motile cilia. We also focus on recent reports that studied cilia motility using zebrafish as a model system and then present the story of *ktu*. For a more detailed review of ciliary signaling, including sensory cilia, in fish, please see the recent article by Wessely and Obara (Wessely and Obara 2008).

9.1.1 Cilia Structure and Functions

Cilia and flagella are composed of a microtubule-based axoneme covered by a plasma membrane that extends from the eukaryotic cell surface (Christensen et al. 2007; Hagiwara et al. 2004; Satir and Christensen 2007). The cilia membrane is continuous with the plasma membrane of the cell body but contains a distinct subset of receptors and other proteins involved in signaling pathways. By classical distinction, cilia are classified as “9+2” (motile) or “9+0” (immotile), referring to the axonemal organization of doublet microtubules (MTs; Fig. 9.1a); “9” refers to the number of peripheral doublet MTs present in the ciliary axoneme and “2” or “0” refers to the presence or absence of a central doublet pair. Motility requires axoneme-associated dynein arms to generate sliding of microtubules to generate motion.

Fig. 9.1 (continued) In kidney, monocilia of a 9+0 structure are present in human and mouse. In contrast, in fish kidney, motile cilia of a 9+2 structure are present. The sperm flagella have a 9+2 structure. Motile cilia of a 9+2 structure line the uterus and fallopian tubes. Cilia with 9+0 axonemes on the mouse node and medaka Kupffer’s vesicle (KV) possess dynein arms and are motile. Zebrafish KV cilia have a 9+2 structure

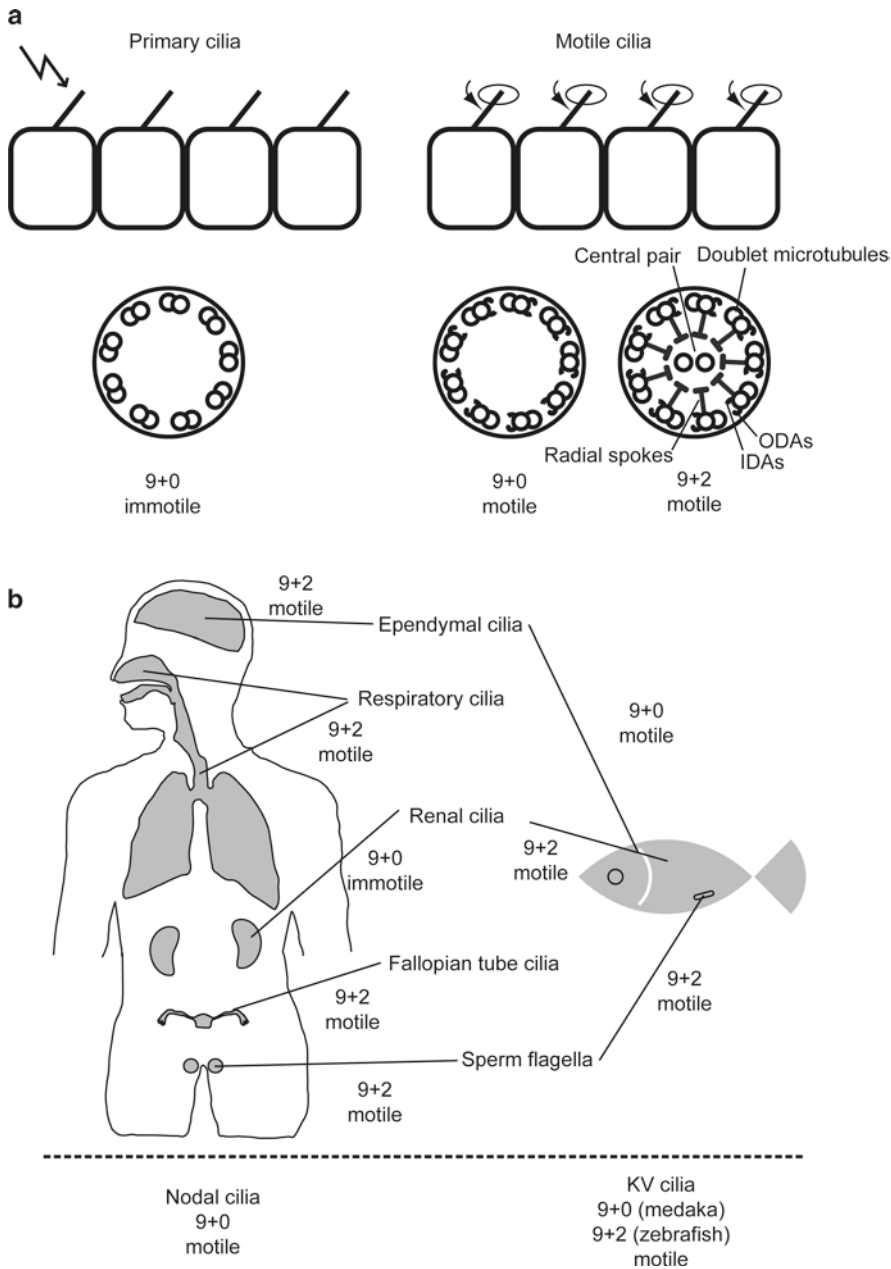


Fig. 9.1 Ciliary structure in vertebrates. **a** Schematic representation of 9+2 and 9+0 axoneme cross sections. The axoneme of 9+2 cilia is composed of nine outer doublets of microtubules surrounding a central pair, whereas 9+0 the latter is not present. Outer dynein arms (ODAs) and inner dynein arms (IDAs) are responsible for generating force for movement. **b** In the brain and spinal cord, the ependymal cells lining the ventricles carry motile cilia with a 9+2 ultrastructure. In the respiratory tract, epithelial cells are covered with motile cilia of 9+2 ultrastructure

In addition to the “9+2” motile cilia, some “9+0” cilia contain dynein arms. For example, cilia with “9+0” axonemes on the mouse node and medaka Kupffer’s vesicle (KV) possess dynein arms and are motile.

Motile cilia are present in a variety of tissues, such as surface epithelial cells lining airways, ependymal cells of the brain ventricles, the embryonic node (or its equivalent organs such as KV), and sperm. The main function of these motile cilia is to move extracellular fluids or substances, for example, in mucociliary clearance of the airways, cerebral spinal fluid across the epithelial surface, and extraembryonic fluid flow (nodal flow or KV flow). Flagellar motility is also required for the swimming motion in sperm motility (Fig. 9.1b).

Primary cilia are nonmotile sensory organelles present on the surface of most eukaryote cells. Recent studies indicate that cilia have crucial roles in the signal transduction pathways that mediate intracellular Ca^{2+} levels, Hedgehog (Hh) signaling, Wnt/planar cell polarity (PCP), and platelet-derived growth factor receptor (PDGFR) signaling. Primary cilia function as mechanical and chemical sensors of the extracellular environment and coordinate a series of signal transduction pathways in a cell (Badano et al. 2006; Christensen et al. 2007; Fliegauf et al. 2007; Gerdes et al. 2009).

9.1.2 Ciliopathy

Dysfunction of cilia gives rise to a broad range of human diseases, including polycystic kidney disease (PKD), nephronophthisis (NPHP), Bardet–Biedl syndrome (BBS), oral-facial-digital type 1 (OFD1), Meckel–Gruber syndrome (MGS), and PCD (Badano et al. 2006). The diverse phenotypes caused by ciliary dysfunction reflect the wide variety of ciliary functions and the broad expression of ciliary proteins in various tissues. For a more detailed view on the function of cilia and ciliopathy, see recent reviews focused on cilia (Badano et al. 2006; Christensen et al. 2007; Fliegauf et al. 2007; Gerdes et al. 2009).

9.1.3 Primary Ciliary Dyskinesia

Primary ciliary dyskinesia (PCD) [Online Mendelian Inheritance in Man (OMIM) accession number, MIM242650] is a collection of heterogeneous disorders characterized by recurrent respiratory tract infections, sinusitis, bronchiectasis, and male infertility. Approximately 50% of the patients exhibit *situs inversus*. The combination of PCD and *situs inversus* is also referred to as Kartagener syndrome (MIM 244400). This disease affects 1 in 20,000 to 1 in 60,000 individuals at birth and is usually inherited as an autosomal recessive trait (Zariwala et al. 2007). PCD disease phenotypes are directly related to motility defects in respiratory cilia, cilia of ependymal cells, cilia of the embryonic node, and sperm.

Currently, seven genes have been linked with PCD: *DNAI1* (MIM 604366) (Pennarun et al. 1999), *DNAH5* (MIM 603335) (Olbrich et al. 2002; Omran et al. 2000), *DNAH11* (MIM 603339) (Bartoloni et al. 2002), *DNAI2* (MIM 605483) (Loges et al. 2008), *TXNDC3* (MIM 607421) (Duriez et al. 2007), *KTU* (MIM 612517) (Omran et al. 2008), *OFD1* (MIM 311200) (Budny et al. 2006), and *RPGR* (MIM 312610) (Moore et al. 2006). The first five genes encode subunits of axonemal dyneins, highlighting the importance of dynein motors for cilia motility. This relationship is consistent with the observation that typical ultrastructural defects in PCD consist of a total or partial absence of dynein arms (Ibanez-Tallon et al. 2003). However, other ultrastructural defects in central tubules, radial spokes, and peripheral microtubule anomalies are reported, suggesting that many ciliary genes underlie the pathogenesis of PCD and still remain to be elucidated. Less frequently, no ultrastructural defects can be identified in patients with PCD (Ibanez-Tallon et al. 2003). In these cases, defects in non-axonemal proteins are most likely involved in PCD. The last two genes in the list above, *OFD1* and *RPGR*, are known to be responsible for other types of ciliopathy. Only a portion of the patients presenting with defects in *OFD1* or *RPGR* display characteristic PCD phenotypes, suggesting that these two genes have a general function or dual roles for cilia. The next section focuses on *KTU*, the first cytoplasmic protein related to PCD (Omran et al. 2008).

9.1.4 Fish as a Ciliary Disease Model and an Experimental Tool

Medaka and zebrafish have become excellent models for the study of human diseases. Their transparent embryos develop outside of the mother's body, which allows for live-imaging analyses of early developmental processes. Furthermore, because the genes and organ structures are well conserved between medaka/zebrafish and humans, studies conducted using medaka and zebrafish have contributed to the understanding of human pathogenic mechanisms (Dooley and Zon 2000) (Table 9.1).

Large-scale mutagenesis projects have been conducted in medaka and zebrafish, providing a vast pool of mutant stocks as a resource for researchers (Driever et al. 1996; Furutani-Seiki et al. 2004; Haffter et al. 1996). The genome project in medaka and zebrafish generated high-quality whole genome sequences, facilitating the positional cloning of mutants with phenotypes of interest (Ahsan et al. 2008; Kasahara et al. 2007; Kobayashi and Takeda 2008; Takeda 2008) (Ensembl database; http://www.ensembl.org/Danio_rerio/), (UTGB/medaka; <http://medaka.utgenome.org/>).

In addition to typical forward genetics, a reverse genetic approach using the morpholino antisense oligonucleotide (MO) approach has been successfully applied to the study of genetic mutations in fish model systems. MOs are useful tools for reverse genetics, which knock down a specific gene's expression rapidly and economically (Nasevicius and Ekker 2000). MOs have been utilized in the study of gene function for the known causative genes involved in human ciliopathy (Beales et al. 2007; Cantagrel et al. 2008; Castleman et al. 2009; Otto et al. 2003;

Table 9.1 The common association of clinical features in zebrafish mutants/morphants

Human gene	<i>PKD1</i> ADPKD	<i>PKD2</i> ADPKD	<i>INVS/INV</i> NPHP2	<i>NPHP5</i> Mo	<i>NPHP6/CEP290</i> NPHP6	<i>ARL13B</i> JBTS8	<i>CC2DA2</i> JSRD
Zebrafish mutant	^a	<i>Curly up</i>	Mo	Mo	Mo	<i>Scorpion</i>	<i>Sentinel</i>
Retinitis pigmentosa	–	–	–	–	–	–	–
Hydrocephalus	–	+	–	+	+	–	–
Kidney cyst	+	+	+	+	+	+	+
Liver disease	(Cyst) ^a	–	–	–	–	–	–
LR defect	–	+	+	–	–	–	–
Infertility	–	–	–	–	–	–	–
Abnormal body curvature	–	+	+	+	+	+	–

References

PKD1: Low et al. (2006)
PKD2: Sun et al. (2004), Bisgrove et al. (2005), Schottenfeld et al. (2007)
INVS/INV: Otto et al. (2003)
NPHP5: Schafer et al. (2008)
NPHP6/CEP290: Schafer et al. (2008)
ARL13B: Cantagrel et al. 2008
CC2DA2: Gordon et al. (2008)
Primary ciliary dyskinesia (PCD) genes are shown in Table 9.2
ADPKD; autosomal dominant polycystic kidney disease; NPHP, nephronophthisis; JBTS, Joubert syndrome; JSRD, Joubert syndrome and related disorders; Mo, morphant

^aTail region of PC1 (PKD1 product) overexpression

Sayer et al. 2006). In some cases, the injection of human wild-type mRNAs rescued the phenotype of their corresponding mutant/morphant embryos (Otto et al. 2003; Streets et al. 2006), indicating evolutionarily conserved functions of disease-related genes in embryogenesis and pathogenesis. Thus, the functional impairment of the patient missense mutations can also be assessed using this system; patient mutant RNAs could be less effective or completely ineffective in rescue experiments when compared with the wild-type human mRNAs. The phenotypes of fish mutants and morphants are often reminiscent of those stemming from human diseases, providing valuable validated resources with which to gain insight into the corresponding human pathology (Drummond 2005).

Fish model systems have also been used for preclinical drug screening. Ectogenesis allows therapeutic compounds to be added directly to the incubation medium of embryos (Zon and Peterson 2005). Tobin and Beales have demonstrated the power of this system by identifying two different compounds, rapamycin and roscovitine, which ameliorate the morphological and functional deficits resulting from renal ciliopathy. This study validated fish as a therapeutic model for human ciliopathies (Tobin and Beales 2008).

9.1.5 Motile Cilia in Fish

In the medaka and zebrafish, cilia in the pronephric system, brain, and KV are motile and essential for proper organ development (Essner et al. 2005; Hojo et al. 2007; Kramer-Zucker et al. 2005; Mochizuki et al. 2005; Bisgrove and Yost 2006). In contrast with the immotile cilia observed in the mammalian kidney, the cilia in the medaka and zebrafish pronephric system are thought to generate urine fluid flow (Kramer-Zucker et al. 2005; Nihei et al., unpublished observation). Although flow in the mammalian metanephros depends upon glomerular blood pressure, in fish it has been reported that cilia motility is required to maintain normal fluid flow, with impairment of motility resulting in a cystic kidney. Furthermore, hydrocephalus, a distension of the brain ventricle, can also stem from impairment of cilia motility (Kramer-Zucker et al. 2005).

KV, a teleost-specific spherical organ attached to the tail region, is functionally equivalent to the mouse node in terms of left–right (LR) specification (Bisgrove et al. 2005; Brummett and Dumont 1978; Hirokawa et al. 2006; Okada et al. 2005). Studies in mice revealed that initial breaking of LR symmetry takes place in the node, with LR asymmetrical signals subsequently transferred to the lateral plate mesoderm. The initial break event in the node that is critical for determination of LR asymmetry is ciliary rotation (Okada et al. 1999). In recent years, these processes have been found to be evolutionarily conserved among vertebrates, including mice, chickens, frogs, and fish (Schweickert et al. 2007). KV contains motile monocilia on its epithelial cells and cilia motility generates a leftward liquid flow. Embryos that are defective in KV flow show abnormal LR development (Essner et al. 2005; Hojo et al. 2007; Kramer-Zucker et al. 2005; Okada et al. 2005; Schweickert et al. 2007).

9.2 Genes Involved in Primary Ciliary Dyskinesia Identified in Zebrafish

9.2.1 *Na, K-ATPase $\alpha 2$ and Ncx4a*

Shu et al. reported that in KV, Na, K-ATPase $\alpha 2$ and its functional partner Ncx4a are involved in cilia motility (Shu et al. 2007). Their investigations involved injection of Na, K-ATPase $\alpha 2$ -MO or Ncx4a-MO into dorsal forerunner cells (DFCs) in the yolk, which give rise to KV at the early blastula stage (128-cell stage) (Amack and Yost 2004). The morphants showed LR defects, whereas KV formation was not affected. In addition, KV cilia were immobilized and counterclockwise fluid flow in KV was perturbed, although the number and the length of cilia were not affected. They further showed that calcium/calmodulin-dependent protein kinase II (CaMKII) is a downstream mediator of Na, K-ATPase $\alpha 2$ - and Ncx4a-dependent calcium signaling. Treatment with KN62, a well-characterized CaMKII inhibitor, could significantly reduce the LR defects induced by Na, K-ATPase $\alpha 2$ -MO and Ncx4a-MO. Furthermore, KN62 treatment of Na, K-ATPase $\alpha 2$ -morphants restored cilia motility, indicating a role for Na, K-ATPase $\alpha 2$ /Ncx4a/CaMKII-mediated Ca^{2+} signaling in KV cilia motility. It was also revealed that Ca^{2+} levels negatively regulate KV cilia motility, although the mechanisms by which Ca^{2+} signaling regulates cilia motility in KV await further investigation. Overall, these results highlight a conserved role for Ca^{2+} signaling in cilia motility among eukaryotes (Shu et al. 2007).

9.2.2 *Leucine-Rich Repeat Containing Protein 50 (Lrrc50)*

The zebrafish LR mutants *switch hitter* (*swt*) and *hu255H* loci were independently cloned by two groups (Sullivan-Brown et al. 2008; van Rooijen et al. 2008). These loci encode a 562-amino-acid protein containing leucine-rich repeats known as Lrrc50. Zebrafish *lrrc50* is highly homologous to the *Chlamydomonas* gene *Oda7*, which is essential for axonemal ODA assembly (Freshour et al. 2007; Kamiya 1988). Sullivan-Brown et al. revealed that cilia in *swt* mutant pronephros were grossly normal in length but were completely immotile. They also showed that *swt* was expressed in multiple tissues that have motile cilia, further supporting the idea that Lrrc50 functions in cilia motility. Ciliary motility in the *lrrc50*^{*hu255H*} pronephros was assessed by pronephric fluid flow in a dye excretion test (Kramer-Zucker et al. 2005). Dye excretion in the *lrrc50*^{*hu255H*} mutant embryos was markedly reduced or absent, indicating that cilia motility in the pronephric duct was also impaired. Ultrastructurally, most mutant cilia lack ODAs, and inner dynein arms (IDAs) are also partially affected. Together with the fact that *Chlamydomonas* Oda7 is required for proper ODA assembly, Lrrc50 is an indispensable component of dynein arm assembly but is dispensable for ciliogenesis.

9.2.3 *Inositol 1,3,4,5,6-Pentakisphosphate 2-Kinase (Ipk1)*

Ipk1, an enzyme catalyzing inositol hexakisphosphate synthesis, is required for zebrafish LR-specific gene expression and morphogenesis (Sarmah et al. 2005). Although gross cilia morphology and number of cilia are not affected in the *ipk1* morphant, KV ciliary beating is inhibited and the length of cilia in the spinal canal, pronephric ducts, and KV is decreased (Sarmah et al. 2007). Ultrastructural analysis by transmission electron microscopy (TEM) failed to detect any structure defects in cilia. The ciliary beating in *ipk1*-morphants were rescued by co-injection of wild-type *ipk1* mRNA, but not by kinase-dead Ipk1, indicating an essential role for Ipk1 kinase activity in this process. With GFP-Ipk1 enriched in centrosomes and basal bodies, it was proposed that Ipk1 plays a previously uncharacterized role in ciliary function.

9.2.4 *Radial Spoke Head Protein 9 (Rsph9)*

RSPH9 has been proposed as a candidate gene for PCD with central MT pair defects (Castleman et al. 2009; Yang et al. 2006). RSPH9 protein shows 28% identity to *Chlamydomonas* radial spoke head 9 (RSP9), which is mutated in *Chlamydomonas* flagella-immotile-mutant *pf17*. Zebrafish was utilized to study the function of RSPH9 (Castleman et al. 2009). RSPH9 morphants showed olfactory-pit cilia motility defects, which is reminiscent of the aberrant beating pattern of RSPH9 patient cilia defective in the central MT pair. Even though the *Chlamydomonas pf17* shows a displacement of central pair rather than deletion, RSPH9/RPS9 is thought to be essential for a correct central pair placement in cilia. Intriguingly, the LR axis was unaffected in *rsph9* morphants, suggesting that the central pair may be dispensable in zebrafish KV cilia. It is worth noting that medaka KV cilia lack the central pair. Consistently, LR defects have not been observed in patients with PCD showing ultrastructural abnormalities in the central MT pair, yet present with normal 9+0 nodal cilia (Stannard et al. 2004).

9.2.5 *Oral-Facial-Digital Type 1 (Ofd1)*

OFD1 is an X-linked dominant human developmental disorder characterized by craniofacial malformations, digital abnormalities, mental retardation, and male lethality (Ferrante et al. 2001). In a Polish family, OFD1 was reported to be accompanied by PCD (Budny et al. 2006). In zebrafish *ofd1* morphants, KV was ciliated, but their cilia were shortened and axonemal configuration was disrupted, leading to perturbed KV flow and a LR defect. Zebrafish morphants also exhibited hydrocephalus, reminiscent of malformations in human OFD1. Based on the structural anomalies of KV cilia in the *ofd1* morphants, it was proposed that Ofd1 facilitates cilia formation and/or maintenance.

9.2.6 *Forkhead Box j1 (Foxj1)*

The transcription factor Foxj1 has been implicated in motile cilia formation in mammals (Brody et al. 2000; Chen et al. 1998). Yu et al. reported that foxj1 is a master regulator of motile ciliogenesis in zebrafish (Yu et al. 2008). *foxj1a*, one of the *foxj1* homologues in zebrafish, is expressed in tissues with motile cilia, such as the floor plate, pronephric ducts, and KV. Analysis of morphants revealed that the development of motile cilia was severely impaired in these tissues. Consistent with the lack of motile cilia, pronephric cysts, hydrocephalus, and LR defects were evident in *foxj1a* morphants. Surprisingly, *foxj1a* mRNA injection induced ectopic cilia with 9+2 arrangement of microtubules and with ODAs on the peripheral doublet microtubules, substantiating the possibility that Foxj1a has a central role in triggering a genetic cascade leading to motile ciliogenesis. They also revealed that Foxj1a controls motile ciliogenesis through the transcription regulation of genes encoding components that are required for motile cilia. In addition to this direct regulation of motile ciliogenesis, *rfx2*, which encodes a transcriptional regulator involved in both sensory and motile cilia formation, is one of the downstream targets of Foxj1a. It is thus likely that *foxj1a* may function partially through *rfx2*. There is an additional zebrafish *foxj1*-like gene, *foxj1b*, which was also shown to participate in motile cilia formation. Stubbs et al. also reported that *Xenopus joxj1* has a similar function (Stubbs et al. 2008). Therefore, the *foxj1* family is likely to function as master control genes for motile ciliogenesis.

9.2.7 *Growth-Arrest-Specific 8, a Dynein Regulatory Complex Subunit*

Using zebrafish morphants, *gas8* was identified as an essential gene for cilia motility (Colantonio et al. 2009). Growth-arrest-specific 8 (Gas8) is a coiled-coil protein that shares significant homology with a *Chlamydomonas* PF2 and a trypanosome protein known as trypanin. Gas8 is a subunit of the dynein regulatory complex (DRC) that is required for cilia motility (Hutchings et al. 2002; Rupp and Porter 2003). DRC regulates axonemal dynein activity in response to signals from the radial spokes and the central pair apparatus. Consistent with the function of Gas8, morphants showed pronephric cysts, hydrocephalus, and LR defects, indicating that Gas8 is required for normal motility of cilia. Furthermore, they examined tethered cilia motility directly using high-speed microscopy and found that most of the morphants displayed immotile tether cilia.

9.2.8 *Fused*

In *Drosophila*, Hh signal transduction is mediated by a cytoplasmic signaling complex that includes the putative serine-threonine kinase Fused (Fu) and the kinesin Costal 2 (Cos2, also known as Cos), yet Fu does not have a conserved role in Hh signaling

in mammals. Mouse *Fu* (also known as *Stk36*) mutants are viable and respond normally to Hh signaling. However, *Fused* (*fu*) was recently found to be essential for construction of the central pair apparatus of motile, 9+2 cilia (Wilson et al. 2009). KV-targeted morphants had disorganized axonemal structures, including loss of or acquisition of an extra central pair of microtubules, resulting in loss of cilia motility and LR defects. This phenotype is consistent with the *fu*(-/-) mouse phenotype lacking a central pair (Wilson et al. 2009), suggesting a conserved role for vertebrate *Fu* in central pair construction. Thus, *Fused* has evolved to have divergent roles in vertebrate Hh signaling and motile ciliogenesis.

9.2.9 *Leucine-Rich Repeat-Containing Six-Like (lrrc6l, Seahorse)*

Two alleles of *seahorse* (*sea*), *sea*^{tg238a} and *sea*^{fa20r}, encode a leucine-rich repeat-containing protein, *lrrc6l*. Mutations in *sea*^{tg238a} and *sea*^{fa20r} do not affect the gross morphology of cilia but rather result in severe ciliary motility defects in the pronephros and neural tube (Serluca et al. 2009). In spite of this phenotype, no difference was found in the axoneme structure between the mutant and wild type. Intriguingly, in contrast to the predominant immotility in pronephric cilia, cilia motility in the mutant KV was variable. In this study, KV motility analysis was based on the movement of injected beads. Direct observation of KV cilia may give us further insight into *sea* function in KV cilia motility.

Another *lrrc6l* mutant insertional allele, *seahorse* (*sea*), was reported to cause pronephric cyst phenotypes, but did not affect ciliary motility (Kishimoto et al. 2008; Sun et al. 2004). Kishimoto et al. provided evidence that *Sea* participates in the Wnt/PCP pathway and interacts with Disheveled (*Dsh*), a key protein of the canonical Wnt and PCP pathways, suggesting a role for *Sea* in the Wnt/PCP pathway. Thus, *Sea* seems to play dual roles in cilia: one in the Wnt/PCP signaling pathway, and the other in the regulation of cilia motility. It will be interesting to identify the *Lrrc6l* functional domains that are required for either cilia motility or the Wnt/PCP signaling pathway. It will also be worthwhile determining which *Lrrc6l* protein-binding partner(s) are involved in these processes.

9.3 Medaka *ktu* Mutant

Because cilia motility defects result in pleiotropic phenotypes, it is important to understand the molecular and cellular basis for the formation and function of motile cilia. Through ciliary mutant analysis using model animals, novel ciliary proteins and additional roles for ciliary proteins are expected to be uncovered. These new insights will greatly contribute to our understanding of ciliogenesis, the roles of

cilia in development, and pathogenesis of ciliopathy. A novel medaka PCD mutant, *ktu*, is one such mutant. In the following section, we describe the story of *ktu*, in which we finally came up with the novel gene essential for ciliary motility from algae to humans.

9.3.1 Medaka Mutant Screening

Systematic screening for mutations among a mutagenized population of model organisms has been a powerful approach for identification of genes and their functions. Furthermore, some of these mutants share similar phenotypes, suggesting that mutated genes can be categorized into particular pathways. Thus, the pathogenic and molecular mechanisms underlying a disease of interest can be analyzed using these mutants. To study the genetic basis of cilia formation and/or function in vertebrate embryos, we carried out mutagenesis screening for ciliary defects in medaka. As described above, mutations in cilia-related genes often result in randomization of the LR axis, suggesting that LR mutants would be candidate ciliary mutants. LR anomalies are easily observable in medaka, because the transparent embryos develop outside the mother's body. Reversed heart looping and liver positioning were used as indexes for screening embryos at 4 days postfertilization.

We screened about 100 F₂ families to isolate medaka mutants with reversed heart looping or disposition of the liver. Ten LR mutants were isolated and used for further studies (Hojo et al. 2007; Yokoi et al. 2007 and unpublished observations). One of these mutants, *kintoun* (*ktu*), showed an intriguing phenotype (Omran et al. 2008). *ktu* homozygous fish are viable, grow to adulthood, and can be bred. However, nearly all homozygous fish gradually develop kidney enlargement (Fig. 9.2a–d). Histological analysis revealed that the mutant fish suffer from PKD (Fig. 9.2e, f). Because PKD is one of the typical phenotypes of ciliary diseases, *ktu* are a strong candidate for being a ciliary mutant.

Furthermore, homozygous male *ktu* exhibited low fertility, another phenotype of ciliary disease. To investigate sperm motility, we recorded swimming sperm using high-speed video-microscopy and found that their waveform was abnormal (Fig. 9.2g, h). Although the waveform of the wild-type sperm showed proper flagellar bending and propagation to the tip of the tail, mutant sperms looked paralyzed because of the affected propagation of the waveform. This observation suggested that *ktu* mutants have motility defects in cilia and flagella.

Mammalian kidney cilia are thought to be immotile and to serve only a sensory function, but previous research in medaka has demonstrated that renal epithelial cells bear single or multiple 9+2 cilia with dynein arms; thus, they are motile (Mochizuki et al. 2005; Nihei et al., unpublished data). Because impairment of cilia motility results in cystic kidneys in zebrafish, PKD in *ktu* could be the result of a cilia motility defect. We then examined ciliary motility in KV and found, using injected fluorescent tracers, that fluid flow is lost in *ktu* KV, although the formation of KV and KV cilia appears normal. To directly assess ciliary motility, high-speed

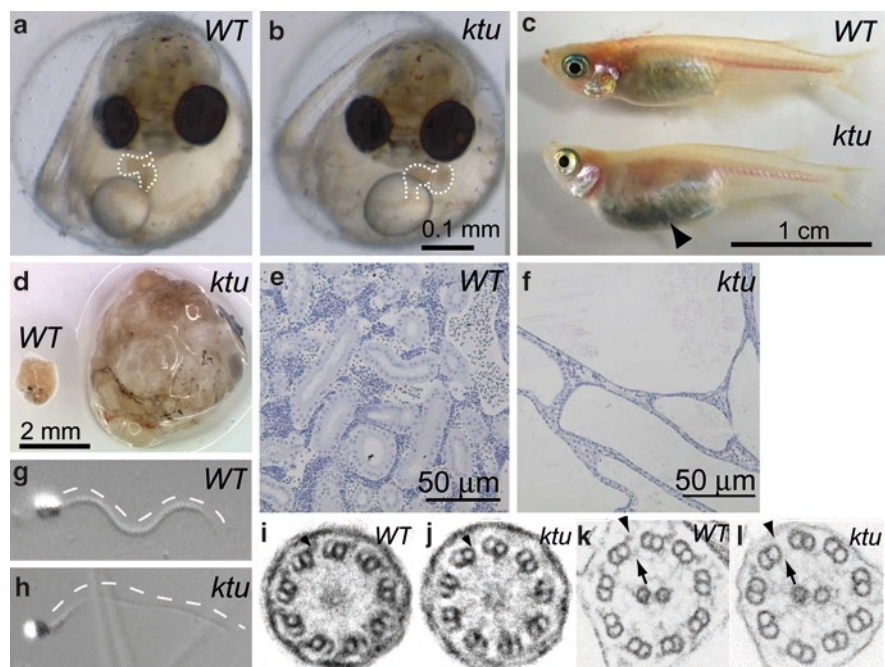


Fig. 9.2 Medaka *kintoun* (*ktu*) mutant. **a, b** The heart looping (dashed line) at 3 days post fertilization (dpf). **c** The belly of the mutant adult (3-month-old) becomes expanded (arrowhead): schematic structure. **d** Cyst formation in *ktu* mutant kidneys. The volume of the affected kidney dramatically increases. **e, f** Polycystic kidney disease (PKD) in *ktu* mutant kidney. **g, h** Swimming sperm. **i-l** Transmission electron micrographs (EM) of Kupffer's vesicle (KV) cilia (**i, j**; 9+0) and sperm flagella (**k, l**; 9+2), showing a partial or complete loss of ODA (arrowheads) and IDA (arrows) in *ktu*. WT, wild type

video-microscopy was applied. In wild-type embryos, the rotation of monocilia on the surface of KV epithelia was observed as previously reported (Okada et al. 2005). In contrast, KV cilia in *ktu* are completely immotile. These data suggested that the *ktu* gene encodes a protein essential for cilia motility.

Ultrastructural studies of KV cilia and sperm flagella revealed that dynein arms (DAs) are missing in the mutant cilia and flagella. In wild-type KV cilia, a 9+0 structure of MTs was observed, and ODAs were attached to the peripheral doublet MTs (Fig. 9.2i). In contrast, *ktu* mutant KV cilia completely lacked ODAs (Fig. 9.2j), indicating that the loss of ODAs likely causes the paralyzed phenotype. Similar defects were observed in sperm flagella. In wild-type sperm, flagella possessed the expected 9+2 structure MTs, with both ODAs and IDAs attached on the peripheral doublet MTs. In contrast, *ktu* mutant sperm flagella revealed the partial absence of ODAs and IDAs (Fig. 9.2k,l). This partial presence of ODAs and IDAs may account for the incomplete loss of motility in mutant sperm. These result indicated that *ktu* is required for proper dynein arm placement in cilia and flagella.

defect in PCD is the total or partial absence of dynein arms. Because the *ktu* mutant also exhibited dynein arm and cilia motility defects, we proposed that the human ortholog *KTU* was a promising candidate gene for human PCD. To investigate this hypothesis, we thus collaborated with a special medical research group in Freiburg led by Dr. Heymut Omran, which had collected genomic DNA from different PCD families.

We screened 112 DNA samples from individuals with PCD originating from distinct families for the presence of a mutation in the *KTU* gene by direct sequencing of the polymerase chain reaction (PCR)-amplified products of three coding exons. We identified homozygous loss-of-function mutations in two consanguineous families (Fig. 9.3c). Consistent with the medaka mutant phenotype, TEM of respiratory cilia and sperm tails identified abnormal axonemal dynein arms. High-speed video-microscopy confirmed complete immotility of respiratory cilia and sperm. Collectively, this evidence showed that, as in the medaka *ktu* mutant, mutations in *KTU* cause PCD with dynein arm defects. These patients with PCD had suffered from chronic otitis media and sinusitis, recurrent pneumonia, and bronchiectasis. Complete *situs inversus* was also observed in two of the three affected individuals with *KTU* mutations, which was consistent with randomization of LR body asymmetry. Interestingly, in contrast to medaka *ktu*, the kidneys of these patients were of normal size and devoid of cysts. This observation probably reflects a difference between species in the origin of kidneys, with a mesonephric origin for fish and a metanephric origin for mammals. The former requires sensory cilia, whereas the latter requires motile cilia for regulation of cell growth in the renal tubule.

9.3.4 *Chlamydomonas PF13 Is a Mutant of ktu Homologue*

Chlamydomonas is a single-celled alga that has been used extensively as a model system for the study of flagella (Fig. 9.4a). We collaborated with two experts on *Chlamydomonas*, Drs. David R. Mitchell and Ritsu Kamiya, and found that the *ktu* homologue in *Chlamydomonas* is *pf13*, a locus required for ODA assembly (Huang et al. 1979). *pf13* mutant cells show a severe swim defect, and their flagella are paralyzed. Consistent with the medaka and human mutant phenotypes, TEM images showed an almost complete absence of ODA and a partial absence of IDA, which likely accounts for the paralyzed phenotype (Kamiya 2002). These results suggested *ktu*/*pf13* function in cilia and flagella is highly conserved in organisms from single-cell algae to vertebrates.

9.3.5 *ktu Evolution and Function*

Database searches identified *ktu* homologous genes in other vertebrates, insects, and *Tetrahymena*, but did not find homologs in *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, or *Dictyostelium discoideum*. Because the latter

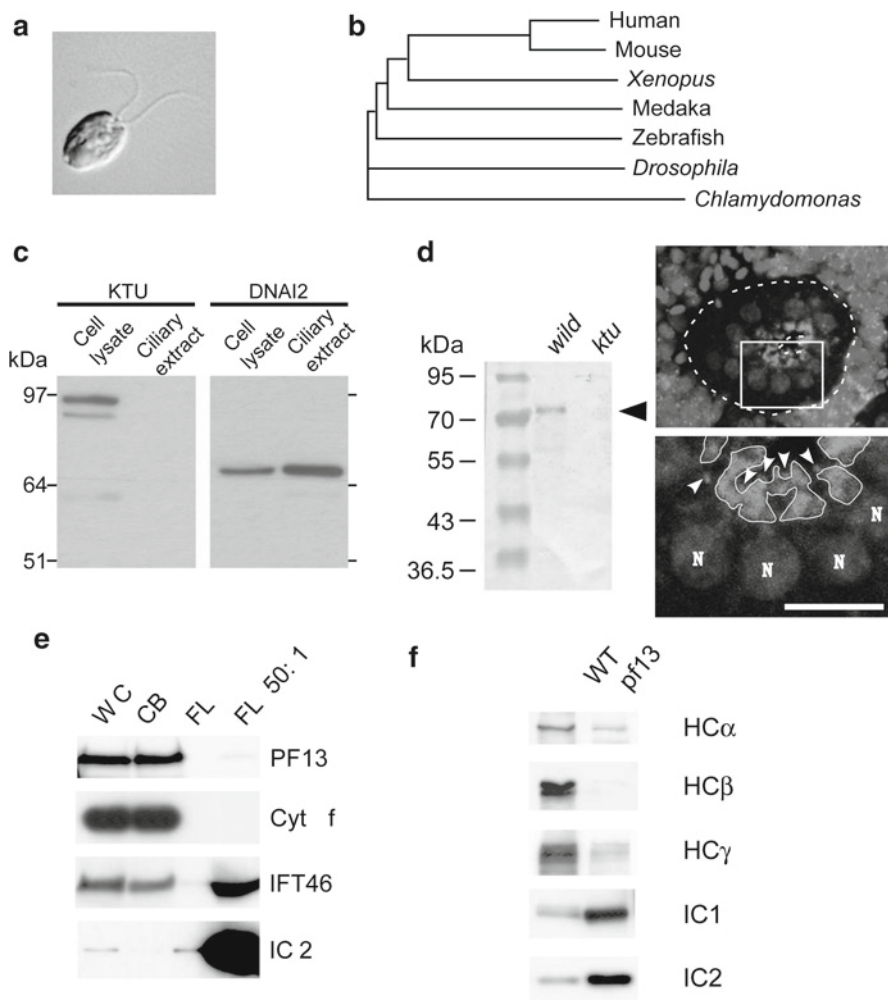


Fig. 9.4 Subcellular localization of *ktu* homologs. **a** *Chlamydomonas*. **b** A phylogenetic tree of Ktu proteins. The tree was made by the neighbor-joining (NJ) method using their conserved N-terminal region and short stretch of the C-terminal end. **c** In control respiratory ciliary extracts, KTU is absent, whereas DNAI2 is abundant. KTU is only present in the cytoplasm (*Human*). **d** Overlay and high-power images of a nephric tubule (dotted lines) double-stained with anti-Ktu (enclosed by white lines) and γ -tubulin (arrowheads) antibodies. Nuclei are indicated by N (*medaka*). **e** PF13 protein in whole cells (WC) and cell bodies (CB) but not in flagella. Absence of cytochrome (Cyt) **f** demonstrates lack of cell body contamination in flagellar fractions. FL, equal number of cells; FL 50:1, a 50-fold excess of flagella (*Chlamydomonas*). **f** In *pf13* mutant cytoplasm, ODA heavy chains (HCs) are depleted but ODA intermediate chains (ICs) are hyperabundant (*Chlamydomonas*)

group of species lack motile cilia, this result is consistent with the concept that *ktu* is specifically required for dynein arm assembly (Fig. 9.4b).

In spite of the functional conservation of *ktu* among the organisms examined, it was still unclear how *ktu* was involved in the dynein arm assembly process as *Ktu* was a totally novel protein lacking any known functional domains. However, examination of subcellular localization hinted that *ktu* may be involved in the cytoplasmic process of cilia assembly. Immunohistochemical analyses in medaka and Western blot analyses in human and *Chlamydomonas* revealed that KTU/PF13 is localized to the cytoplasm (Fig. 9.4c–e). Specifically, *ktu* is located in the apical cytoplasm around the γ -tubulin-positive pericentriolar region in medaka renal epithelial cells (Fig. 9.4d). It had been hypothesized that cilia-specific proteins are targeted to the ciliary base where individual axonemal components are preassembled and then loaded into intraflagellar transport (IFT) particles (Rosenbaum and Witman 2002). The cytoplasmic localization of *ktu* suggests that it is required for dynein arm assembly or transport within the cytoplasm.

Biochemical analyses using *Chlamydomonas* provided important insight into this proposed function of *ktu*/PF13 as a facilitator of dynein preassembly, with experimental results demonstrating specific defects in the assembly process between intermediate and heavy chains in the cytoplasm (Fig. 9.4f). It was previously proposed that, in *Chlamydomonas*, dynein arms are preassembled in the cytoplasm before transport into the flagellar compartment (Fowkes and Mitchell 1998). Three complexes, an intermediate-chain–heavy-chain (IC–HC) complex, a light-chain (LC) complex, and a docking complex (DC), are thought to be preassembled in the cytoplasm (Fig. 9.5b). In this process, ODA subunits HC and IC exist as a preassembled complex in a cytoplasmic precursor pool, and that absence of the IC subunit, in turn, prevents preassembly of the IC–HC complex (Fowkes and Mitchell 1998). We found that ODA HCs are depleted from the *pf13* mutant cytoplasm, whereas ODA ICs accumulate above normal levels (Fig. 9.4f). These observations indicate that PF13 is needed for ODA heavy chain stability, possibly by facilitating the assembly step of HCs with other subunits as proposed in Fig. 9.5b.

Biochemical analyses in mice have provided further insight into *ktu* function. Immunoprecipitation experiments and glutathione *S*-transferase (GST) pull-down assays using mouse *Ktu* (m*Ktu*) demonstrated that m*Ktu* interacts with at least one of the intermediate chains, DNAI2 (IC2 in *Chlamydomonas*), which shows defective assembly in human and *Chlamydomonas* mutant cilia (Fig. 9.5b). Medaka *ktu* is also expected to share a similar function, as m*Ktu* rescued the phenotype of medaka *ktu* mutants.

To gain mechanistic insight into *Ktu* function, a next logical step was the isolation of *Ktu* binding partners. Using a powerful and sensitive high-throughput mass spectrometry (MS) technique, we performed a global analysis of proteins interacting with *Ktu*. In this analysis, immunoprecipitates from mouse testis extracts were subjected to matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) MS. Intriguingly, this analysis identified heat-shock protein (Hsp) 70 as one of the main interacting proteins (see Fig. 9.5a). Among the molecular chaperones examined, Hsp70 and Hsp90 are highly expressed in mouse testis (data not shown), but only Hsp70 co-immunoprecipitates with m*Ktu* (Fig. 9.5a).

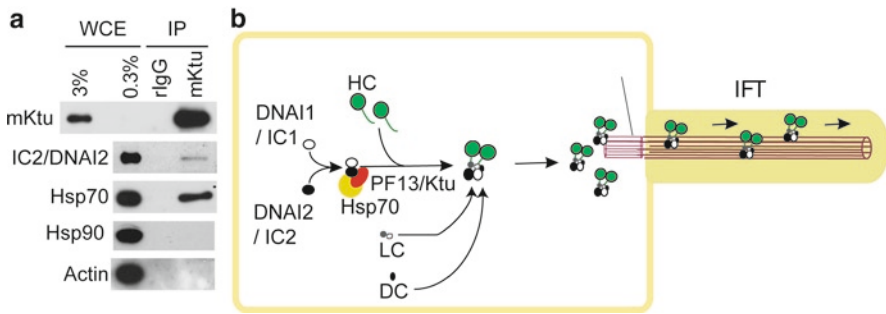


Fig. 9.5 Ktu binds to dyneins and heat-shock protein (Hsp) 70. **a** Immunoprecipitates with anti-mKtu or rabbit (*r*) IgG (control) from wild-type mouse testis extracts probed with anti-DNAI2, anti-Hsp70, anti-Hsp90, and anti-actin. **b** A hypothesized role of PF13/Ktu in ODA assembly. WCE, whole cell extract; IP, immunoprecipitate; IC2, intermediate chain 2; DNAI2, dynein axonemal intermediate 2; Hsp70, heat-shock protein 70; Hsp90, heat-shock protein 90; IC1, intermediate chain 1; HC, heavy chain; LC, light chain; DC, docking complex; IFT, intraflagellar transport

The N-terminal half and a short stretch at the C-terminus of *ktu* homologs share sequence similarity across evolutionarily distinct species. The conserved N-terminal region shows a weak similarity to NOP17, a yeast nucleolar protein involved in assembly of pre-rRNA processing complexes (Gonzales et al. 2005). Recently, this distantly related protein was identified as a cofactor of Hsp90, an evolutionarily conserved chaperone protein in yeast (Zhao et al. 2005). By analogy with Nop17 in the Hsp90 complex, we suggested that Ktu/Pf13 may facilitate dynein preassembly as a co-chaperone with Hsp70.

Although there have been many advances in our understanding of the structure and composition of IFT particles and the molecular motors that move them, virtually nothing is known about the cytoplasmic processes involved in the assembly and transport of dynein complexes. *ktu* is the first cytoplasmic protein confirmed to have a role in this process and is thus a good starting point for future study.

9.4 Perspective

Our study on *ktu* has demonstrated that mutagenesis screening is still a very useful tool for the discovery of new aspects in cilia. Some LR mutants awaiting analysis may reveal unexpected aspects of ciliogenesis or ciliary functions.

A current list of PCD genes is summarized in Table 9.2 and Fig. 9.6. Although most of the PCD genes identified in humans (Fig. 9.6, gray) are axonemal components, fish PCD genes (Fig. 9.6, black; and Table 9.1) have shown more variety, including cytoplasmic proteins. This observation suggests that fish PCD models will facilitate our understanding of the pathogenesis of human PCD.

Table 9.2 (continued)

Analysis in fish				Other organisms					References	
Gene	Mutant	Mutant/ morphant phenotype	Gross cilia morphology defects	Ciliary ultrastr- ructure (TEM)	Subcellular localization	Human disease	Mouse KO	<i>Chl</i> mutant		Proposed function
<i>gas8</i>	–	Hydrocephalus LR defect otolith biogenesis	Normal	–	Cilia (a subunit of dynein regulatory complex)	–	–	<i>pf2</i>	Dynein regulation	Colantonio et al. (2009)
<i>rsph9</i>	–	Dysmotility in olfactory-pit cilia	–	–	Cilia (radial spoke)	PCD	–	<i>pf17</i>	Link the peripheral doublet microtubules with the central pair.	Castleman et al. (2009)
<i>Fused</i>	–	LR defect	–	Loss or acquisition of extra central pair	Cytosol	–	Yes	–	Central pair construction	Wilson et al. (2009)
<i>Seahorse</i>	<i>Sea^{tg238}a</i> <i>Sea^{fb20r}</i>	Pronephric cyst LR defect CTD	Normal	Normal	Cytosol (punctate)	–	–	–	Scaffold protein?	Serluca et al. (2009)
<i>ofdl1</i>	–	Hydrocephalus LR defect CTD Otolith biogenesis CE defect	Shorter length	Abnormal axonemal configuration	Centrosomes/ basal bodies	OFD1 PCD	Yes	–	–	Ferrante et al. (2001)
<i>ktu/PF13</i>	<i>ktu</i>	Mesonephric cyst LR defect CTD	Normal	ODA, IDA defect	Pericentriolar region	PCD	Yes	<i>pf13</i>	Co-chaperone	Omran et al. (2008)

KO, knockout; *Chl*, *Chlamydomonas*; CTD, curly-tail-down; CE, convergent extension; *InsP6*, inositol hexakisphosphate

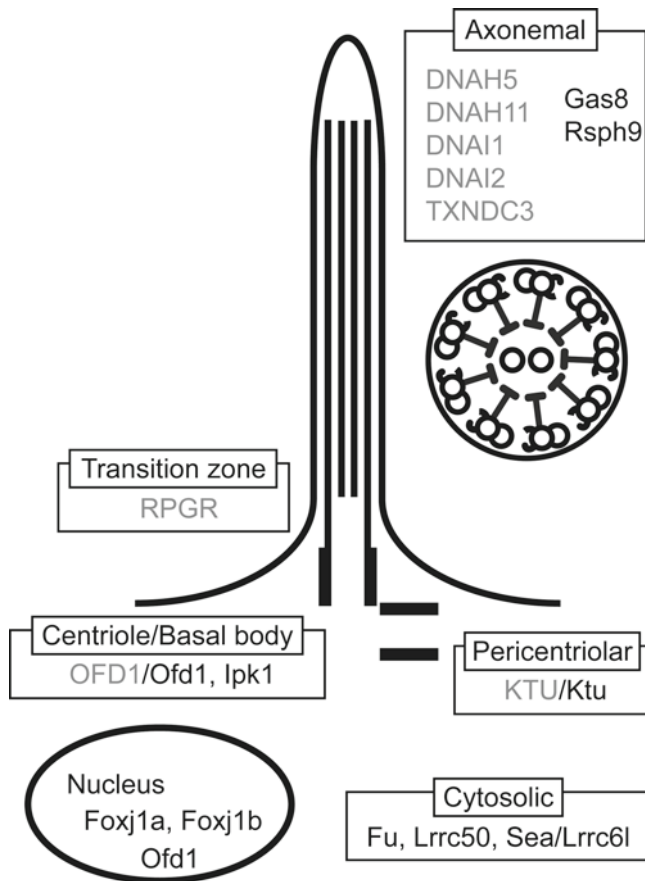


Fig. 9.6 Primary ciliary dyskinesia (PCD) genes. PCD genes identified in humans are shown in *gray letters* and fish PCD genes are shown in *black letters*. *DNAH5*, dynein axonemal heavy chain 5; *DNAH11*, dynein axonemal heavy chain 11; *DNAI1*, dynein axonemal intermediate chain 1; *DNAI2*, dynein axonemal intermediate chain 2 (IC2 in *Chlamydomonas*); *TXNDC3*, thioredoxin domain-containing 3; *RPGR*, retinitis pigmentosa GTPase regulator; *OFD1/Ofd1*, oral-facial-digital syndrome 1; *Gas8*, growth-arrest-specific 8; *rsph9*, radial spoke head protein 9; *Ipk1*, inositol 1,3,4,5,6-pentakisphosphate 2-kinase; *Foxj1a*, Forkhead box j1a; *Foxj1b*, Forkhead box j1b; *Fu*, fused.; *Lrrc50*, leucine-rich repeat-containing protein 50; *Sea/Lrrc6l*, seahorse/leucine-rich repeat-containing 6-like; *KTU/ktu*, kintoun (mKtu in mouse, PF13 in *Chlamydomonas*)

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References

- Ahsan B, Kobayashi D, Yamada T, Kasahara M, Sasaki S, Saito TL, Nagayasu Y, Doi K, Nakatani Y, Qu W, Jindo T, Shimada A, Naruse K, Toyoda A, Kuroki Y, Fujiyama A, Sasaki T, Shimizu A, Asakawa S, Shimizu N, Hashimoto S, Yang J, Lee Y, Matsushima K, Sugano S, Sakaizumi M, Narita T, Ohishi K, Haga S, Ohta F, Nomoto H, Nogata K, Morishita T, Endo T, Shin IT,

- Takeda H, Kohara Y, Morishita S (2008) UTGB/medaka: genomic resource database for medaka biology. *Nucleic Acids Res* 36:D747–D752
- Amack JD, Yost HJ (2004) The T box transcription factor no tail in ciliated cells controls zebrafish left–right asymmetry. *Curr Biol* 14:685–690
- Badano JL, Mitsuma N, Beales PL, Katsanis N (2006) The ciliopathies: an emerging class of human genetic disorders. *Annu Rev Genomics Hum Genet* 7:125–148
- Bartoloni L, Blouin J-L, Pan Y, Gehrig C, Maiti AK, Scamuffa N, Rossier C, Jorissen M, Armengot M, Meeks M, Mitchison HM, Chung EMK, Delozier-Blanchet CD, Craigen WJ, Antonarakis SE (2002) Mutations in the DNAH11 (axonemal heavy chain dynein type 11) gene cause one form of situs inversus totalis and most likely primary ciliary dyskinesia. *Proc Natl Acad Sci USA* 99:10282–10286
- Beales PL, Bland E, Tobin JL, Bacchelli C, Tuysuz B, Hill J, Rix S, Pearson CG, Kai M, Hartley J, Johnson C, Irving M, Elcioglu N, Winey M, Tada M, Scambler PJ (2007) IFT80, which encodes a conserved intraflagellar transport protein, is mutated in Jeune asphyxiating thoracic dystrophy. *Nat Genet* 39:727–729
- Bisgrove BW, Yost HJ (2006) The roles of cilia in developmental disorders and disease. *Development (Camb)* 133:4131–4143
- Bisgrove BW, Snarr BS, Emrazian A, Yost HJ (2005) Polaris and polycystin-2 in dorsal forerunner cells and Kupffer's vesicle are required for specification of the zebrafish left-right axis. *Dev Biol* 287:274–288
- Brody SL, Yan XH, Wuerffel MK, Song SK, Shapiro SD (2000) Ciliogenesis and left-right axis defects in forkhead factor HFH-4-null mice. *Am J Respir Cell Mol Biol* 23:45–51
- Brummett AR, Dumont JN (1978) Kupffer's vesicle in *Fundulus heteroclitus*: a scanning and transmission electron microscope study. *Tissue Cell* 10:11–22
- Budny B, Chen W, Omran H, Fliegau M, Tzschach A, Wisniewska M, Jensen LR, Raynaud M, Shiochet SA, Badura M, Lenzner S, Latos-Bielenska A, Ropers HH (2006) A novel X-linked recessive mental retardation syndrome comprising macrocephaly and ciliary dysfunction is allelic to oral-facial-digital type I syndrome. *Hum Genet* 120:171–178
- Cantagrel V, Silhavy JL, Bielas SL, Swistun D, Marsh SE, Bertrand JY, Audollent S, Attie-Bitach T, Holden KR, Dobyns WB, Traver D, Al-Gazali L, Ali BR, Lindner TH, Caspary T, Otto EA, Hildebrandt F, Glass IA, Logan CV, Johnson CA, Bennett C, Brancati F, Valente EM, Woods CG, Gleeson JG (2008) Mutations in the cilia gene ARL13B lead to the classical form of Joubert syndrome. *Am J Hum Genet* 83:170–179
- Castleman VH, Romio L, Chodhari R, Hirst RA, de Castro SCP, Parker KA, Ybot-Gonzalez P, Emes RD, Wilson SW, Wallis C, Johnson CA, Herrera RJ, Rutman A, Dixon M, Shoemark A, Bush A, Hogg C, Gardiner RM, Reish O, Greene NDE, O'Callaghan C, Purton S, Chung EMK, Mitchison HM (2009) Mutations in radial spoke head protein genes RSPH9 and RSPH4A cause primary ciliary dyskinesia with central-microtubular-pair abnormalities. *Am J Hum Genet* 84:197–209
- Chen J, Knowles HJ, Hebert JL, Hackett BP (1998) Mutation of the mouse hepatocyte nuclear factor/forkhead homologue 4 gene results in an absence of cilia and random left-right asymmetry. *J Clin Invest* 102:1077–1082
- Christensen ST, Pedersen LB, Schneider L, Satir P (2007) Sensory cilia and integration of signal transduction in human health and disease. *Traffic* 8:97–109
- Colantonio JR, Vermot J, Wu D, Langenbacher AD, Fraser S, Chen J-N, Hill KL (2009) The dynein regulatory complex is required for ciliary motility and otolith biogenesis in the inner ear. *Nature (Lond)* 457:205–209
- Dooley K, Zon LI (2000) Zebrafish: a model system for the study of human disease. *Curr Opin Genet Dev* 10:252–256
- Driever W, Solnica-Krezel L, Schier AF, Neuhauss SC, Malicki J, Stemple DL, Stainier DY, Zwartkruis F, Abdelilah S, Rangini Z, Belak J, Boggs C (1996) A genetic screen for mutations affecting embryogenesis in zebrafish. *Development (Camb)* 123:37–46
- Drummond IA (2005) Kidney development and disease in the zebrafish. *J Am Soc Nephrol* 16:299–304

- Duriez B, Duquesnoy P, Escudier E, Bridoux A-M, Escalier D, Rayet I, Marcos E, Vojtek A-M, Bercher J-F, Amselem S (2007) A common variant in combination with a nonsense mutation in a member of the thioredoxin family causes primary ciliary dyskinesia. *Proc Natl Acad Sci USA* 104:3336–3341
- Essner JJ, Amack JD, Nyholm MK, Harris EB, Yost HJ (2005) Kupffer's vesicle is a ciliated organ of asymmetry in the zebrafish embryo that initiates left-right development of the brain, heart and gut. *Development (Camb)* 132:1247–1260
- Ferrante MI, Giorgio G, Feather SA, Bulfone A, Wright V, Ghiani M, Selicorni A, Gammara L, Scolari F, Woolf AS, Sylvie O, Bernard L, Malcolm S, Winter R, Ballabio A, Franco B (2001) Identification of the gene for oral-facial-digital type I syndrome. *Am J Hum Genet* 68:569–576
- Fliegauf M, Benzing T, Omran H (2007) When cilia go bad: cilia defects and ciliopathies. *Nat Rev Mol Cell Biol* 8:880–893
- Fowkes ME, Mitchell DR (1998) The role of preassembled cytoplasmic complexes in assembly of flagellar dynein subunits. *Mol Biol Cell* 9:2337–2347
- Freshour J, Yokoyama R, Mitchell DR (2007) *Chlamydomonas* flagellar outer row dynein assembly protein Oda7 interacts with both outer row and II inner row dyneins. *J Biol Chem* 282:5404–5412
- Furutani-Seiki M, Sasado T, Morinaga C, Suwa H, Niwa K, Yoda H, Deguchi T, Hirose Y, Yasuoka A, Henrich T, Watanabe T, Iwanami N, Kitagawa D, Saito K, Asaka S, Osakada M, Kunimatsu S, Momoi A, Elmasri H, Winkler C, Ramialison M, Loosli F, Quiring R, Carl M, Grabber C, Winkler S, Del Bene F, Shinomiya A, Kota Y, Yamanaka T, Okamoto Y, Takahashi K, Todo T, Abe K, Takahama Y, Tanaka M, Mitani H, Katada T, Nishina H, Nakajima N, Wittbrodt J, Kondoh H (2004) A systematic genome-wide screen for mutations affecting organogenesis in medaka, *Oryzias latipes*. *Mech Dev* 121:647–658
- Gerdes JM, Davis EE, Katsanis N (2009) The vertebrate primary cilium in development, homeostasis, and disease. *Cell* 137:32–45
- Gonzales FA, Zanchin NI, Luz JS, Oliveira CC (2005) Characterization of *Saccharomyces cerevisiae* Nop17p, a novel Nop58p-interacting protein that is involved in Pre-rRNA processing. *J Mol Biol* 346:437–455
- Gorden NT, Arts HH, Parisi MA, Coene KL, Letteboer SJ, van Beersum SE, Mans DA, Hikida A, Eckert M, Knutzen D, Alswaid AF, Ozyurek H, Dibooglu S, Otto EA, Liu Y, Davis EE, Hutter CM, Bammler TK, Farin FM, Dorschner M, Topçu M, Zackai EH, Rosenthal P, Owens KN, Katsanis N, Vincent JB, Hildebrandt F, Rubel EW, Raible DW, Knoers NV, Chance PF, Roepman R, Moens CB, Glass IA, Doherty D (2008) CC2D2A is mutated in Joubert syndrome and interacts with the ciliopathy-associated basal body protein CEP290. *Am J Hum Genet* 83:559–571
- Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, Kane DA, Odenthal J, van Eeden FJ, Jiang YJ, Heisenberg CP, Kelsh RN, Furutani-Seiki M, Vogelsang E, Beuchle D, Schach U, Fabian C, Nusslein-Volhard C (1996) The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development (Camb)* 123:1–36
- Hagiwara H, Ohwada N, Takata K (2004) Cell biology of normal and abnormal ciliogenesis in the ciliated epithelium. *Int Rev Cytol* 234:101–141
- Hirokawa N, Tanaka Y, Okada Y, Takeda S (2006) Nodal flow and the generation of left-right asymmetry. *Cell* 125:33–45
- Hojo M, Takashima S, Kobayashi D, Sumeragi A, Shimada A, Tsukahara T, Yokoi H, Narita T, Jindo T, Kage T, Kitagawa T, Kimura T, Sekimizu K, Miyake A, Setiamarga D, Murakami R, Tsuda S, Ooki S, Kakihara K, Naruse K, Takeda H (2007) Right-elevated expression of charon is regulated by fluid flow in medaka Kupffer's vesicle. *Dev Growth Differ* 49:395–405
- Huang B, Piperno G, Luck DJ (1979) Paralyzed flagella mutants of *Chlamydomonas reinhardtii*. Defective for axonemal doublet microtubule arms. *J Biol Chem* 254:3091–3099
- Hutchings NR, Donelson JE, Hill KL (2002) Trypanin is a cytoskeletal linker protein and is required for cell motility in African trypanosomes. *J Cell Biol* 156:867–877
- Ibanez-Tallon I, Heintz N, Omran H (2003) To beat or not to beat: roles of cilia in development and disease. *Hum Mol Genet* 12:R27–R35

- Kamiya R (1988) Mutations at twelve independent loci result in absence of outer dynein arms in *Chlamydomonas reinhardtii*. *J Cell Biol* 107:2253–2258
- Kamiya R (2002) Functional diversity of axonemal dyneins as studied in *Chlamydomonas* mutants. *Int Rev Cytol* 219:115–155
- Kasahara M, Naruse K, Sasaki S, Nakatani Y, Qu W, Ahsan B, Yamada T, Nagayasu Y, Doi K, Kasai Y, Jindo T, Kobayashi D, Shimada A, Toyoda A, Kuroki Y, Fujiyama A, Sasaki T, Shimizu A, Asakawa S, Shimizu N, Hashimoto S-i, Yang J, Lee Y, Matsushima K, Sugano S, Sakaizumi M, Narita T, Ohishi K, Haga S, Ohta F, Nomoto H, Nogata K, Morishita T, Endo T, Shin-I T, Takeda H, Morishita S, Kohara Y (2007) The medaka draft genome and insights into vertebrate genome evolution. *Nature (Lond)* 447:714–719
- Kishimoto N, Cao Y, Park A, Sun Z (2008) Cystic kidney gene seahorse regulates cilia-mediated processes and Wnt pathways. *Dev Cell* 14:954–961
- Kobayashi D, Takeda H (2008) Medaka genome project. *Brief Funct Genomic Proteomic* 7:415–426
- Kramer-Zucker AG, Olale F, Haycraft CJ, Yoder BK, Schier AF, Drummond IA (2005) Cilia-driven fluid flow in the zebrafish pronephros, brain and Kupffer's vesicle is required for normal organogenesis. *Development (Camb)* 132:1907–1921
- Loges NT, Olbrich H, Fenske L, Mussaffi H, Horvath J, Fliegauf M, Kuhl H, Baktai G, Peterffy E, Chodhari R, Chung EMK, Rutman A, O'Callaghan C, Blau H, Tiszlavicz L, Voelkel K, Witt M, Zietkiewicz E, Neesen J, Reinhardt R, Mitchison HM, Omran H (2008) DNAI2 mutations cause primary ciliary dyskinesia with defects in the outer dynein arm. *Am J Hum Genet* 83:547–558
- Low SH, Vasanth S, Larson CH, Mukherjee S, Sharma N, Kinter MT, Kane ME, Obara T, Weimbs T (2006) Polycystin-1, STAT6, and P100 function in a pathway that transduces ciliary mechanosensation and is activated in polycystic kidney disease. *Dev Cell* 10:57–69
- Mochizuki E, Fukuta K, Tada T, Harada T, Watanabe N, Matsuo S, Hashimoto H, Ozato K, Wakamatsu Y (2005) Fish mesonephric model of polycystic kidney disease in medaka (*Oryzias latipes*) *pc* mutant. *Kidney Int* 68:23–34
- Moore A, Escudier E, Roger G, Tamalet A, Pelosse B, Marlin S, Clement A, Geremek M, Delaisi B, Bridoux AM, Coste A, Witt M, Duriez B, Amselem S (2006) RPGR is mutated in patients with a complex X linked phenotype combining primary ciliary dyskinesia and retinitis pigmentosa. *J Med Genet* 43:326–333
- Nasevicius A, Ekker SC (2000) Effective targeted gene 'knockdown' in zebrafish. *Nat Genet* 26:216–220
- Okada Y, Nonaka S, Tanaka Y, Saijoh Y, Hamada H, Hirokawa N (1999) Abnormal nodal flow precedes situs inversus in *iv* and *inv* mice. *Mol Cell* 4:459–468
- Okada Y, Takeda S, Tanaka Y, Belmonte J-CI, Hirokawa N (2005) Mechanism of nodal flow: a conserved symmetry breaking event in left-right axis determination. *Cell* 121:633–644
- Olbrich H, Haffner K, Kispert A, Volkel A, Volz A, Sasmaz G, Reinhardt R, Hennig S, Lehrach H, Konietzko N, Zariwala M, Noone PG, Knowles M, Mitchison HM, Meeks M, Chung EMK, Hildebrandt F, Sudbrak R, Omran H (2002) Mutations in DNAH5 cause primary ciliary dyskinesia and randomization of left-right asymmetry. *Nat Genet* 30:143–144
- Omran H, Haffner K, Volkel A, Kuehr J, Ketelsen U-P, Ross U-H, Konietzko N, Wienker T, Brandis M, Hildebrandt F (2000) Homozygosity mapping of a gene locus for primary ciliary dyskinesia on chromosome 5p and identification of the heavy dynein chain DNAH5 as a candidate gene. *Am J Respir Cell Mol Biol* 23:696–702
- Omran H, Kobayashi D, Olbrich H, Tsukahara T, Loges NT, Hagiwara H, Zhang Q, Leblond G, O'Toole E, Hara C, Mizuno H, Kawano H, Fliegauf M, Yagi T, Koshida S, Miyawaki A, Zentgraf H, Seithe H, Reinhardt R, Watanabe Y, Kamiya R, Mitchell DR, Takeda H (2008) Ktu/PF13 is required for cytoplasmic pre-assembly of axonemal dyneins. *Nature (Lond)* 456:611–616
- Otto EA, Schermer B, Obara T, O'Toole JF, Hiller KS, Mueller AM, Ruf RG, Hoefele J, Beekmann F, Landau D, Foreman JW, Goodship JA, Strachan T, Kispert A, Wolf MT, Gagnadoux MF, Nivet H, Antignac C, Walz G, Drummond IA, Benzing T, Hildebrandt F (2003) Mutations in

- INVS encoding inversin cause nephronophthisis type 2, linking renal cystic disease to the function of primary cilia and left-right axis determination. *Nat Genet* 34:413–420
- Pennarun G, Escudier E, Chapelin C, Bridoux AM, Cacheux V, Roger G, Clement A, Goossens M, Amselem S, Duriez B (1999) Loss-of-function mutations in a human gene related to *Chlamydomonas reinhardtii* dynein IC78 result in primary ciliary dyskinesia. *Am J Hum Genet* 65:1508–1519
- Rosenbaum JL, Witman GB (2002) Intraflagellar transport. *Nat Rev Mol Cell Biol* 3:813–825
- Rupp G, Porter ME (2003) A subunit of the dynein regulatory complex in *Chlamydomonas* is a homologue of a growth arrest-specific gene product. *J Cell Biol* 162:47–57
- Sarmah B, Latimer AJ, Appel B, Wente SR (2005) Inositol polyphosphates regulate zebrafish left-right asymmetry. *Dev Cell* 9:133
- Sarmah B, Winfrey VP, Olson GE, Appel B, Wente SR (2007) A role for the inositol kinase Ipk1 in ciliary beating and length maintenance. *Proc Natl Acad Sci USA* 104:19843–19848
- Satir P, Christensen ST (2007) Overview of structure and function of mammalian cilia. *Annu Rev Physiol* 69:377–400
- Sayer JA, Otto EA, O'Toole JF, Nurnberg G, Kennedy MA, Becker C, Hennies HC, Helou J, Attanasio M, Fausett BV, Utsch B, Khanna H, Liu Y, Drummond I, Kawakami I, Kusakabe T, Tsuda M, Ma L, Lee H, Larson RG, Allen SJ, Wilkinson CJ, Nigg EA, Shou C, Lillo C, Williams DS, Hoppe B, Kemper MJ, Neuhaus T, Parisi MA, Glass IA, Petry M, Kispert A, Gloy J, Ganner A, Walz G, Zhu X, Goldman D, Nurnberg P, Swaroop A, Leroux MR, Hildebrandt F (2006) The centrosomal protein nephrocystin-6 is mutated in Joubert syndrome and activates transcription factor ATF4. *Nat Genet* 38:674–681
- Schafer T, Putz M, Lienkamp S, Ganner A, Bergbreiter A, Ramachandran H, Gieloff V, Gerner M, Mattonet C, Czarnecki PG, Sayer JA, Otto EA, Hildebrandt F, Kramer-Zucker A, Walz G (2008) Genetic and physical interaction between the NPHP5 and NPHP6 gene products. *Hum Mol Genet* 17:3655–3662
- Schottenfeld J, Sullivan-Brown J, Burdine RD (2007) Zebrafish curly up encodes a Pkd2 ortholog that restricts left-side-specific expression of southpaw. *Development (Camb)* 134:1605–1615
- Schweickert A, Weber T, Beyer T, Vick P, Bogusch S, Feistel K, Blum M (2007) Cilia-driven leftward flow determines laterality in *Xenopus*. *Curr Biol* 17:60–66
- Serluca FC, Xu B, Okabe N, Baker K, Lin S-Y, Sullivan-Brown J, Konieczkowski DJ, Jaffe KM, Bradner JM, Fishman MC, Burdine RD (2009) Mutations in zebrafish leucine-rich repeat-containing six-like affect cilia motility and result in pronephric cysts, but have variable effects on left-right patterning. *Development (Camb)* 136:1621–1631
- Shu X, Huang J, Dong Y, Choi J, Langenbacher A, Chen J-N (2007) Na⁺/K-ATPase $\alpha 2$ and Ncx4a regulate zebrafish left–right patterning. *Development (Camb)* 134:1921–1930
- Stannard W, Rutman A, Wallis C, O'Callaghan C (2004) Central microtubular agenesis causing primary ciliary dyskinesia. *Am J Respir Crit Care Med* 169:634–637
- Streets AJ, Moon DJ, Kane ME, Obara T, Ong ACM (2006) Identification of an N-terminal glycogen synthase kinase 3 phosphorylation site which regulates the functional localization of polycystin-2 in vivo and in vitro. *Hum Mol Genet* 15:1465–1473
- Stubbs JL, Oishi I, Izpisua Belmonte JC, Kintner C (2008) The forkhead protein Foxj1 specifies node-like cilia in *Xenopus* and zebrafish embryos. *Nat Genet* 40:1454–1460
- Sullivan-Brown J, Schottenfeld J, Okabe N, Hostetter CL, Serluca FC, Thiberge SY, Burdine RD (2008) Zebrafish mutations affecting cilia motility share similar cystic phenotypes and suggest a mechanism of cyst formation that differs from pkd2 morphants. *Dev Biol* 314:261–275
- Sun Z, Amsterdam A, Pazour GJ, Cole DG, Miller MS, Hopkins N (2004) A genetic screen in zebrafish identifies cilia genes as a principal cause of cystic kidney. *Development (Camb)* 131:4085–4093
- Takeda H (2008) Draft genome of the medaka fish: a comprehensive resource for medaka developmental genetics and vertebrate evolutionary biology. *Dev Growth Differ* 50(suppl 1): S157–S166
- Tobin JL, Beales PL (2008) Restoration of renal function in zebrafish models of ciliopathies. *Pediatr Nephrol* 23:2095–2099

- van Rooijen E, Giles RH, Voest EE, van Rooijen C, Schulte-Merker S, van Eeden FJ (2008) LRRC50, a conserved ciliary protein implicated in polycystic kidney disease. *J Am Soc Nephrol* 19:1128–1138
- Wessely O, Obara T (2008) Fish and frogs: models for vertebrate cilia signaling. *Front Biosci* 13:1866–1880
- Wilson CW, Nguyen CT, Chen M-H, Yang J-H, Gacayan R, Huang J, Chen J-N, Chuang P-T (2009) Fused has evolved divergent roles in vertebrate Hedgehog signalling and motile ciliogenesis. *Nature (Lond)* 459:98–102
- Yang P, Diener DR, Yang C, Kohno T, Pazour GJ, Dienes JM, Agrin NS, King SM, Sale WS, Kamiya R, Rosenbaum JL, Witman GB (2006) Radial spoke proteins of *Chlamydomonas* flagella. *J Cell Sci* 119:1165–1174
- Yokoi H, Shimada A, Carl M, Takashima S, Kobayashi D, Narita T, Jindo T, Kimura T, Kitagawa T, Kage T, Sawada A, Naruse K, Asakawa S, Shimizu N, Mitani H, Shima A, Tsutsumi M, Hori H, Wittbrodt J, Saga Y, Ishikawa Y, Araki K, Takeda H (2007) Mutant analyses reveal different functions of fgfr1 in medaka and zebrafish despite conserved ligand-receptor relationships. *Dev Biol* 304:326
- Yu X, Ng CP, Habacher H, Roy S (2008) Foxj1 transcription factors are master regulators of the motile ciliogenic program. *Nat Genet* 40:1445–1453
- Zariwala MA, Knowles MR, Omran H (2007) Genetic defects in ciliary structure and function. *Annu Rev Physiol* 69:423–450
- Zhao R, Davey M, Hsu YC, Kaplanek P, Tong A, Parsons AB, Krogan N, Cagney G, Mai D, Greenblatt J, Boone C, Emili A, Houry WA (2005) Navigating the chaperone network: an integrative map of physical and genetic interactions mediated by the hsp90 chaperone. *Cell* 120:715–727
- Zon LI, Peterson RT (2005) In vivo drug discovery in the zebrafish. *Nat Rev Drug Discov* 4:35–44

Chapter 10

p53-Deficient Medaka Created by TILLING

Yoshihito Taniguchi

Abstract Because loss-of-function experiments in medaka and zebrafish had relied on morpholino, whose effect is transient and lasts only a few days post fertilization, developmental biology has been the major target of research using these small laboratory fish. With the advent of targeting-induced local lesions in genome (TILLING) technology, the teleost has reemerged as a model animal in which the specific genes can be disrupted. The potential research field is now expanded to slow and time-consuming biological phenomena such as carcinogenesis, aging, and degeneration. p53 is a multifunctional transcription factor involved in such various biological processes. Among other vertebrates, medaka is especially suited for the TILLING approach because of the compact genome and inbred strains, which makes amplicon design and screening fast and efficient. We have generated several lines of *p53* mutant medaka, including two nonsense mutants. These lines showed the impaired transcriptional regulation of the cell cycle and apoptosis and the increased incidence of spontaneous tumorigenesis. The mutant medaka isolated by TILLING will occupy an important and valuable position in various research fields in the future.

10.1 Mutant Isolation by TILLING

p53 is a transcription factor that regulates the expression of genes involved in cell-cycle arrest and apoptosis, thus preventing genomic abnormalities and consequent tumorigenesis. It is an important tumor suppressor, which is stabilized and activated in response to a wide variety of genotoxic stresses (Kruse and Gu 2009). p53 is also involved in many other biological processes such as organismal aging, metabolism, and development (Vousden and Prives 2009). However, the role of p53 in fish as

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“a guardian of the genome” has been questioned, because mutations in the p53 gene have only rarely been found in naturally occurring or induced tumors in teleosts (Krause et al. 1997), whereas other studies show that p53 plays a general role in tumorigenesis in fish (Berghmans et al. 2005).

Small laboratory fish such as zebrafish and medaka are attractive vertebrate animal models well suited for genetic studies. They have drawn increasing attention as a model for cancer research for a number of reasons, such as their relative transparency, which allows in vivo imaging by fluorescence, and their small body size, which is suitable for drug screening (Feitsma and Cuppen 2008). Cancer studies using medaka range from spontaneous tumor development in nature to artificial chemical mutagenesis and to induction of tumors by transgenes (Aoki and Matsudaira 1977; Masahito et al. 1989; Winkler et al. 1994). Until now, what has been lacking is a genetic model for loss-of-function experiments that can help us elucidate the process of tumorigenesis and other biological phenomena.

Reverse genetics is an approach in which the modification of a gene of interest is achieved irrespective of its phenotype. Recently, two new technologies emerged to generate gene-disrupted fish: targeting-induced local lesions in genome (TILLING) and zinc-finger nuclease. The latter utilizes a nuclease fused to the tandem zinc-finger module engineered so that it binds to a unique site on the genome, which then creates a DNA double-strand break in the target gene (Doyon et al. 2008; Meng et al. 2008). TILLING is the process of screening mutagenized various plant and animal species by polymerase chain reaction (PCR) amplification to identify mutations in genes of interest (Moens et al. 2008). We describe here the TILLING-based disruption of medaka genes and phenotypic analysis of *p53*-deficient medaka.

10.2 p53 Gene in Medaka

10.2.1 Structure and Conservation

Both the cDNA and the genomic sequence for medaka (*Oryzias latipes*) *p53* have been published (Chen et al. 2001; Krause et al. 1997). The *p53* gene has been isolated in several species of teleost fish, including medaka, zebrafish, fugu, green puffer fish, stickleback, barbel, catfish, flounder, trout, platyfish, and swordtails. All p53 proteins from teleosts to mammals share common features, including a transactivation domain at the N-terminus, a DNA-binding domain at the center, and a tetramerization domain at the C-terminus (Fig. 10.1a). The most conserved among these are the DNA-binding domain. An isoform of *p53* that is transcribed from the alternative promoter in intron 4 is known in zebrafish (Chen et al. 2005), but to date such a transcript has not been described for medaka. p53 acts as a transcriptional activator and upregulates genes involved in cell-cycle arrest or apoptosis in response to genotoxic stress. Some, but not all, *p53* target genes can be found in

10.3 Generation of p53 Mutant Fish

10.3.1 Generation and Screening of Medaka Mutant Library

The detailed protocol for the generation of a mutant medaka library for TILLING has been described previously (Taniguchi et al. 2006). Briefly, founder fish were repeatedly mutagenized with *N*-ethyl-*N*-nitrosourea (ENU), crossed with wild-type females, and the progeny were used to establish a permanent cryopreserved resource of 5,771 F₁ males. A specific locus test using the albino mutant showed the appearance of white-eyed embryos at a rate of 1 in 272, which is in line with previously observed frequencies (Furutani-Seiki et al. 2004). The sequencing of the genome revealed that the average ENU-induced mutation frequency for the library was 1 mutation per 345 kb.

We screened the mutant medaka library for the amplicon covering exons 5, 6, and 7 in the p53 gene. These exons encode the highly conserved DNA-binding domain, where mutations are known to cluster in human tumors. We chose to use dideoxy resequencing of the PCR-amplified target sequences for mutation discovery, as it is fast and reliable (Taniguchi et al. 2006). Of 3.9 Mbp, we found seven ENU-induced mutations, including three missense mutations, one splice site, one intronic, and two nonsense mutations (see Fig. 10.1; Table 10.1). The p53^{Y186X} allele is a T-to-A transversion that alters Y186 (tyrosine) to a stop codon, whereas the p53^{E241X} allele is a rare G-to-T transversion that results in the alteration of E241 (glutamic acid) to a stop codon. [We noticed two variants of the p53 gene, one with a serine at position 22 as reported by Chen et al. (2001), and one without a serine as reported by Krause et al. (1997), resulting in 352 and 351 amino acid proteins, respectively. The number of amino acid positions described here is according to Krause et al.] Both mutant alleles are presumed to result in a

Table 10.1 The p53 mutations retrieved from the mutant medaka library

Number	Sequence context	Amino acid change	Type of mutation
1	TCCCTTTTCT (C>T) CATCGACTGT		Intronic
2	TGGCCAGTA (T>A) TTTGAAGACC	Y186X	Truncation
3	CTACATGTGT (A>G) ACAGCTCGTG	N220D	Substitution
4	TACATGTGTA (A>G) CAGCTCGTGC	N220S	Substitution
5	GTGTAACAGC (T>C) CGTGTCATGGG	S222P	Substitution
6	TCTGGAAACC (G>T) AGTAAGTTTA	E241X	Truncation
7	GGAAACCGAG (T>C) AAGTTTAGTC		Splice site

The sequence context represents the 5'- and 3'-genomic sequences surrounding the mutation found in the screen. Shown in *parentheses* are the nucleic acid change. Note that each individual mutant fish has only one of seven mutations, and not all seven. [Taniguchi et al. 2006. Copyright (2006) BioMed Central, USA]

truncated protein that terminates prematurely in the midst of a DNA-binding domain. The missense mutations found in our screen, although not disrupting the overall structure of protein, may be interesting. All three missense mutations were at highly conserved residues within the DNA-binding region. N220S and N220D are of particular interest, because N220 (asparagine) is located next to the Zn-binding cysteine in loop 3 (see Fig. 10.1a), which is important for the stabilization of p53 folding (Cho et al. 1994).

The *p53* mutants were recovered from frozen sperm archived by in vitro fertilization. As expected, the genotyping using caudal fin tissue from the F₂ offspring revealed that the ratio of wild-type fish to mutant heterozygotes was about 1:1 (data not shown). As the mutation frequency in the current library is approximately 1 per 345 kb, and the genome size of medaka is 700 Mb, each fish retrieved from the ENU-mutagenized library could be expected to contain about 2,000 mutations. The number of mutations introduced into any coding region was estimated to be about 80. According to the screen results obtained to date, we assume that each mutant fish carries an average of at least four or five loss-of-function mutations. To eliminate these extra mutations (except for the mutation of interest), we usually perform three or four backcrosses before intercrossing to obtain homozygous animals. Because *p53* mutants are expected to be used for many purposes, we backcrossed *p53*^{Y186X/+} and *p53*^{E241X/+} to *wild-type* cab (one of the southern strains of medaka) ten times, then intercrossed the resulting heterozygotes to produce homozygotes. HdrR is another strain derived from the southern population, which was subjected to extensive brother–sister mating. It is considered to be the most genetically homogeneous strain among the available inbred strains of medaka and was used to determine the whole genome sequence. We have also bred *p53*-deficient mutants with HdrR.

10.3.2 Functional Analysis of *p53* Mutant Medaka

Impaired target gene induction upon DNA damage is one of the hallmarks of *p53* deficiency (Harms et al. 2004). When *p53*^{Y186X/Y186X} or *p53*^{E241X/E241X} homozygous embryos were irradiated with γ -rays, the increase of target genes was not observed, whereas control embryos showed an upregulation of *p21* and *Mdm2* transcription levels (Fig. 10.2a; data not shown). The impaired *p21* induction after γ -irradiation was also seen in the mutants that carry a mutation at the splicing donor site of intron 7 (data not shown). Bax is the key regulator of apoptosis, which is transcribed by *p53* in response to DNA damage. Although the upregulation of *Bax* mRNA was not evident in our experimental condition (6 h after 20 Gy γ -irradiation; see Fig. 10.2a), apoptosis was observed 6–12 h after irradiation in *p53*-proficient primary cultured cells or embryos (Fig. 10.2b, c). *p53* deficiency again diminished the apoptotic response in homozygous mutants. These results are consistent with the complete loss-of-function phenotype of *p53*.

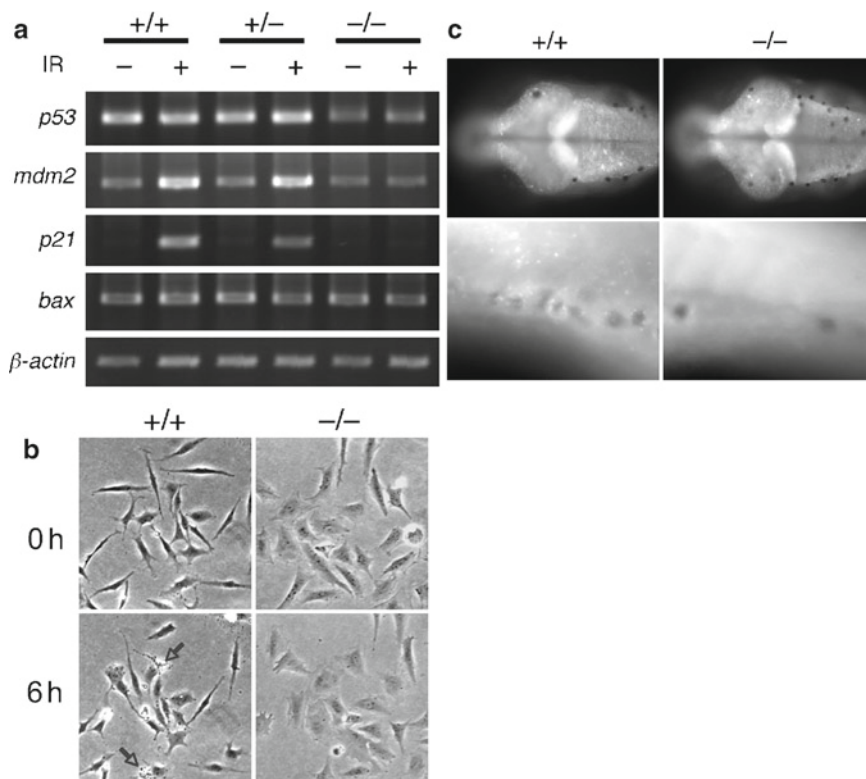


Fig. 10.2 Radiation-induced *p53* target gene induction and apoptosis. **a** Induction of *p53* target genes in the medaka embryos of the indicated genotype was examined by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) 6 h after 20 Gy γ -ray irradiation (IR). **b** Primary culture cells derived from the embryos of the indicated genotype were irradiated with 10 Gy γ -rays, and the apoptotic cell death was monitored by time-lapse microscopy. **c** Embryos 2 days post hatching (2 dpf) were irradiated with 20 Gy γ -radiation and stained with acridine orange 12 h after irradiation. Upper panels, dorsal view of the brain region (100 $\times$); lower panels, lateral view of the trunk (400 $\times$). +/+, wild type; +/-, *p53* E241X heterozygote; -/-, *p53* E241X homozygote. [From Taniguchi et al. 2006. Copyright (2006) BioMed Central, USA]

10.3.3 Tumor Development

It is known that spontaneous tumorigenesis is rather rare in medaka (Masahito et al. 1989). However, in the absence of functional p53 protein, medaka showed an increased tumor development rate for both Y186X and E241X alleles (Fig. 10.3). When *p53* homozygous mutants were monitored for survival over 1 year, a dramatic tumor predisposition was observed with a median lifespan of 311 days for *p53*^{Y186X/Y186X}, and 228 days for *p53*^{E241X/E241X}, whereas most of the wild-type littermates lived for more than 1 year. Notably, *p53*^{E241X/E241X} mutants developed a wide variety of tumor types, originating in the kidney (where tumors appeared to derive from the interstitial

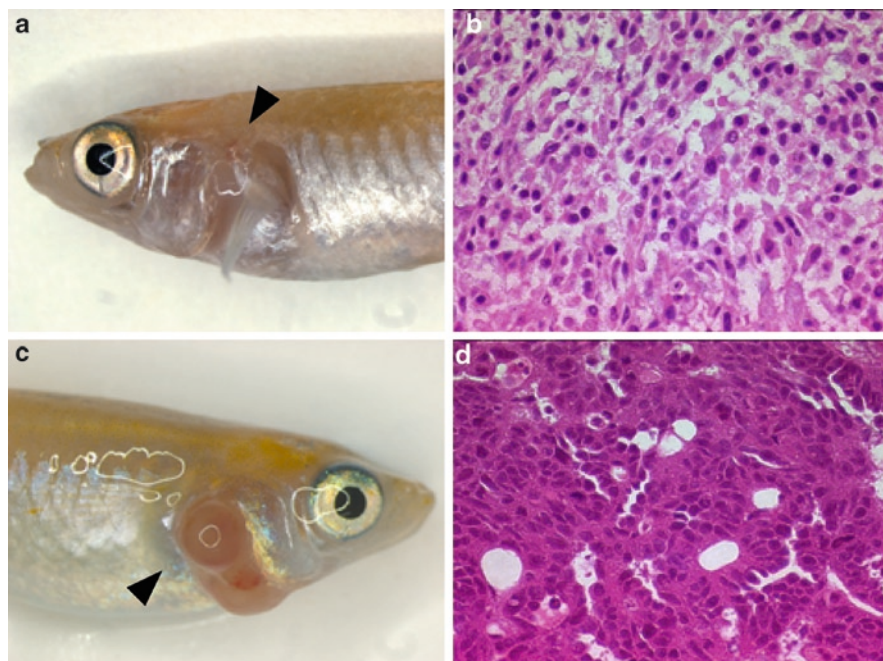


Fig. 10.3 Typical solid tumor that arose in the thymus (**a, b**) and the gill (**c, d**) of *p53*-deficient medaka. **a, c** Stereoscopic view. **b, d** Hematoxylin and eosin staining ($\times 100$). [From Taniguchi et al. 2006. Copyright (2006) BioMed Central, USA]

lymphoid cells, not from epithelial origin), retina, brain, intestine, gill, thymus, and testis. On the other hand, $p53^{Y186X/Y186X}$ mutants tended to develop no obvious solid tumors; rather, they became progressively pale, lean, and weak until they stopped moving at the bottom of the tank and died. We currently do not know the exact cause of death of aged $p53^{Y186X/Y186X}$ mutants. Further stereoscopic and histological studies are required to understand the mechanism of higher mortality of this mutant.

Interestingly, the phenotypes observed in the $p53^{E241X/E241X}$ mutants changed after backcrossing them to the *wild-type* fish six times (five times following artificial insemination). The 67 $p53^{E241X/E241X}$ and 60 $p53^{Y186X/Y186X}$ homozygous fish were observed for spontaneous tumorigenesis. The survival curve of the $p53^{E241X/E241X}$ mutants shifted to the right and was similar to that of the $p53^{Y186X/Y186X}$ homozygous mutant fish. The first death was observed at 167 days for the $p53^{E241X/E241X}$ mutants and at 152 days for the $p53^{Y186X/Y186X}$ mutants; the median lifespan was 416 days for $p53^{E241X/E241X}$ and 364 days for $p53^{Y186X/Y186X}$. Both mutants showed a precipitous fall in survival rate at 1 year after birth. Moreover, the plethora of tumor types that was characteristic of $p53^{E241X/E241X}$ was not as pronounced as before. These results suggest that the phenotypic difference observed between two alleles of *p53* in the young generation was not caused by the functional difference between the E241- and

Y186-truncated proteins. When the *p53* heterozygous medaka were first generated using cryopreserved sperm carrying the *p53* mutation and the unfertilized egg from the *wild-type* female, there had been thousands of ENU-induced point mutations on the genome that deviated from the original sequence. In the course of backcrosses, these mutations are replaced by the chromosomes from the *wild-type* animals. These facts argue that the previously observed differences between the *p53*^{E241X/E241X} and the *p53*^{Y186X/Y186X} phenotypes could result from mutations in the gene(s) that, together with the *p53* deficiency, influenced the onset and spectrum of the tumors, providing a unique opportunity to explore the gene functions that affect the onset of tumorigenesis and the tumor types.

It was reported that peripheral nerve sheath tumors predominate in the *p53* mutant zebrafish isolated by TILLING (Berghmans et al. 2005). In spite of the high incidence of tumors observed in *p53*-deficient medaka compared to their *p53*-proficient littermates, this unusual type of tumor has never developed in medaka. Because the mutant retrieved from the zebrafish TILLING archive had a M214K missense mutation and not a nonsense mutation, it is possible that this specific mutation has a unique function that influences the type of tumor. Alternatively, the difference in the tumor spectrum may be caused by the presence of organism-specific gene(s) that are differentially involved in tumor susceptibility. Tissue-specific tumor development in different species is also described in mammals. For example, the wide variety of cancer types such as soft tissue sarcoma and breast cancer are often seen in Li-Fraumeni syndrome, the inherited genetic disorder caused by *p53* mutation in the human, whereas T-cell lymphomas preferentially develop in mice deficient in the *p53* gene (Donehower et al. 1992; Li et al. 1988). The comparative approach using medaka and zebrafish may be useful for understanding such mechanisms of organism-specific differences caused by the same gene.

10.4 Protocols

10.4.1 Housing the Fish

The fish were kept at 26°C in circulating water. They were fed brine shrimp twice daily. No special precautions were taken for raising the embryos and fry, as no morphological abnormalities or high mortality rate was found during embryogenesis. Because the fish start to die after half a year, the next generation must be isolated before that happens.

10.4.2 Identification of the Genotype

Genotyping may be required when the contamination of the *p53*-deficient population with other genotype is suspected, or when *p53*-deficient medaka are crossed

with other mutants or transgenic lines. We use the direct sequencing method to determine the genotype of individual fish. However, other methods, such as the Cel-I cleavage assay or high-resolution melting (HRM) can also be used for this purpose.

10.4.2.1 Genomic DNA Extraction

A small part of the caudal fin was lysed in 100 μ l buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 1% sodium dodecyl sulfate (SDS), and 200 μ g/ml protease K (Sigma-Aldrich) at 55°C for 1 h. Then, 200 μ l ethanol was added to the lysate, which was incubated on ice for 20 min. The DNA was precipitated by centrifugation at 15,000 rpm for 10 min, then rinsed with 70% ethanol. The pellet was briefly air-dried and dissolved in 100 μ l TE (10 mM Tris pH 7.5, 1 mM EDTA pH 8.0).

10.4.2.2 PCR Amplification of the *p53* Locus

For PCR amplification of the region encompassing the mutation, the primers 5'-TGTTACATTTTATAGCTGTGGAGCA-3' (forward) and 5'-GGCTGAAAA CAGCACAACC-3' (reverse) were used; this will amplify a 390-bp fragment. The PCR was carried out using a touchdown thermocycling program. It consisted of an initial incubation of 92°C for 1 min, a first round of 12 cycles of 92°C for 60 s, 65°C for 20 s with a decrement of 0.5°C per cycle, 72°C for 30 s, a second round of 20 cycles of 92°C for 20 s, 58°C for 20 s, and 72°C for 30 s, and a final extension step of 72°C for 3 min. This reaction contained a 1- μ l aliquot of genomic DNA from the fin, 0.2 μ M forward primer and 0.2 μ M reverse primer, 200 μ M of each dNTP, 0.25 U ExTaq polymerase (Takara Bio, Japan), and 1 $\times$ PCR buffer supplied by the manufacturer for a total volume of 10 μ l. Several samples were tested for the presence of the proper PCR fragment on a 1% agarose gel.

10.4.2.3 Sequencing

The PCR products were directly used as a template for the sequencing reactions without purification. The primers were usually used up in the PCR reaction and did not interfere with the sequencing reaction. Sequencing reactions, containing 0.33 μ l BigDye v3.1 (Applied Biosystems), 1.83 μ l 5 $\times$ dilution buffer (Applied Biosystems), and 0.5 μ M reverse primer for a total volume of 10 μ l, were performed using 40 cycles of 92°C for 10 s, 50°C for 5 s, and 60°C for 1 min. Sequencing products were purified with CleanSeq (Agencourt) and were run on an ABI 3100 capillary-based DNA sequencer.

10.4.3 *p53 Target Gene Induction*

Four days post fertilization, the embryos were irradiated with 20 Gy γ -rays using ^{137}Cs (0.02 Gy/s; Gammacell 40, Atomic Energy of Canada Limited Industrial Products, Ontario). Six hours later, the embryos were frozen in liquid nitrogen and RNA was extracted by Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA was synthesized from each genotype using SuperScript III (Invitrogen). The RNA was treated with RNase-free DNase I (Takara Bio, Japan) at 37°C for 30 min, and was purified by phenol/chloroform extraction followed by ethanol precipitation. The mRNA expression levels were determined by PCR reactions (94°C for 1 min; predetermined cycles of 94°C for 30 s, 55°C for 20 s, 72°C for 30 s) using the primers 5'-ACTACCTCATGAAGATCCTG-3' and 5'-TTGCTGATCCACATCTGCTG-3' for *β -actin*, 5'-CAGCTGTACGACCAGAAGCA-3' and 5'-AGTTGTCGCTGTCCGACTCT-3' for *Mdm2*, 5'-CAGCCAGCTGGCCCAGTATTTGAAGACCC-3' and 5'-TTAATTGCTCTTCAGCTTC-3' for *p53*, 5'-CTGCTCCAAAGCGGATTC-3' and 5'-GCGGCATCCAGACCATTTCGT-3' for *p21*, and 5'-CTCAATGCCTCCAGCAGATT-3' and 5'-CACATCTTGCGAATGACCAC-3' for *Bax*. The numbers of cycles used were *β -actin*, 15; *Mdm2*, 24; and *p53*, *p21*, and *Bax*, 26.

10.4.4 *Apoptosis Assay*

For time-lapse monitoring of irradiated cells, primary cell cultures derived from *p53^{E241X/E241X}* and *p53^{+/+}* embryos were inoculated in a 35-mm dish at a density of 1.5×10^5 and irradiated with 10 Gy γ -rays. The cells were monitored for fragmentation of the nucleus using an IX81 inverted microscope (Olympus, Japan) controlled by IPLab software (BD Biosciences, Rockville, MD, USA) for 8 h after irradiation. For acridine orange (AO) staining, 2-dpf embryos were irradiated with 20 Gy γ -rays. The eggshells were removed by hatching enzyme, and 12 h after irradiation, the embryos were stained live with 17 $\mu\text{g/ml}$ AO for 45 min. The embryos were then washed with phosphate-buffered saline (PBS)(-) for 10 min and fixed in 4% paraformaldehyde in PBS(-) for more than 1 h. The image was acquired using fluorescence microscopy, IX81 (Olympus, Japan).

10.5 Medaka in TILLING

Medaka eggs are difficult to handle compared to zebrafish eggs because of the hard and thick chorion and the sticky filaments on the surface; this makes it difficult for the injection needles to penetrate, obstructing the experiments using morpholino. On the other hand, there are several substantial advantages to using medaka with respect to the generation of mutants by TILLING, as described next.

10.5.1 Design of Amplicons

According to the genome draft published in 2007, the size of the medaka genome is 700 Mb, less than half of that of zebrafish (Kasahara et al. 2007). The compactness of the medaka genome is largely the result of the short introns and other noncoding regions, which facilitates the relatively easy and efficient design of PCR-based amplicons. Because of the physical limitations of the capillary sequencer for providing a clear signal, a single amplicon can cover only 800 bp at most. In the case of the medaka *p53* gene, a single amplicon of 673 bp in length can cover the three critical exons that encode the DNA-binding domain, whereas three separate amplicons must be designed to screen the zebrafish *p53* gene. The same is true for many other genes (Fig. 10.4).

10.5.2 Strains to Be Used

Several inbred strains have been established for medaka from both southern and northern populations. These strains should be extremely useful when employing the TILLING technique, because the mutant is based on single nucleotide changes, and the detection system relies on the separation of homozygous *wild-type* alleles from heterozygous mutant alleles. When outbred strains are used to construct mutant libraries, it can be difficult to discriminate the interspersed single nucleotide polymorphisms (SNPs) from the bona fide ENU-induced mutations, especially if the Cel-I cleavage system or HRM is used where SNPs complicate the analysis of the heteroduplex signals.

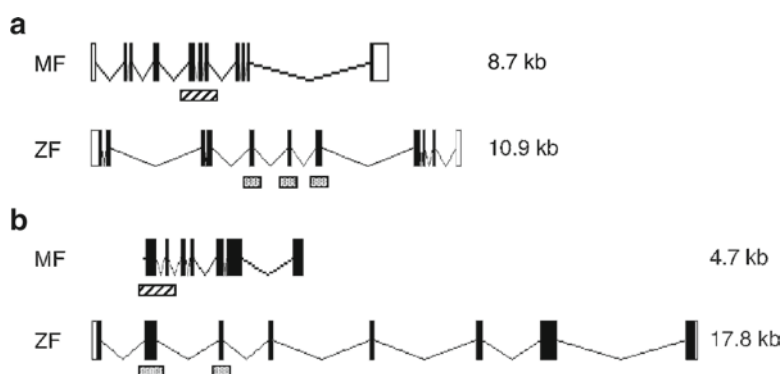


Fig. 10.4 Comparison of amplicons for medaka and zebrafish genes. The amplicons designed to screen for *p53* (a) and *DKC1* (b) medaka (MF) genes are shown in *striped bars*. The corresponding regions in zebrafish (ZF) genes are shown in *dotted bars*. *Black boxes*, coding exons; *white boxes*, noncoding exons; *thin lines*, intronic regions

10.5.3 Cryopreservation of the Library

Medaka sperm is harder than that of zebrafish, and cryopreservation is thus much easier in medaka. Motility and the ability of sperm to fertilize eggs are usually preserved after thawing. These features allow us to maintain the mutant medaka library as a semipermanent sperm stock. Another point that benefits from construction of sperm archive is that, because the animal is killed at the time of cryopreservation, a large amount of genomic DNA can be extracted from the rest of the body. This extraction eliminates the need for nested-PCR, which is often used in screening of a live stock zebrafish library, where only a minute amount of DNA is available from the fin.

10.5.4 Isolated Mutants

Morpholino antisense oligonucleotides are the nuclease-resistant synthetic compound that is widely used for loss-of-function analysis in the fish research field; they are injected into fertilized eggs at the one-cell stage. Because the effect of morpholinos is transient, the use of this technology has been confined to early development, which is a particular problem for medaka research, as it usually takes 7–10 days for medaka embryos to hatch, compared to 2 days for zebrafish. Thus, there is a limited time window during which development can be analyzed by morpholinos in medaka.

Gene disruption by TILLING expands the possibilities of research not only in developmental biology but also in many new fields. The classical forward genetics approach misses many gene disruptants in which mutants appear asymptomatic at first glance. Table 10.2 shows the part of the results of our screen. Using the TILLING mutants, we are particularly interested in the time-consuming and slowly progressing biological processes such as tumorigenesis, degeneration, and aging. Two other areas that could benefit from TILLING technology are aquaculture and drug discovery. The research on immune responses, endocrine system, and reproduction using the teleost as a model animal will be of particular importance in aquaculture, and in the establishment of disease models, which can be utilized in drug screening.

10.6 Future Perspectives

TILLING offers an efficient means to isolate teleost mutants using a reverse genetics approach. However, not all genes are amenable to this method. For example, small genes are hard to disrupt by TILLING because of the small probability of any meaningful point mutations being introduced in the coding region. Noncoding RNA and regulatory regions such as promoters are also very difficult to disrupt by

Table 10.2 The medaka mutants isolated by targeting-induced local lesions in genome (TILLING) screen

No.	Gene	Amplicons		Mutations					Total
		Number	Genome (bp)	CDS (bp)	Nonsense	Splice	Missense	Silent	
1	<i>MSH2</i>	1	1,238	716	2	1	7	2	20
2	<i>AmhrII</i>	1	778	357	2	1	6	4	28
3	<i>p53</i>	1	673	395	2	1	3	0	7
4	<i>PERK</i>	2	1,500 ^a	754	2	0	9	5	35
5	<i>TERT</i>	2	885/1,136	445/631	1	2	23	9	61
6	<i>IL12Rb2</i>	1	848	405	1	1	10	5	28
7	<i>FoxnI</i>	1	691	296	1	1	3	1	19
8	<i>SSB2</i>	1	809	728	1	0	na	na	1
9	<i>AID</i>	1	899	565	1	0	12	9	29
10	<i>Hoxc6a</i>	1	1,232	708	1	0	12	4	35
11	<i>ATM</i>	1	976	610	1	0	12	3	25
12	<i>Lepr</i>	1	848	355	1	0	9	7	33
13	<i>Zmpste24</i>	1	693	351	1	0	7	3	19
14	<i>Notch1b</i>	1	496	339	1	0	7	2	12
15	<i>PINK1</i>	1	683	386	1	0	6	5	14
16	<i>Parkin</i>	1	892	556	1	0	4	4	11
17	<i>Blm</i>	2	643	495	1	0	4	0	6
18	<i>ATF6</i>	1	693	319	1	0	3	2	12
19	<i>Notch1a</i>	1	848	602	0	1	21	3	30
20	<i>ATP13A2</i>	1	1,251 ^b	665	0	1	16	5	36
21	<i>Sir1</i>	2	792/445	512/228	0	1	12	0	15
22	<i>STAT4</i>	1	1,255	458	0	1	8	3	44
23	<i>Foxp3</i>	1	1,113	301	0	1	7	1	29
24	<i>TTK</i>	3	844/883/1,311	417/523/559	0	0	31	11	64
25	<i>HtrA2</i>	2	617/1,066 ^b	517/433	0	0	21	5	31
26	<i>GPR151</i>	1	893	893	0	0	16	5	21

(continued)

Table 10.2 (continued)

No.	Gene	Amplicons		Mutations							Total
		Number	Genome (bp)	CDS (bp)	Nonsense	Splice	Missense	Silent	Intron		
27	<i>Ttpo3</i>	1	847	486	0	0	13	11	15	39	
28	<i>Cyclin B3</i>	2	900 ^b /868	468	0	0	12	10	26	48	
29	<i>BiP</i>	1	924 ^b	519	0	0	12	1	6	19	
30	<i>Irela</i>	1	738	364	0	0	11	1	5	17	
31	<i>Myostatin</i>	2	651/689	378/374	0	0	10	6	7	23	
32	<i>Pumilio2</i>	1	1,029	640	0	0	9	1	12	22	
33	<i>Progranulin</i>	1	728	474	0	0	8	4	2	14	
34	<i>Atg10</i>	1	685	424	0	0	8	1	1	10	
35	<i>ESCO2</i>	1	802	518	0	0	6	7	5	18	
36	<i>Mak3</i>	1	821	362	0	0	6	4	12	22	
37	<i>Pumilio1</i>	1	1,031	516	0	0	6	3	5	14	
38	<i>DKC1</i>	1	973 ^b	319	0	0	6	2	24	32	
39	<i>PTEN</i>	1	881	381	0	0	3	8	1	12	
40	<i>MGMT</i>	1	358	137	0	0	0	1	5	6	

The results of screening for 40 genes are listed together with the number of amplicons screened for each gene, the length of genomic (genome) and coding (CDS) sequences contained in each amplicon, and the number and the type of mutations discovered in the screen. Note that these are the results of high-throughput sequencing carried out at RIKEN Genomic Sciences Research Complex and may contain false-positive heterozygous signals
na, not available

^aThe amplicon contains a stretch of nucleotides of unknown length whose sequence has not been determined

^bIndicates that the sequence was determined from both ends of amplified polymerase chain reaction (PCR) products

a point mutation. Attempts to disrupt genes consisting of dispersed exons would be very inefficient, because one must design multiple short amplicons covering each exon. Amino acid composition of target protein is another important factor that should be taken into account. Hydrophobic residues often cannot be converted into a nonsense mutation because of the fixed combination of trinucleotides. For example, valine is encoded by GTN, where N is any nucleotide, but no change in a single nucleotide in this sequence would result in a stop codon. Thus, multipass membrane proteins such as G protein-coupled receptors would be a difficult target. Other classes of genes for which special precautions must be taken when designing amplicons are those that encode the protein with a functional domain at the N-terminus. Even if one luckily finds a nonsense mutation in the central or C-terminal portion, the resulting truncated protein may remain active without the C-terminus. Alternatively, if there is a molecular interface at the N-terminus, such a truncated version of the protein could exert a dominant negative effect on its binding partner.

All this having been said, what limits the screen the most in a practical sense is cost. Although the Cel-I-based method, TGCE, and HRM have better cost performance compared to conventional capillary sequencing, they are indirect methods that detect the heteroduplex formed between *wild-type* and mutant alleles, and are limited in that they can target only a small portion of the genome at a time. The gene-disrupting mutations can be found in only about one-half the cases when the current library is screened for the genes of interest (see Table 10.2), suggesting the need for screening as large an area as possible unless the new library is constructed with a dramatic increase in mutation frequency. The next generation sequencer, which will be able to produce a few gigabases of sequence at one run, may be applied to TILLING and solve this problem. The recently developed barcode technology in which each PCR fragment is ligated to an adaptor that has a unique sequence embedded within itself would be able to discriminate which mutation came from which sample (Craig et al. 2008); this will make the identification of mutants much easier and faster. With multiple medaka mutants on hand, understanding the molecular network in vertebrate animals will be enhanced greatly in the future.

References

- Aoki K, Matsudaira H (1977) Induction of hepatic tumors in a teleost (*Oryzias latipes*) after treatment with methylazoxymethanol acetate: brief communication. J Natl Cancer Inst 59:1747–1749
- Berghmans S, Murphey RD, Wienholds E, Neuberg D, Kutok JL, Fletcher CD, Morris JP, Liu TX, Schulte-Merker S, Kanki JP, Plasterk R, Zon LI, Look AT (2005) tp53 Mutant zebrafish develop malignant peripheral nerve sheath tumors. Proc Natl Acad Sci USA 102:407–412
- Chen S, Hong Y, Scherer SJ, Schartl M (2001) Lack of ultraviolet-light inducibility of the medaka fish (*Oryzias latipes*) tumor suppressor gene p53. Gene (Amst) 264:197–203
- Chen J, Ruan H, Ng SM, Gao C, Soo HM, Wu W, Zhang Z, Wen Z, Lane DP, Peng J (2005) Loss of function of def selectively up-regulates Delta113p53 expression to arrest expansion growth of digestive organs in zebrafish. Genes Dev 19:2900–2911

- Cho Y, Gorina S, Jeffrey PD, Pavletich NP (1994) Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. *Science* 265:346–355
- Craig DW, Pearson JV, Szelinger S, Sekar A, Redman M, Corneveaux JJ, Pawlowski TL, Laub T, Nunn G, Stephan DA, Homer N, Huentelman MJ (2008) Identification of genetic variants using bar-coded multiplexed sequencing. *Nat Methods* 5:887–893
- Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr, Butel JS, Bradley A (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature (Lond)* 356:215–221
- Doyon Y, McCammon JM, Miller JC, Faraji F, Ngo C, Katibah GE, Amora R, Hocking TD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Amacher SL (2008) Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat Biotechnol* 26:702–708
- Feitsma H, Cuppen E (2008) Zebrafish as a cancer model. *Mol Cancer Res* 6:685–694
- Furutani-Seiki M, Sasado T, Morinaga C, Suwa H, Niwa K, Yoda H, Deguchi T, Hirose Y, Yasuoka A, Henrich T, Watanabe T, Iwanami N, Kitagawa D, Saito K, Asaka S, Osakada M, Kunimatsu S, Momoi A, Elmasri H, Winkler C, Ramialison M, Loosli F, Quiring R, Carl M, Grabher C, Winkler S, Del Bene F, Shinomiya A, Kota Y, Yamanaka T, Okamoto Y, Takahashi K, Todo T, Abe K, Takahama Y, Tanaka M, Mitani H, Katada T, Nishina H, Nakajima N, Wittbrodt J, Kondoh H (2004) A systematic genome-wide screen for mutations affecting organogenesis in medaka, *Oryzias latipes*. *Mech Dev* 121:647–658
- Harms K, Nozell S, Chen X (2004) The common and distinct target genes of the p53 family transcription factors. *Cell Mol Life Sci* 61:822–842
- Kasahara M, Naruse K, Sasaki S, Nakatani Y, Qu W, Ahsan B, Yamada T, Nagayasu Y, Doi K, Kasai Y, Jindo T, Kobayashi D, Shimada A, Toyoda A, Kuroki Y, Fujiyama A, Sasaki T, Shimizu A, Asakawa S, Shimizu N, Hashimoto S, Yang J, Lee Y, Matsushima K, Sugano S, Sakaizumi M, Narita T, Ohishi K, Haga S, Ohta F, Nomoto H, Nogata K, Morishita T, Endo T, Shin IT, Takeda H, Morishita S, Kohara Y (2007) The medaka draft genome and insights into vertebrate genome evolution. *Nature (Lond)* 447:714–719
- Krause MK, Rhodes LD, Van Beneden RJ (1997) Cloning of the p53 tumor suppressor gene from the Japanese medaka (*Oryzias latipes*) and evaluation of mutational hotspots in MNNG-exposed fish. *Gene (Amst)* 189:101–106
- Kruse JP, Gu W (2009) Modes of p53 regulation. *Cell* 137:609–622
- Li FP, Fraumeni JF Jr, Mulvihill JJ, Blattner WA, Dreyfus MG, Tucker MA, Miller RW (1988) A cancer family syndrome in twenty-four kindreds. *Cancer Res* 48:5358–5362
- Masahito P, Aoki K, Egami N, Ishikawa T, Sugano H (1989) Life-span studies on spontaneous tumor development in the medaka (*Oryzias latipes*). *Jpn J Cancer Res* 80:1058–1065
- Meng X, Noyes MB, Zhu LJ, Lawson ND, Wolfe SA (2008) Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nat Biotechnol* 26:695–701
- Moens CB, Donn TM, Wolf-Saxon ER, Ma TP (2008) Reverse genetics in zebrafish by TILLING. *Brief Funct Genomic Proteomic* 7:454–459
- Taniguchi Y, Takeda S, Furutani-Seiki M, Kamei Y, Todo T, Sasado T, Deguchi T, Kondoh H, Mudde J, Yamazoe M, Hidaka M, Mitani H, Toyoda A, Sakaki Y, Plasterk RH, Cuppen E (2006) Generation of medaka gene knockout models by target-selected mutagenesis. *Genome Biol* 7:R116
- Vousden KH, Prives C (2009) Blinded by the light: the growing complexity of p53. *Cell* 137:413–431
- Winkler C, Wittbrodt J, Lammers R, Ullrich A, Scharlt M (1994) Ligand-dependent tumor induction in medakafish embryos by a Xmrk receptor tyrosine kinase transgene. *Oncogene* 9:1517–1525

Chapter 11

Medaka Spontaneous Mutants for Body Coloration

Shoji Fukamachi

Abstract People take a keen interest in colors on their body surface. Colors are also important in wild animals for successful survival and reproduction; for example, camouflage, mimicry, and sexual attraction for mating. Studies of body coloration have mainly been conducted using the mouse model, where more than 300 coat-color loci have been described. Zebrafish is another unique model for investigating pattern (stripe) formation in the skin. Medaka is a fish with a boring-brown coloration from which only tens of spontaneous mutants are available. However, the mutants have a rather long research history, from which several intriguing achievements have been made. I summarize these studies in this chapter.

11.1 Introduction: Body Coloration in Vertebrates

Body surfaces of animals are pigmented, often with complex color patterns (Fig. 11.1). These colors in the skin, hairs, feathers, scales, etc. are highly divergent even between closely related species, and sometimes play indispensable roles for successful survival and reproduction in nature. For example, cryptic pigmentation helps animals to hide from predators (whereas some species, such as glass fish and frogs, have no pigment in the skin and are transparent), and nuptial coloration increases the chance of mating. Humans also have keen interests in their skin, hair, and eye colors, which is reflected in the huge market for cosmetics. They also trade color variants of pet animals at high prices (e.g., a single Koi carp can cost up to several hundred thousand U.S. dollars). Skin protection against ultraviolet (UV) light is another important role of body colors; that is, albino people suffer from a higher risk of skin cancer.

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Fig. 11.1 (color online) Colors on animal body surfaces. [Images courtesy of www.wallpaperlink.com (macaw), www.photolibary.jp (beetle), www.0014suizokukan.com (sea slug), and www.fishing-forum.org (filefish)]

In vertebrates, the skin (including hairs, plumes, scales) is pigmented by cells called chromatophores. The chromatophores contain light-absorbing pigments or light-reflecting structures within the cell organelles called chromatosomes. As the chromatosomes absorb or reflect light at certain wavelengths, the cell looks pigmented. The chromatophores are neural crest derivatives (Sauka-Spengler and Bronner-Fraser 2008), and therefore animals other than vertebrates have different means of skin pigmentation (see Messenger 2001).

The chromatophores are classified into five types based on the color of chromatosomes: black melanophores, orange xanthophores, white leucophores, blue cyanophores, and silver iridophores. Given the relatively recent discovery of the cyanophore (Goda and Fujii 1995), further explorations may identify additional types of chromatophores. Lower vertebrates can bidirectionally transport chromatosomes, causing peripheral dispersion or perinuclear aggregation of the pigments. This intracellular movement changes the cell color, and the skin color as a whole, which is called a physiological body-color change (Nery and Castrucci 1997). Mammals and birds, where chromatosomes are only peripherally transported to be secreted into the skin, hairs, and feathers, can change body color but more slowly, by increasing pigment synthesis or cell numbers (morphological body-color change; Sugimoto 2002).

Medaka has four types of chromatophores, whereas the zebrafish or mouse has three or only one type(s), respectively. Therefore, comparative studies between medaka and other model organisms would provide unique clues for understanding the molecular bases of body-color diversity.

11.2 History of Medaka Body-Color Mutants

Descriptions of medaka mutants can be traced back to the 18th century (Fig. 11.2). A Japanese naturalist, Zuiken Kurimoto (1756–1834), described in his encyclopedia *Risshi Gyofu* that “Medaka. A bright-red variant infrequently occurs, which is

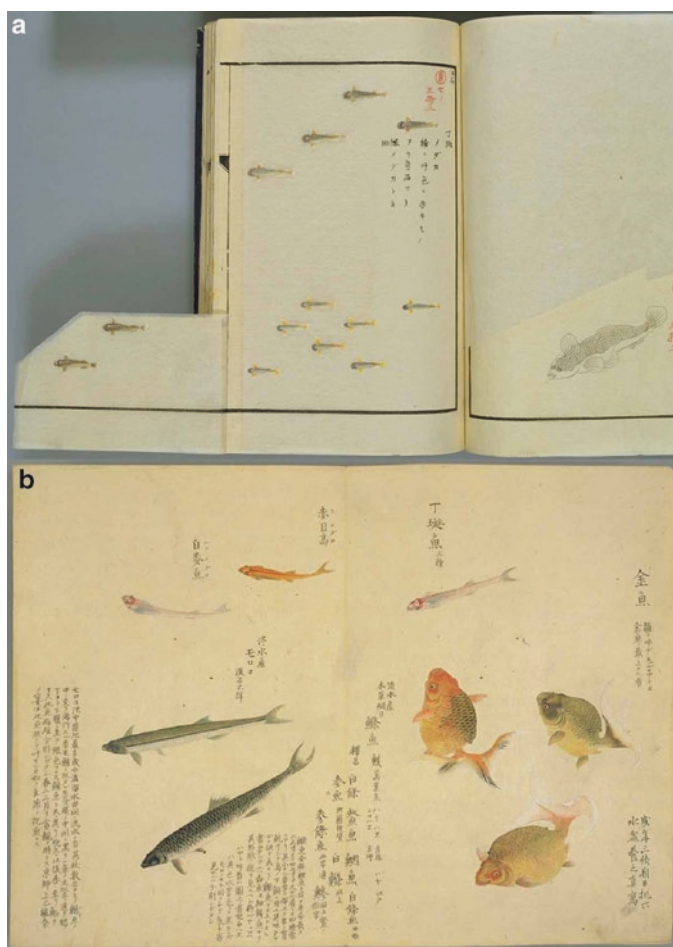


Fig. 11.2 (color online) Medaka mutants in ancient encyclopedias. **a** The *b* mutants in *Risshi-gyofu* (bottom). **b** The *b* (top middle) and *b-r* (top left) mutants in *Baien-gyofu*. (Images courtesy of National Diet Library)

called hi-medaka. Precious.” Another naturalist, Motohisa Mōri (1815–1881), also described the hi-medaka in his encyclopedia *Baien Gyofu* together with another body-color variant, shiro (white)-medaka. Thus, people in Japan have been breeding these body-color variants (mutants; see below) for at least 200 years.

In 1921, Tatsuo Aida (1871–1957) reported Mendelian inheritance of these traits in the journal *Genetics*. He crossed the hi-, shiro-, and wild-type medaka, raised more than ten thousands of F_1 and F_2 fish in about 40 tanks settled at his home, and scored their phenotypes. His results demonstrated that (1) the hi-medaka is caused by a single recessive mutation (b) that causes hypopigmentation of melanophores, (2) the shiro-medaka has an additional recessive mutation (r) that causes hypopigmentation of xanthophores, (3) the r mutation is sex linked and on the X chromosome, and (4) buchi(mottled)-medaka, another color variant with variegated patterns, is caused by another recessive allele of the b locus (B' ; dominance hierarchy is $b < B'$). Considering that Mendel's work (1866) was rediscovered in 1900 and that Morgan reported the sex-linked inheritance of *Drosophila* eye colors in 1910, Aida's experiments (started in 1913) were a cutting edge for that period.

Next, an astonishing professor, Hideo Tomita (1931–1998), appeared. Wearing a straw hat and overalls on the campus of Nagoya University, he conducted extensive crossing using about 500 pottery tanks. He purchased hi-medaka from commercial breeders, released them in a pool, gazed over their swimming for a while, and somehow selected individuals to be used in his experiments (K. Naruse of National Institute for Basic Biology, personal communication). As his achievements show (see below), many of the selected fish actually carried a mutation. Considering that most of his mutant phenotypes are caused by a recessive allele (as he himself described; Tomita 1992; see also Kelsh et al. 2004), it is both legendary and mysterious that he saw these in the swimming of heterozygous founders. Nevertheless, his screening was highly successful, isolating about 50 pigmentation and about 30 nonpigmentation mutants. These spontaneous mutants have been providing precious opportunities for present researchers to study mutation rates (Shima and Shimada 1991), unstable DNA fragments (Koga et al. 1995), or sex differentiation (Wada et al. 1998), or to establish transparent strains (Wakamatsu et al. 2001; Fukamachi et al. 2008). Progressive genetic studies are now revealing genes responsible for his and other ancient medaka mutants. Focusing on the pigmentation mutants, I summarize here the achievements to date.

11.3 Medaka Body-Color Mutants

11.3.1 The *i*-Locus Mutants

The *i*-locus mutants [i (i^l), i^t , i^o , and i^b], all of which were isolated by Tomita alone, have typical albino phenotypes with red eyes and white skin (oculocutaneous albinism, OCA). OCAs in mammals and birds (which have only one type of chromatophores, the melanocytes) are described as “lack of melanin.” In medaka

OCA, however, hypopigmentation occurs in not only melanophores but also xanthophores; that is, hypopigmentation of melanophores alone causes the orange skin of hi-medaka (Fig. 11.2).

A mutation on *Tyrosinase* (*Tyr*), which encodes a rate-limiting enzyme for melanin synthesis, is known for the mouse *c*-locus albino mouse (Shibahara et al. 1990). A series of experiments using the medaka *i*-locus mutants, including Mendelian inheritance of the phenotype (Yamamoto 1969), lack of *Tyr* activity in vivo (Tomita 1975), mutations on a medaka *Tyr* ortholog (Koga et al. 1995), and rescue of the *i* phenotype by the heterogeneous/homogeneous *Tyr* gene (Hyodo-Taguchi et al. 1997; Inagaki et al. 1998), demonstrated that *Tyr* plays an indispensable role for melanin synthesis throughout vertebrates and has a novel function for xanthophore regulation in fish. Considering that xanthophores do not synthesize pigments, but rather take in pigments (carotenoids) from outside, it remains unknown how this catalytic enzyme regulates xanthophore pigmentation.

*i*¹ was the first mutation identified in the medaka genome (Koga et al. 1995). Another breakthrough of this work was the finding that the mutation is caused by insertion of an active transposon, *Tol1*. Interestingly, *i*⁴ and *i*^b mutations are also caused by an insertion of another active transposon, *Tol2* (*i*⁶ carries a deletion). Furthermore, at least three more Tomita mutants (*eyeless*, *reduced scale-3*, and *Double anal fins*) have been known to harbor a transposon or repetitive element as their mutations (Loosli et al. 2001; Kondo et al. 2001; Ohtsuka et al. 2004). Therefore, quite a few genes in the medaka genome seem to be mutated by such unstable DNA fragments, which may have helped Tomita's mutagen-free screening. Many researchers are now using the *Tols* (particularly *Tol2*) as tools for genetic engineering such as mutagenesis and transgenesis (Kawakami 2007; Koga et al. 2008).

11.3.2 The *i*³ Mutant

Tomita isolated another OCA medaka, *i*³, whose phenotype is indistinguishable from that of the *i* mutant. A cross between *i* and *i*³ mutants can complement the OCA phenotype, and the loci are not linked on a chromosome (Tomita 1992). *Tyr* activity is incompletely suppressed in *i*³ and expression of mouse *Tyr* could rescue the phenotype, suggesting that the *i*³ gene plays a role in melanin synthesis by regulating *Tyr* (Hyodo-Taguchi et al. 1997).

The *i*³ mutation was identified as a four-base deletion on a medaka ortholog of the mouse *pink-eyed dilution* (*p*) gene (Fukamachi et al. 2004b). The *p* gene is also called *oca2*, because mutations on a human ortholog cause OCA type 2 (the OCA caused by a mutation on *Tyr* is called OCA1). The *p* gene had been thought to regulate transport of tyrosine, the substrate of melanin, and it actually encodes a putative membrane transporter (Brilliant et al. 1991). However, recent experiments indicated that the *p* protein controls intracellular pH for appropriate processing and trafficking of *Tyr* into the melanosomes where melanin is synthesized (Ni-Komatsu

and Orlow 2006). Medaka *p* is transcribed in not only the melanized skin and eyes but also amelanotic gonads, indicating an additional function or functions other than melanin synthesis (Fukamachi et al. 2004b).

There are interesting studies reporting that the *p* gene is mutated in hypopigmented cavefish (Protas et al. 2006) and is responsible for differences in skin, hair, and eye color among human populations (Sulem et al. 2007). Thus, the *p* gene for OCA2 is also involved in natural color variations.

11.3.3 The *b*-Locus Mutants

The ancient *b* mutant (hi-medaka) has orange skin as a result of colorless melano-phores and pigmented xanthophores. Tyr is fully active in the *b* mutant. Some researchers, however, could rescue the hypopigmentation by *Tyr* injection (Matsumoto et al. 1992; Ono et al. 1997), whereas others could not (Hyodo-Taguchi et al. 1997); thus the function of the *b*-gene product in relation to Tyr remains unclear.

The eyes of the *b* mutant are black, but slight amelanosis actually occurs in the choroid membrane (but not the retinal pigment epithelium), and also in the peritoneum (Hirose and Matsumoto 1993). Many spontaneous or induced mutants have been isolated for the *b* locus, most of which exhibit apparent hypopigmentation, interestingly, in both the skin and eyes (i.e., OCA), in contrast to the original *b* and a few other spontaneous mutants (*B'*, *b^d*, *b^{dl}*, and *b^v*; Shimada et al. 2002). The strongest allele, *b^{g8}*, causes the OCA phenotype similar to *i* and *i³*, but its eyes and xanthophores are still faintly pigmented.

The *b* gene was identified by the first positional cloning in medaka (Fukamachi et al. 2001). Similarly to *i³*, the *b* gene encodes a putative transporter with 12 transmembrane domains, Solute carrier family 45, member 2 (*Slc45a2*), whose actual function in melanogenesis is yet to be identified. Mutations on mouse/human orthologs also cause OCA (i.e., OCA type 4; Newton et al. 2001), but the symptom is milder than OCA1 and OCA2, as is the case in medaka (see above).

From the medaka mutants that exhibit the typical OCA4 phenotypes, a deletion (*b^{g8}*), duplication (*b^v*), or substitution (*b^{c1}*, *b^{d2}*, *b^{d4}*, *b^{d8}*, *b^{g21}*) were identified on a coding region of *Slc45a2*. By contrast, the original *b* mutation causing the skin-specific albinism was identified on a promoter that controls the cutaneous, but not ocular, expression of *Slc45a2* (Fukamachi et al. 2008). The mutation of another ancient allele, *B'* (see above), remains unidentified, but it possesses the *b* mutation on the promoter. Intriguingly, *B'*-like fish recently emerged from a laboratory stock of the *b* mutant, the d-rR.YHNI strain (A. Froschauer, Technische Universität Dresden, personal communication). These observations seem to indicate that *B'* is an incomplete revertant of *b*. Further explorations on the *b* locus might reveal an additional mutation that causes the late-onset and incomplete melanogenesis in the *B'* skin.

Mutations on *Slc45a2* have been identified in color variants of various species, including horse, chicken, quail, and suncus, and *SLC45A2* is most rapidly evolving among European people (Sabeti et al. 2007). Thus, *Slc45a2* is also involved in natural color variations as is the *p* gene.

11.3.4 The Leucophore Free Mutant

The *leucophore free* (*lf*) mutant, isolated by Tomita, lacks leucophores throughout life. Taking advantage of the location of the *lf* locus on the sex chromosome, a strain (Qurt) in which genetic sex can be identified by observing 2-day-old embryos was established; that is, leucophores appear only in $X^{lf}Y^+$ males but not in $X^{lf}X^{lf}$ females (Wada et al. 1998). The *lf* mutation was also used to eliminate leucophores from the “see-through” strains (Wakamatsu et al. 2001; Fukamachi et al. 2008).

lf lacks not pigments in leucophores but leucophores themselves, indicating its indispensable role in leucophore differentiation (Fukamachi et al. 2006). Because leucophores do not exist in mouse or zebrafish, identification and functional comparison of the *lf* gene would provide a unique clue for development and evolution of chromatophores. A high-resolution linkage map around the *lf* locus is already available (Fukamachi et al. 2006).

11.3.5 The White Leucophore Mutant

Leucophores in medaka embryos are colored pale pink rather than white. However, this Tomita mutant exhibits white embryonic leucophores. The *white leucophore* (*wl*) mutation, together with the *b*, *lf*, *i^b*, and *guanineless* (*gu*) mutations, was used to establish the T5 tester strain for the specific-locus test (Shimada and Shima 2001). The test revealed transgenerational genetic instability of the *wl* locus; that is, γ -ray irradiation of sperm cells sometimes induces mosaic mixtures of the pale-pink (wild-type) and white (*wl*) leucophores in embryos (Shimada and Shima 2004). The *wl* locus has been mapped on LG9 but is yet to be identified (Fukamachi and Shimada, unpublished data).

11.3.6 The Color Interfere Mutant

This intriguing mutant was isolated by a Japanese fancier in 1951, and autosomal recessive inheritance of the phenotype was observed by Aida (unpublished; see Yamamoto 1975). The skin of the *color interfere* (*ci*) mutant is pale gray as a result of a several-fold increase in the number and size of leucophores and a concomitant decrease in xanthophores (Takeuchi 1969). The color difference between *ci* and wild-type medaka is maximized in a dark environment, where wild-type (but not *ci*)

fish decrease leucophores (and increase melanophores) for background adaptation of the skin (i.e., morphological body-color change; Sugimoto et al. 2000).

The *ci* locus is linked to the *i* locus (Yamamoto and Oikawa 1973), and was identified to encode a peptide hormone, somatolactin alpha (SLa; Fukamachi et al. 2004a). Although various functions of SLa have been suggested since its discovery (Ono et al. 1990), medaka SLa seems to function predominantly in body-color regulation; that is, neither the *ci* nor the transgenic *ci* medaka that ectopically expresses *SLa* shows apparent defects besides the skin color (Fukamachi et al. 2009). Expression of *SLa* is distinctly upregulated or downregulated by a dark or bright environment to turn the skin color dark or pale, respectively, suggesting its role in cryptic coloration (Fukamachi et al. 2004a).

Recent experiments indicated another role of SLa, nuptial coloration (Fukamachi et al. 2009). When a male medaka is given a choice between two females, the male usually approaches both females equally. However, only when one of the choices is *ci*, males significantly prefer to approach the other female (Fig. 11.3). Furthermore, the mating preference of males is maximally biased in a choice between *ci* and



Fig. 11.3 (color online) Medaka strains used in mate-choice experiments. From the top: *ci*, b^{s8} , OIMA1-GFP, wild type, OIMA1-DsRed2, and i^1 . Although even the apparently unnatural red/green medaka can normally attract mates, *ci* meet considerable difficulties in mating despite its ordinary fertility and fecundity

SLa-transgenic females, indicating an important role of *SLa* in mate attraction via body color. This finding uniquely explains the reason why tetrapods lost *SLa* during evolution; that is, it may have become dispensable for common ancestors of land vertebrates, because they acquired means for mate attraction other than the nuptial coloration, such as calls in birds and frogs and pheromones in mammals.

Downstream genes of *SLa* are unidentified, except that a receptor for *SLa* (*SLR*) was identified by in vitro binding experiments (Fukada et al. 2005). However, this *SLR* was revealed to be a paralog of *growth-hormone receptor* (*GHR*) acquired only in teleosts by genome duplication (Fukamachi and Meyer 2007). By contrast, *SLa* is not a paralog of *GH*; the teleost-specific genome duplication duplicated ancestral *SL* into *SLa* and *SL beta* (*SLb*; Zhu et al. 2004). Hence, common ancestors of vertebrates had *SL* but not *SLR*. The ancestral *GHR* may have functioned as a dual receptor for both *GH* and *SL* (i.e., *GHSLR*), which later subfunctionalized into *GHR* and *SLR* in teleosts. Experiments using “living fossils,” such as coelacanth, lungfish, polypterus, and sturgeon, would be helpful to address this hypothesis.

11.3.7 The Pale Gray Eyes Mutant

Recent backcrossing of hi-medaka (similar to Tomita) isolated a spontaneous mutant, *pale gray eyes* (*pge*) (Yu et al. 2006). The mutation is homozygous lethal and strongly suppresses pigmentation of the eyes and all chromatophores in the skin. The locus was mapped on a telomeric region of LG14, where the *Vacuolar protein sorting 11 homolog* (*Vps11*) gene, whose transcription is reduced in the *pge* mutant, is located. Considering that *Vps* proteins function for vesicle docking/fusion to lysosome-like vacuoles, protein trafficking to chromatosomes would be defected by the *pge* mutation.

11.4 Future Perspectives

Thus, the medaka body-color mutants, which were isolated decades ago by Tomita and other Japanese, are now providing unique platforms for studying the molecular bases of body-color regulation. Furthermore, the mutants are also contributing to other fields of study, including genetic instability (*i* and *wl*), the transparent strains for developmental experiments (*i³*, *b*, *lf*, and *gu*), and behavioral sexual selection (*ci*). The forward-genetic studies will no doubt be accelerated by the draft genome sequence (Kasahara et al. 2007), and further exciting findings should be provided from this colorful collection.

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References

- Aida T (1921) On the inheritance of color in a fresh-water fish, *Aplocheilichthys latipes* Temmick and Schlegel, with special reference to sex-linked inheritance. *Genetics* 6:554–573
- Brilliant MH, Gondo Y, Eicher EM (1991) Direct molecular identification of the mouse *pink-eyed unstable* mutation by genome scanning. *Science* 252:566–569
- Fukada H, Ozaki Y, Pierce AL et al (2005) Identification of the salmon somatolactin receptor, a new member of the cytokine receptor family. *Endocrinology* 146:2354–2361
- Fukamachi S, Meyer A (2007) Evolution of receptors for growth hormone and somatolactin in fish and land vertebrates: lessons from the lungfish and sturgeon orthologues. *J Mol Evol* 65:359–372
- Fukamachi S, Shimada A, Shima A (2001) Mutations in the gene encoding B, a novel transporter protein, reduce melanin content in medaka. *Nat Genet* 28:381–385
- Fukamachi S, Sugimoto M, Mitani H et al (2004a) Somatolactin selectively regulates proliferation and morphogenesis of neural-crest derived pigment cells in medaka. *Proc Natl Acad Sci USA* 101:10661–10666
- Fukamachi S, Asakawa S, Wakamatsu Y et al (2004b) Conserved function of medaka pink-eyed dilution in melanin synthesis and its divergent transcriptional regulation in gonads among vertebrates. *Genetics* 168:1519–1527
- Fukamachi S, Wakamatsu Y, Mitani H (2006) Medaka double mutants for *color interfere* and *leucophore free*: characterization of the xanthophore–somatolactin relationship using the *leucophore free* gene. *Dev Genes Evol* 216:152–157
- Fukamachi S, Kinoshita M, Tsujimura T et al (2008) Rescue from oculocutaneous albinism type 4 using medaka *slc45a2* cDNA driven by its own promoter. *Genetics* 178:761–769
- Fukamachi S, Yada T, Meyer A, Kinoshita M (2009) Effects of constitutive expression of somatolactin alpha on skin pigmentation in medaka. *Gene (Amst)* 442:81–87
- Fukamachi S, Kinoshita M, Aizawa K, Oda S, Meyer A, Miani H (2009) Dual control by a single gene of secondary sexual characters and mating preferences in medaka. *BMC Biol* 7:64
- Goda M, Fujii R (1995) Blue chromatophores in two species of callionymid fish. *Zool Sci* 12:811–813
- Hirose E, Matsumoto J (1993) Deficiency of the gene *B* impairs differentiation of melanophores in the medaka fish, *Oryzias latipes*: fine structure studies. *Pigment Cell Res* 6:45–51
- Hyodo-Taguchi Y, Winkler C, Kurihara Y et al (1997) Phenotypic rescue of the albino mutation in the medakafish (*Oryzias latipes*) by a mouse tyrosinase transgene. *Mech Dev* 68:27–35
- Inagaki H, Koga A, Bessho Y et al (1998) The *tyrosinase* gene from medakafish: transgenic expression rescues albino mutation. *Pigment Cell Res* 11:283–290
- Kasahara M, Naruse K, Sasaki S (2007) The medaka draft genome and insights into vertebrate genome evolution. *Nature (Lond)* 447:714–719
- Kawakami K (2007) Tol2: a versatile gene transfer vector in vertebrates. *Genome Biol* 8:S7
- Kelsh RN, Inoue C, Momoi A et al (2004) The Tomita collection of medaka pigmentation mutants as a resource for understanding neural crest cell development. *Mech Dev* 121:841–859
- Koga A, Inagaki H, Bessho Y et al (1995) Insertion of a novel transposable element in the tyrosinase gene is responsible for an albino mutation in the medaka fish, *Oryzias latipes*. *Mol Gen Genet* 249:400–405
- Koga A, Cheah FS, Hamaguchi S et al (2008) Germline transgenesis of zebrafish using the medaka Tol1 transposon system. *Dev Dyn* 237:2466–2474
- Kondo S, Kuwahara Y, Kondo M et al (2001) The medaka *rs-3* locus required for scale development encodes ectodysplasin-A receptor. *Curr Biol* 11:1202–1206
- Loosli F, Winkler S, Burgdorf C et al (2001) Medaka eyeless is the key factor linking retinal determination and eye growth. *Development (Camb)* 128:4035–4044
- Matsumoto J, Akiyama T, Hirose E et al (1992) Expression and transmission of wild-type pigmentation in the skin of transgenic orange-colored variants of medaka (*Oryzias latipes*) bearing the gene for mouse tyrosinase. *Pigment Cell Res* 5:322–327
- Mendel G (1866) Versuche über Pflanzen-Hybriden. *Verh Naturforsch Ver Brünn* 4:3–47

- Messenger JB (2001) Cephalopod chromatophores: neurobiology and natural history. *Biol Rev Camb Philos Soc* 76:473–528
- Morgan TH (1910) Sex limited inheritance in *Drosophila*. *Science* 32:120–122
- Nery LE, Castrucci AM (1997) Pigment cell signalling for physiological color change. *Comp Biochem Physiol A Physiol* 118:1135–1144
- Newton JM, Cohen-Barak O, Hagiwara N et al (2001) Mutations in the human orthologue of the mouse *underwhite* gene (*uw*) underlie a new form of oculocutaneous albinism, OCA4. *Am J Hum Genet* 69:981–988
- Ni-Komatsu L, Orlow SJ (2006) Heterologous expression of tyrosinase recapitulates the misprocessing and mistrafficking in oculocutaneous albinism type 2: effects of altering intracellular pH and pink-eyed dilution gene expression. *Exp Eye Res* 82:519–528
- Ohtsuka M, Kikuchi N, Yokoi H et al (2004) Possible roles of *zic1* and *zic4*, identified within the medaka *Double anal fin* (*Da*) locus, in dorsoventral patterning of the trunk-tail region (related to phenotypes of the *Da* mutant). *Mech Dev* 121:873–882
- Ono M, Takayama Y, Rand-Weaver M et al (1990) cDNA cloning of somatotactin, a pituitary protein related to growth hormone and prolactin. *Proc Natl Acad Sci USA* 87:4330–4334
- Ono H, Hirose E, Miyazaki K et al (1997) Transgenic medaka fish bearing the mouse *tyrosinase* gene: expression and transmission of the transgene following electroporation of the orange-colored variant. *Pigment Cell Res* 10:168–175
- Protas ME, Hersey C, Kochanek D et al (2006) Genetic analysis of cavefish reveals molecular convergence in the evolution of albinism. *Nat Genet* 38:107–111
- Sabeti PC, Varilly P, Fry B et al (2007) Genome-wide detection and characterization of positive selection in human populations. *Nature (Lond)* 449:913–918
- Sauka-Spengler T, Bronner-Fraser M (2008) A gene regulatory network orchestrates neural crest formation. *Nat Rev Mol Cell Biol* 9:557–568
- Shibahara S, Okinaga S, Tomita Y et al (1990) A point mutation in the tyrosinase gene of BALB/c albino mouse causing the cysteine–serine substitution at position 85. *Eur J Biochem* 189:455–461
- Shima A, Shimada A (1991) Development of a possible nonmammalian test system for radiation-induced germ-cell mutagenesis using a fish, the Japanese medaka (*Oryzias latipes*). *Proc Natl Acad Sci USA* 88:2545–2549
- Shimada A, Shima A (2001) High incidence of mosaic mutations induced by irradiating paternal germ cells of the medaka fish, *Oryzias latipes*. *Mutat Res* 495:33–42
- Shimada A, Shima A (2004) Transgenerational genomic instability as revealed by a somatic mutation assay using the medaka fish. *Mutat Res* 552:119–124
- Shimada A, Fukamachi S, Wakamatsu Y et al (2002) Induction and characterization of mutations at the *b* locus of the medaka, *Oryzias latipes*. *Zool Sci* 19:411–417
- Sugimoto M (2002) Morphological color changes in fish: regulation of pigment cell density and morphology. *Microsc Res Tech* 58:496–503
- Sugimoto M, Uchida N, Hatayama M (2000) Apoptosis in skin pigment cells of the medaka, *Oryzias latipes* (Teleostei), during long-term chromatic adaptation: the role of sympathetic innervation. *Cell Tissue Res* 301:205–216
- Sulem P, Gudbjartsson DF, Stacey SN et al (2007) Genetic determinants of hair, eye and skin pigmentation in Europeans. *Nat Genet* 39:1443–1452
- Takeuchi T (1969) A study of genes in the gray medaka, *Oryzias latipes*, in reference to body color. *Biol J Okayama Univ* 15:1–24
- Tomita H (1975) Mutant genes in the medaka. In: Yamamoto T (ed) *Medaka (killifish) biology and strains*. Yagakusha, Tokyo
- Tomita H (1992) The lists of the mutants and strains of the medaka, common gambusia, silver crucian carp, goldfish and golden venus fish maintained in the Laboratory of Freshwater Fish Stocks, Nagoya University. *Fish Biol J Medaka* 4:45–47
- Wada H, Shimada A, Fukamachi S et al (1998) Sex-linked inheritance of the *lf* locus in the medaka fish (*Oryzias latipes*). *Zool Sci* 15:123–126
- Wakamatsu Y, Pristiyazhnyuk S, Kinoshita M et al (2001) The see-through medaka: a fish model that is transparent throughout life. *Proc Natl Acad Sci USA* 98:10046–10050

- Yamamoto T (1969) Inheritance of albinism in the medaka, *Oryzias latipes*, with special reference to gene interaction. *Genetics* 62:797–809
- Yamamoto T (ed) (1975) *Medaka (killifish) biology and strains*. Keigaku, Tokyo
- Yamamoto T, Oikawa T (1973) Linkage between albino gene (i) and color interferer (ci) in the medaka, *Oryzias latipes*. *Jpn J Genet* 48:315–329
- Yu JF, Fukamachi S, Mitani H et al (2006) Reduced expression of vps11 causes less pigmentation in medaka, *Oryzias latipes*. *Pigment Cell Res* 19:628–634
- Zhu Y, Stiller JW, Shaner MP et al (2004) Cloning of somatolactin alpha and beta cDNAs in zebrafish and phylogenetic analysis of two distinct somatolactin subtypes in fish. *J Endocrinol* 182:509–518

Chapter 12

Craniofacial Traits

Minori Shinya

Abstract Every species exhibits distinctive craniofacial morphologies; thus, these features have been traditionally used as one of the basic species classificatory criteria. Human beings, for example, distinguish individuals by their facial appearance, implying that there are also some individual differences in craniofacial morphology within the species *Homo sapiens*. Craniofacial morphology is a complex but interesting trait that reveals morphological variety within a species while maintaining the particular morphology of the species. Although common features are main topics of study in developmental biology, the diversity or polymorphisms are essential factors for consideration with regard to evolution. It is also simply interesting to contemplate how differences between individuals within a species are generated during developmental or life processes. Recently, genetic backgrounds responsible for such variations have been analyzed using quantitative trait loci (QTL) analysis methods in which phenotypes are treated as quantitative traits. In this chapter, we focus on the craniofacial morphology of the medaka and introduce our approach for the genetic dissection of the underlying mechanisms that generate morphological diversity in the medaka head region.

12.1 Introduction

When your relatives or friends show you their newborn baby, you bless the baby and then, perhaps, comment on the baby's looks, and in particular with respect to the baby's facial appearance; for example, "She is beautiful and takes after her father!" We take it for granted that a child takes after his or her parents and that siblings resemble each other. On the other hand, we would be shocked if the facial appear of an unrelated person was similar to our own. These behaviors attest to a kind of common

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knowledge empirical validity of the important role of heredity in determining one's craniofacial morphology. At the same time, we realize that environmental factors also affect morphological appearance, based on observations that even monozygotic twins look somewhat different, and that these differences become more prominent with age. Multiple genetic factors and environmental variables account for the large degree of variability in human craniofacial morphology. Logical questions have been posed to address variations in cranial morphology, such as the extent to which a genetic component influences to craniofacial morphology, how these traits are inherited, and the identity of these genetic regulatory factors.

To address these questions, genetic studies have been performed on the inheritance of human facial characteristics in twins, siblings, or between parents and their offspring. Many of these studies have reported relatively high heritability estimate values for most facial measurements. An example is presented in Fig. 12.1. Baydas et al. (2007) studied sibling pairs and estimated a high heritability, 0.71, for upper-to-lower facial height index (nasion–anterior nasal spine distance divided by ans–menton distance). Two other studies involving twins (Savoye et al. 1998) and parents and their offspring (Johannsdottir et al. 2005) also supported a high genetic component for this facial index.

Unfortunately, further analysis of human linkages is difficult, in part because of sample heterogeneity, limited sample numbers, and a significant impact of environmental factors on craniofacial phenotypes. To control for these effects, animal models are often used to genetically dissect the developmental pathways that operate in concert to govern multifactorial traits. Craniofacial skeletons, especially jaw apparatuses, have been genetically analyzed in mice and east African cichlid fishes. In mice, inbred strains and recombinant inbred strains have been used to identify chromosomal locations with significant effects on mandibular shape and size and other craniofacial measurements (Cheverud et al. 1997; Dohmoto et al. 2002; Klingenberg et al. 2001, 2004; Nishimura et al. 2003). In cichlids, linkage analyses were performed using F_2 hybrid progeny between two closely related Lake Malawi species.

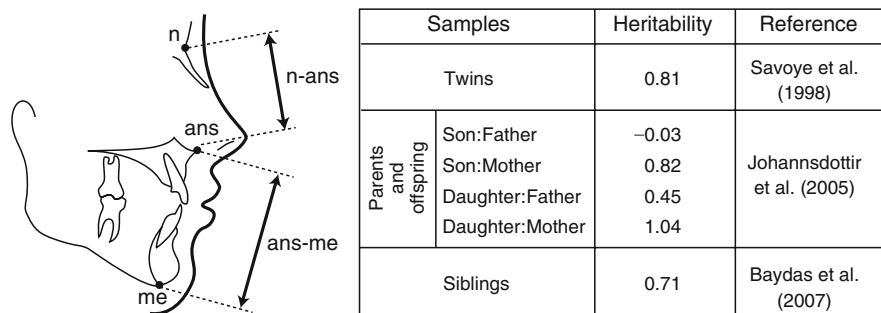


Fig. 12.1 Heritability of human craniofacial traits. *Left figure*: lateral cephalometric landmarks and an example trait measurement. Upper-to-lower facial height index is defined as the nasion–anterior nasal spine distance divided by ans–menton distance. *Right figure*: heritability estimated in three previous studies. *ans*, anterior nasal spine; *me*, menton; *n*, nasion

These analyses identified several regions of the cichlid genome responsible for functionally important shape differences in the oral jaw apparatus (Albertson et al. 2003). However, there are either few genetic studies on gross craniofacial morphology, and very few genes have been identified as genetic factors for craniofacial traits.

The medaka fish is suitable for genetic studies of both Mendelian and multifactorial traits because the species has a short life cycle and is very fertile. In addition, several inbred strains are available (reviewed in Naruse et al. 2004a). Inbred strains of medaka differ in terms of various traits, including body shape, behavior, and susceptibility to chemical- or radiation-induced tumorigenesis (Hyodo-Taguchi 1990). Furthermore, gross brain morphology was shown to vary greatly between five inbred strains with different genotypes (Ishikawa et al. 1999). The availability of a dense and accurate genetic linkage map (Naruse et al. 2004b; Kimura et al. 2005; see Chap. 2), in combination with a number of inbred strains that display a variety of diverse phenotypes, facilitates the identification of the genetic loci responsible for specific complex traits in medaka fish and possibly for craniofacial morphology.

12.2 Craniofacial Morphology in Medaka

Examples of the medaka craniofacial structure are presented in Fig. 12.2a–c. One immediately obvious feature is that the eyes are large relative to the overall body size. Conversely, the oral apparatus is rather small and superior, opening dorsally

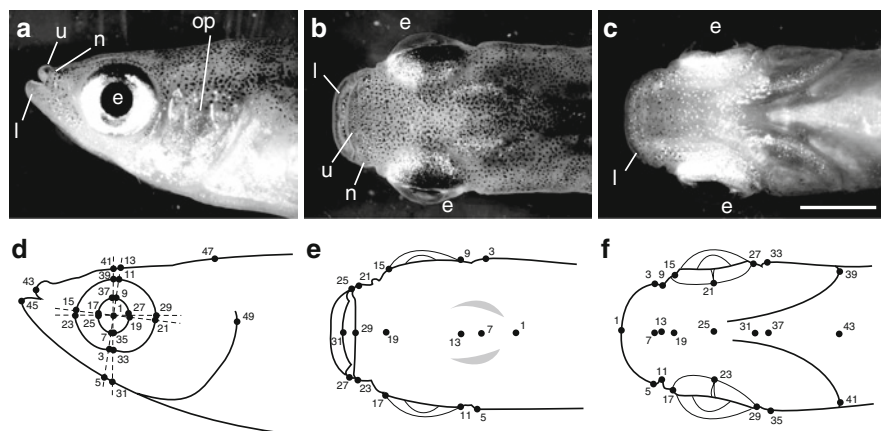


Fig. 12.2 Craniofacial morphology of the medaka. **a–c** Digital images of the medaka head region in the lateral (**a**), dorsal (**b**), and ventral (**c**) views. **d–f** Landmarks in the schematic representations of medaka head region in the lateral (**d**), dorsal (**e**), and ventral (**f**) views. Dots indicate odd-numbered landmarks, which were denoted as such for convenience. *e*, eye; *l*, lower jaw; *n*, nose; *op*, operculum; *u*, upper jaw. Bar 3 mm

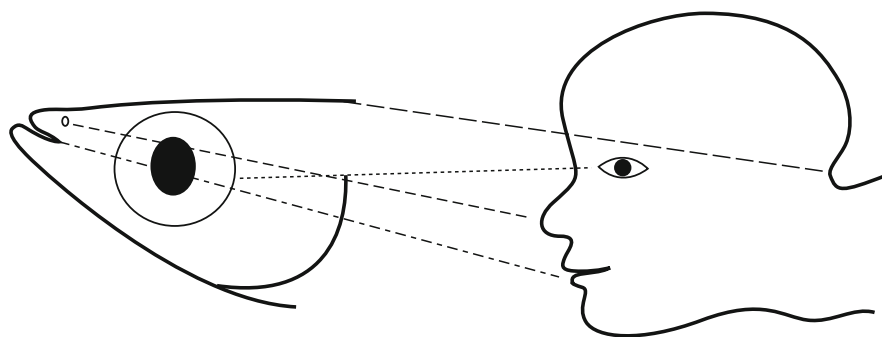


Fig. 12.3 Comparison of the facial feature arrangement between medaka (*left*) and human (*right*). Lines represent corresponding parts of the head region

as the lower jaw protrudes towards the dorsal (Fig. 12.2a). The snout length is also relatively short in the medaka. The anterior edge of the lower jaw corresponds to the anterior edge of the head region, and the nostrils are located in front of each eye, just behind the mouth (Fig. 12.2b). Laterally, the region behind the eye is called the operculum. Comparing the location of the major facial parts in the head region (Fig. 12.3), we can relate the medaka facial arrangement as if a human face had been stretched dorsally and anteriorly by moving the mouth up.

Are there any morphological variations in the craniofacial structures of medaka? This is a quite primitive but important question to address if medaka are to be used as a model animal for the genetic study of craniofacial morphology. In short, the answer is “Yes.” There are more than a hundred closed colonies of natural populations in medaka that have been collected from all over Japan and maintained for many generations in Japanese research laboratories. When some of the colonies were compared, a number of colony-specific, that is, region-specific, craniofacial morphologies were apparent (Fig. 12.4). A colony from Hagi exhibits an upper jaw at a more ventral position and a shorter snout than is present in many other colonies and inbred lines. An Ohdate colony looks somewhat like Hagi, but the anterior tip of the upper jaw tends to point more dorsally and Ohdate eyes are located more ventrally than those of Hagi medaka. These observations tell us that, similar to what is observed in humans, there is indeed diversity in the craniofacial morphology of the medaka.

12.3 Genetic Analysis of Quantitative Traits

Craniofacial morphology reveals continuous phenotypic variation within a population. Such traits are referred to as “quantitative traits” and include those traits that are distinguishable from qualitative traits, which involve discrete distributions. The phenotype of a quantitative trait is typically represented by a numerical value. It is generally thought that quantitative traits are usually governed by several or multiple genes and environmental factors, whereas many qualitative traits follow the

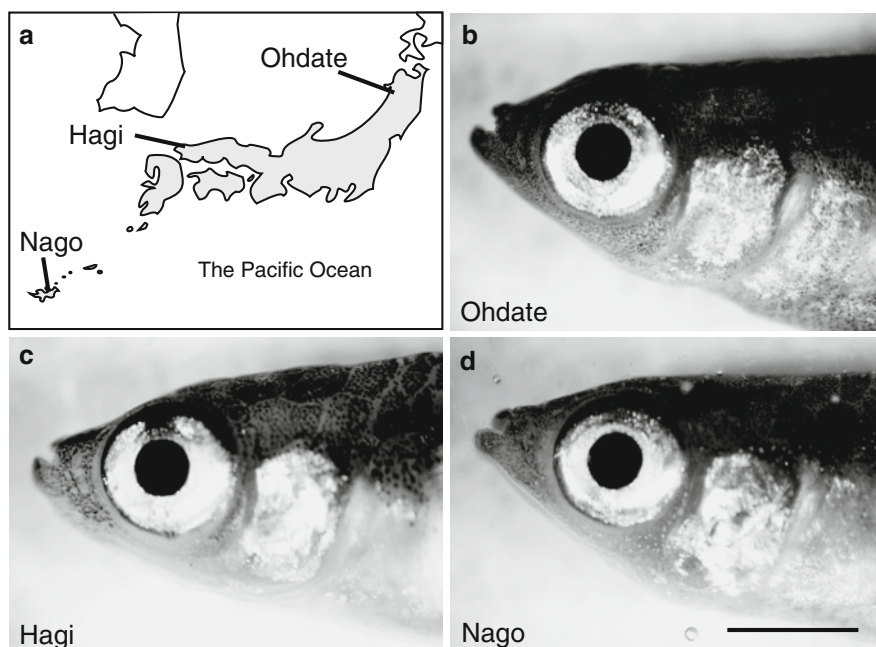


Fig. 12.4 Diversity in the craniofacial morphology of the medaka. **a** A regional map around Japan, showing Nago in Okinawa, Hagi in Yamaguchi, and Ohdate in Akita Prefectures. **b–d** Images of the medaka closed colonies that originated from Ohdate (**b**), Hagi (**c**), and Nago (**d**). Colony-specific morphological features are apparent. Bar 3 mm

Mendelian law of segregation. Monogenic traits provided the early and encouraging successes in the association of a particular genotype with a phenotype in the human, and in domesticated animals and plant populations. In medaka, great progress has been made on the identification of the specific genes that are responsible for monogenic mutants.

In contrast, defining the genetic basis for quantitative traits has proved to be more challenging because of the following three points. First, quantitative traits are influenced by environmental factors, so that phenotypic values differ even between individuals with an identical genetic background as occurs with clones. Second, because phenotypic values are continuous, we cannot determine the genotype of a trait from its phenotype. Finally, it is difficult to detect the effects of a single candidate gene on a quantitative trait, because not only are multiple genes involved, but also interactions between gene products affect the phenotype.

However, the recent accumulation of knowledge and technology has opened up new avenues for the mapping and positional cloning of quantitative traits. A large number of genetic markers have now been established, making it feasible to rapidly map any given trait in medaka (Kimura et al. 2005; Naruse et al. 2004b). From the analytical aspect, various statistical tools for quantitative trait loci (QTL) mapping have been reported, including interval mapping, composite interval mapping, and

multiple trait mapping (Isobe et al. 2007; Kao et al. 1999; Lander et al. 1987; Zeng 1993, 1994). Genomic sequencing has either been completed or is in progress for many species, including medaka (Kasahara et al. 2007). Based on existing sequence data, a large number of the single nucleotide polymorphisms (SNPs) have been identified, which provide an extremely dense genetic map. These SNPs have facilitated the development of new genome-based methods, such as linkage disequilibrium analysis (Hong and Julius 2007; Pletcher et al. 2004). In summary, several new strategies using new statistical and bioinformatic techniques have been reported for identification of the causative underlying quantitative traits (Burgess-Herbert et al. 2008; Flaherty et al. 2005).

There are two key considerations that impact the successful genetic analysis of craniofacial morphology. The first is the precision of phenotype quantification. This aspect has been greatly improved through the development of new techniques and equipment for measurements. The second key consideration is the selection of a proper model animal for analysis. Domesticated animal models enable us to control environmental influences such that the variance contributed to environmental factors can be made as small as possible. In general, a large sample of individuals must be collected to represent a total population, provide an observable number of recombinants, and allow a thorough assessment of the trait under investigation. Therefore, an ideal model animal would be prolific with a short generation time. The availability of inbred lines of a model animal also has important advantages because of the uniform genetic background in a strain. The use of inbred lines allows accurate QTL mapping, or the deliberate crossing of inbred lines to construct the more specialized resources used in linkage studies. These resources include congenic strains, consomics, and recombinant inbred lines. Medaka meets all these requirements, indicating that it is an excellent model animal for the statistical genetic analysis of complex traits.

12.4 Quantification Method

Conducting genetic studies on craniofacial morphology requires quantification of the phenotype. As the first step toward quantifying shape, it must be decided whether shape will be characterized in three dimensions or in two dimensions. The craniofacial region of the medaka is strongly curved both anteroposteriorly and mediolaterally, making it highly three dimensional. Therefore, it is more precise to quantify the medaka morphology in three dimensions. On the other hand, as it is difficult or time-consuming to survey a small model animal such as the medaka under a microscope, it is more convenient and efficient to make measurements using two-dimensional images of the samples. Nowadays, several challenging systems are under development, for example, computed tomography (CT) scan for small model animals, and an efficient system for reconstructing a three-dimensional structure from serial continuous slices of an embedded sample. This new technology is promising for future quantification of craniofacial morphology in three dimensions.

Whichever dimensions are chosen, the simplest and rather classical measurements for shape are a set of linear distances, relative sizes of specific features or elements, or specific ratios of two distances (Cheverud et al. 1997; Dohmoto et al. 2002; Leamy et al. 1997; Nishimura et al. 2003; Sherwood et al. 2008). There are also statistical methods with which a principal component analysis is performed on linear distances or landmark axes (Mezey et al. 2005). Furthermore, geometric morphometrics is an effective method for measuring differences in morphological traits. This technique enables us to separate shape from size and to handle only the element of shape (Zelditch et al. 2004). This separation is achieved by introduction of a coherent mathematical theory of shape, which requires articulating a precise definition of the concept. Zelditch et al. (2004) have described several software and other resources for geometric morphometrics studies. Successful genetic analyses of morphological traits using this method have also been reported (Albertson et al. 2003; Klingenberg et al. 2001, 2004).

In the analysis introduced in this chapter, craniofacial morphology in the medaka was quantified by one of the classical methods: ratios of linear distances between two landmarks. This measurement focuses on the proportions of a shape, independent from absolute size. Four-month-old adult medaka were used in the analysis. Lateral, dorsal, and ventral head images of each medaka (see Fig. 12.2a–c) were captured under a stereo microscope using a CCD camera. Craniofacial landmarks were identified on each image as shown in Fig. 12.2d–f. The linear distances between landmark combinations were calculated from the coordinates of craniofacial landmarks. Ratios of different craniofacial distances were then calculated to generate phenotypic values. In total, 444 traits were quantified: 158 lateral view traits, 157 dorsal view traits, and 129 ventral view traits. With this method, the calculated value is affected directly by the landmarks used. Thus, when one of the landmarks varies greatly, the variation of the ratio will largely depend on the landmark. Although there is potential weakness in the selected measurement and no guarantee that variations in shape are captured completely, ratios are still used by many researchers, as the method is simple and enables the direct quantification of differences that are identified through careful observations.

12.5 Diversity and Inheritance of Craniofacial Traits in Medaka

Figure 12.5 shows the distributions of some craniofacial traits in the inbred medaka strains HNI and Hd-rR, established from a northern Japanese population and a southern Japanese population, respectively. These examples demonstrate the variances in each inbred population. As individuals of an inbred line are (almost) homozygous and are governed by the same genetics components, a phenotypic variance observed in an inbred line is a variance generated by environment (called the environmental variance). What we do next for the genetic analyses of traits is the comparison of distributions between the HNI and Hd-rR strains. Figure 12.5a shows

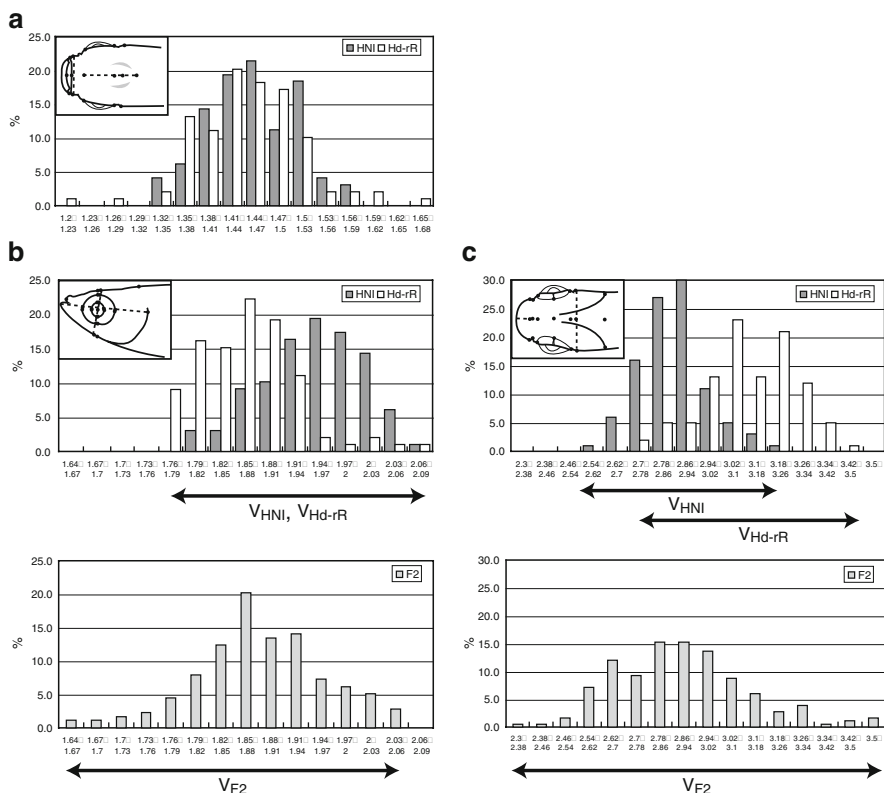


Fig. 12.5 Distribution of craniofacial traits in medaka. **a** A histogram of D67 (points 1–19/21–23) in HNI and Hd-rR strains. **b** Histograms of L33 (points 45–49/5–13) in HNI and Hd-rR strains (upper) and an F_2 population (lower). **c** Distribution of V15 (points 33–35/1–19) in HNI and Hd-rR strains (upper) and an F_2 population (lower). Black, white, and gray bars indicate data from HNI ($n=100$), Hd-rR ($n=100$), and F_2 ($n=184$) populations, respectively. Arrows indicate variances. V_{HNI} , variance in the HNI population; $V_{\text{Hd-rR}}$, variance in the Hd-rR population; V_{F_2} , variance in the F_2 population

the distributions of a trait named D67 (points 1–19/21–23, representing a ratio of the length between points 1 and 19 to the length between points 21 and 23) that reflects the ratio of the length between the anterior edge of eyes and the end of the head region to the width between the noses. Although these two medaka populations are known to differ genetically (Kasahara et al. 2007; Sakaizumi et al. 1983; Takehana et al. 2003), the histogram of D67 for HNI population overlaps almost completely with that of Hd-rR. Phenotypic differences owing to genetic factors were not detected for the D67 trait. In contrast, the lateral trait L33 (points 45–49/5–13) and ventral trait V15 (points 33–35/1–19) exhibited separate distributions in the two populations (Fig. 12.5b,c, upper panels). These differences were statistically significant. For L33, which represents a proportion of the head length to height at the eye

level, HNI showed a larger value than Hd-rR. V15, the ratio of the head width to snout length, exhibited a clear difference between the lines, and was smaller in HNI than in Hd-rR. These two medaka lines were grown under controlled laboratory conditions that were kept as nearly identical as possible. Therefore, we could interpret that the phenotypic differences observed in L33 and V15 reflect the genetic differences between HNI and Hd-rR inbred lines. In other words, some of the differences in genetic factors between the two lines govern the phenotypic differences found in traits L33 and V15.

In our analysis, a total of 444 traits were quantified in 100 individuals (50 males and 50 females) from each of the inbred strains, and were examined for phenotypic differences using Student's *t* tests. Of the 140 gender-independent traits, 125 (89.3%) differed significantly between these two strains, whereas 254 of the 304 gender-dependent traits (83.6%) differed in either male or female medaka (Kimura et al. 2007). Of these traits, 145 (47.7%) differed in both sexes, 59 (19.4%) differed only in males, and 50 (16.4%) differed only in females. Thus, our phenotypic survey shows that 379 of 444 traits differ significantly between HNI and Hd-rR. From these results, we now understand that, at least to a certain extent, medaka craniofacial phenotypes are controlled genetically and are highly heritable.

The next question to be addressed was the degree to which genetic factors influence the diversity of morphological traits. This degree is represented as “heritability.” In quantitative genetics, the phenotypic variance is considered as the sum of environmental variance and genetic variance (the variance owing to genetic factors). Broad-sense heritability is defined as a ratio of the genetic variance to the total variance (phenotypic variance). This heritability can be estimated using the variance in an F_1 population generated from the cross of two inbred lines or in their parental lines and the variance in an F_2 population obtained by crossing the F_1 individuals. As there is no genetic variance in each of the F_1 and parental populations, the variances observed in those populations are equal to the environmental variance. The variance in an F_2 population is usually larger than that observed in the F_1 and parental populations (for examples, see Fig. 12.5b, c, lower panels), as it consists of both the environmental and the genetic variances. Considering the variance in an F_2 population as the phenotypic variance, the genetic variance is represented by

$$\text{Genetic variance} = \text{variance in } F_2 - \text{variance in } F_1 \text{ or their parental lines.}$$

Thus, the heritability can be calculated by dividing the above genetic variance by the variance in F_2 . To estimate the heritability in our study, the craniofacial traits that differed significantly between strains HNI and Hd-rR were further quantified in 50 F_1 and 184 F_2 progeny. Many of the craniofacial traits were highly heritable. The greatest heritability for a lateral view trait was 0.768 for L37 (points 13–47/13–43), for a dorsal view trait was 0.870 for D34 (points 21–23/19–31), and for a ventral view trait was 0.893 for both V1 (points 1–43/1–19) and V4 (points 19–43/1–19). A high degree of heritability was evident in many of the lateral view traits, with 67.6% of traits showing a heritability value greater than 0.5. Only 43.2% (38 of 88 traits) of the dorsal and ventral view traits showed such a high degree of heritability.

12.6 Identification of QTLs

In the foregoing sections, we have described the establishment of diversity resulting from genetic factors in the craniofacial morphology of the medaka. So, what are the genetic factors involved in establishing craniofacial diversity? Which alleles are involved and how do these alleles of the polymorphism affect the observed morphological variations? To gain insight into these questions, the trait loci need to be initially mapped to particular chromosomal regions. Interval mapping (Lander et al. 1987) is one of the commonly used QTL mapping methods, in which the individual genotype of a trait is estimated statistically based on its phenotypic value and genotypes of genetic markers, with associations detected between the trait and particular genomic regions. Here, we utilized this statistical method to map craniofacial traits that exhibited strain differences independent of gender. Because the interval mapping method assumes a normal distribution for a given trait, traits subjected for the analysis were further selected based on their distribution in the F_2 population. After selection, the final list of analyzed traits included 31 of the lateral view traits, 35 of the dorsal view traits, and 23 of the ventral view traits. The 184 F_2 progeny were genotyped with 304 genetic markers covering the entire medaka genome with a 5.9-centimorgan (cM) interval on average. Taken together with the genotype and phenotype data, QTL analysis was performed using MAPMAKER/QTL 1.1, a program for computed interval mapping. The significance threshold of the logarithm of the odds (LOD) score was 2.8, based on the average intermarker distance in this study and the size of the medaka genome.

A total of 66 of 89 traits (74.2%) were successfully localized in the medaka genome: 17 lateral view traits (17/31; 54.8%), 34 dorsal view traits (34/35; 97.1%), and 15 ventral view traits (15/23; 65.2%). Eight traits were mapped with maximum LOD scores of 5.0 or greater. There were three lateral view traits: L23 (points 41–43/33–39), L29 (points 43–49/5–13), and L33; three dorsal view traits: D13 (points 19–31/29–31), D14 (points 19–29/29–31), and D29 (points 1–31/21–23); and two ventral view traits: V14 (points 27–29/1–19) and V15. Table 12.1 shows each mapped position, maximum LOD score, the percentage of the phenotypic variance explained by a QTL (%Var), and the phenotype analysis of the three genotypes of the nearest marker for each trait. L33 was mapped to LG9 with the highest LOD score of 6.2. LOD scores were highest in the middle part of the chromosome (Fig. 12.6a). Phenotype analysis revealed that the ratio of head length to head height was greater in homozygotes with the HNI allele near the given QTL. The lack of a significant difference in a trait between Hd-rR homozygotes and heterozygotes indicated that the Hd-rR allele was dominant at this locus. Because the HNI strain showed a greater ratio than did the Hd-rR strain (see Fig. 12.5b), the QTL in LG9 is expected to function positively for the trait L33. The other trait with the highest LOD score was V15. V15 mapped to the distal part of LG11 (Fig. 12.6b). Fish homozygous for HNI alleles near the mapped locus had the largest head width-to-snout length ratio; those homozygous for Hd-rR alleles had the shortest proportion. Heterozygotes were intermediate, suggesting either no or limited dominance

Table 12.1 Traits mapped with a logarithm of the odds (LOD) ≥ 5 for each of the three genotypes of the nearest marker to the quantitative trait loci

Trait	LG	Nearest marker	LOD	N/N ^b		N/R ^c		R/R ^d		<i>P</i> ^e
				Mean $\pm$ SD	% Var ^a	Mean $\pm$ SD		Mean $\pm$ SD		
L23	14	MM04G02K	5.2	1.018 $\pm$ 0.056	13.0	1.040 $\pm$ 0.067		1.078 $\pm$ 0.051 ^{fg}		0.0000078
L29	9	MF01SSA025F06	5.4	1.827 $\pm$ 0.066	13.1	1.763 $\pm$ 0.067 ^f		1.784 $\pm$ 0.077 ^f		0.0000054
L33	9	MF01SSA025F06	6.2	1.931 $\pm$ 0.068	15.9	1.862 $\pm$ 0.073 ^f		1.873 $\pm$ 0.075 ^f		0.0000013
D13	12	MF01SSA068F11	5.3	2.48 $\pm$ 0.42	12.6	2.31 $\pm$ 0.46		2.04 $\pm$ 0.35 ^{fg}		0.0000061
D14	12	MF01SSA068F11	5.3	1.48 $\pm$ 0.42	12.6	1.31 $\pm$ 0.46		1.04 $\pm$ 0.35 ^{fg}		0.0000060
D29	22	OLa3003a	5.0	1.945 $\pm$ 0.068	12.5	1.987 $\pm$ 0.077 ^f		2.023 $\pm$ 0.072 ^{fg}		0.0000016
V14	11	MF01SSA045F05	6.0	2.92 $\pm$ 0.21	15.8	2.78 $\pm$ 0.18 ^f		2.70 $\pm$ 0.21 ^f		0.0000014
V15	11	MF01SSA045F05	6.2	3.00 $\pm$ 0.22	16.2	2.87 $\pm$ 0.18 ^f		2.78 $\pm$ 0.20 ^{fg}		0.0000010

^aPercentage of the phenotypic variance that can be explained by the quantitative trait loci (QTL)

^bHomozygous for the HNI allele

^cHeterozygous for HNI and Hd-rR alleles

^dHomozygous for the Hd-rR allele

^e*P* value of one-way analysis of variance

^fSignificantly different from N/N mean (*P* < 0.05)

^gSignificantly different from N/R mean (*P* < 0.05)

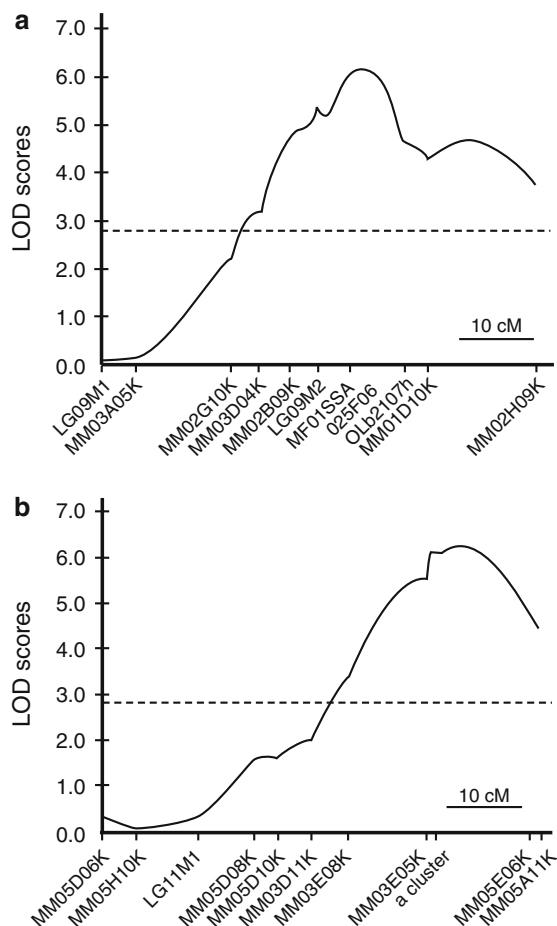


Fig. 12.6 Results of quantitative trait loci (QTL) mapping. **a** Plot of the logarithm of the odds (LOD) scores on LG9 for L33. **b** Plot of the LOD scores on LG11 for V15. *x*-axis, position of LG; *y*-axis, LOD score. Dotted lines indicate the location of 2.8 along the *y*-axis, the threshold for LOD score significance

between the alleles. This locus seems to affect the trait negatively, as the HNI strain, but not the Hd-rR strain, had a shorter head width-to-snout length ratio (see Fig. 12.5c). The value of %Var reflects the potential ease of identification of a gene responsible for the trait variation. Generally speaking, the higher the %Var, the easier the positional cloning. Unfortunately, we did not identify any traits with an extremely high phenotypic variance that might account for the total variance: the highest was 16.2% in V15.

So far as we know, this is the first demonstration that QTLs can be identified in medaka by genetic analysis of the F_2 progeny. A number of studies have analyzed morphological phenotypes as quantitative traits in mice (Cheverud et al. 1997;

Dohmoto et al. 2002; Nishimura et al. 2003; Lang et al. 2005). The LOD scores in their reports were approximately 5.0 using more than 100 mice (Dohmoto et al. 2002; Nishimura et al. 2003), and 7.3, at the highest, by examining 200 F_2 mice (Lang et al. 2005). The ability to identify QTLs is affected by a sample size, with an increase in sample size resulting in an increase in the probability of finding QTLs with high LOD scores. We analyzed 184 F_2 samples in this study, and mapped, at most, five genomic regions per trait. The highest LOD score in our study was 6.2, just slightly lower than the LOD score of 7.3 obtained in the study of 200 F_2 mice (Lang et al. 2005). Thus, the medaka are an alternative animal model for the genetic analysis of morphological traits, with the benefits of being less expensive and easier to prepare a large number of samples. We hope that many researchers will initiate research in this interesting and progressing field using medaka as a model system.

12.7 Future Prospects

The next step in the identification of genes responsible for medaka craniofacial traits is to narrow the critical region (fine mapping). Congenic strains and subcongenics derived from congenic strains are powerful tools for fine mapping. However, the traditional congenic breeding strategies will take a few years to complete in medaka. Analysis of a great number of QTLs is currently too time consuming an endeavor. Therefore, we are now constructing a rapid system for generation of congenic strains that combines the speed-congenic method reported in mice (Markel et al. 1997) and the sperm freezing technique used in medaka. This system will generate a congenic strain in approximately 1 year, which should be tremendously useful for future genetic analyses of quantitative traits.

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References

- Albertson RC, Streelman JT, Kocher TD (2003) Directional selection has shaped the oral jaws of Lake Malawi cichlid fishes. *Proc Natl Acad Sci USA* 100:5252–5257
- Baydas B, Erdem A, Yavuz I et al (2007) Heritability of facial proportions and soft-tissue profile characteristics in Turkish Anatolian siblings. *Am J Orthod Dentofacial Orthop* 131:504–509
- Burgess-Herbert SL, Cox A, Tsaih SW et al (2008) Practical applications of the bioinformatics toolbox for narrowing quantitative trait loci. *Genetics* 180:2227–2235

- Cheverud JM, Routman EJ, Irschick DJ (1997) Pleiotropic effects of individual gene loci on mandibular morphology. *Evolution* 51:2006–2016
- Dohmoto A, Shimizu K, Asada Y et al (2002) Quantitative trait loci on chromosomes 10 and 11 influencing mandible size of SMXA RI mouse strains. *J Dent Res* 81:501–504
- Flaherty L, Herron B, Symula D (2005) Genomics of the future: identification of quantitative trait loci in the mouse. *Genome Res* 15:1741–1745
- Hong LS, Julius W (2007) Fine mapping of multiple interacting quantitative trait loci using combined linkage disequilibrium and linkage information. *J Zhejiang Univ Sci B* 8:787–791
- Hyodo-Taguchi Y (1990) Inbred strains of the medaka and their characteristics. In: Egami N, Yamagami K, Shima A (eds) *Biology of the medaka*. Tokyo University Press, Tokyo
- Ishikawa Y, Yoshimoto M, Yamamoto N et al (1999) Different brain morphologies from different genotypes in a single teleost species, the medaka (*Oryzias latipes*). *Brain Behav Evol* 53:2–9
- Isobe S, Nakaya A, Tabata S (2007) Genotype matrix mapping: searching for quantitative trait loci interactions in genetic variation in complex traits. *DNA Res* 14:217–225
- Johannsdottir B, Thorarinsson F, Thordarson A et al (2005) Heritability of craniofacial characteristics between parents and offspring estimated from lateral cephalograms. *Am J Orthod Dentofacial Orthop* 127:200–207
- Kao CH, Zeng ZB, Teasdale RD (1999) Multiple interval mapping for quantitative trait loci. *Genetics* 152:1203–1216
- Kasahara M, Naruse K, Sasaki S et al (2007) The medaka draft genome and insights into vertebrate genome evolution. *Nature (Lond)* 447:714–719
- Kimura T, Yoshida K, Shimada A et al (2005) Genetic linkage map of medaka with polymerase chain reaction length polymorphisms. *Gene (Amst)* 363:24–31
- Kimura T, Shimada A, Sakai N et al (2007) Genetic analysis of craniofacial traits in the medaka. *Genetics* 177:2379–2388
- Klingenberg CP, Leamy LJ, Routman EJ et al (2001) Genetic architecture of mandible shape in mice: effects of quantitative trait loci analyzed by geometric morphometrics. *Genetics* 157:785–802
- Klingenberg CP, Leamy LJ, Cheverud JM (2004) Integration and modularity of quantitative trait locus effects on geometric shape in the mouse mandible. *Genetics* 166:1909–1921
- Lander ES, Green P, Abrahamson J et al (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* 1:174–181
- Lang DH, Sharkey NA, Mack HA et al (2005) Quantitative trait loci analysis of structural and material skeletal phenotypes in C57BL/6J and DBA/2 second-generation and recombinant inbred mice. *J Bone Miner Res* 20:88–99
- Leamy LJ, Routman EJ, Cheverud JM (1997) A search for quantitative trait loci affecting asymmetry of mandibular characters in mice. *Evolution* 51:957–969
- Markel P, Shu P, Ebeling C et al (1997) Theoretical and empirical issues for marker-assisted breeding of congenic mouse strains. *Nat Genet* 17:280–284
- Mezey JG, Houle D, Nuzhdin SV (2005) Naturally segregating quantitative trait loci affecting wing shape of *Drosophila melanogaster*. *Genetics* 169:2101–2113
- Naruse K, Hori H, Shimizu N et al (2004a) Medaka genomics: a bridge between mutant phenotype and gene function. *Mech Dev* 121:619–628
- Naruse K, Tanaka M, Mita K et al (2004b) A medaka gene map: the trace of ancestral vertebrate proto-chromosomes revealed by comparative gene mapping. *Genome Res* 14:820–828
- Nishimura I, Drake TA, Lusis AJ et al (2003) ENU large-scale mutagenesis and quantitative trait linkage (QTL) analysis in mice: novel technologies for searching polygenic determinants of craniofacial abnormalities. *Crit Rev Oral Biol Med* 14:320–330
- Pletcher MT, McClurg P, Batalov S et al (2004) Use of a dense single nucleotide polymorphism map for in silico mapping in the mouse. *PLoS Biol* 2:e393
- Sakaizumi M, Moriwaki K, Egami N (1983) Allozymic variation and regional differentiation in wild populations of the fish, *Oryzias latipes*. *Copeia* 1983:311–318

- Savoye I, Loos R, Carels C et al (1998) A genetic study of anteroposterior and vertical facial proportions using model-fitting. *Angle Orthod* 68:467–470
- Sherwood RJ, Duren DL, Havill LM et al (2008) A genomewide linkage scan for quantitative trait loci influencing the craniofacial complex in baboons (*Papio hamadryas* spp.). *Genetics* 180:619–628
- Takehana Y, Nagai N, Matsuda M et al (2003) Geographic variation and diversity of the cytochrome b gene in Japanese wild populations of medaka, *Oryzias latipes*. *Zool Sci* 20:1279–1291
- Zelditch ML, Swiderski DL, Sheets HD et al (2004) Geometric morphometrics for biologists: a primer. Elsevier Academic, London
- Zeng ZB (1993) Theoretical basis for separation of multiple linked gene effects in mapping quantitative trait loci. *Proc Natl Acad Sci USA* 90:10972–10976
- Zeng ZB (1994) Precision mapping of quantitative trait loci. *Genetics* 136:1457–1468

Chapter 13

Double Anal Fin (Da): A Medaka Mutant Exhibiting a Mirror-Image Pattern Duplication of the Dorsal–Ventral Axis

Masato Ohtsuka, Hiroyuki Takeda, and Atsuko Shimada

Abstract A medaka *Double anal fin (Da)* mutant, spontaneously isolated from the wild-type, exhibits a unique ventralizing phenotype consisting of a mirror-image duplication across the lateral midline in the dorsal trunk–tail region. No similar mutation has been reported in other model organisms. Hence, the *Da* mutant provides a unique model system for investigation of the mechanisms controlling the overall dorsal–ventral patterning of the vertebrate trunk–tail region. In this chapter, we introduce (1) a brief history of the *Da* mutant; (2) details of the morphological phenotype of *Da*; (3) positional cloning of the *Da* mutation; (4) the *Da* mutant in relation to fish evolution; and (5) *Da* as a potential model for human disease.

13.1 History of the *Da* Mutant

The *Double anal fin (Da)* is a medaka mutant in which the dorsal half of the tail shows a mirror-image phenotype of the ventral half across the lateral midline (Fig. 13.1). *Da* was originally isolated from wild medaka progeny collected at Toyokawa, Aichi Prefecture, Japan, in 1966 (Tomita 1975) and harbors an autosomal semidominant mutation that arose spontaneously. The homozygote mutant fish mature, behave, and reproduce normally.

Since its discovery, phenotypic and genetic analyses of *Da* have been conducted. Initially however, only the external appearance and some genetic features such as the correlation between genotype and the number of dorsal fin rays were described.

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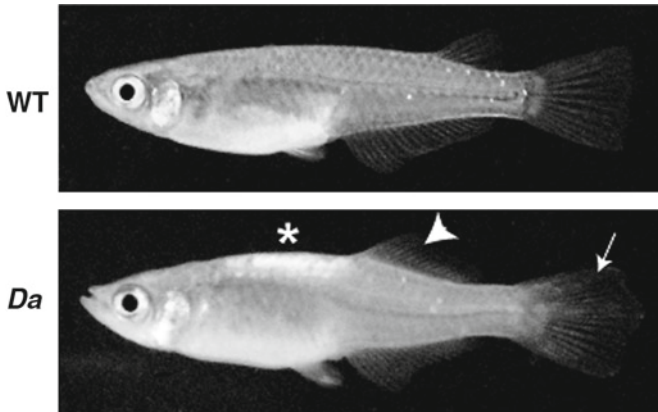


Fig. 13.1 Adult wild-type (WT, *upper*) and *Da* mutant (*lower*) medaka. The dorsal half of the *Da* trunk and tail region shows a mirror-image phenotype of the ventral half across the lateral midline. The *Da* dorsal fin (*arrowhead*) is similar in shape to its anal fin, the caudal fin exhibits a rhombic shape (*arrow*), and the iridophores are abundant at the dorsal (*asterisk*) and ventral sides of the trunk region

In 1990, Ishikawa published a detailed morphological study on the internal structures of *Da*, including the muscles and skeleton of the caudal region (Ishikawa 1990). Tamiya et al. have also examined the melanocytes, the dorsal fin hold, and dorsal myotomes in the tail at various stages in the *Da* embryo (Tamiya et al. 1997). Because of its dramatic and unique phenotype, *Da* has been regarded as a good model for the study of vertebrate dorsal–ventral patterning. In the 1990s, however, there were no reports on the underlying molecular mechanisms resulting in the unique *Da* phenotype, and a candidate gene was difficult to predict from known genes. In addition, no zebrafish mutants obtained by large-scale mutagenesis showed a similar mirror-image pattern duplication of the dorsal–ventral axis (Hammerschmidt et al. 1996).

To address this issue, our research group (K. Ozato's lab in Nagoya University and M. Kimura and H. Inoko's laboratory in Tokai University, Japan) decided in the mid-1990s to attempt to isolate the causative gene for the *Da* phenotype using a positional cloning approach. By mapping the *Da* locus both genetically and physically, a set of candidate genes were eventually identified in 2004 (Ohtsuka et al. 2004a). Although the list of candidate genes described below contains known genes, further genetic and developmental analyses of *Da* in relation to the cascade of causative genes may provide new insights into not only critical aspects of the gene regulation required for proper dorsal–ventral patterning but also the morphogenetic evolution of fish. These investigations are currently being conducted in the laboratory of H. Takeda at Tokyo University, Japan.

13.2 Morphological Phenotypes of the *Da* Mutant

The most drastic *Da* phenotypes alter the external medaka appearance and involve the localization of pigment cells and the shape of the fins as a result of apparent mirror-image duplication of the dorsal–ventral axis. Interestingly, such phenotypes appear only in the trunk–tail region, while the head region appears normal. Although the causative gene(s) for the *Da* phenotype could be involved in dorsal–ventral patterning, this phenotype is distinct from certain zebrafish mutants in which defects in early dorsal–ventral specification are exhibited as most of these mutants show hypogenesis or dysgenesis of the dorsal or ventral structures rather than mirror-image duplication. This section introduces the major phenotypes of the homozygous *Da* mutant.

13.2.1 Embryonic Phenotypes

The unique phenotypes of the *Da* embryo can be roughly classified into five features: (1) localization of the pigment cells; (2) shape of the dorsal fin fold; (3) shape of the dorsal myotome; (4) caudal structures, including the terminal axis of the notochord; and (5) positioning of the neuromasts.

Pigment cells in *Da* mutants first appear by stereomicroscopic observations at around stage 30 (stages 29–31), and become evident after stage 31 (Tamiya et al. 1997). In the wild-type medaka embryo, the dorsal melanocytes of the tail region are arranged in a single line along the dorsal midline, whereas those in the ventral side are arranged in two lines along the ventral midline. In contrast, the dorsal melanocytes in the *Da* mutant are arranged in two lines in the same manner as the ventral melanocytes (see arrows in Fig. 13.2a). The dorsal and ventral melanocytes in *Da* localize to the dorsal- and ventralmost portion of the myotomes, respectively. Because this unique localization is the earliest visible external *Da* phenotype, Tamiya et al. have argued that it could be used as a marker to screen for *Da* mutants in the early stages of development (Tamiya et al. 1997). In the genetic mapping studies described below, experiments in our laboratory have used the melanocyte arrangement phenotype as well as the dorsal fin fold phenotype as markers for embryonic phenotyping (Ohtsuka et al. 1999). In addition to the melanophores, the location of other chromatophores, including xanthophores and leucophores, are altered in *Da*. Numerous xanthophores and leucophores normally appear in a single row along the dorsal midline in wild-type medaka, but only a few of these chromatophores are seen at stage 39 of the *Da* embryo (Ohtsuka et al. 2004a).

The median fins, including the dorsal, caudal, and anal fins, develop directly from the continuous epidermal fold surrounding the tail region. In medaka, this fold first appears at stage 29 (Tamiya et al. 1997). In the wild-type medaka embryo, the anterior end of the ventral fin fold at stage 29 is situated near the anus, whereas the dorsal fin, which appears at stage 31, is situated at the seventh myotome located

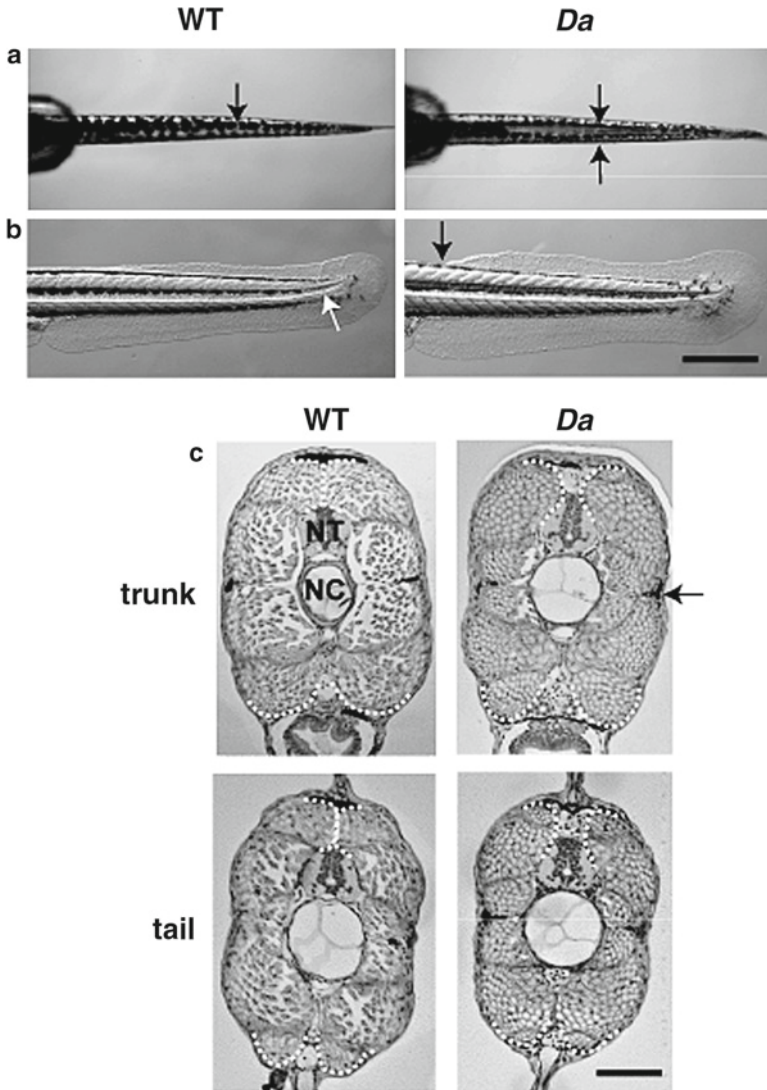


Fig. 13.2 Embryonic phenotypes of the *Da* mutant. **a** Distribution of melanocytes in the tail region of *Da* and wild-type (WT) medaka at embryonic stage 39 (dorsal view). In the wild-type fish, the dorsal melanocytes are arranged in a single line along the dorsal midline (arrow). In contrast, those in the *Da* mutant fish are arranged in two lines in the same manner as ventral melanocytes (arrows). **b** Anterior end of the dorsal fin fold at stage 39 (lateral view). In the wild-type, the anterior end of the dorsal fin fold is located at the seventh myotome, posterior to the anal myotome, whereas in *Da* it extends to the level of the anal myotome (black arrow). White arrow, terminal axis bending. **c** Cross sections at the level of the trunk (upper) and tail (lower) regions at embryonic stage 39. In the wild-type, the tops of the left and right myotome elongate dorsally and ultimately cover the neural tube (NT), whereas dorsal myotome elongation fails to proceed in *Da*, resulting in a morphology of the most dorsal myotome that appears very similar to that of the most ventral myotome (dotted lines). Arrow, lateral septum; NC, notochord. Bars **b** 500 μ m; **c** 1 mm

posterior to the anal myotome. The ventral fin fold in *Da* mutants develops with the same spatial and temporal manner as wild-type embryos. However, at stage 31, the anterior end of the dorsal fin fold in the *Da* mutant is situated at the fourth myotome located posterior to the anal myotome. Interestingly also, it extends in the anterior direction and finally reaches the level of the anal myotome at stage 39 (see black arrow in Fig. 13.2b).

In fish, myotomes derived from the somites are divided into dorsal and ventral myotomes at the lateral septum (see arrow in Fig. 13.2c) along the dorsal–ventral axis. The dorsal myotome localizes to the left and right sides of the neural tube from developmental stages 26 to 34. At these stages, there are no morphological differences between wild-type and *Da* mutant medaka. In the wild-type fish, the top of the left and right myotomes elongates dorsally, finally covering the neural tube at stage 36. However, dorsal myotome elongation fails to occur in *Da*, resulting in the base of the dorsal fin fold coming in contact with the neural tube. This phenotype is apparent at stage 39, with the *Da* dorsal myotomes growing and then protruding in the dorsal direction and not covering the neural tube (Tamiya et al. 1997) (Fig. 13.2c).

Mutant phenotypes also appear in the epichordal region of the *Da* embryo (Ishikawa 1990, 2000). The first phenotypic differences are seen at stages 31–32. In *Da*, the rudiments of epurals 1, 2, and 3 differentiate at the dorsal side of the caudal region. However, in the wild-type embryos, epurals 1 and 2 differentiate at later stages, and epural 3 never appears. In contrast, rudiments of the parhypural, lower hypural plate, and upper hypural plate, which differentiate at ventral side of the wild-type caudal region at stage 32, are not affected in *Da* (see Fig. 13.3c, d for adult caudal skeletons). At stage 32, the epichordal region of *Da* grows and the notochord extends straight into the tail, whereas the notochord terminus of the wild-type begins to bend dorsally (see white arrow in Fig. 13.2b). Additional musculature and fin rays also appear in the epichordal region of the *Da* mutants.

Neuromasts, which make up a small cluster of sensory cells that are part of the lateral line system, are used to detect water movement. These cells are normally localized in the lateral and ventral regions in wild-type medaka. However, in *Da* mutants, several neuromasts are mislocated at the dorsal tail region at embryo stage 39 (Ohtsuka et al. 2004a).

13.2.2 Adult Phenotypes

The characteristic mutant phenotypes of the adult *Da* consist of (1) an unpaired fin; (2) mislocalization of the iridophore; (3) an altered body shape; and (4) an irregular axial skeleton. These phenotypes are described below.

The dorsal fin of the adult *Da* is similar in shape to the anal fin, as if this mutant has two anal fins (see arrowhead in Fig. 13.1). The *Da* (*Double anal fin*) nomenclature in fact refers to this severe phenotype. In the adult male *Da*, the papillar processes that are present on the anal fin of wild-type males as secondary sexual

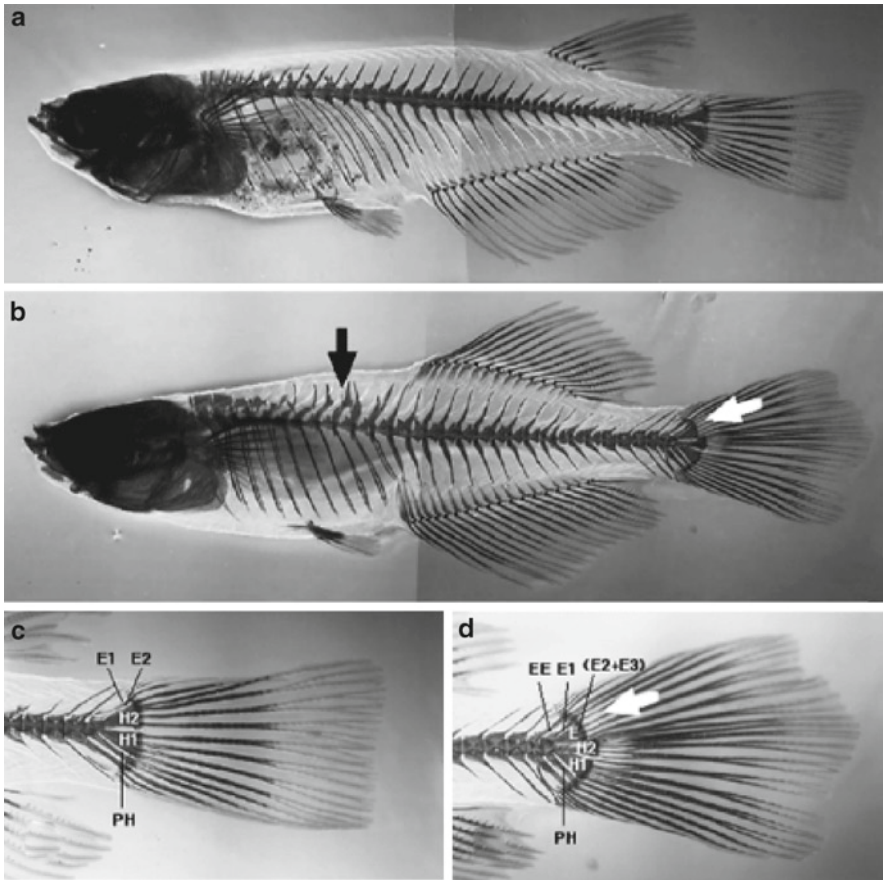


Fig. 13.3 Skeletal elements of the wild-type (**a**, **c**) and *Da* mutant (**b**, **d**) adult medaka. In the trunk axial skeleton, the neural arches of the *Da* mutant are irregularly shaped, and the neural spines are deformed and larger (*black arrow*) than those of the wild-type. The *Da* caudal fin also has ectopic structures such as extra epurals and extra fin rays in the epichordal region (*white arrow* in **b** and **d**). *E1*, epural 1; *E2*, epural 2; *E3*, epural 3; *EE*, extra epural; *H1*, lower hypural plate; *H2*, upper hypural plate; *PH*, parhypural. [Refer to Ishikawa (1990) for further details]

characteristics also appear on the dorsal fin (Tomita 1975). In the case of the caudal fin, there is no obvious phenotypic difference in the hypochordal region. However, the skeletal and muscular patterns in the epichordal region are ventralized in *Da* (Ishikawa 1990). *Da* also has ectopic structures such as extra epurals (see white arrow in Fig. 13.3b, d), extra interradial muscles, and an extra fin ray in the epichordal region. These structures seem to exhibit a mirror-image of those in the ventral region of the *Da* mutants, resulting in a caudal fin with a rhombic shape, including both external and internal symmetry along the dorsal–ventral axis (arrow in Fig. 13.1).

The iridophores, which are normally found in the abdominal region of wild-type medaka, are abundant at the dorsal side as well as the ventral side of the *Da* trunk

region (Ohtsuka et al. 1999) (see asterisk in Fig. 13.1). Because the *Da* mutant exhibits a similar silver color at the dorsal side and the ventral side, it reflects light brilliantly when swimming. In the tail region of the adult medaka, the body shape through the dorsal region is different between wild-type and *Da* medaka (Tamiya et al. 1997). The dorsal half is more rounded and the ventral half more slender in the wild-type, while both the dorsal and ventral halves exhibit a slender shape in *Da*; that is, they are symmetrical along the dorsal–ventral axis. This body shape can be mostly attributed to the shape of the myotome. Thus, the adult *Da* mutant exhibits a teardrop body shape that is typical of fast-swimming fish that reside in the deeper midlayers of the ocean, rather than that of the slowly swimming fish which reside near the ocean’s surface.

The *Da* axial skeleton also exhibits a unique morphological phenotype. The trunk neural arches of the *Da* are irregularly shaped and, in some cases, the midline fusion of several neural arches is disturbed, resulting in spina bifida occulta (Ohtsuka et al. 2004a). The neural spines of *Da* are also deformed and larger than those of wild-type medaka (see black arrow in Fig. 13.3b). The epipleurals, which are normally attached to the proximal region of the ribs in the wild-type, are truncated or missing in *Da*. However, the centrums, ribs, and hemal spines of appear normal in this mutant.

Most of the aforementioned phenotypes in the dorsal region of *Da* seem to be a mirror-image duplication of the ventral region, rather than an overproduction of the dorsal structures discussed previously by Ishikawa (1990). The mutated phenotypes are pleiotrophic and become evident during late embryogenesis. Although it is important to clarify the primary defects and secondary characteristics that result from the *Da* mutation, they are difficult to predict based on the observed phenotype. In amphibians and fish, the neural crest cells, which migrate and differentiate to form a wide variety of cell types including the pigment cells, are also thought to be involved in the localization and differentiation of the fin fold during the early developmental stages (Smith et al. 1994; Tucker and Slack 2004). Based on the unique localization of the pigment cells and the morphology of the unpaired fin, which appear as early *Da* phenotypes, one can hypothesize that neural crest-derived cells contain the primary *Da* defects. To test this hypothesis, it was important to isolate the causative gene for the *Da* phenotype. A summary of our 8-year effort to identify the *Da* gene is presented in the next section. From the expression pattern of the *Da* causative gene, a somite derivative rather than a neural crest derivative presents as the tissue containing the primary defects, as described below.

13.3 Positional Cloning of the *Da* Mutant Gene

The unique *Da* phenotypes prompted us to attempt to isolate the causative gene underlying the observed developmental changes. Because it was difficult to predict candidate genes based on the *Da* phenotype, we undertook a forward genetic approach. At the time that this project was initiated, however, no medaka-specific

genomic resources, such as genetic linkage maps or a genomic library, were available. We therefore used a positional cloning approach while such genomic resources were under development.

13.3.1 Genetic Mapping of the *Da* Mutation

The mutation resulting in the *Da* phenotype exhibits autosomal semidominant inheritance. Heterozygote mutants show a very mild phenotype in the dorsal fin and are easily distinguishable from homozygotes [e.g., heterozygotes (*BBDa+RR*) have a large dorsal fin with 7–14 fin rays, whereas the wild-type (*BB++RR*) fish have a normal dorsal fin with 5–7 fin rays] (Tomita 1975). We used the HNI inbred strain established from a northern medaka population for obtaining backcross progeny because *Da* is derived from a southern medaka population and a considerable number of polymorphisms useful for genetic mapping were expected to be detected between the HNI and the *Da* strains. In our investigations, the penetrance of the mutant phenotypes in the backcross progeny obtained from the cross between HNI and *Da* was found to be 100%. Forty-two backcross progeny were then used as a primary mapping population with which to construct a genetic linkage map.

We used three types of polymorphic DNA markers for genetic mapping: random amplified polymorphic DNA (RAPD) markers, sequence-tagged site (STS) markers converted from RAPD markers, and known genes (Ohtsuka et al. 1999). The RAPD method is polymerase chain reaction (PCR) based and uses short arbitrary primers with low annealing temperatures. Because it amplifies random genomic DNA fragments and can be used to detect polymorphisms without the need for prior genomic sequence information, rapid generation of a framework linkage map is possible even in genetically uncharacterized species. With this method, we constructed a genetic linkage map of the medaka based on female meiosis and successfully mapped the *Da* locus to the linkage group (LG) VIII (LG20 in the current map; Naruse et al. 2000) (Fig. 13.4a).

To further map this region with fine resolution, a total of 625 backcross progeny were used for segregation analyses (Ohtsuka et al. 1999). We ultimately obtained the two flanking markers stsM02-5 and stsB07-3 [0.32 and 0.80 centimorgans (cM), respectively, from the *Da* locus] and used these markers for the initiation of chromosome walking (M02-5, the nearest marker, was obtained on the first day that we initiated the RAPD analysis). Although the known developmental genes *shh* and *eng2* were mapped in close proximity to the *Da* locus (2.64 cM), no candidate genes could be predicted from the synteny. It is worth noting in this regard that we utilized a near-isogenic line of the *Da* mutant (NIL; the mutated *Da* gene was introduced from the original *Da* fish into the HNI background by nine generations of backcrossing) to efficiently isolate RAPD markers linked to the *Da* locus. The NIL was developed by Dr. Mitsuru Sakaizumi (Niigata University, Japan).

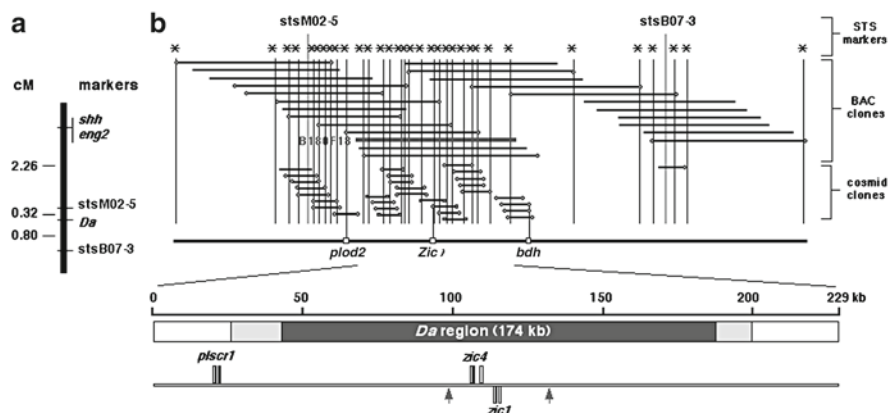


Fig. 13.4 Genetic and physical mapping of the *Da* regions. **(a)** Genetic mapping. The region covering the *Da* locus maps to linkage group (LG) VIII (corresponding to LG 20 on the current map). **(b)** Genomic contig with bacterial artificial chromosome (BAC) and cosmid clones. Chromosome walking was initiated from the markers *stsM02-5* and *stsB07-3*. The complete nucleotide sequence (229 kb) was determined from the BAC clone B180F18. Only three genes within this BAC clone were identified. Genetic mapping excluded the *p1scr1* gene from the 174-kb *Da* region, leaving only the *zic1* and *zic4* genes as candidates within this region. Insertions specific to the *Da* mutant (shown in arrows) are candidates for the *Da* mutation. Sequence-tagged (STS) markers, derived from cosmid and BAC end sequences and used for ordering clones, are indicated by the asterisks

13.3.2 Chromosome Walking and Physical Mapping of the *Da* Locus

Chromosome walking was initiated from the markers *stsM02-5* and *stsB07-3* by screening cosmid (Ohtsuka et al. 2002) and bacterial artificial chromosome (BAC) genomic libraries (Matsuda et al. 2001) (Fig. 13.4b). The cosmid library was generated from the HNI strain and screened by PCR, whereas the BAC library was generated from the Hd-rR strain derived from a southern population and screened by hybridization. The clones obtained were ordered using restriction digest fragment patterning and by PCR amplification of STS markers derived from the cosmid and BAC end sequences. Because of the large insert size of the BAC clones (>200 kb), the *Da* region was covered in only two rounds of walking. This procedure was completed within 2 months. The resultant BAC and cosmid contig spanned approximately 1 Mb, with a physical distance between the *stsM02-5* and *stsB07-3* markers of 817 kb in the current medaka genome sequence.

Homology searching of a total of 83 BAC and cosmid end sequences produced only three fragments exhibiting high levels of homology to the known genes procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysine hydroxylase) 2 (*plod2*), zinc finger of the cerebellum 1 (*zic1*), and 3-hydroxybutyrate dehydrogenase (*bdh*). All three genes are located on chromosome 3 in humans, whereas human orthologs for *shh* and *eng2*, which were closely linked to the *Da* gene in medaka,

are located on chromosome 7. These findings indicate that chromosome rearrangements between these regions occurred during medaka or human evolution (Ohtsuka et al. 2004b).

Using polymorphic STS markers derived from the cosmid and BAC end sequences and seven recombinant backcross progeny in which the recombination occurred between stsM02-5 and stsB07-3, the region critical for the *Da* mutation was narrowed to a 174-kb region that was covered by only one BAC clone, B180F18 (Ohtsuka et al. 2004a) (see Fig. 13.4b). We next determined the complete genomic sequence of this BAC clone using shotgun sequencing.

13.3.3 Identification of Candidate Genes

Three genes, *phospholipid scramblase 1* (*plscr1*), *zic1*, and *zic4*, were identified within the 228,508 bp of the B180F18 BAC insert by homology searching. Among these candidates, *plscr1* was excluded from the 174 kb of the smallest *Da* region, leaving *zic1* and *zic4* as candidates. This number of genes was lower than we had expected, because approximately 5 genes would be expected within the 174-kb region, based on a total of approximately 20,000 genes in the complete 700 Mb of the medaka genome (Kasahara et al. 2007) and assuming that the genes are evenly distributed. However, comparative genomic analyses of this region (Ohtsuka et al. 2004b) and a bioinformatics approach undertaken within our research program (Ohtsuka et al. 2004c) failed to identify any additional protein-coding genes in this region. We therefore concluded that this region represents a gene-poor region and that the 2 *zic* genes were the only candidates for the *Da* gene, although there remained a possibility that a gene regulatory sequence for another gene locating outside of this region or other noncoding genes might be present within this 174 kb region.

The hypothesis that *zic* genes are causative for the *Da* phenotype was supported by our gene expression data, knockdown analysis, and the phenotype of the *zic1*-knockout mouse (Ohtsuka et al. 2004a). Interestingly, the *zic1* and *zic4* genes exhibited a bidirectional (head-to-head) gene configuration. In the wild-type medaka, *zic1* is expressed in the head region, dorsal spinal cord, and dorsal somite derivatives. The expression pattern of *zic4* is almost identical to that of *zic1*. Expression of the somite in medaka becomes detectable between developmental stages 19 and 21 and is gradually restricted to the dorsalmost region and the region surrounding the spinal cord (dorsal sclerotome) at stages 33–34. Intense expression of both *zic* genes is present in the region dorsal to the terminal axis bending at stage 35. In stage 39, *zic1* expression is detected in the region dorsal to myotome elongation (underneath the dorsal epidermis), and *zic1*-expressing cells are present in the mesenchyme of the dorsal fin fold. In the *Da* medaka, expression of both *zic* genes in the derivatives of the dorsal somite and the region dorsal to the terminal axis bending is lost or severely reduced, whereas expression in the head region and the dorsal spinal cord is similar to that observed in wild-type medaka. Many of the *Da* phenotypes (in the dorsal myotome, trunk neural arches, dorsal fin fold, and tail termini) could be explained

by the loss of *zic* gene expression corresponding to the *Da* mutation because wild-type *zic* genes are expressed in the same regions displaying mutated phenotypes in the *Da* medaka. In this case, these phenotypes are thought to be primary defects, whereas mislocalization of pigment cells and neuromasts may be secondary characteristics caused by the defects in the dorsal mesenchyme or myotome.

A subsequent morpholino-based knockdown experiment indicated that *zic1* is responsible for the *Da* phenotypes (Ohtsuka et al. 2004a). Although the penetrance was incomplete, 16.2% of *zic1*-morpholino-injected embryos exhibited some of the *Da* early phenotypes, including an abnormal pigmentation pattern, anterior shift in the position of the dorsal fin fold, and a failure of normal dorsal myotome elongation. The *zic4*-morpholino, however, failed to induce the *Da* early phenotypes, indicating that *Zic4* has little function in the determination of the *Da* phenotype.

As already described, the *Da* mutation results in neural arch defects such as spina bifida occulta (Ohtsuka et al. 2004a). This observation seems to correspond with the phenotypes of the *zic1*-knockout mouse, which displays vertebral arch phenotypes leading to spina bifida occulta (Aruga et al. 1999). It would be interesting in this regard if the irregular hair color over the region of spina bifida occulta in the knockout mouse was homologous to the abnormal pigment pattern observed in the *Da* dorsal region.

In summary, the foregoing findings, based on expression data, knockdown analyses, and the phenotype of the *zic1*-knockout mouse, strongly suggest that *zic* genes, particularly *zic1*, are causative for the *Da* mutant phenotype and that the loss of *zic* expression in the dorsal somite derivatives and tail region directly results in these mutant phenotypes.

13.3.4 Mapping the Specific *Da* Mutation

Following extensive examination, there was no obvious mutation found, such as an insertion/deletion or nonsynonymous substitution, within the coding regions of the *zic1* and *zic4* genes (Ohtsuka et al. 2004a). This finding was not that surprising, however, because *zic* genes appear to function normally in the head region of the *Da* mutant medaka. Hence, the *Da* phenotype may be the result of a mutation that leads to the loss of somite-specific transcription of the *zic* genes, rather than one which disrupts the protein itself. Although several single nucleotide polymorphisms (SNPs) are found in the *zic* gene introns and the intergenic region, none are located in the evolutionally conserved region. We also detected two *Da*-specific insertions, one located 15.8 kb downstream of the *zic1* gene and another located 7 kb downstream of the *zic4* gene (see arrows in Fig. 13.4b). The former is a 324-bp insertion (AB164417) that we believe is unlikely to cause the *Da* phenotypes. The latter is an insertion that was previously estimated to be 3–4 kb based on genomic Southern analyses, but the actual size of this insertion is now known to be greater than 50 kb (Moriyama et al., unpublished data). This insertion is a strong candidate for causative genomic change resulting in the *Da* mutation, which could disrupt a

somite-specific enhancer or inhibit its activity. Nucleotide sequencing of this insertion and a search for a somite-specific enhancer is currently underway in the laboratory of H. Takeda at Tokyo University.

13.4 *Da* Phenotypes and Fish Evolution

Terminal axis bending, a characteristic acquired during fish evolution, is used to classify fish species. However, the genes involved in this process have not, to our knowledge, been characterized. Caudal fin classifications can be divided into proto-cercal, heterocercal, and homocercal types, and are believed to have evolved in this order (Ledent 2002). These evolutionary changes recapitulate during ontogeny, as in the case of wild-type medaka. In the *Da* mutant, however, terminal axis bending is arrested, presumably because of the loss of *zic* expression, resulting in a caudal fin with an isocercal-like structure in which the ural vertebra shows no inflexion. From an evolutionary point of view, we could speculate that expression of *zic1* and *zic4* in this region was acquired during fish evolution. Based on this scenario, and on the features of the *Da* phenotype, we hypothesize that the dorsal region of ancient fish was identical to the ventral region, and that *zic* expression in the dorsal somite and/or tail termini enabled the development of a distinct dorsal structure. If this is indeed the case, the phenotypes of the *Da* mutant may be a manifestation of ancestral regression. Dorsalization caused by the acquisition of *zic* expression in somite derivatives and/or tail termini might be a driving force in fish diversification. Therefore, it will be interesting to examine the expression of the *zic* gene cascade in species such as the lamprey or more primitive organisms such as amphioxus.

13.5 *Da* as a Model System for Human Disease

Neural tube defects are human birth defects caused by an incomplete closure of the neural tube. Spina bifida occurs when the fetal spinal column fails to close during the first month of pregnancy, and this is the most common neural tube defect in humans (Mitchell et al. 2004). As a result, a part of the spinal cord and its membranes is exposed through a gap in the backbone, often causing partial paralysis of the legs as a result of nerve damage. Although the *Da* medaka do not exhibit such severe defects, they do show a similar although milder phenotype in the neural arch and neural spines (see Fig. 13.3b), resulting in spina bifida occulta (Ohtsuka et al. 2004a). The *Da* mutants possess trunk neural arches with irregular shapes and the midline fusions of some neural arches are disturbed. In addition, the neural spines are enlarged and deformed. These phenotypes indicate that the *Da* medaka is a potential animal model for spina bifida.

The *zic1* knockout mouse also exhibits spina bifida occulta (Aruga et al. 1999). However, in contrast to the *Da* mutants, spinous processes, probably corresponding

to the neural spines of the medaka, are missing in this knockout mouse. This difference could be the result of the loss of *zic1* expression in the dorsal neural tube as well as in the dorsal somite of the knockout mouse. Because *zic1* expression in the dorsal neural tube seems to be required for the formation of spinous processes, the *Da* phenotype suggests that *zic1* expression in the somite derivatives is required for late patterning of the spinous processes. Hence, the *Da* medaka may be a useful model to distinguish between *zic* functions in the somite derivatives and in the central nervous system, with reference to the pathogenetic mechanisms of spina bifida.

It has been reported that several individuals diagnosed with Dandy-Walker malformation (DWM) possess 3q2 interstitial deletions in the region of the human genome encompassing the *ZIC1* and *ZIC4* genes. Grinberg et al. (Grinberg et al. 2004) have shown that heterozygous loss of *Zic1* and *Zic4* in mice results in a DWM-like phenotype. As DWM is characterized by cerebellar hypoplasia and other malformations in the brain, the expression of these genes in the brain must be critical for the pathogenesis of DWM. Although the causative genes of DWM are the same as those of the *Da* phenotype, no brain phenotypes are evident in *Da* as the expression of *zic1* and *zic4* is intact in the brain of this medaka mutant. However, occasional cases of DWM associated with spina bifida have also been reported (Shuto et al. 1999) which may be caused by the abnormal *zic1* and *zic4* expression in the trunk region.

13.6 Conclusions and Future Perspectives

In this chapter, we have presented a brief history of the studies thus far on the medaka *Da* mutant including a detailed description of its morphological phenotypes, an outline of the positional cloning we undertook in our own laboratory and subsequent identification of the causative genes, the current understanding of the *Da* medaka in terms of fish evolution, and possibilities for using the *Da* medaka as a human disease model. The unique mirror-image duplication phenotypes of the dorsal–ventral axis in *Da* initiated extensive research on this mutant not only from a developmental standpoint, but also as it relates to evolution and human disease. Given that the causative genes have now been identified, the molecular mechanisms underlying these unique phenotypes need to be clarified. For example, further elucidation of the molecular cascade regulating *zic* gene expression in somite derivatives may provide vital clues as to how dorsal identity is determined in the trunk–tail region.

In addition, it is important to distinguish between primary and secondary defects as described here. From the expression pattern of the *zic1* and *zic4* genes in medaka, we speculate that defects in the somite derivatives are the primary *Da* mutant characteristics, and that the external features such as altered fin morphology and mislocation of pigment cells derived from the neural crest cells are secondary characteristics. If this is indeed the case, tissue interactions may occur between the somite derivatives and the surface ectoderm, which have never been reported previously. Transplantation experiments between wild-type and *Da* medaka could

aid our understanding of this issue. Indeed, there are preliminary experimental data that support this hypothesis (Kawanishi et al., unpublished data).

Although the *Da* phenotypes are unique, there may be other fish that exhibit similar phenotypes. Some fish species, such as the codfish, have isocercal caudal fins in which the vertebrae extend in a straight line to the end of the fin. With aquarium fish, the external morphology of the double tail strain of a betta fish (*Betta splendens*) seems to exhibit mirror-image duplication of the dorsal–ventral axis, similar to that observed in the *Da* medaka. Because the evolution of gene enhancers may play a pivotal role in morphological evolution, it would be of interest in the future to examine the expression patterns and somite-enhancer activities of the *zic* genes in these fish strains, as well as the expression of the *zic* gene cascade. In this regard, the somite-enhancer sequence in wild-type medaka will soon be identified by ongoing research being conducted in the laboratory of H. Takeda utilizing modified BAC transgenesis.

References

- Aruga J, Mizugishi K, Koseki H et al (1999) *Zic1* regulates the patterning of vertebral arches in cooperation with *Gli3*. *Mech Dev* 89:141–150
- Grinberg I, Northrup H, Ardinger H et al (2004) Heterozygous deletion of the linked genes *ZIC1* and *ZIC4* is involved in Dandy-Walker malformation. *Nat Genet* 36:1053–1055
- Hammerschmidt M, Pelegri F, Mullins MC et al (1996) *dino* and *mercedes*, two genes regulating dorsal development in the zebrafish embryo. *Development (Camb)* 123:95–102
- Ishikawa Y (1990) Development of caudal structures of a morphogenetic mutant (*Da*) in the teleost fish, medaka (*Oryzias latipes*). *J Morphol* 205:219–232
- Ishikawa Y (2000) Medakafish as a model system for vertebrate developmental genetics. *BioEssays* 22:487–495
- Kasahara M, Naruse K, Sasaki S et al (2007) The medaka draft genome and insights into vertebrate genome evolution. *Nature (Lond)* 447:714–719
- Ledent V (2002) Postembryonic development of the posterior lateral line in zebrafish. *Development (Camb)* 129:597–604
- Matsuda M, Kawato N, Asakawa S et al (2001) Construction of a BAC library derived from the inbred Hd-rR strain of the teleost fish, *Oryzias latipes*. *Genes Genet Syst* 76:61–63
- Mitchell LE, Adzick NS, Melchionne J et al (2004) Spina bifida. *Lancet* 364:1885–1895
- Naruse K, Fukamachi S, Mitani H et al (2000) A detailed linkage map of medaka, *Oryzias latipes*: comparative genomics and genome evolution. *Genetics* 154:1773–1784
- Ohtsuka M, Makino S, Yoda K et al (1999) Construction of a linkage map of the medaka (*Oryzias latipes*) and mapping of the *Da* mutant locus defective in dorsoventral patterning. *Genome Res* 9:1277–1287
- Ohtsuka M, Kikuchi N, Nogami M et al (2002) Rapid screening of a novel arrayed medaka (*Oryzias latipes*) cosmid library. *Mar Biotechnol (NY)* 4:173–178
- Ohtsuka M, Kikuchi N, Yokoi H et al (2004a) Possible roles of *zic1* and *zic4*, identified within the medaka *Double anal fin (Da)* locus, in dorsoventral patterning of the trunk-tail region (related to phenotypes of the *Da* mutant). *Mech Dev* 121:873–882
- Ohtsuka M, Kikuchi N, Ozato K et al (2004b) Comparative analysis of a 229-kb medaka genomic region, containing the *zic1* and *zic4* genes, with Fugu, human, and mouse. *Genomics* 83:1063–1071
- Ohtsuka M, Horiuchi S, Kulski JK et al (2004c) CHOP: visualization of ‘wobbling’ and isolation of highly conserved regions from aligned DNA sequences. *Nucleic Acids Res* 32:W55–W58

- Shuto T, Sekido K, Ohtsubo Y et al (1999) Dandy-Walker syndrome associated with occipital meningocele and spinal lipoma: case report. *Neurol Med Chir (Tokyo)* 39:544–547
- Smith M, Hickman A, Amanze D et al (1994) Trunk neural crest origin of caudal fin mesenchyme in the zebrafish *Brachydanio rerio*. *Proc R Soc Lond* 256:137–145
- Tamiya G, Wakamatsu Y, Ozato K (1997) An embryological study of ventralization of dorsal structures in the tail of medaka (*Oryzias latipes*) *Da* mutants. *Dev Growth Differ* 39:531–538
- Tomita H (1975) Mutant genes in the medaka. In: Yamamoto T (ed) *Medaka (killifish) biology and strains*. Keigaku, Tokyo
- Tucker AS, Slack JM (2004) Independent induction and formation of the dorsal and ventral fins in *Xenopus laevis*. *Dev Dyn* 230:461–467

Part III
Germ Cells, Sex Determination,
and Reproduction

Chapter 14

Interaction of Germ Cells and Gonadal Somatic Cells During Gonadal Formation

Minoru Tanaka

Abstract The gonad is composed of many cell types, including germ cells. The different cell lineages likely associate with one another during formation of the gonadal primordium and during sex differentiation. However, the details of these interactions in the context of gonad formation and sex differentiation have not been clarified. Recently, cell tracing experiments in transgenic medaka and analysis of medaka mutants lacking expression of the type II *Amh* receptor (*amhrII*) have provided information about the cellular interactions between the several cell lineages in the gonad. Here, I give an overview of gonad development and discuss the role of *Amh* signaling during sex differentiation and maintenance of sex in the gonads.

14.1 Development of Gonads

14.1.1 Movement of Primordial Germ Cells

Germ cells are the only cell type capable of transmitting genetic information to the next generation. During embryonic development in many vertebrates, germ cells are distinguished from somatic cell lineages as primordial germ cells (PGCs), which contain a germ cell-specific intracellular structure called the nuage (McMillan 2007). This electron-dense intracellular structure is known as the germplasm in *Xenopus* and *Drosophila*, P-granules in nematodes, and the chromatoid body in mammals. Recent investigation reveals that this structure is composed of multiple proteins with RNAs and that some components are conserved among animals (Hashimoto et al. 2004).

Nanos proteins are a component of the nuage-like structure in many vertebrates. Genomic and syntenic analyses have revealed that medaka has four different *nanos* genes (*nanos1a*, *1b*, 2, and 3). In terms of synteny and expression pattern, *nanos3*

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is the most conserved among vertebrates and is exclusively expressed in PGCs (Aoki et al. 2009; Kurokawa et al. 2006). The loss of zygotic Nanos3 protein function results in degeneration of PGCs during development, indicating the essential function of *nanos3* on germ cell development (Köprunner et al. 2001). Knockdown of medaka *nanos3* led to abnormal PGC migration and gonads without germ cells (Kurokawa et al. 2007).

Nanos3 is a marker for PGCs in medaka, and its expression in PGCs is dependent on its 3'-untranslated region (3'-UTR), because injection of green fluorescent protein (GFP) RNA fused to the *nanos* 3'-UTR results in localization of fluorescence to PGCs (Kurokawa et al. 2006). The *nanos* 3'-UTR is also important in other vertebrates, such as zebrafish, in which miRNAs appear to regulate *nanos* expression via the 3'-UTR (Mishima et al. 2006).

Medaka PGCs are first detectable by *nanos3* expression as early as gastrulation stage, near the yolk in the middle of the blastoderm. During epiboly, PGCs migrate toward the posterior of the embryo by at least three different mechanisms: *sdf1b* (previously called *sdf1a*)-dependent movement toward the marginal region, passive convergent movement towards the embryonic body, and *sdf1b*-dependent posterior movement (Kurokawa et al. 2006; Herpin et al. 2008). A repulsive signal is also involved in PGC migration (Sasado et al. 2008). Transplantation experiments suggest that medaka PGCs acquire the ability to migrate and differentiate into germ cells by the gastrulation stage (Saito et al. 2006).

Interestingly, the localization of Nanos proteins changes intracellularly in migrating PGCs as they approach the gonadal primordium (Aoki et al. 2008). Immediately after the establishment of PGCs at gastrulation stage, Nanos protein is localized to a single perinuclear granule in the PGCs. As posterior migration proceeds, this granule breaks down into small particles. Time-lapse imaging reveals that the granules move within the cytoplasm, joining and separating from one another. Other proteins in the medaka nuage-like structure, Tdrd1 and Olvas (the medaka Vasa homolog), also form granules in germ cells. These proteins autonomously form granules in cultured cells and shuttle between granules and the cytoplasm (Nagao et al. 2008). Similarly, the zebrafish germ cell-specific proteins Granuto and Tdrd7 move dynamically in PGCs (Strasser et al. 2008). The gel-like character of the granule may facilitate the intracellular dynamics of the components (Brangwynne et al. 2009).

By the time PGCs reach the gonadal primordium and become enclosed by *sox9b*-expressing supporting cells, Nanos3, Olvas, and Tdrd1 granules form a hollow area in the middle (Aoki et al. 2008), suggesting a change in the function of the nuage-like structure. These intracellular changes may reflect the maturation of PGCs before they develop into gonocytes or germline stem cells with the ability to differentiate into either female or male gametes.

14.1.2 Development of Gonadal Somatic Cells

PGCs move on the lateral plate mesoderm along the embryonic body (stages 21–28) before they reach the gonadal primordium. Prepatterning of the lateral plate

mesoderm becomes apparent at this stage (Nakamura et al. 2006). *Sdf1b* expression is found in a large region of the lateral plate mesoderm and overlaps with the *wt1* expression domain in the anterior region but excludes the *gata2* expression domain. As embryogenesis proceeds, *sdf1b* expression is confined to the most posterior part of the lateral plate mesoderm. This restriction in expression is essential for the guidance of PGCs toward the posterior lateral plate mesoderm (Reichman-Fried et al. 2004; Kurokawa et al. 2006).

The most posterior region of the lateral plate mesoderm appears to form a gonadal field (Nakamura et al. 2006). Cell-tracing experiments have shown that at least two different types of gonadal somatic precursors that express *ftz-f1* (the medaka homolog of mammalian *Sf1/Ad4BP*) or *sox9b* arise from the posterior lateral plate mesoderm. Until the late segmentation stage, gonadal fields retain the potential to regenerate somatic precursors after elimination of somatic precursors by laser ablation. The *ftz-f1*-expressing cells maintain *sdf1b* expression and wait at the posterior end, where they contact PGC progenitors. Gonadal fields are essential not only for the differentiation of gonadal somatic cells, but also for posterior PGC migration and coordination of PGC and gonadal somatic precursor contact.

After they meet, somatic precursors and PGCs move from the posterior end of the lateral plate mesoderm along the developing hindgut to reach the dorsal region of the gut, where they form a single gonadal primordium. In the gonadal primordium, the PGCs (called gonocytes or germ cells) are enclosed by *sox9b*-expressing cells.

14.2 Sexual Dimorphism of Gonads

Sex determination in medaka is controlled by sex chromosomes. In the presence of the *DMY/dmrt1bY* gene on the Y chromosome, medaka gonads initiate testicular development (see Chaps. 17 and 19). The *sox9b*-expressing cells that enclose germ cells eventually differentiate into either male (Sertoli cells) or female (granulosa cells) supporting cells, depending on the genetic context. Approximately 1 day after the gonadal primordium forms, flattened cells appear in the middle, and the gonadal primordium splits into right and left lobes (Nakamura et al. 2008).

DMY/dmrt1bY expression begins in *sox9b*-expressing cells as early as the onset of gonadal primordium formation, similar to the early expression of the testis determination gene *Sry* in mice. However, the expression of *sox9b*, which is just downstream of *Sry* in the mouse male sex determination pathway (Sekido and Lovell-Badge 2008), persists in medaka female gonads. Another mammalian male-specific marker, the anti-Müllerian hormone (*amh/mis*) gene, is also expressed in the supporting cells of both sexes of medaka. Notably, sexual dimorphism of medaka gonads is not manifested morphologically until *amh* expression becomes apparent following expression of *DMY/dmrt1bY*. With the onset of *amh* gene expression, germ cells in the female gonad increase in number and produce follicles with oocytes. This process contrasts sharply with the male gonads, which retain features of the gonadal primordium and do not undergo spermatogenesis until about 1 month after hatching (Sato and Egami 1972; Kanamori et al. 1985; Saito et al. 2007).

Expression of the *aromatase* gene (*cyp19a1/P450arom*) is detected exclusively in the female gonads as early as 5 days post hatching (5 dph) (Suzuki et al. 2004; Nakamura et al. 2009). Aromatase catalyzes the conversion of testosterone into the female steroid hormone estrogen and is a marker of granulosa cells, the steroidogenic supporting cells in the mammalian ovary. Mammalian ovaries contain two types of steroidogenic cells that are generated from *sox9b*-expressing cells: theca cells, which are present in the stromal region and the outer layer of the follicles, and granulosa cells, the supporting cells that enclose oocytes. However, cell lineage analysis has revealed that, in medaka, *aromatase* (*cyp19a1*)-expressing cells are not granulosa cells; rather, they are precursors of theca cells that first emerge from the ventral side of the developing female gonads and subsequently the surrounding follicles. Interestingly, germ cells are required for the maintenance of *aromatase* (*cyp19a1*)-expressing cells (Nakamura et al. 2009).

Simultaneous with the emergence of *aromatase* (*cyp19a1*)-expressing cells is the appearance of an estrogen precursor-producing cell type that expresses *P450c17* in both female and male gonads. Similar to *aromatase* (*cyp19a1*)-expressing cells, these cells are in the outer layer of the follicles. There are two types of steroidogenic theca cells in the follicles that express either *P450c17* or *aromatase* (*cyp19a1*) in the adult ovary. Granulosa cells do not express the *aromatase* (*cyp19a1*) gene until they become cuboidal, just a couple of days before the maturation of oocytes (Nakamura et al. 2009).

14.3 Germ Cell Behavior During Sex Differentiation

As a result of the exponential increase in the number of germ cells in female gonads, female gonads are larger than male gonads at hatching. *DMY/dmrt1bY* is expressed only in supporting cells, indicating that germ cells are sexually controlled by the somatic cells. Live imaging of fluorescent germ cells has shown that germ cells in female gonads undergo several rounds of successive, synchronous cell division (type II divisions) (Saito et al. 2007). The resultant germ cells form cysts, with the germ cells within a single cyst connected by intercellular bridges. In addition to synchronously dividing germ cells, another population of less frequently dividing germ cells (type I divisions) is found in the gonads of both sexes. After undergoing type I divisions, each germ cell is enclosed by *sox9b*-expressing supporting cells.

Phenotypic analysis of the medaka *zenzai* mutant provides information about the functional differences between the two types of division. *Zenzai* mutants possess fewer germ cells than wild-type medaka at hatching and gradually lose germ cells as they grow, finally becoming sterile. In female *zenzai* mutants, germ cells that normally undergo type I divisions are binucleate and show signs of cell death, whereas the clustered cystic germ cells that undergo type II division are normal and express meiotic markers. This result indicates that the type I division is related to the process of germ cell maintenance and self-renewal, whereas the type II division occurs in germ cells that are committed to gametogenesis. In addition, these data

suggest that at early stages of gonad development, the transition between the two types of germ cell proliferation is regulated by sexual dimorphic signals from somatic cells.

14.4 Amh Signaling Controls Germ Cell Division

AMH/MIS belongs to the transforming growth factor-beta (TGF β) superfamily of proteins and is secreted from Sertoli cells (male supporting cells) during sex differentiation in mammalian gonads. The expression of AMH is critical for regression of the Müllerian ducts, which are the primordia of the upper vagina, oviduct, and other female reproductive organs, thereby ensuring male development of reproductive organs. Because of its Sertoli cell-specific expression, *amh* has been used as an indicator of male development in mammals. However, in other vertebrates such as chick and teleosts (Oreal et al. 1998; von Hofsten et al. 2005; Shiraishi et al. 2008), *amh* is detected in the developing gonads of both female and male animals. Because no anatomical structure corresponding to the Müllerian duct is present in teleosts, the function of *amh* signaling during gonadal development has remained enigmatic.

The medaka *hotei* mutant was isolated by a chemical mutagenesis screen (Morinaga et al. 2004) and was shown to have an A-to-G substitution in the type II Amh receptor (*amhrII*) (Morinaga et al. 2007). The mutation maps to exon 9 and replaces a conserved tyrosine residue in a kinase domain of the type II receptor with a serine residue. *Hotei* mutants have an enlarged abdomen because of gonadal hypertrophy resulting from an abnormally large number of germ cells. Interestingly, approximately half the genetically male *hotei* mutants (XY) exhibit female secondary sex characteristics. These sex-reversed *hotei* mutants possess ovarian structures with oocytes and female-specific gene expression, such as *foxl2* and *aromatase* (*cyp19a1*) expression.

Germ cell proliferation in *hotei* mutants begins at the same time as normal female-specific type II division, at around stage 35. Interestingly, both *amhrII* and the gene encoding its ligand, *amh*, are expressed in supporting cells but not in germ cells, indicating that *amh* signaling acts cell autonomously and indirectly regulates germ cell proliferation. Furthermore, our results showed that sex reversal caused by the mutation of *amhrII* is mediated through germ cells, as *hotei* mutants artificially depleted of germ cells do not exhibit sex reversal.

14.5 Reciprocal Regulation Between Germ Cells and Somatic Cells

The phenotype of *hotei* mutants is consistent with the results of our previous studies of germ cell-deficient medaka that were generated by disrupting PGC migration. These animals display secondary male sex characteristics, secrete male steroid

hormones (11-keto-testosterone), and express some male-specific genes in the gonad. This masculinization resulting from loss of germ cells occurred in all germ cell-deficient medaka, irrespective of their genetic sex. Thus, germ cells play a critical role in normal female development, whereas gonadal somatic cells predispose the gonad to male development in the absence of germ cells (Kurokawa et al. 2007). This finding suggests that interactions between germ cells and gonadal somatic cells are needed for proper sex differentiation.

The molecular mechanisms of masculinization during normal sex differentiation are not yet understood in detail, but we have found that *amh* expression is not maintained in the absence of germ cells. The loss of Amh signaling in *hotei* mutants increases germ cell number, ultimately resulting in feminization of the gonads. Given that Amh signaling functions in supporting cells in an autocrine manner, it is possible that Amh signaling modulates reciprocal signals between germ cells and somatic cells to control germ cell proliferation (Fig. 14.1). In this scenario, germ cells proliferate very rapidly in *hotei* mutants, causing an imbalance between germ cells and somatic cells. Some germ cells escape masculinization by gonadal somatic cells and adopt the default feminized fate. The observation that half the XY *hotei* mutants are feminized could be explained by this stochastic process of imbalancing.

The male gonad in wild-type medaka animals morphologically resembles the gonadal primordium, whereas the female gonad is larger because of an increase in type II germ cell divisions. It is interesting to note that *DMY/dmrt1bY* can stop the cell cycle at G₂ phase (Herpin et al. 2007), supporting a model in which the proper balance between the number of germ cells and somatic cells is important for normal sex differentiation in wild-type medaka. This balancing between germ cells and

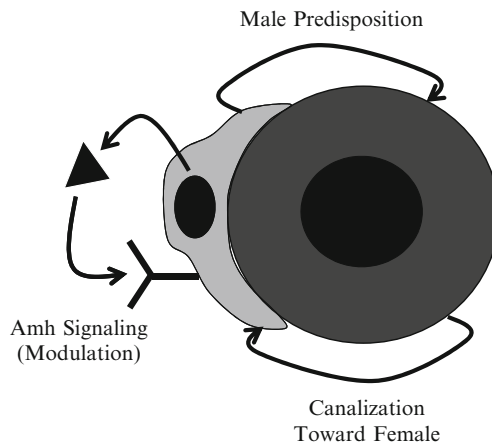


Fig. 14.1 Amh signaling functions in an autocrine fashion in supporting cells to modulate reciprocal communication between germ cells and gonadal somatic cells. Disruption of this signal leads to excessive germ cell proliferation, ultimately resulting in feminization of the gonads (Tanaka 2008; Saito and Tanaka 2009)

somatic cells does not affect the initial process of sex determination; rather, it is involved in sex differentiation or maintenance of gonadal sex.

In the absence of germ cells, gonadal somatic cells ultimately exhibit male characteristics, even though the initiation of feminization is normal in XX medaka. Female-specific [*aromatase* (*cyp19a1*)-expressing] theca cells and female supporting cells (granulosa cells expressing *foxl2*) differentiate with normal timing in these animals. However, expression of both *aromatase* and *foxl2* is not maintained; rather, it is markedly reduced without germ cells. In transgenic medaka in which GFP reporter expression is controlled by the *aromatase* (*cyp19a1*) promoter, once the *aromatase* transcripts are lost, GFP fluorescence is maintained for at least 10 days in the cells that would normally have expressed *aromatase*. This event does not reflect a loss of *aromatase*-expressing cells; instead, feminization of the cells is not retained (Kurokawa et al. 2007; Nakamura et al. 2009). This finding also suggests that once sex is determined and developmental processes are initiated in the gonad, interactions between germ cells and somatic cells are required for further development and/or maintenance of gonadal sex (Tanaka 2008; Saito and Tanaka 2009).

It will be very interesting to know if the interaction that is observed in medaka is true for other animals. In another teleost fish, zebrafish, all the gonads develop ovarian structures first, but those with few germ cells undergo oocyte degeneration and begin male differentiation (Takahashi 1977; Wang et al. 2007; Siegfried and Nusslein-Volhard 2008). Even in mice, the loss of oocytes in the adult ovary results in masculinization of supporting cells, although whether this effect is a direct consequence of oocyte loss remains controversial (Guigon and Magre 2006). In mice, the follicular structures turn into seminiferous tubule-like structures with the onset of *Sox9* expression. It would be interesting to investigate what happens to mouse gonads when the germ cells are completely removed before PGCs come in contact with gonadal precursor cells.

References

- Aoki Y, Nagao I, Saito D, Ebe Y, Kinjo M, Tanaka M (2008) Temporal and spatial localization of three germline-specific proteins in medaka. *Dev Dyn* 237:800–807
- Aoki Y, Nakamura S, Ishikawa Y, Tanaka M (2009) Expression and syntenic analyses of four *nanos* genes in medaka. *Zool Sci* 26:112–118
- Brangwynne CP, Eckmann CR, Courson DS, Rybarska A, Hoege C, Gharakhani J, Julicher F, Hyman AA (2009) Germline P granules are liquid droplets that localize by controlled dissolution/condensation. *Science* 26:1729–1732
- Guigon CJ, Magre S (2006) Contribution of germ cells to the differentiation and maturation of the ovary: insight from models of germ cell depletion. *Biol Reprod* 74:450–458
- Hashimoto Y, Maegawa S, Nagai T, Yamaha E, Suzuki H, Yasuda K, Inoue K (2004) Localized maternal factors are required for zebrafish germ cell formation. *Dev Biol* 268:152–161
- Herpin A, Rohr S, Riedel D, Klüver N, Raz E, Scharl M (2007) Specification of primordial germ cells in medaka (*Oryzias latipes*). *BMC Dev Biol* 7:3
- Herpin A, Fischer P, Liedtke D, Kluever N, Neuner C, Raz E, Scharl M (2008) Sequential SDF1a and b-induced mobility guides medaka PGC migration. *Dev Biol* 15:319–327

- Kanamori A, Nagahama Y, Egami N (1985) Development of the tissue architecture in the gonads of the medaka *Oryzias latipes*. *Zool Sci* 2:695–706
- Köprunner M, Thisse C, Thisse B, Raz E (2001) A zebrafish *nanos*-related gene is essential for the development of primordial germ cells. *Gene Dev* 15:2877–2885
- Kurokawa H, Aoki Y, Nakamura S, Ebe Y, Kobayashi D, Tanaka M (2006) Time-lapse analysis reveals different modes of primordial germ cell migration in the medaka *Oryzias latipes*. *Dev Growth Differ* 48:209–221
- Kurokawa H, Saito D, Nakamura S, Katoh-Fukui Y, Ohta K, Aoki Y, Baba T, Morohashi K, Tanaka M (2007) Germ cells are essential for sexual dimorphism in the medaka gonad. *Proc Natl Acad Sci USA* 104:16958–16963
- McMillan DB (2007) Fish histology: female reproductive systems. Springer, Dordrecht
- Mishima Y, Giraldez AJ, Takeda Y, Fujiwara T, Sakamoto H, Schier AF, Inoue K (2006) Differential regulation of germline mRNA in soma and germ cells by zebrafish miR-430. *Curr Biol* 16:2135–2142
- Morinaga C, Tomonaga T, Sasado T, Suwa H, Niwa K, Yasuoka A, Henrich T et al (2004) Mutations affecting gonadal development in medaka, *Oryzias latipes*. *Mech Dev* 121:829–839
- Morinaga C, Saito D, Nakamura S, Sasaki T, Asakawa S, Shimizu N, Mitani H, Furutani-Seiki M, Tanaka M, Kondoh H (2007) The *hotei* mutation of medaka in the anti-Müllerian hormone receptor causes the dysregulation of germ cell and sexual development. *Proc Natl Acad Sci USA* 104:9691–9696
- Nagao I, Aoki Y, Tanaka M, Kinjo M (2008) Analysis of the molecular dynamics of medaka nuage proteins by fluorescence correlation spectroscopy and fluorescence recovery after photobleaching. *FEBS J* 275:341–349
- Nakamura S, Kobayashi D, Aoki Y, Yokoi H, Ebe Y, Wittbrodt J, Tanaka M (2006) Identification and lineage tracing of two populations of somatic gonadal precursors in medaka embryos. *Dev Biol* 295:678–688
- Nakamura S, Aoki Y, Saito D, Kuroki Y, Fujiyama A, Naruse K, Tanaka M (2008) *Sox9b/sox9a2*-EGFP transgenic medaka reveals the morphological reorganization of the gonads and a common precursor of both the female and male supporting cells. *Mol Reprod Dev* 75:472–476
- Nakamura S, Kurokawa H, Asakawa S, Shimizu N, Tanaka M (2009) Two distinct types of theca cells in the medaka gonad: germ cell-dependent maintenance of *cyp19a1*-expressing cells. *Dev Dyn* 238:2652–2657
- Oreal E, Pieau C, Tattei M-G, Josso N, Picard J-Y, Carre-Eusebe D, Magre S (1998) Early expression of *AMH* in chicken embryonic gonads precedes testicular *SOX9* expression. *Dev Dyn* 212:522–532
- Reichman-Fried M, Minina S, Raz E (2004) Autonomous modes of behavior in primordial germ cell migration. *Dev Cell* 6:589–596
- Saito D, Tanaka M (2009) Comparative aspects of gonadal differentiation in medaka: a conserved role of developing oocytes in sexual canalization. *Sex Dev* 3:99–107
- Saito T, Fujimoto T, Maekawa S, Inoue K, Tanaka M, Arai K, Yamaha E (2006) Visualization of primordial germ cells in vivo using GFP-*nos1* 3'UTR mRNA. *Int J Dev Biol* 50:691–700
- Saito D, Morinaga C, Aoki Y, Nakamura S, Mitani H, Furutani-Seiki M, Kondoh H, Tanaka M (2007) Proliferation of germ cells during gonadal sex differentiation in medaka: insights from germ cell depleted mutant *zenzai*. *Dev Biol* 310:280–290
- Sasado T, Yasuoka A, Abe K, Mitani H, Furutani-Seiki M, Tanaka M, Kondoh H (2008) Distinct contributions of CXCR4b and CXCR7/RDC1 receptor systems in regulation of PGC migration revealed by medaka mutants *kazura* and *yanagi*. *Dev Biol* 320:328–329
- Satoh N, Egami N (1972) Sex differentiation of germ cells in the teleost, *Oryzias latipes*, during normal embryonic development. *J Embryol Exp Morphol* 28:385–395
- Sekido R, Lovell-Badge R (2008) Sex determination involves synergistic action of SRY and SF1 on a specific *Sox9* enhancer. *Nature (Lond)* 453:930–934

- Shiraishi E, Yoshinaga N, Miura T, Yokoi H, Wakamatsu Y, Abe S-I, Kitano T (2008) Müllerian inhibiting substance is required for germ cell proliferation during early gonadal differentiation in medaka (*Oryzias latipes*). *Endocrinology* 149:1813–1819
- Siegfried KR, Nusslein-Volhard C (2008) Germline control of female sex determination in zebrafish. *Dev Biol* 324:277–287
- Strasser MJ, Mackenzie NC, Dumstrei K, Nakkrasae LL, Stebler J, Raz E (2008) Control over the morphology and segregation of zebrafish germ cell granules during embryonic development. *BMC Dev Biol* 8:58
- Suzuki A, Tanaka M, Shibata N, Nagahama Y (2004) Expression of aromatase mRNA and effects of aromatase inhibitor during ovarian development in the medaka, *Oryzias latipes*. *J Exp Zool* 301:266–273
- Suzuki H, Tsuda M, Kiso M, Saga Y (2008) Nanos3 maintains the germ cell lineage in the mouse by suppressing both Bax-dependent and -independent apoptotic pathways. *Dev Biol* 318:133–142
- Takahashi H (1977) Juvenile hermaphroditism in the zebrafish *Brachydanio rerio*. *Bull Fac Fish Hokkaido Univ* 28:57–65
- Tanaka M, Saito D, Morinaga C, Kurokawa H (2008) Cross talk between germ cells and gonadal somatic cells is critical for sex differentiation of the gonads in the teleost fish, medaka (*Oryzias latipes*). *Dev Growth Differ* 50:273–278
- von Hofsten J, Larsson A, Olsson PE (2005) Novel steroidogenic factor-1 homolog (*ffld*) is coexpressed with anti-Müllerian hormone (*AMH*) in zebrafish. *Dev Dyn* 233:595–604
- Wang XW, Bartfai R, Sleptsova-Freidrich I, Orban L (2007) The timing and extent of ‘juvenile ovary’ phase are highly variable during zebrafish testis differentiation. *J Fish Biol Suppl A* 70:33–44

Chapter 15

Frequent Turnover of Sex Chromosomes in the Medaka Fishes

Yusuke Takehana

Abstract The mechanisms that govern the decision to become male or female are diverse, ranging from environmental to strict sex chromosomal systems. In vertebrates, only two sex-determining genes have been isolated: *SRY/Sry* from mammals and *DMY* from the medaka, *Oryzias latipes*. In contrast to the widespread distribution of *SRY/Sry* in mammals, *DMY* is absent in most *Oryzias* species, suggesting that closely related species have different sex-determining genes. Recent studies have demonstrated that *Oryzias* species have different sex determination systems (XX/XY and ZZ/ZW). Furthermore, the sex chromosomes differ in their origin and degree of differentiation. These findings suggest the repeated creation of new sex chromosomes from autosomes during evolution of *Oryzias* fishes, possibly in association with the formation of new sex-determining genes.

15.1 Introduction

Almost all vertebrates have two sexes, male and female. Despite such universal occurrence, sex is determined by a variety of mechanisms. Among the major vertebrate groups, sex determination is controlled by highly divergent genetic and environmental factors: these include male heterogamety (XX/XY system), as typified by mammals; female heterogamety (ZZ/ZW system), in birds and snakes; and environmental sex determination (e.g., temperature-dependent sex determination in alligators). Some vertebrates with genetic sex determination have highly differentiated sex chromosomes. The X or Z chromosomes are large and gene rich, whereas the Y or W chromosomes are smaller, highly heterochromatic, and contain few genes. In mammals, the sex-determining gene *SRY/Sry* is located on the Y chromosome (Gubbay et al. 1990; Sinclair et al. 1990). However, no equivalent genes have

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been found in nonmammalian vertebrates until recently. Thus, the molecular mechanisms that control such diverse sex determination systems are largely unknown.

Fish possess a variety of sex determination systems, ranging from environmental to strictly genetic sex determination, such as the XX/XY and ZZ/ZW systems (Devlin and Nagahama 2002). Because of this variety, they are an ideal group for evolutionary studies of sex determination and sex chromosomes. Furthermore, most species with genetically determined sex have no cytogenetically distinct sex chromosomes, except for some species with heteromorphic sex chromosomes. The first sex-determining gene to be found in nonmammalian vertebrates was isolated from the medaka, *Oryzias latipes*. This species has an XX/XY sex determination system in which the *DMY* gene was identified as a Y-specific sex-determining gene (Matsuda et al. 2002, 2007). This discovery was a breakthrough that has allowed more detailed examination of the diversity of molecular mechanisms of sex determination. Given this, the medaka and its related species are an excellent model system for analyzing the genetic and molecular mechanisms underlying the evolution of sex determination and sex chromosomes.

In this chapter, I survey recent studies involving sex determination systems and sex chromosomes in medaka fishes. Then, I discuss the possible mechanisms underlying these diverse sex determination systems.

15.2 Evolutionary Origin of the Medaka Sex-Determining Gene, *DMY*

The *DMY* gene encodes a putative transcription factor belonging to the family of DM-domain proteins. All members of this gene family have the conserved DNA-binding motif, originally found in two proteins, DSX in *Drosophila melanogaster* and MAB-3 in *Caenorhabditis elegans* (Raymond et al. 1998). One of these genes, *DMRT1* (DM-related transcription factor 1), has been implicated in male sexual development in vertebrates (Zarkower 2001). *DMY* and *DMRT1* share a high similarity (approximately 80% at the amino acid level), suggesting that *DMY* arose through an interchromosomal duplication of the autosomal *DMRT1* gene (Matsuda et al. 2002; Nanda et al. 2002). Kondo et al. (2006) sequenced Y-specific and adjacent regions and compared these to the X chromosome. Their results indicate that the Y-specific region, including *DMY*, on linkage group 1 (LG1) is derived from the duplication of a 43-kb fragment from LG9. *DMY* appears to be the only functional gene in the Y-specific region because all other co-duplicated genes have degenerated. The Y-specific region has accumulated large stretches of repetitive sequences and duplicated pieces of DNA from elsewhere in the genome, thereby growing to 258 kb. These findings suggest that *DMY* originated from a duplicated copy of autosomal *DMRT1* and has since acquired a new function as a sex-determining gene.

In contrast to the widespread distribution of the *Sry* gene in mammals (Graves 2002), the *DMY* gene is absent in other fish species, even in closely related

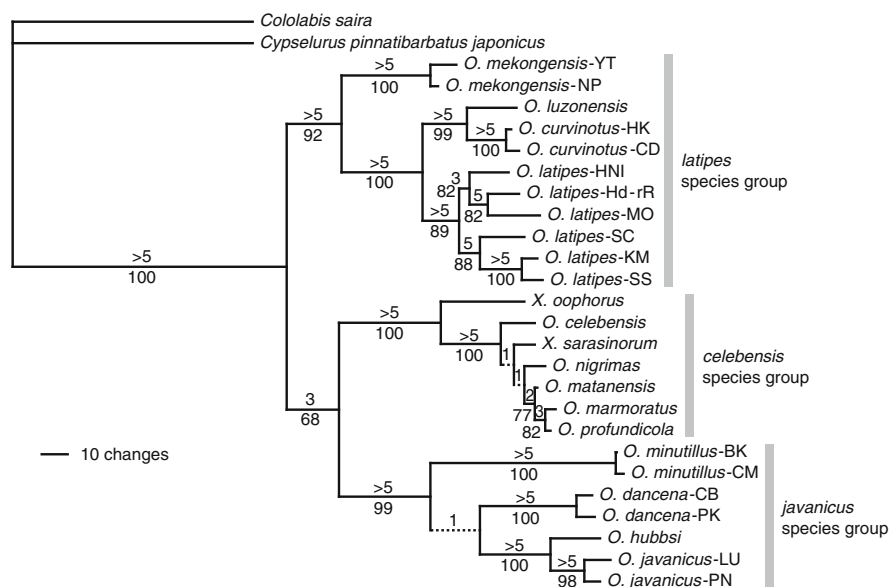


Fig. 15.1 Molecular phylogeny of medaka species inferred from nuclear and mitochondrial DNA sequences (Takehana et al. 2005). Numbers above and below the internal branches indicate decay indices and bootstrap values, respectively. Medaka fishes are divided into three monophyletic groups: *javanicus*, *latipes*, and *celebensis* species groups. *O.*, *Oryzias*; *X.*, *Xenopoecilus*

species of the same genus (Kondo et al. 2003). The genus *Oryzias* contains about 20 species and has been divided into three monophyletic species groups: *latipes*, *javanicus*, and *celebensis* (Fig. 15.1; Takehana et al. 2005). Only two species (*O. latipes* and *Oryzias curvinotus*) in the *latipes* group have the *DMY* gene on the homologous Y chromosome (Matsuda et al. 2003; Kondo et al. 2004). Furthermore, molecular phylogenetic analyses of the *DMY* and *DMRT1* genes from *Oryzias* species suggest that the duplication of *DMRT1* (generating the *DMY* gene) occurred within the *latipes* group lineage (Kondo et al. 2004). These findings suggest that *DMY* has evolved recently, and that *Oryzias* fishes in the remaining species groups (*javanicus* and *celebensis*) possess a different sex-determining gene or genes.

15.3 Sex Determination Systems in *Oryzias*

Identification of the sex-determining genes in these *Oryzias* species first requires an understanding of the sex determination system. One method for elucidating the sex determination system involves progeny testing of sex-reversed fish obtained from androgen- or estrogen-treated parents (Hamaguchi et al. 2004). Among the androgen-treated males that have an XX/XY system, the sex-reversed males

produce all-female progeny because both parents are XX. In contrast, among the estrogen-treated females that have a ZZ/ZW system, sex-reversed females produce all-male progeny, because both parents are ZZ. Using this test, it was demonstrated that *Oryzias luzonensis*, *Oryzias mekongensis*, and *Oryzias dancena* have an XX/XY system, whereas *Oryzias hubbsi* has a ZZ/ZW system (Hamaguchi et al. 2004; Takehana et al. 2007a, b).

An alternate strategy for identifying the sex determination system involves segregation analysis using sex-linked markers. In the medaka, more than 1,000 expression sequence tags (ESTs) have been mapped to all chromosomes. The majority of these EST primers can also be used in closely related species. The medaka ESTs were randomly amplified via polymerase chain reaction (PCR) to identify nucleotide polymorphisms in the amplified fragments between the parents. For those markers displaying polymorphisms between the parents, the F_1 and/or backcross progeny were genotyped and analyzed to determine whether such polymorphisms co-segregated with phenotypic sex. As a result, sex-linked DNA markers were successfully isolated in six species: *O. luzonensis*, *O. mekongensis*, *O. dancena*, *Oryzias minutillus*, *O. hubbsi*, and *Oryzias javanicus* (Tanaka et al. 2004; Takehana et al. 2007a, b, 2008; Nagai et al. 2008). Sex-linked markers in the former four species displayed male-heterogametic inheritance, while those in the latter two species displayed female-heterogametic inheritance. These findings demonstrate that both XX/XY and ZZ/ZW sex determination systems exist in the genus *Oryzias*.

15.4 The Origins of Sex Chromosomes in *Oryzias*

Using isolated sex-linked markers, a linkage map of the sex chromosomes was constructed in each species. Figure 15.2 shows the sex-linkage maps of *O. dancena* and *O. hubbsi* (Takehana et al. 2007a,b). All sex-linked ESTs for these species were located on LG10 and LG5 of *O. latipes*, respectively. Furthermore, the gene order was completely conserved between the sex-linkage map and the physical map of *O. latipes*, which suggests that the sex chromosomes of *O. dancena* and *O. hubbsi* are homologous to the autosomes (LG10 and LG5) of *O. latipes*, whose sex chromosomes are located on LG1. Fluorescence in situ hybridization (FISH) mapping of the sex-linked markers on the metaphase chromosomes confirmed there was no homology among these different sex chromosomes (Fig. 15.3), suggesting they originated independently. Similar results were obtained in four other species: *O. luzonensis*, *O. mekongensis*, *O. javanicus*, and *O. minutillus* (Tanaka et al. 2004; Takehana et al. 2008; Nagai et al. 2008). In summary, the sex chromosomes for *O. luzonensis*, *O. mekongensis*, *O. dancena*, *O. hubbsi*, *O. javanicus*, and *O. minutillus* were homologous to autosomes LG12, LG2, LG10, LG8, LG5, and LG16 of *O. latipes*, respectively (Fig. 15.4), suggesting that the different sex chromosomes in each *Oryzias* species have evolved independently.

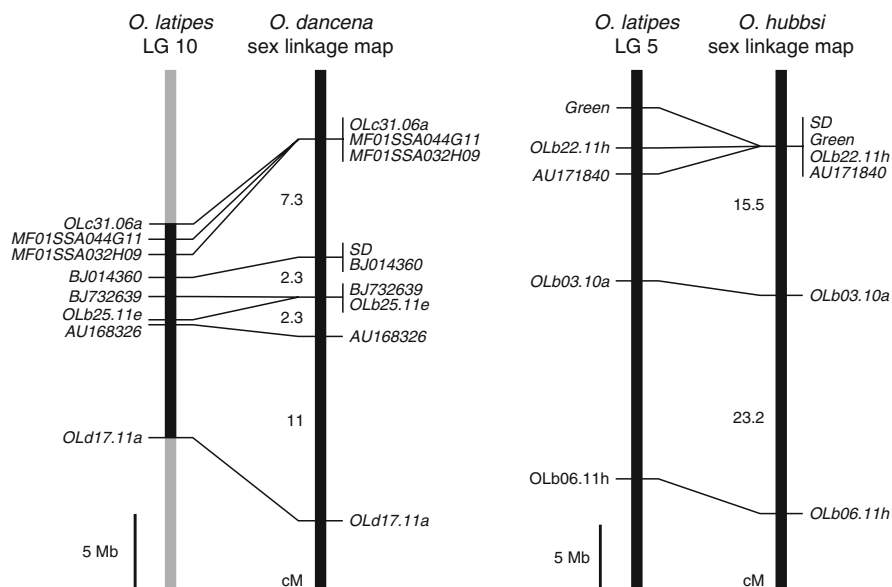


Fig. 15.2 Sex-linkage maps of *Oryzias dancena* and *Oryzias hubbsi*, and corresponding physical maps of *Oryzias latipes* (Takehana et al. 2007a, b). Lines between the chromosomes connect the positions of the orthologous gene pairs. The distances between flanking markers are shown in centimorgans (cM) or as physical length (Mb). Map positions for genes and distances in *O. latipes* were obtained from the draft genome sequence data (<http://dolphin.lab.nig.ac.jp/medaka/>)

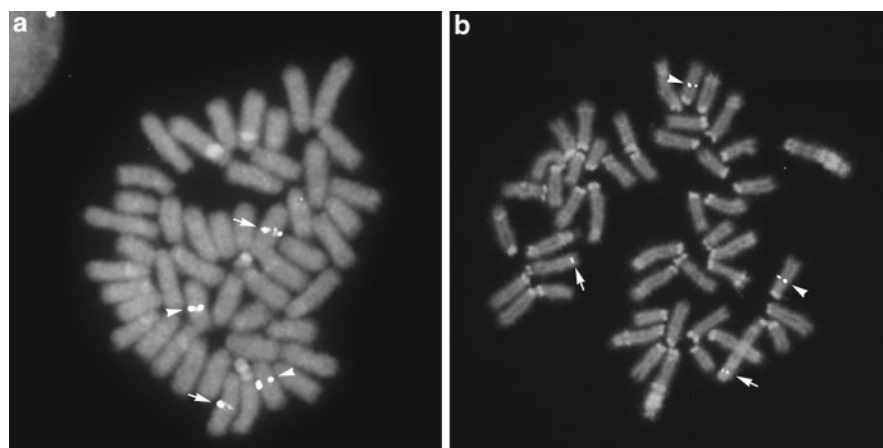


Fig. 15.3 Fluorescence in situ hybridization (FISH) mapping of sex-linked markers on meta-phase chromosomes. (a) Chromosomal location of the *Oryzias dancena* sex chromosomal marker *BJ014360* (fosmid *Od38_01*, arrowheads) and the *O. latipes* sex chromosomal marker *SLI* (BAC *Md0173J11*, arrows) on male chromosomes of *O. dancena* (Takehana et al. 2007a). (b) Chromosomal location of the *O. hubbsi* sex chromosomal marker *OLb22.11h* (fosmid *F10_01*, arrows) with the *O. dancena* sex chromosomal marker, *Od38_01* (arrowheads) on female chromosomes of *O. hubbsi* (Takehana et al. 2007b)

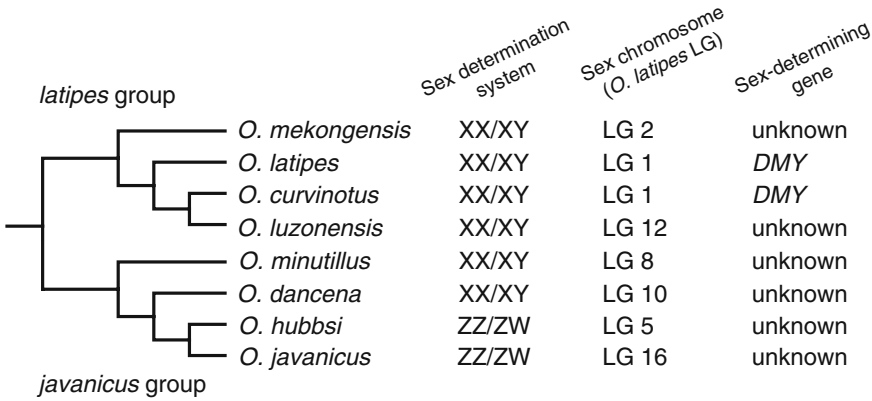


Fig. 15.4 Summary of sex determination systems and sex chromosomes in *Oryzias* species. The sex chromosome in each species is shown as the homologous linkage group (LG) in *O. latipes*

15.5 Differentiation of Sex Chromosomes in *Oryzias*

Standard models for the evolution of sex chromosomes hypothesize that the first step is the occurrence of a novel single locus on an autosome. Heterozygosity at this locus leads to the development of one sex, and homozygosity to the other sex, thereby establishing a proto-sex chromosome system (Rice 1996; Charlesworth et al. 2005). Therefore, at this stage of evolution, both sex chromosomes are morphologically homomorphic. Heteromorphic sex chromosomes are thought to arise through suppression of recombination around the sex-determining gene, maintaining the nonrecombining region in a constant heterozygous state, and leading to numerous deletions of functional genes and accumulation of the repetitive sequences in a sex-specific chromosome (Y or W). This degenerative process spreads the sex-specific region over almost the entire chromosome. During this process, several genes with sex-specific advantages (such as spermatogenesis) accumulate on the chromosome, as in the human Y chromosome (Skaletsky et al. 2003; Graves 2006).

Morphologically homomorphic sex chromosomes have been observed in several species of medaka, including *O. latipes*, *O. luzonensis*, and *O. dancena* (Matsuda et al. 1998; Takehana et al. 2007a; Tanaka et al. 2007), suggesting that these sex chromosomes are at an early stage of the differentiation process. In these species, sex-reversed XY females and XX males are fully fertile, and YY individuals are viable (Yamamoto 1961; Takehana et al. 2007a; Tanaka et al. 2007). Furthermore, crossing-over of the sex chromosomes occurs over almost the entire length of the chromosome (Matsuda et al. 1999; Kondo et al. 2001; Takehana et al. 2007a; Tanaka et al. 2007). Based on these results, it was hypothesized that there are no functional differences between the X and Y sex chromosomes, other than the sex-determining gene, in these species. In fact, the sequencing around the sex-determining region on the X and Y chromosomes in *O. latipes* has confirmed that the Y-specific region

spans 258 kb and that *DMY* appears to be the only functional gene in the Y-specific region (Kondo et al. 2006). Thus, the Y chromosome contains an extra 258-kb sequence compared with the X chromosome in *O. latipes*.

Conversely, *O. hubbsi* and *O. javanicus* have heteromorphic ZW sex chromosomes, suggesting that differentiation of the sex chromosomes has progressed to a stage at which it becomes cytogenetically visible. In *O. hubbsi*, the W chromosome is larger than the Z chromosome. Two 4'-6-diamidino-2-phenylindole (DAPI)-positive heterochromatin blocks are located near the telomeric region on the W chromosome alone (Fig. 15.5; Takehana et al. 2007b). Furthermore, a telomeric inversion has occurred in either the Z or W chromosome. Similarly, a strong DAPI-positive band is located in the centromeric region of the W chromosome in *O. javanicus* (Fig. 15.6; Takehana et al. 2008). Together, these data suggest that suppression of recombination around the sex-determining locus has permitted subsequent heterochromatization with accumulation of repetitive sequences in the non-recombining region of these W chromosomes. However, a large section of the W chromosome (except for the region around the sex-determining locus) recombines with the Z chromosome in each species, indicating that these recombining regions are pseudo-autosomal. Thus, it is likely that the W chromosome-specific regions are very small and that the rest of the W chromosome retains its original function in these species. Taken together, the data suggest that the sex chromosomes in *O. hubbsi* and *O. javanicus* are slightly differentiated. In summary, *Oryzias* species have several different sex chromosomes that differ in their degree of differentiation.

15.6 Possible Mechanisms for Producing Different Sex Chromosomes

The lack of conservation of sex chromosomes among closely related species is likely to be common in fish. For example, the three-spine stickleback (*Gasterosteus aculeatus*) has a heteromorphic XY sex chromosome pair, corresponding to LG19, whereas the nine-spine stickleback (*Pungitius pungitius*) has a heteromorphic pair that correspond to LG12 (Ross et al. 2009). In addition, neither LG19 nor LG12 markers are sex linked in two other species, *Culaea inconstans* and *Apeltes quadracus*, suggesting independent origins for these sex chromosomes. Different origins of sex chromosomes have also been reported in tilapia (Cnaani et al. 2008) and salmonids (Woram et al. 2003; Phillips et al. 2001, 2005, 2007).

One explanation for the evolution of different sex chromosomes is transposition or translocation of a small region, including the sex-determining gene, throughout the genome without adjacent markers, resulting in the evolution of different sex chromosomes among species. For example, two salmonids, *Oncorhynchus kisutch* and *Oncorhynchus tshawytscha*, share common Y-specific markers adjacent to the sex-determining locus at the chromosome telomeres. However, a large part of the chromosome does not appear to be homologous, thereby supporting this hypothesis (Phillips et al. 2005). In contrast, the sex-determining loci are not located in the telomeric

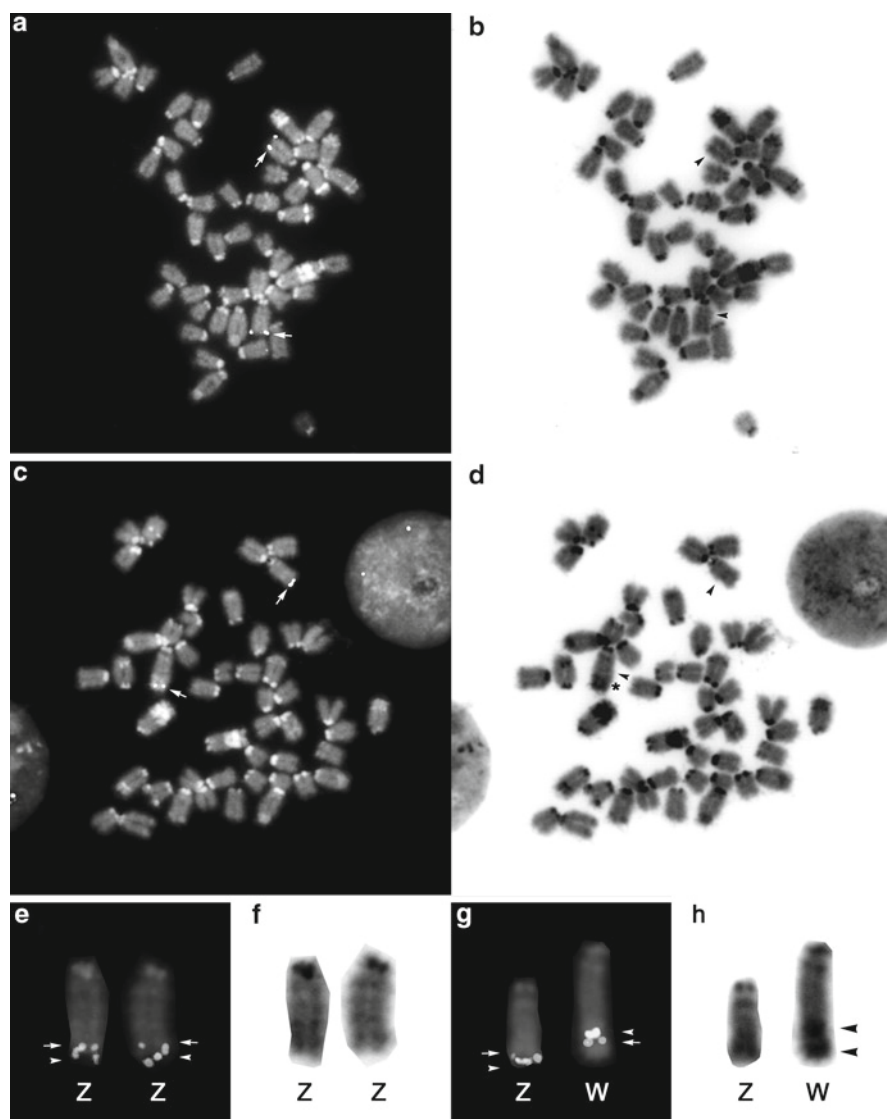


Fig. 15.5 Heteromorphic sex chromosomes of *Oryzias hubbsi* (Takehana et al. 2007b). **a, c** FISH using a fosmid clone (F10_01) containing a sex-linked expressed sequence tag (EST), *OLb22.11h*, on metaphase spreads from a male (**a**) and a female (**c**). Arrows indicate specific hybridization signals. **b, d** Inverted 4'-6-diamidino-2-phenylindole (DAPI) picture of the metaphase in **a** and **c**, respectively. Arrowheads indicate sex chromosomes. The W chromosome has specific heterochromatin densely stained with DAPI (asterisk). **e, g** FISH mapping of two sex-linked markers, *OLb22.11h* (arrows) and *AU171840* (arrowheads) on the ZZ sex chromosomes in the male (**e**) and the ZW chromosomes in the female (**g**). The order of signals on the W chromosome is reversed on the Z chromosome. **f, h** Inverted DAPI picture of the metaphase in **e** and **g**, respectively. Arrowheads indicate the two DAPI-stained heterochromatin blocks on the W chromosomes

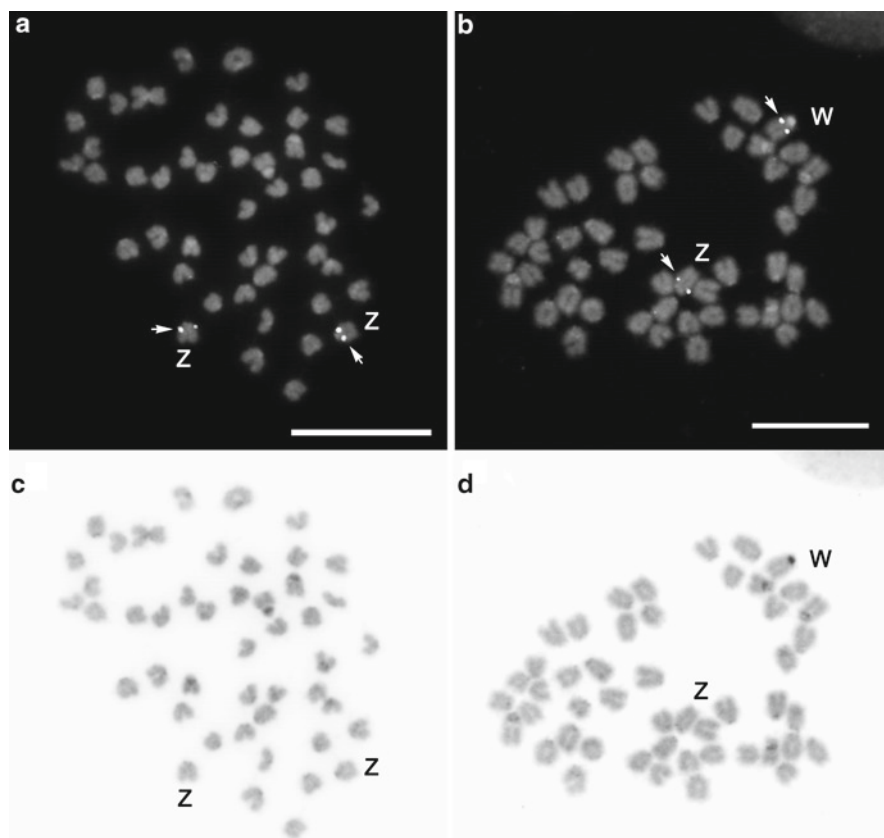


Fig. 15.6 Heteromorphic sex chromosomes of *Oryzias javanicus* (Takehana et al. 2008). **a, b** FISH mapping of a fosmid clone (F11_01) containing a sex-linked expressed sequence tag (EST), *U77376*, on the metaphase spreads from male (**a**) and female (**b**). Arrows indicate specific hybridization signals. **c, d** Inverted DAPI picture of the metaphase in **a** and **b**, respectively. The W chromosome has a specific DAPI band on the centromere. Bars **a, b** 10 μ m

region of the sex chromosomes in most *Oryzias* species. Furthermore, no sex-linked markers are conserved among *Oryzias* species, suggesting that such transposition or translocation of the sex-determining region is unlikely in this genus.

Another explanation for the independent origins of sex chromosomes is that *Oryzias* species have evolved different primary sex-determining genes on different sex chromosomes. Under this scenario, an autosomal gene or a duplicated gene copy may acquire a new mutation that consistently gives rise to either male or female development; this results in a novel sex-determining gene and the emergence of new sex chromosomes from autosomes. In fact, it is assumed that *DMY* emerged recently as the master male sex-determining gene within the *latipes* group lineage, through a duplication of the autosomal *DMRT1* gene (Kondo et al. 2004). Furthermore, this duplication event appears to have occurred in the common

ancestor of *O. latipes*, *O. curvinotus*, and *O. luzonensis*. However, *DMY* is absent from the *O. luzonensis* genome, and its XY sex chromosomes are homologous with an autosome (LG 12) in *O. latipes*, suggesting that another sex-determining gene has supplanted *DMY* within the *O. luzonensis* lineage (Tanaka et al. 2007). Thus, different sex-determining genes can evolve even among closely related species in the genus *Oryzias*.

Although only two sex-determining genes have been isolated in vertebrates, a number of genes have been identified as having roles in the sexual development pathway through studies of human sexual anomalies and/or functional analyses in mice (Koopman 2001). Some of these genes, such as *DMRT1*, also display sex-specific expression patterns in nonmammalian vertebrates and are thought to be involved in sexual development. Furthermore, loss-of-function and gain-of-function mutations in such genes lead to sex-reversal phenotypes, suggesting a potential ability to become the primary sex-determining gene. Therefore, mutations in these genes, or in duplicated copies of such genes, may lead to a novel function as the sex-determining gene in *Oryzias* species that do not possess *DMY*. Identification of sex-determining genes in these *Oryzias* species should reveal which genes and what type of mutations are involved in producing novel sex-determining genes and may help to elucidate the conserved mechanisms in sex determination and differentiation.

15.7 Evolutionary Basis for the Frequent Occurrence of New Sex Chromosomes

Fish have clearly evolved a number of different sex chromosomes. In contrast, the majority of mammals share common sex chromosomes and the sex-determining gene *SRY/Sry*. Why is sex determination so diverse in fish compared with mammals? One hypothesis for this difference involves the fertility of sex-reversed individuals. In fish, sex-reversed individuals obtained by androgen or estrogen treatment are generally fully fertile (Devlin and Nagahama 2002). Furthermore, loss-of-function mutations in the *DMY* gene result in fertile XY females in *O. latipes* (Otake et al. 2006, 2008). Similarly, *DMY*-transgene manipulation produces fertile XX males (Matsuda et al. 2007). Conversely, complete sex reversal is impossible in mammals because a deficiency in *SRY/Sry* results in abnormal sexual development. It is also notable that *Sry*-transgenic mice are sterile. Sterility in mammals is likely to be associated with functional differentiation of the XY sex chromosomes, such as the accumulation of spermatogenic genes on the Y chromosome and the evolution of X-chromosome dosage compensation. This change suggests that, even if sex-reversed males were created by mutation of an autosomal gene, the highly differentiated sex chromosomes would prevent this male from producing offspring. Thus, it would be difficult for a new Y chromosome, which has acquired a new masculinizing mutation, to override the original Y chromosome in mammalian species. In other words, males could not be produced without the Y chromosome in these species. Therefore, the existence of poorly differentiated or

undifferentiated sex chromosomes in fish may have allowed the evolutionary lability of genetic sex determination. *Oryzias* species that have different “young” sex chromosomes may provide an informative model for studying the evolutionary process of turnover of the sex determination mechanisms.

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References

- Charlesworth D, Charlesworth B, Marais G (2005) Steps in the evolution of heteromorphic sex chromosomes. *Heredity* 95:118–128
- Cnaani A, Lee BY, Zilberman N et al (2008) Genetics of sex determination in tilapiine species. *Sex Dev* 2:43–54
- Devlin RH, Nagahama Y (2002) Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture* 208:191–364
- Graves JAM (2002) The rise and fall of *SRY*. *Trends Genet* 18:259–264
- Graves JAM (2006) Sex chromosome specialization and degeneration in mammals. *Cell* 124:901–914
- Gubbay J, Collignon J, Koopman P et al (1990) A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature (Lond)* 346:245–250
- Hamaguchi S, Toyazaki Y, Shinomiya A et al (2004) The XX–XY sex-determination system in *Oryzias luzonensis* and *O. mekongensis* revealed by the sex ratio of the progeny of sex-reversed fish. *Zool Sci* 21:1015–1018
- Kondo M, Nagao E, Mitani H et al (2001) Differences in recombination frequencies during female and male meioses of the sex chromosomes of the medaka, *Oryzias latipes*. *Genet Res* 78:23–30
- Kondo M, Nanda I, Hornung U et al (2003) Absence of the candidate male sex-determining gene *dmrt1b(Y)* of medaka from other fish species. *Curr Biol* 13:416–420
- Kondo M, Nanda I, Hornung U et al (2004) Evolutionary origin of the medaka Y chromosome. *Curr Biol* 14:1664–1669
- Kondo M, Hornung U, Nanda I et al (2006) Genomic organization of the sex-determining and adjacent regions of the sex chromosomes of medaka. *Genome Res* 16:815–826
- Koopman P (2001) The genetics and biology of vertebrate sex determination. *Cell* 105:843–847
- Matsuda M, Matsuda C, Hamaguchi S et al (1998) Identification of the sex chromosomes of the medaka, *Oryzias latipes*, by fluorescence *in situ* hybridization. *Cytogenet Cell Genet* 82:257–262
- Matsuda M, Sotoyama S, Hamaguchi S et al (1999) Male-specific restriction of recombination frequency in the sex chromosomes of the medaka, *Oryzias latipes*. *Genet Res* 73:225–231
- Matsuda M, Nagahama Y, Shinomiya A et al (2002) *DMY* is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature (Lond)* 417:559–563
- Matsuda M, Sato T, Toyazaki Y et al (2003) *Oryzias curvinotus* has *DMY*, a gene that is required for male development in the medaka *O. latipes*. *Zool Sci* 20:159–161
- Matsuda M, Shinomiya A, Kinoshita M et al (2007) The *DMY* gene induces male development in genetically female (XX) medaka fish. *Proc Natl Acad Sci USA* 104:3865–3870
- Nagai T, Takehana Y, Hamaguchi S et al (2008) Identification of the sex-determining locus in the Thai medaka, *Oryzias minutilus*. *Cytogenet Genome Res* 121:137–142
- Nanda I, Kondo M, Hornung U et al (2002) A duplicated copy of *DMRT1* in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. *Proc Natl Acad Sci USA* 99:11778–11783

- Otake H, Shinomiya A, Matsuda M et al (2006) Wild-derived XY sex-reversal mutants in the medaka, *Oryzias latipes*. *Genetics* 173:2083–2090
- Otake H, Hayashi Y, Hamaguchi S et al (2008) The Y chromosome that lost the male-determining function behaves as an X chromosome in the medaka fish, *Oryzias latipes*. *Genetics* 179:2157–2162
- Phillips RB, Konkol NR, Reed KM et al (2001) Chromosome painting supports lack of homology among sex chromosomes in *Oncorhynchus*, *Salmo*, and *Salvelinus* (Salmonidae). *Genetica* 111:119–123
- Phillips RB, Morasch MR, Park LK et al (2005) Identification of the sex chromosome pair in coho salmon (*Oncorhynchus kisutch*): lack of conservation of the sex linkage group with chinook salmon (*Oncorhynchus tshawytscha*). *Cytogenet Genome Res* 111:166–170
- Phillips RB, DeKoning J, Morasch MR et al (2007) Identification of the sex chromosome pair in chum salmon (*Oncorhynchus keta*) and pink salmon (*Oncorhynchus gorbuscha*). *Cytogenet Genome Res* 116:298–304
- Raymond CS, Shamu CE, Shen MM et al (1998) Evidence for evolutionary conservation of sex-determining genes. *Nature (Lond)* 391:691–695
- Rice WR (1996) Evolution of the Y sex chromosome in animals. *Bioscience* 46:331–343
- Ross JA, Urton JR, Boland J et al (2009) Turnover of sex chromosomes in the stickleback fishes (Gasterosteidae). *PLoS Genet* 5:e1000391
- Sinclair AH, Berta P, Palmer MS et al (1990) A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature (Lond)* 346:240–244
- Skaletsky H, Kuroda-Kawaguchi T, Minx PJ et al (2003) The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature (Lond)* 423:825–837
- Takehana Y, Naruse K, Sakaizumi M (2005) Molecular phylogeny of the medaka fishes genus *Oryzias* (Belontiiformes: Adrianichthyidae) based on nuclear and mitochondrial DNA sequences. *Mol Phylogenet Evol* 36:417–428
- Takehana Y, Demiyah D, Naruse K et al (2007a) Evolution of different Y chromosomes in two medaka species, *Oryzias dancena* and *O. latipes*. *Genetics* 175:1335–1340
- Takehana Y, Naruse K, Hamaguchi S et al (2007b) Evolution of ZZ/ZW and XX/XY sex-determination systems in the closely related medaka species, *Oryzias hubbsi* and *O. dancena*. *Chromosoma (Berl)* 116:463–470
- Takehana Y, Hamaguchi S, Sakaizumi M (2008) Different origins of ZZ/ZW sex chromosomes in closely related medaka fishes, *Oryzias javanicus* and *O. hubbsi*. *Chromosome Res* 16:801–811
- Tanaka K, Takehana Y, Naruse K et al (2007) Evidence for different origins of sex chromosomes in closely related medaka fishes: Substitution of the master sex-determining gene. *Genetics* 177:2075–2081
- Woram RA, Gharbi K, Sakamoto T et al (2003) Comparative genome analysis of the primary sex-determining locus in salmonid fishes. *Genome Res* 13:272–280
- Yamamoto T (1961) Progenies of sex-reversal females mated with sex-reversal males in the medaka, *Oryzias latipes*. *J Exp Zool* 146:163–179
- Zarkower D (2001) Establishing sexual dimorphism: conservation amidst diversity? *Nat Rev Genet* 2:175–185

Chapter 16

Function of the Medaka Male Sex-Determining Gene

Manfred Schartl

Abstract Substantial genetic and experimental evidence has accumulated that the medaka male sex-determining gene on the Y chromosome is *dmrt1bY/dmY*. The gene emerged approximately 5–10 million years ago from a duplication of the autosomal *dmrt1a* gene. The *dmrt1bY* gene encodes a nuclear protein of 36 kDa that binds to specific DNA sequences via the conserved DM domain containing two so-called intertwined zinc fingers. Transcriptional regulation by a *Dmrt1a* and *Dmrt1bY* binding site in the *dmrt1bY* promoter and posttranscriptional regulation through a highly conserved element in the 3'-UTR contribute to a specific expression in the somatic cells of the developing male gonad anlage during the sex determination stage and a massive downregulation in the Sertoli cells of the adult testes. The initiating process of sexual development of the undifferentiated gonad toward either ovary or testes, namely the inhibition of proliferation of the primordial germ cells in males, is mediated by *Dmrt1bY*. The molecular mechanisms by which *Dmrt1bY* brings about this effect are unknown so far.

16.1 Introduction

The medaka is a gonochoristic species with a genetic sex determination system of the XY type. There is some evidence that the presence of the Y chromosome is deterministic for the development of a male fish rather than the dosage of an X-linked factor being responsible for determining the onset of female or male development. One case was reported that a fish with a sex chromosome constitution XXY developed as a fertile male (Yamamoto 1963). In nuclear transplantation experiments, a triploid fish was obtained that also had a XXY genotype and became

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a phenotypic male, although it was infertile, probably because of the impossibility of gamete production in uneven ploidy organisms (Bubenshchikova et al. 2007).

The region, which is specific for the Y chromosome, is only 258 kb long and it contains only one structural gene, which can give rise to a functional protein (Kondo et al. 2006). This gene is called *dmrt1bY* (Nanda et al. 2002) or *dmY* (Matsuda et al. 2002). The medaka sex-determining gene is the result of a segmental gene duplication event that happened approximately 5–10 million years ago (MYA) (Kondo et al. 2004). A fragment of linkage group 9 containing the *dmrt1* gene was duplicated and inserted into one chromosome from linkage group 1, which thereby became the Y chromosome. Following the nomenclature rules for designating duplicate genes in fish (http://zfinfo.org/zf_info/nomen.html), the autosomal copy is *dmrt1a* and the Y chromosomal copy is *dmrt1b* (Voff et al. 2003). The suffix “Y” is added to the name of the 1b copy to indicate its chromosomal location and to facilitate recognition of its synonym, *dmY*, for the same gene, which is historically the priority name.

The only other species that has *dmrt1bY* is the sister taxon of *Oryzias latipes*, namely *Oryzias curvinotus* (see Chap. XY). The Y chromosomes of both species are homologous (Kondo et al. 2004), and it is assumed that also the *dmrt1bY*s exert the same function.

The function of *dmrt1bY* as the master regulator of male development is inferred from several facts. The genetic arguments are that it is the single functional gene in the entire male-specific region on the Y chromosome (Kondo et al. 2006) and that mutations in *dmrt1bY* cause XY male-to-female sex-reversal (Matsuda et al. 2002; Otake et al. 2006). In fish of XX genotype that were injected with a construct consisting of a 56-kb *dmrt1bY* coding region and a 60-kb upstream and 1.4-kb downstream noncoding region, full male development was induced in about 20% (Matsuda et al. 2007), which supports the notion that expression of *dmrt1bY* is sufficient for male development.

16.2 Structure of *dmrt1bY*

Both the autosomal *dmrt1a* and the Y-chromosomal *dmrt1bY* gene consist of six exons with exon 1 being noncoding (Fig. 16.1). Exon–intron borders are conserved between the two copies. The coding sequence of both *dmrt1* genes is highly conserved (92.4% for the HNI strain). In contrast, the introns have considerably diverged. The overall similarity is less than 50%, and no blocks of higher conservation have been identified that would indicate shared regulatory elements. This condition is especially true for intron 5, which is much larger in *dmrt1bY* as a result of the insertion of a large sequence from elsewhere in the genome (Kondo et al. 2006).

Also, the 5'-flanking regions of the *dmrt1*s are very different. After the duplication event, the *dmrt1bY* upstream region was enlarged by the insertion of several blocks of repetitive DNA. At least some of these sequences are supposed to be functional because they carry putative transcription factor-binding sites

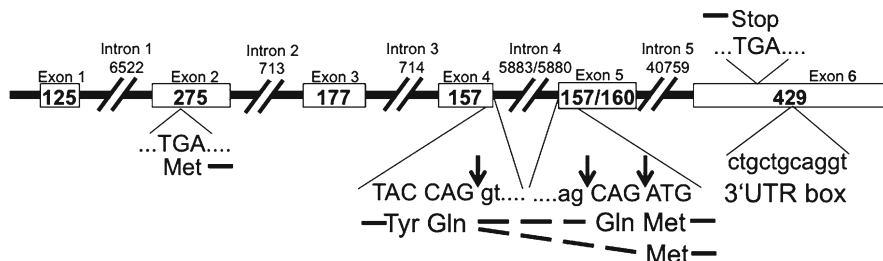


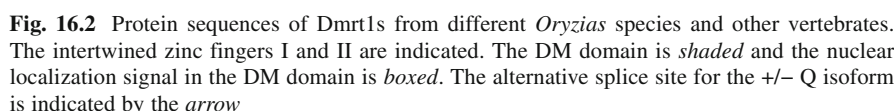
Fig. 16.1 Genomic organization of the *dmrt1bY* gene of medaka (HNI strain). Numbers within boxes represent exons and numbers above introns indicate the size in base pairs (bp). The start and stop codons, as well as the alternative splice site (+/- Q forms) and the motif in the 3'-untranslated region (3'-UTR) involved in gonad-specific expression, are depicted

(Herpin et al., in manuscript) and may contribute to the differences in expression of both gene copies (see following).

A single transcript size of 1.3 kb (*dmrt1bY*) or 1.5 kb (*dmrt1a*), respectively, is produced. The mRNA has a relatively limited half-life of approximately 6 h (Hornung et al. 2007). Because of the presence of two alternative splice acceptor sites for exon 5, the protein sequence can have an additional amino acid (glutamine) at this position (Weiss and Scharlt, unpublished data). The same situation was also found in the *Tetraodon nigroviridis* genome (Lutfalla et al. 2003). Whether this has any functional meaning is unclear. Both isoforms were isolated from a cDNA of transcripts from the *dmrt1bY* genes of medaka and *Oryzias curvinotus* as well as from the autosomal *dmrt1(a)s* of the different *Oryzias* species (Fig. 16.2). In *Tetraodon*, both variants are produced at equal levels in the testes and also in the juvenile female gonad, which may indicate that the alternative splicing is not instrumental in sexual differentiation (Lutfalla et al. 2003). This alternative splicing is not a general situation in fish, as the zebrafish gene can only code for the variant with the single glutamine. In medaka, in addition to the frequent +/- Q variants the only other alternative splicing product found so far was a single cDNA sequence that was lacking exon 5. Conceptual translation resulted in a predicted protein that would have a different and shorter carboxy-terminal domain as the result of a frameshift created at the new exon 4/exon 6 border (Weiss and Scharlt, unpublished data).

The scarcity of alternative splice variants in medaka is in contrast to reports from zebrafish (Postlethwait and Rodriguez, unpublished data) (Guo et al. 2005) about multiple alternatively spliced mRNAs.

The *dmrt1a* gene encodes a protein of 279 amino acids. The *dmrt1bY* gene translates into a product of 267 amino acids (northern medaka, HNI strain) or 273 amino acids (southern medaka, Carbio strain) as the result of nucleotide differences that lead to differences in the sequence and length of the distal carboxy-terminus. Dmrt1a has an apparent size of 37 kDa and Dmrt1bY a slightly lower molecular weight of 36 kDa (Kobayashi et al. 2004). Both proteins are predicted to have the typical domain structure shared by all members of the gene family from flies



(*Drosophila*, Doublesex protein) and worms (*Caenorhabditis elegans*, Mab3 protein) to mammals. There are no apparent differences in the two medaka proteins compared to all other known Dmrt1 protein sequences from fish and other vertebrates including mammals that would indicate a different biochemical function or activity. The amino-terminus in medaka is, as in all other vertebrates except mammals, a short stretch of amino acids for which no secondary or domain structure is predicted; this is followed by the highly conserved DM domain, which is involved in binding to specific DNA motifs. The interaction with the nucleic acid sequence is mediated by an unusual zinc-finger domain that consists of two intertwined Zn²⁺-binding motifs (site I: Cys-Cys-His-Cys; site II: His-Cys-Cys-Cys) (Zhu et al. 2000). For the *Drosophila* and human proteins, it was shown that they bind to the minor groove of the DNA double helix but do not induce bending of the substrate (Zhu et al. 2000). This finding is different from the SRY protein, the male sex-determining factor of most mammals, which also binds to the minor groove of the DNA but has the ability to sharply bend the DNA. Evidently this difference occurs because SRY contacts the minor groove with a different DNA-binding domain, namely an HMG box.

The zinc fingers are followed by a stretch of amino acids that form a “nascent” alpha helix. In the medaka Dmrt1bYs, the first five of these are less conserved whereas the remainder of this region is identical in all vertebrates. From the analysis of the *Drosophila* and human homologs, it can be predicted that also the medaka Dmrt1 have to dimerize for binding to the DNA and that the alpha-helical carboxy-terminal part of the DM domain is required for DNA recognition (Zhu et al. 2000). The DNA target sequence determined for binding to the mammalian protein (Murphy et al. 2007) is present in the medaka genome several times, but it is unknown which of these are functional binding motifs for a transcription factor activity of Dmrt1a and Dmrt1bY. Consistent with such a function, both medaka proteins localize to the nucleus (Hornung et al. 2007). In addition to a predicted highly conserved nuclear localization motif in the DM domain (Lys-Gly-His-Lys-Arg) (Ying et al. 2007), there is a second (not further specified) motif in the carboxy-terminal part of the protein that cooperates in addressing the Dmrt1s to the nuclear compartment (Hornung et al. 2007).

The following carboxy-terminal sequence has a proline/serine-rich domain and is much less conserved than the DM domain (see sequence comparison in Fig. 16.2). Most prominently, a stretch of 41 amino acids present in all tetrapod sequences is missing in medaka as in all other teleost species. This sequence is encoded in the tetrapods by the second half of exon 4, which is missing in fish. None of the known protein domains, families, and functional sites, or the associated patterns and profiles, are predicted by the currently available bioinformatics tools. The biochemical function is unclear. However, it has been proposed that this part of the protein is indispensable for the male-determining function of Dmrt1bY. In all natural sex-reversal mutants analyzed so far that have an altered protein sequence, the DM domain is intact but the carboxy-terminal part is missing (Otake et al. 2006). In the *Drosophila* protein outside the DM domain in the carboxy-terminal tail, a second dimerization domain has been identified (An et al. 1996). As this part

is not conserved on the amino acid sequence level, it is difficult to predict whether this function is conserved in the medaka Dmrt1 proteins.

The Dmrt1a and Dmrt1bY proteins differ at several amino acid positions. There is also considerable sequence variation between the homologous genes of Northern (HNI strain) and Southern (Carbio and HdrR strain) medaka and of *Oryzias curvinotus*. Most of the differences are at positions that are not highly conserved and thus may not have any influence on the function of the protein. Interestingly, the Dmrt1bY protein of *O. curvinotus* is more similar to the autosomal Dmrt1a proteins and does not share most of the changes with the Dmrt1bYs isolated from the different medaka populations, most intriguingly two larger deletions in the amino-terminal domain. However, there is evidence from molecular evolutionary analyses that the DM domain of Dmrt1bY has acquired amino acid changes since it diverged from Dmrt1a that are consistent with an evolution toward a new function (Zhang 2004). The only difference that is shared by all Dmrt1bY sequences at a highly conserved position in the autosomal Dmrt1s is a serine-to-threonine exchange at amino acid position 26. The hypothesis has been put forward that this could influence the DNA sequence recognition and binding (Zhang 2004), but experimental data to substantiate this are lacking. Moreover, a serine-to-threonine exchange is generally regarded as a conservative change, and this part of the zinc-finger domain where the difference is located shows, in general, some variation among species.

16.3 Expression of *dmrt1bY*

The autosomal *dmrt1a* gene is strongly expressed in the testes. The first transcripts are found between 12 and 20 days after hatching (Kobayashi et al. 2004; Nanda et al. 2002; Winkler et al. 2004). The mRNA is localized to the Sertoli cells, whereas in germ cells no expression of the gene can be detected. This finding is somewhat unusual, as in many other organisms including fish there is expression in the germ cells [mouse (Raymond et al. 2000); chicken (Smith et al. 2003); Japanese frog (Aoyama et al. 2003); rice field eel (Huang et al. 2005); and zebrafish (Guo et al. 2005)]. The situation in medaka is, on the other hand, similar to *Takifugu rubripes*, where Dmrt1 is present in Sertoli cells but absent from germ cells (Yamaguchi et al. 2006). A weak expression was seen in the ovary by reverse transcription-polymerase chain reaction (RT-PCR) (Ohmuro-Matsuyama et al. 2003) and could be localized by in situ hybridization to early differentiating oocytes (Winkler et al. 2004). Because of the lack of a Dmrt1a-specific antibody, the protein has not been studied yet.

Transcripts from the *dmrt1bY* gene can be detected from the mid-blastula transition onward (Hornung et al. 2007), when zygotic transcription starts; this is long before the sex determination stage, which is around hatching. The transcripts are not localized to a specific tissue or organ, and it is not known whether they are translated. By RNA in situ hybridization, the first and only local concentration of mRNA is seen in the somatic cells of the embryonic gonad at stage 36. Shortly

thereafter, the protein was shown to appear in the same cell type (Kobayashi et al. 2004). Thus, *dmrt1bY* expression precedes the first appearance of morphological sex differences, that is, the lower number of primordial germ cells (PGCs) in males at hatching stage (Sato and Egami 1972). The timing and location of *dmrt1bY* expression is in perfect agreement with its function as the primary male sex-determining gene.

Importantly, the level of *dmrt1bY* expression at the sex determination stage appears to be critical for its function. Mutants that have reduced expression levels fail to develop as males and become sex-reversed XY females (Matsuda et al. 2002; Otake et al. 2006).

The expression of *dmrt1bY* persists in the somatic compartment of the testes in the Sertoli cells and the epithelial cells of the intratesticular ducts (Kobayashi et al. 2004; Nanda et al. 2002). The level of the mRNA compared to the autosomal *dmrt1a* transcript decreases considerably, which in adult testes is about 50 times higher than *dmrt1bY* (Hornung et al. 2007).

Strangely, there is a high level of *dmrt1bY* transcripts in spleen. As females do not possess this gene, the expression in spleen may have no functional meaning, and it is not known whether these transcripts are ever translated. So far the *dmrt1* genes from all other vertebrates are specifically and exclusively expressed in gonads. This “ectopic” expression in spleen of a “testes” gene may be the result of the diversification process of the promoters of the *dmrt1a* and *dmrt1bY* genes, for example, that *dmrt1bY* may have lost a spleen-specific silencer or gained an enhancer for expression in this organ.

In sex-reversed (estrogen-treated) XY females, *dmrt1bY* is expressed in the ovaries at the same level as in testes (Nanda et al. 2002) from follicular cells of vitellogenic phase oocytes (Suzuki et al. 2005). Also, the early expression in the somatic cells of the undifferentiated gonad is not affected (Suzuki et al. 2005), which is consistent with the function of a master regulator gene for male gonad development. The activity of an upstream regulatory gene has not to be suppressed by the sex-reverting hormone treatment; this has to switch off only all those genes that have a function in the differentiation of the testes and would interfere with ovarian development.

16.4 Transcriptional Regulation of *dmrt1bY*

The 5'-flanking regions of *dmrt1bY* and *dmrt1a*, which putatively contain the promoter and transcriptional control elements, are quite divergent. Most prominently the 5'-flanking region of *dmrt1bY* is enlarged by the insertion of several repeats, which are absent from *dmrt1a* (Herpin et al., in manuscript). One of these repeats, which is present elsewhere in the medaka genome as a transposable element more than 50 times, contains a predicted perfect binding site for Dmrt1 according to the consensus sequence determined for the mammalian homolog (Murphy et al. 2007). Both Dmrt1a and Dmrt1bY proteins bind specifically to this site in the *dmrt1bY* promoter. In promoter assays, this binding leads to a transcriptional downregulation,

indicating that *Dmrt1bY* exerts a feedback inhibition on its own expression and that *Dmrt1a* can shut down the expression of its Y-chromosomal counterpart (Herpin et al., in manuscript). It should be noted that the opposite regulation, however, does not take place as this binding site is not present in the promoter of the autosomal *dmrt1a* gene. The downregulation of the *dmrt1bY* promoter may explain the much higher expression levels of the autosomal copy in the adult testes.

The action of the *Dmrt1* proteins on the *dmrt1bY* promoter shows that one function of *Dmrt1bY* is that of a transcriptional repressor. This finding does not exclude that in a different promoter context or in the presence of different cofactors the protein can also act as a transcriptional activator.

16.5 Posttranscriptional Control of *dmrt1bY* Expression

A major regulation of *dmrt1bY* and *dmrt1a* expression occurs on the posttranscriptional level. Both transcripts contain a conserved 11-bp sequence motif (CUG CUGCAGGU) in the 3'-untranslated region (UTR). One or more proteins bind specifically to this element and mediate two effects to the mRNA. In vitro reporter gene assays revealed a two- to threefold upregulation of translation in the medaka spermatogonial cell line SG3 compared to medaka embryonic stem cell-like cell line MES1 and medaka fibroblast (OL-17) cells. Importantly, the sequence also confers stability of the mRNA in the embryonic gonad primordium but degradation in all other organs and tissues. During stages 24–26 it stabilizes reporter mRNAs injected into the one-cell stage embryo in the pregonadal somatic mesoderm as well as in PGCs. At stages 34–36, mRNA stabilization is restricted to a subset of the PGC population, which comprises about half of this cell type (Herpin et al. 2009). The action of the 3'-UTR motif in adult medaka, as well as the identity of the binding protein(s), are still unknown.

Interestingly this sequence motif is highly conserved in the 3'-UTRs of homologous *dmrt1* genes from other *Oryzias* species and also from many organisms ranging from insects to mice and humans.

16.6 Regulation of PGC Proliferation by *Dmrt1bY*

The first recognizable morphological difference during development between male and female is a difference in PGC numbers at the hatching stage and thereafter (see Chap. 14). Although in the female hatchling PGCs start to proliferate and enter meiosis, the PGC number remains constant in males for a period up to 10–15 days after hatching (dah). Only thereafter do they resume proliferation and differentiate into male germ cells, accompanied by the differentiation of the somatic component of the gonad primordium toward testes. Expression of *dmrt1bY* in the somatic cells of the embryonic gonad precedes this process. Thus, it was reasonable to expect that *dmrt1bY* functions by controlling the proliferation of PGCs in the male embryo

at the sex determination state. Support for this hypothesis came from the effects of knocking down *dmrt1bY* expression. After transient inhibition of *dmrt1bY* expression during embryonic development up to hatching, an increase in PGC numbers was seen in XX female embryos (Herpin et al. 2007; Paul-Prasanth et al. 2006). Using bromodeoxyuridine (BrdU) staining it was revealed that the PGCs in the knockdown fish have a higher proliferation rate, comparable to those in control XX gonads (Herpin et al. 2007). Ectopic overexpression of Dmrt1bY in embryos and in cell culture leads to strong accumulation cells in the G₂ phase of the cell cycle. Interestingly, not only the Dmrt1bY overexpressing cell but also nontransfected cells showed this growth arrest effect (Herpin et al. 2007). Thus, it can be inferred that the function of *dmrt1bY* is to mediate a G₂ arrest in a cell-autonomous and non-cell-autonomous manner. During the sex determination stage, the non-cell-autonomous effect is crucial to maintain the proliferative silence of the PGCs in male embryos. In the adult testes, the cell-autonomous action of Dmrt1bY (and Dmrt1a) becomes more prevalent. Sertoli cells are postmitotic cells in the male gonad, and both Dmrt1s may be involved in the induction and maintenance of this state, which is supported by the fact that mouse mutants with a defective *dmrt1* gene show an overproliferation of Sertoli cells and develop testicular tumors (Fahrioglu et al. 2007; Kim et al. 2007; Raymond et al. 2000).

16.7 Dmrt1bY and Sex Reversal

In nature and in laboratory strains, spontaneous XX female-to-male sex reversal has been observed (Aida 1936; Nanda et al. 2003; Shinomiya et al. 2004; Yamamoto 1975). Those fish are rare in the wild populations (1%), but in some laboratory strains their frequency can reach 20%. Their occurrence is intriguing, because they demonstrate that Dmrt1bY function is totally dispensable for male sex determination. Interestingly, at high temperatures, which increase the frequency of the XX males under laboratory conditions (Sato et al. 2005), a precocious expression of *dmrt1a* exactly at the time, when usually *dmrt1bY* is active, was observed (Hattori et al. 2007).

The underlying cause, in particular an involvement of *dmrt1a*, that leads to XX males collected from the wild is unclear, whereas the naturally occurring XY females could be traced back either to mutations, leading to carboxy-terminal truncations of the Dmrt1bY, or to reduced expression of *dmrt1bY* at the sex determination stage (Otake et al. 2006).

16.8 Putative Targets and Interactors of Dmrt1bY

So far neither a target gene on which Dmrt1bY would act as a transcription factor nor any interacting protein has been identified, although we have to anticipate with confidence that they exist. Possible target genes have first to be searched in the

known factors that are involved in regulation of sexual development and act around the time when sex determination takes place in medaka around hatching.

Sox9b (earlier designated *sox9a2*) is expressed in one of the two distinct somatic gonadal cell lineages already before formation of the gonadal primordium (Nakamura et al. 2006, 2008). It is then detectable in the somatic cells of the developing gonads of both sexes and, importantly, becomes reduced in the female gonads (Klüver et al. 2005; Nakamoto et al. 2005). *Sox 9a*, the co-ortholog of *Sox 9b* appears not to be expressed at the critical stage of gonad determination (Klüver et al. 2005; Yokoi et al. 2002).

Similar to *sox 9b/sox9a2*, the orphan nuclear receptor steroidogenic factor SF-1/ftz-f1 is expressed early on in the pregonadal soma in the common precursors of Sertoli and granulosa cells (Nakamura et al. 2006) and is present during the critical period of *Dmrt1bY* action.

The anti-Müllerian hormone (*Amh*) and its receptor *Amh-rII* play an important role in the reciprocal cross-talk of the germ cells and the somatic cells, which is a decisive process in sex determination (Tanaka et al. 2008). Both are coexpressed during early gonad development in the somatic cells of both sexes, with the *amh-rII* gene becoming downregulated later in the ovary (Klüver et al. 2007). As *Dmrt1bY*, the components of this signaling system regulate germ cell numbers.

The role of *Wtl* in the *Dmrt1bY*-governed sex determination process of medaka is not clear. Knockdown of either *wtl1a* or *wtl1b* expression had no influence on the somatic cells of the gonad primordium and the germ cells at the sex determination stage. The double knockdown of both *wtl* genes resulted, however, in a reduction of PGC numbers, indicating a synergistic role of both genes for PGC maintenance and/or survival (Klüver et al. 2009). The effect on the PGCs appears to occur before *dmrt1bY* is expressed; therefore, a link to *Dmrt1bY* function is difficult to see at the moment.

16.9 Conclusion, Questions, and Future Directions

Since *dmrt1bY* was found in 2002 (Matsuda et al. 2002; Nanda et al. 2002), much evidence has accumulated that it is responsible for determining in males the development of the undifferentiated gonad toward a testes. On the other hand, the ease with which XX males can be produced shows that the function of *Dmrt1bY* is not necessary for male development, but it is sufficient, as the transgenic experiments convincingly have proven.

Do the *dmrt1a* and *dmrt1bY* genes perform the same job? Taking the distinct expression patterns into account, the answer appears to be “no.” Temporal and spatial expression only overlap in Sertoli cells of the adult testes. But, even here the 50-fold-higher expression of *dmrt1a* compared to *dmrt1bY* indicates that the main function in this cell type is executed by the autosomal gene. Do the two proteins have similar or the same biochemical properties? From their structure this appears likely, but experimental proof is missing so far. There is a good indication, however,

namely that in temperature-induced sex reversal *dmrt1a* is expressed in the XX fish exactly at the time when usually *dmrt1bY* is expressed in the undifferentiated gonad. This finding could indicate that the autosomal gene is recruited here to fulfil the male-determining function, which in the normal male embryo is executed by the Y chromosomal *dmrt1bY*.

On the other hand, there are findings that would speak against *dmrt1a* being able to substitute for *dmrt1bY* function. Although in transient transgenic fish overexpression of *dmrt1bY* under control of the CMV promoter resulted in the production of fertile XX males, the overexpression of *dmrt1a* did not lead to sex reversal (Matsuda et al. 2007).

For enhancing our understanding of the function of the male sex determining gene, *dmrt1bY*, and its autosomal counterpart, *dmrt1a*, it is essential to find its direct and indirect effector and targets. It will also be important to find partners with which it interacts on the protein–protein level.

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References

- Aida T (1936) Sex reversal in *Aplocheilus latipes* and a new explanation of sex differentiation. *Genetics* 21:136–153
- An W, Cho S, Ishii H, Wensink PC (1996) Sex-specific and non-sex-specific oligomerization domains in both of the doublesex transcription factors from *Drosophila melanogaster*. *Mol Cell Biol* 16:3106–3111
- Aoyama S, Shibata K, Tokunaga S, Takase M, Matsui K, Nakamura M (2003) Expression of Dmrt1 protein in developing and in sex-reversed gonads of amphibians. *Cytogenet Genome Res* 101:295–301
- Bubenshchikova E, Kaftanovskaya E, Motosugi N, Fujimoto T, Arai K, Kinoshita M, Hashimoto H, Ozato K, Wakamatsu Y (2007) Diploidized eggs reprogram adult somatic cell nuclei to pluripotency in nuclear transfer in medaka fish (*Oryzias latipes*). *Dev Growth Differ* 49:699–709
- Fahrioglu U, Murphy MW, Zarkower D, Bardwell VJ (2007) mRNA expression analysis and the molecular basis of neonatal testis defects in Dmrt1 mutant mice. *Sex Dev* 1:42–58
- Guo Y, Cheng H, Huang X, Gao S, Yu H, Zhou R (2005) Gene structure, multiple alternative splicing, and expression in gonads of zebrafish Dmrt1. *Biochem Biophys Res Commun* 330:950–957
- Hattori RS, Gould RJ, Fujioka T, Saito T, Kurita J, Strussmann CA, Yokota M, Watanabe S (2007) Temperature-dependent sex determination in Hd-rR medaka *Oryzias latipes*: gender sensitivity, thermal threshold, critical period, and DMRT1 expression profile. *Sex Dev* 1:138–146
- Herpin A, Schindler D, Kraiss A, Hornung U, Winkler C, Scharl M (2007) Inhibition of primordial germ cell proliferation by the medaka male determining gene Dmrt1 bY. *BMC Dev Biol* 7:99
- Herpin A, Nakamura S, Wagner TU, Tanaka M, Scharl M (2009) A highly conserved cis-regulatory motif directs differential gonadal synexpression of Dmrt1 transcripts during gonad development. *Nucleic Acids Res* 37:1510–1520
- Hornung U, Herpin A, Scharl M (2007) Expression of the male determining gene *dmrt1bY* and its autosomal orthologue *dmrt1a* in medaka. *Sex Dev* 1:197–206

- Huang X, Guo Y, Shui Y, Gao S, Yu H, Cheng H, Zhou R (2005) Multiple alternative splicing and differential expression of *dmrt1* during gonad transformation of the rice field eel. *Biol Reprod* 73:1017–1024
- Kim S, Bardwell VJ, Zarkower D (2007) Cell type-autonomous and non-autonomous requirements for *Dmrt1* in postnatal testis differentiation. *Dev Biol* 307:314–327
- Klüver N, Kondo M, Herpin A, Mitani H, Scharl M (2005) Divergent expression patterns of *Sox9* duplicates in teleosts indicate a lineage specific subfunctionalization. *Dev Genes Evol* 215:297–305
- Klüver N, Pfennig F, Pala I, Storch K, Schlieder M, Froschauer A, Gutzeit HO, Scharl M (2007) Differential expression of anti-Müllerian hormone (*amh*) and anti-Müllerian hormone receptor type II (*amhrII*) in the teleost medaka. *Dev Dyn* 236:271–281
- Klüver N, Herpin A, Braasch I, Driessle J, Scharl M (2009) Regulatory back-up circuit of medaka *Wt1* co-orthologs ensures PGC maintenance. *Dev Biol* 325:179–188
- Kobayashi T, Matsuda M, Kajiura-Kobayashi H, Suzuki A, Saito N, Nakamoto M, Shibata N, Nagahama Y (2004) Two DM domain genes, *DMY* and *DMRT1*, involved in testicular differentiation and development in the medaka, *Oryzias latipes*. *Dev Dyn* 231:518–526
- Kondo M, Nanda I, Hornung U, Schmid M, Scharl M (2004) Evolutionary origin of the medaka Y chromosome. *Curr Biol* 14:1664–1669
- Kondo M, Hornung U, Nanda I, Imai S, Sasaki T, Shimizu A, Asakawa S, Hori H, Schmid M, Shimizu N, Scharl M (2006) Genomic organization of the sex-determining and adjacent regions of the sex chromosomes of medaka. *Genome Res* 16:815–826
- Lutfalla G, Roest Crollius H, Brunet FG, Laudet V, Robinson-Rechavi M (2003) Inventing a sex-specific gene: a conserved role of *DMRT1* in teleost fishes plus a recent duplication in the medaka *Oryzias latipes* resulted in *DMY*. *J Mol Evol* 57(suppl 1):S148–S153
- Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Morrey CE, Shibata N, Asakawa S, Shimizu N, Hori H, Hamaguchi S, Sakaizumi M (2002) *DMY* is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature (Lond)* 417:559–563
- Matsuda M, Shinomiya A, Kinoshita M, Suzuki A, Kobayashi T, Paul-Prasanth B, Lau EL, Hamaguchi S, Sakaizumi M, Nagahama Y (2007) *DMY* gene induces male development in genetically female (XX) medaka fish. *Proc Natl Acad Sci USA* 104:3865–3870
- Murphy MW, Zarkower D, Bardwell VJ (2007) Vertebrate DM domain proteins bind similar DNA sequences and can heterodimerize on DNA. *BMC Mol Biol* 8:58
- Nakamoto M, Suzuki A, Matsuda M, Nagahama Y, Shibata N (2005) Testicular type *Sox9* is not involved in sex determination but might be in the development of testicular structures in the medaka, *Oryzias latipes*. *Biochem Biophys Res Commun* 333:729–736
- Nakamura S, Kobayashi D, Aoki Y, Yokoi H, Ebe Y, Wittbrodt J, Tanaka M (2006) Identification and lineage tracing of two populations of somatic gonadal precursors in medaka embryos. *Dev Biol* 295:678–688
- Nakamura S, Aoki Y, Saito D, Kuroki Y, Fujiyama A, Naruse K, Tanaka M (2008) *Sox9b/sox9a2-EGFP* transgenic medaka reveals the morphological reorganization of the gonads and a common precursor of both the female and male supporting cells. *Mol Reprod Dev* 75:472–476
- Nanda I, Kondo M, Hornung U, Asakawa S, Winkler C, Shimizu A, Shan Z, Haaf T, Shimizu N, Shima A, Schmid M, Scharl M (2002) A duplicated copy of *DMRT1* in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. *Proc Natl Acad Sci USA* 99:11778–11783
- Nanda I, Hornung U, Kondo M, Schmid M, Scharl M (2003) Common spontaneous sex-reversed XX males of the medaka, *Oryzias latipes*. *Genetics* 163:245–251
- Ohmuro-Matsuyama Y, Matsuda M, Kobayashi T, Ikeuchi T, Nagahama Y (2003) Expression of *DMY* and *DMRT1* in various tissues of the medaka (*Oryzias latipes*) [corrected]. *Zool Sci* 20:1395–1398
- Otake H, Shinomiya A, Matsuda M, Hamaguchi S, Sakaizumi M (2006) Wild-derived XY sex-reversal mutants in the medaka, *Oryzias latipes*. *Genetics* 173:2083–2090
- Paul-Prasanth B, Matsuda M, Lau EL, Suzuki A, Sakai F, Kobayashi T, Nagahama Y (2006) Knock-down of *DMY* initiates female pathway in the genetic male medaka, *Oryzias latipes*. *Biochem Biophys Res Commun* 351:815–819

- Raymond CS, Murphy MW, O'Sullivan MG, Bardwell VJ, Zarkower D (2000) Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. *Genes Dev* 14:2587–2595
- Sato T, Endo T, Yamahira K, Hamaguchi S, Sakaizumi M (2005) Induction of female-to-male sex reversal by high temperature treatment in medaka, *Oryzias latipes*. *Zool Sci* 22:985–988
- Satoh N, Egami N (1972) Sex differentiation of germ cells in the teleost, *Oryzias latipes*, during normal embryonic development. *J Embryol Exp Morphol* 28:385–395
- Shinomiya A, Otake H, Togashi K, Hamaguchi S, Sakaizumi M (2004) Field survey of sex-reversals in the medaka, *Oryzias latipes*: genotypic sexing of wild populations. *Zool Sci* 21:613–619
- Smith CA, Katz M, Sinclair AH (2003) DMRT1 is upregulated in the gonads during female-to-male sex reversal in ZW chicken embryos. *Biol Reprod* 68:560–570
- Suzuki A, Nakamoto M, Kato Y, Shibata N (2005) Effects of estradiol-17beta on germ cell proliferation and DMY expression during early sexual differentiation of the medaka *Oryzias latipes*. *Zool Sci* 22:791–796
- Tanaka M, Saito D, Morinaga C, Kurokawa H (2008) Cross talk between germ cells and gonadal somatic cells is critical for sex differentiation of the gonads in the teleost fish, medaka (*Oryzias latipes*). *Dev Growth Differ* 50:273–278
- Volff JN, Zarkower D, Bardwell VJ, Scharl M (2003) Evolutionary dynamics of the DM domain gene family in metazoans. *J Mol Evol* 57:S241–S249
- Winkler C, Hornung U, Kondo M, Neuner C, Duschl J, Shima A, Scharl M (2004) Developmentally regulated and non-sex-specific expression of autosomal dmrt genes in embryos of the medaka fish (*Oryzias latipes*). *Mech Dev* 121:997–1005
- Yamaguchi A, Lee KH, Fujimoto H, Kadomura K, Yasumoto S, Matsuyama M (2006) Expression of the DMRT gene and its roles in early gonadal development of the Japanese pufferfish *Takifugu rubripes*. *Comp Biochem Physiol Part D Genomics Proteomics* 1:59–68
- Yamamoto TO (1963) Induction of reversal in sex differentiation of YY zygotes in the medaka, *Oryzias latipes*. *Genetics* 48:293–306
- Yamamoto T-O (1975) Medaka (killifish) biology and strains. Keigaku, Tokyo
- Ying M, Chen B, Tian Y, Hou Y, Li Q, Shang X, Sun J, Cheng H, Zhou R (2007) Nuclear import of human sexual regulator DMRT1 is mediated by importin-beta. *Biochim Biophys Acta* 1773:804–813
- Yokoi H, Kobayashi T, Tanaka M, Nagahama Y, Wakamatsu Y, Takeda H, Araki K, Morohashi K, Ozato K (2002) Sox9 in a teleost fish, medaka (*Oryzias latipes*): evidence for diversified function of Sox9 in gonad differentiation. *Mol Reprod Dev* 63:5–16
- Zhang J (2004) Evolution of DMY, a newly emergent male sex-determination gene of medaka fish. *Genetics* 166:1887–1895
- Zhu L, Wilken J, Phillips NB, Narendra U, Chan G, Stratton SM, Kent SB, Weiss MA (2000) Sexual dimorphism in diverse metazoans is regulated by a novel class of intertwined zinc fingers. *Genes Dev* 14:1750–1764

Chapter 17

The Sex-Determining Gene in Medaka

Masaru Matsuda

Abstract Because the sex of most vertebrate species is determined by genetic information, these species must possess a sex-determining gene in their genome. The first sex-determining gene among vertebrates was identified in mammals. Thereafter, for more than a decade, no other sex-determining gene was found. The second sex-determining gene in vertebrates was identified in medaka (*Oryzias latipes*). This gene is designated *Dmy* or *DMRT1bY* and has a DNA-binding motif that is different from that of the mammalian sex-determining gene *SRY* or *Sry*. In this review, I have focused on the isolation of the sex-determining gene of medaka. The identification of the sex-determining gene has revealed the diversity among the sex chromosomes of medaka. This knowledge has provided important clues that are useful to study the diversity of sex-determining genes in vertebrates and to identify the sex-determining genes in other nonmammalian vertebrates.

17.1 Introduction

In vertebrates, two sex-determining systems are present: the genetic sex-determining system and the environmental sex-determining system. In the genetic sex-determining system, gonadal sex is determined by either a single or a number of genetic factors. In other words, gonadal sex depends on the combination of sex chromosomes that a zygote possesses. On the other hand, in the environmental sex-determining system, gonadal sex is determined by one or more environmental factors, such as temperature or pH.

In mammals, the sex-determining gene *SRY* is located on the Y chromosome and was first identified during a deletion analysis of the human Y chromosome

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(Sinclair et al. 1990). Furthermore, *Sry*, the mouse homolog of the *SRY*, is sufficient for male development in transgenic mice (Koopman et al. 1991). Some nonmammalian vertebrates also possess a male heterogametic (XX–XY) sex-determining system but no *Sry* homolog, and the sex-determining gene of nonmammalian vertebrates has yet to be identified.

Because most vertebrates have a genetic sex-determining system, they possess the sex-determining gene on either one or both sex chromosomes. Therefore, the positional cloning technique was considered a conventional option for the identification of the sex-determining gene in these animals; this technique was successfully used to identify the sex-determining gene in humans (Sinclair et al. 1990). The experimental animal model employed in this technique should possess a genetic sex-determining system and a genome that can be easily mapped. Among nonmammalian vertebrates, the teleost fish medaka (*Oryzias latipes*) satisfies both the aforementioned requirements.

Medaka possesses a male heterogametic XX–XY sex-determining system, similar to that found in mammals (Aida 1921). In the d-rR strain of medaka, a cross between a female (X^rX^r) and a heterozygous orange-red male (X^rY^R) gives rise to a progeny consisting of equal numbers of white females (X^rX^r) and orange-red males (X^rY^R). Because the dominant allele (*R*) is located on the Y chromosome, the homozygous recessive (*rr*) condition of the gene locus for the phenotypic orange-red body color occurs only in white females, whereas the heterozygous (*rR*) condition with the dominant allele for the same locus occurs only in orange-red males. Hence, *r* and *R* can be used as markers for the identification of the genotypic sex, that is, XX and XY. Yamamoto (1953) reported the first sex reversals. Treatment with steroid sex hormones during the larval stage and well-calculated mating experiments were used to produce XY females, XX males, YY males, and even YY females by using the d-rR strain of medaka (Yamamoto 1975). However, in medaka, as in many other fish species, the sex chromosomes are not morphologically differentiated (Uwa and Ojima 1981). Although recombination occurs between the X and Y chromosomes near the sex-determining region, the recombination rates in male meiosis are significantly lower than those in female meiosis (Kondo et al. 2001; Matsuda et al. 1999; Yamamoto 1961).

The large interstrain diversity among inbred strains of medaka provides ideal conditions for a comparative study. Wild populations of medaka have been divided into four genetically distinct groups – the Northern, Southern, East Korean, and the China-West Korean populations – which show large genomic diversity (Matsuda et al. 1997b, c; Sakaizumi et al. 1983; Takehana et al. 2003, 2004, 2005). The nucleotide sequences of the exons and introns of nuclear DNA in these populations differ by approximately 1% and 3%, respectively (Kasahara et al. 2007). Furthermore, several inbred strains have been derived from these populations, for example, the Hd-rR strain from the Southern population and the HNI strain from the Northern population (Hyodo-Taguchi and Egami 1985; Hyodo-Taguchi and Sakaizumi 1993).

17.2 Identification of the Sex-Determining Gene

As many attempts to identify the sex-determining gene in nonmammalian vertebrates have failed, the question arises why this identification was successful in medaka. One reason is that we did not focus on the differences between the X and Y chromosomes but on the differences between the strains (Matsuda et al. 2002).

A Y-congenic strain, Hd-rR.Y^{HNI}, was created to highlight the genetic differences between the sex chromosomes of inbred strains (Fig. 17.1) (Matsuda et al. 1997a). In this strain, the sex-determining region on the Y chromosome was derived from the HNI strain, and the genetic background derived from the Hd-rR strain. Using this strain, we isolated sex-linked DNA markers (Matsuda et al. 1997a, 1998) and constructed genetic and cytogenetic maps (Matsuda et al. 1998; Sato et al. 2001) and a bacterial artificial chromosome (BAC) genomic library (Matsuda et al. 2001).

Recombinant sex chromosomes are necessary for genetic mapping of the sex chromosomes. However, the recombination frequency of sex chromosomes exhibits a male-specific restriction (Kondo et al. 2001; Matsuda et al. 1999; Yamamoto 1961). In medaka, sex chromosome-specific recombination restriction has been

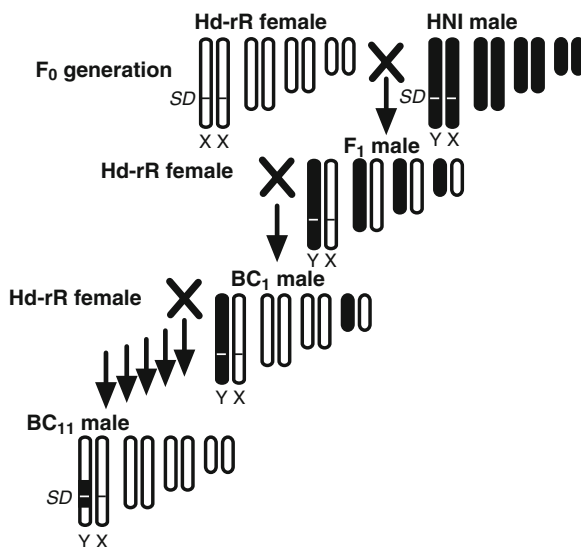


Fig. 17.1 Construction of the Y-congenic strain Hd-rR.Y^{HNI}. The Y-congenic strain was derived from Hd-rR females and an HNI male. Chromosomes of the Hd-rR strain are schematically represented by *open bars*; those of the HNI strain are represented by *black bars*. The largest chromosomes represent the sex chromosomes. F₁ hybrid males were obtained by crossing an Hd-rR female with an HNI male. An F₁ hybrid male was mated with an Hd-rR female to obtain a backcross progeny (BC₁). Further, a BC₁ male was mated with an Hd-rR female to obtain a BC₂ generation. The progenies produced by backcrossing with the Hd-rR females were denoted as BC_n, where *n* is the number of backcross generations. In all the males in the backcross generations, the sex-determining region on the Y chromosome was derived from the HNI strain

observed not only in XY males but also in XX males and YY males. To construct a fine genetic map of the sex chromosomes of medaka, the progeny of the sex-reversed XY females should be screened. This screening results in the identification of many recombinant sex chromosomes.

By using recombinant breakpoint analysis of the sex-reversed XY congenic medaka and by constructing a fine genetic map of the Y chromosome, we located a unique gene in the short sex-determining region on the Y chromosome (Fig. 17.2). This gene comprises six exons, encodes a putative protein consisting of 267 amino

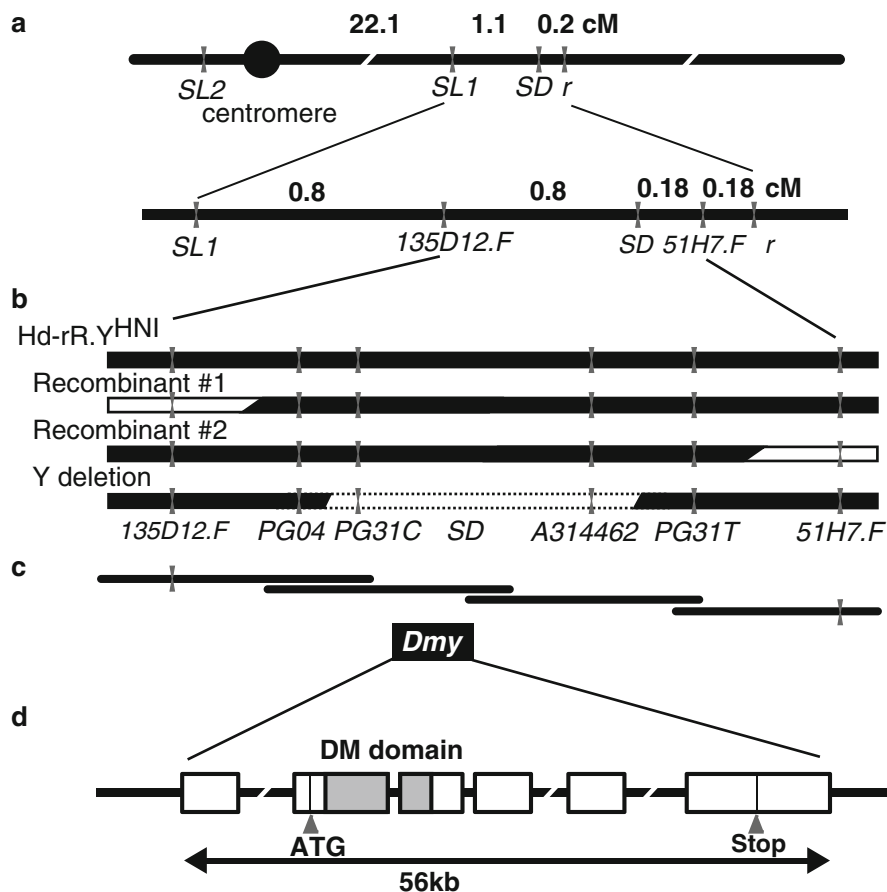


Fig. 17.2 Positional cloning strategy of the sex-determining region and subsequent identification of *Dmy*. **a** A genetic map of the sex-determining region. *SL1* and *SL2* are sex-linked DNA markers; *r* is the gene for body color; *SD* represents the sex-determining locus. **b** Y chromosomes of the congenic Hd-rR.Y^{HNI} strain, recombinant, and Y-deletion medaka. Black bars, chromosomal regions derived from the HNI strain; open bars, chromosomal regions derived from the Hd-rR strain. **c** A minimum bacterial artificial chromosome (BAC) contig. Horizontal bars indicate BAC clones. The *Dmy* gene is located on the BAC clone. **d** Structure of *Dmy* showing its exons (open boxes), the DM domain (gray boxes), and introns (horizontal lines). Translation start and stop sites are indicated by ATG and Stop, respectively. The *Dmy* gene spans a region of approximately 56 kb on the Y chromosome

acids, and contains a DM domain. The DM domain was originally described as a DNA-binding motif shared between *doublesex (dsx)* in *Drosophila melanogaster* and *male abnormal 3 (mab-3)* in *Caenorhabditis elegans*. This Y-specific DM-domain gene was designed *Dmy* (Matsuda et al. 2002) or *Dmrt1b(Y)* (Nanda et al. 2002; Volff et al. 2003).

17.3 Function of *Dmy*

To determine the conditions in which *Dmy* contributes to sex determination in medaka, we had to perform a loss-of-function experiment and a gain-of-function experiment.

For the former experiment, we assumed that because mutations in the sex-determining gene are viable and cause a simple sex reversal, they must be maintained in wild populations. This assumption has been confirmed by screening wild populations of medaka (Matsuda et al. 2002; Otake et al. 2006; Shinomiya et al. 2004). Shinomiya et al. (2004) surveyed wild populations in 40 localities and 69 laboratory stocks, which were originally derived from wild populations and subsequently maintained in the laboratory. In this survey 26 XY females were identified from 13 localities. Otake et al. (2006) reported that 12 fish from 6 localities exhibited a frameshift mutation in the third exon of the protein-coding region of *Dmy*; this mutation resulted in the truncation of the *Dmy* protein. All offspring that inherited this mutant allele of *Dmy* were females. Other mutants exhibited very low expressions of *Dmy*, and thus gave rise to a progeny with a high proportion of XY females. These results further strengthen the assumption that *Dmy* is required for normal male development in medaka (Matsuda et al. 2002).

To demonstrate that *Dmy* is sufficient for normal male development in medaka, we injected one-cell embryos of medaka with a genomic DNA fragment carrying *Dmy*. This fragment contained approximately 56 kb of a coding region, approximately 60 kb of an upstream noncoding region, and approximately 1.4 kb of a downstream noncoding region (Matsuda et al. 2007). In the F_0 generation, we obtained 57 orange-red (XY) and 58 white (XX) adult-stage transgenic fish. Of the 58 white fish, 13 (22.4%) exhibited male secondary sex characters. Of these 13 white males, 8 were fertile. In addition, overexpression of *Dmy* cDNA under the control of the cytomegalovirus (CMV) promoter led to XX sex reversal. These data show that *Dmy* is sufficient to induce male development in XX medaka.

When considered together with previous data, these findings indicate that *Dmy* is the sex-determining gene of medaka. Thus, *Dmy* is the first sex-determining gene detected in nonmammalian vertebrates. *Dmy* mRNA and protein are expressed specifically in the somatic cells that surround the germ cells in the early gonadal primordium, before morphological sex differences are discernible (Kobayashi et al. 2004). When the germ cells were localized in the coelomic epithelium below the nephric duct, *Dmy*-specific signals were found to be localized in the somatic cells surrounding the germ cells at stage 36, that is, 2 to 3 days before hatching. However,

somatic cells surrounding the germ cells do not express *Dmy* during the early migratory period. Expression of *Dmy* persists in cells of the Sertoli cell lineage, from supporting cells to Sertoli cells. In other words, *Dmy* expression persists in the adult testis (Kobayashi et al. 2004). In contrast, the function of *Dmy* in adult testis is yet unclear.

17.4 Lessons from the Sex-Determining Gene in Medaka

17.4.1 Sex Reversal in Fishes

Dmy determines the sex of medaka; that is, the presence or absence of *Dmy* determines the sex of the fish. However, in medaka, as in other fish species, sex reversal can occur because of other genetic or nongenetic factors. Sex reversal is also known to occur in many gonochoristic fish species. Male and female medaka can be easily distinguished genetically by identification of the sex-determining gene. Conversely, the genetic identification of sex is not possible in other fish species; in such cases, mating experiments are required to confirm the genetic sex. Therefore, medaka is a good experimental model for the investigation of the mechanisms underlying sex reversal resulting from genetic or nongenetic factors.

17.4.1.1 Sex Reversal by Steroid Hormones

Artificially induced sex reversal in genotypic medaka males was first reported in 1953 (Yamamoto 1953). Treatment of XY males with estrogen induces ovarian development in the males, whereas treatment of XX females with androgen induces testicular development in the females. Immersing fertilized XY eggs in water containing estrogen induces sex reversal in the embryo (Iwamatsu et al. 2005); in this case, neither *Dmy* expression nor the number of germ cells is affected during the early developmental stages (Scholz et al. 2003; Suzuki et al. 2005). These results suggest that male sex differentiation of germ cells, which is triggered by the expression of *Dmy* in the supporting cell lineage, proceeds even in estrogen-treated XY individuals until hatching, and that early sexual differentiation is not altered by estrogen.

17.4.1.2 Sex Reversal by Gene Mutations

Sex reversal occurs because of dysfunction of not only the *Dmy* gene but also genes involved in the sexual differentiation of the gonads.

Anti-Müllerian hormone (Amh), also known as Müllerian inhibiting substance/factor (Mis/Mif), is essential for the early development of the mammalian gonads

(Park and Jameson 2005; Tilmann and Capel 2002). Amh is a secreted intercellular signaling protein belonging to the transforming growth factor- β (TGF- β) superfamily. Amh functions primarily through anti-Müllerian hormone receptor type II (AmhrII). Amh expression is also detected in lower vertebrates that lack a Müllerian duct. In a medaka mutant termed *hotei*, obtained by treatment with the mutagen *N*-ethyl-*N*-nitrosourea (ENU), the highly conserved amino acid tyrosine at position 390 in the kinase domain in AmhrII is substituted by a cysteine residue. This mutant shows excessive germ cell proliferation and male-to-female sex reversal (Morinaga et al. 2007). In both XX and XY *hotei* homozygotes, the number of germ cells increases by 20 days after hatching, analogous to the germ cells in normal ovaries. Hypertrophy of the gonad occurs during the posthatching period. Further, half the *hotei* homozygotes XY develop into females, but the mechanism underlying this remains unclear.

In medaka, *cxc4*, a chemoattractant receptor gene, is involved in the migration of primordial germ cells at the gastrulation stage; thus, the process of gastrulation is severely impaired by the inhibition of this gene. Germ cell-deficient medaka morphants were produced by the injection of morpholino antisense oligonucleotides that were directed against *cxc4* (*cxc4*MO) (Kurokawa et al. 2007). Of the adult XX medaka morphants, 76% exhibited female-to-male sex reversal and developed male secondary sex characters. Gonadal somatic cells of these germ cell-deficient XX medaka morphants expressed male-specific genes. These results indicate that a deficiency or reduction in germ cells leads the development of male-specific gonadal somatic cells, followed by the development of male secondary sex characters.

The naturally occurring mutant of medaka, *scl* (Sato et al. 2008), does not exhibit any secondary sexual characters. The gene responsible for the generation of this type of mutant is *P450c17-I*; it encodes the steroidogenic enzyme P450c17-I, which converts progesterone to androgen. In this mutant, the amount of steroid hormones is very low in both XX and XY individuals. In XX *scl* mutants, ovarian development initially proceeds normally with many intact oocytes; however, during the later stages of development, the gonad is diverted to the testicular pathway. Because the development of male secondary sex characters depends on the presence of androgen, these fish do not exhibit male secondary sex characters, but interestingly, spermatogenesis proceeds normally.

17.4.1.3 Sex Reversal by High Temperature

In the absence of any genetic factor, exposure of the XX embryo to high temperatures can divert the development of the XX gonad toward the testicular pathway. These results suggest that, similar to androgen treatment of embryos, exposure of embryos to high temperatures leads the XX bipotential gonad to develop into a testis.

Sato et al. (2005) have reported that incubation of fertilized eggs at high temperatures until hatching induces female-to-male sex reversal. In the Hd-rR strain, 24% of XX medaka developed into males when incubated at 32°C, whereas no sex

reversals occurred when the incubation was performed at 27°C, which is the normal temperature under laboratory conditions. In contrast, in the case of the HNI strain, incubation of the embryos at 27 and 32°C induced sex reversal in 11% and 50% of XX medaka, respectively. These XX sex-reversed males had normal testes and were fully fertile. These results illustrate the strain-dependent differences in medaka with regard to temperature-dependent sex reversal and suggest that these differences reflect the intraspecific diversity in wild populations (Sato et al. 2005). Evaluation of this intraspecific diversity may clarify the mechanism of temperature-dependent sex reversal.

Hattori et al. (2007) have also shown that high-temperature treatment of medaka embryos induces female-to-male sex reversal. In their study, fertilized eggs were exposed to temperatures ranging from 17 to 34°C for fixed durations either shortly after fertilization (8- to 16-cell stage; embryonic stages 5–6) or from mid-embryogenesis (during the stage of heart development; stage 36) until hatching. Genotypic (XX) females exposed to high temperatures from stages 5 to 6 onward showed increasing rates of sex reversal into phenotypic males at temperatures above 27°C; this rate reached 100% at 34°C. Thermal manipulation of sex was ineffective after stage 36, indicating that gonadal fate in medaka is determined much before the stage of histological differentiation (stage 39). High temperature induced *DMRT1* expression in genotypic females, which was observed from stage 36 onward (Hattori et al. 2007).

17.4.2 Sex Chromosomes and Sex-Determining Systems

17.4.2.1 Differences Between X and Y

No cytogenetic difference has been identified between the X and Y chromosomes in medaka (Matsuda et al. 1998; Uwa and Ojima 1981). However, analysis of the genomic organization of the Y chromosome has revealed that the Y-specific region spans 258 kb, and the male sex-determining gene *Dmy* appears to be the only functional gene in the Y-specific region (Kondo et al. 2006). This finding implies that in comparison with the X chromosome, the Y chromosome of medaka contains an additional 258-kb sequence (Fig. 17.3a).

A survey of wild populations revealed the frequent occurrence of fish whose phenotypic sex was not correlated with the presence of *Dmy* in the genome. These wild-derived XY sex-reversal mutants have been genetically analyzed (Otake et al. 2006); for example, when XY females found in Awara were mated with normal XY males, XY females were produced. YY individuals were produced from the XY females by conducting well-calculated mating experiments; both the Y chromosomes in the offspring were derived from the Awara XY females. This YY organism developed as a female in the same manner as normal XX females. This result suggests that the Y chromosome in the XY Awara females (Y^{Awara}), which contains a frameshift mutation in the third exon of *Dmy* that results in the truncation of the

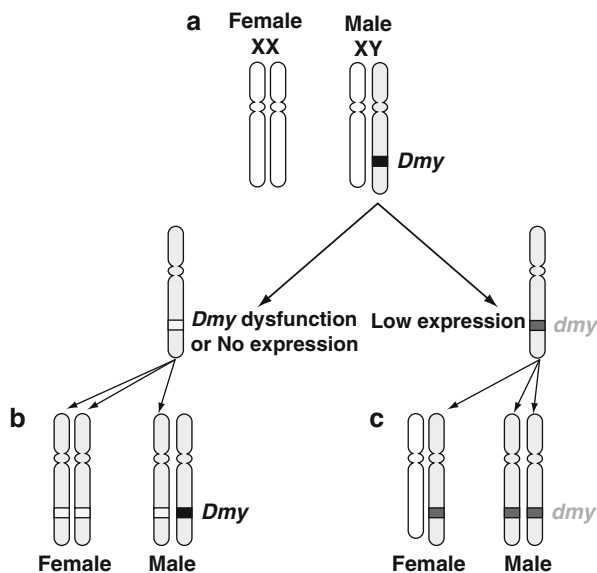


Fig. 17.3 Variation in the sex chromosomes found in wild populations. **a** Wild-type sex chromosomes. *White chromosomes*, X chromosomes; *gray chromosomes*, Y chromosomes. **b** *Dmy* dysfunction makes the Y chromosome behave like the X chromosome. Y chromosomes that harbor a dysfunctional *Dmy* are functionally equivalent to X chromosomes. Thus, the differences between the Y and X chromosomes are quite small. **c** Low *dmy* expression converts the XX–XY system to the ZZ–ZW system. One copy of a low-expressing *dmy* results in the development of a female, whereas two copies result in the development of a male

Dmy protein, is functionally equivalent to a normal X chromosome (Fig. 17.3b). When a $Y^{Awara}Y^{Awara}$ female is mated with a $Y^{Awara}Y$ male, the resulting progeny contains equal numbers of males and females. It has been assumed that the sequences of the Y^{Awara} and the normal Y chromosomes differ by a single base pair, which suggests that the differences between heterogametic sex chromosomes within a species are sometimes quite small. If the differences between the X and Y chromosomes in our inbred strain had also been small, it would have been more difficult to identify the sex-determining gene of medaka.

17.4.2.2 Conversion of the XX–XY System to the ZZ–ZW System of Sex Determination

Decreased *Dmy* expression leads to the conversion of the male heterogametic sex-determining system (XX–XY) to the female heterogametic sex-determining system (ZZ–ZW).

Screening of wild populations of medaka has led to the discovery of many *Dmy* mutants. *dmy^{Kesen}* is one such mutant and is expressed at low levels. Therefore, XY medaka that inherit the *dmy^{Kesen}* mutation develop into females. However, YY

medaka, in which both Y chromosomes are derived from the *dmy*^{Kesen} mutants, develop into males because the *Dmy* expression level reaches its threshold value in these individuals. Thus, one copy of *dmy*^{Kesen} in the genome leads to the development of a female, whereas two copies result in the development of a male. In other words, this phenomenon represents a female heterogametic sex-determining system (Fig. 17.3c).

17.4.2.3 Making the Sex Chromosome

Because the X chromosome is almost identical to the Y chromosome, except that it lacks the sex-determining gene *Dmy*, and because sex-reversed XX males produce normal sperm, we can assume that addition of *Dmy* to the XX genome would result in the development of medaka males.

We obtained two germline transmission lines, whose genome contains the *Dmy* genome integrated in XX background, by injecting the *Dmy* genomic region in the one-cell embryo. As expected, these XX medaka developed into males and no significant differences were observed as compared with the development of the normal XY medaka. In transgenic lines, an autosome in which *Dmy* has been integrated behaves as a new Y chromosome and a homologous chromosome of the new Y chromosome behaves like a new X chromosome. In contrast, the original X-chromosome pair behaves as does a pair of autosomes.

Further, linkage analyses show that linkage groups 23 and 5 function as sex chromosomes in these transgenic lines, respectively. This is the first example wherein a sex chromosome was artificially generated.

17.5 Conclusion

Identification of the sex-determining gene of medaka has shown that the X and Y chromosomes differ by only a single gene, which is located on a Y-specific 256-kb region. Furthermore, a single nucleotide substitution causes *Dmy* dysfunction; Y chromosomes that harbor the mutated *Dmy* gene are functionally equivalent to X chromosomes. Therefore, only a single nucleotide difference exists between the mutated Y chromosome (equivalent to the X chromosome) and the wild-type Y chromosome. Furthermore, reduced expression levels of *Dmy*, which are believed to occur as a result of a slight modification of the nucleotide sequence of the *Dmy* promoter region from the wild-type sequence, converts the male heterogametic sex-determining system into the female heterogametic sex-determining system. These findings suggest that a slight difference between the sex chromosomes determine the sex of an individual and that sex-determining genes and sex-determining systems in fish are inconsistent. It could be a common rule among fishes that many sex-determining genes might exist in a single species itself.

References

- Aida T (1921) On the inheritance of color in a freshwater fish, *Aplocheilus latipes* Temminck and Schlegel, with special reference to sex-linked inheritance. *Genetics* 6:554–573
- Hattori RS, Gould RJ, Fujioka T, Saito T, Kurita J, Strüssmann CA, Yokota M, Watanabe S (2007) Temperature-dependent sex determination in Hd-rR medaka *Oryzias latipes*: gender sensitivity, thermal threshold, critical period, and *DMRT1* expression profile. *Sex Dev* 1:138–146
- Hyodo-Taguchi Y, Egami N (1985) Establishment of inbred strains of the medaka, *Oryzias latipes* and the usefulness of the strains for biomedical research. *Zool Sci* 2:305–316
- Hyodo-Taguchi Y, Sakaizumi M (1993) List of inbred strains of the medaka, *Oryzias latipes*, maintained in the Division of Biology, National Institute of Radiological Sciences. *Fish Biol J Medaka* 5:29–30
- Iwamatsu T, Kobayashi H, Sato M (2005) *In vivo* fertilization and development of medaka eggs initiated by artificial insemination. *Zool Sci* 22:119–123
- Kasahara M, Naruse K, Sasaki S, Nakatani Y, Qu W, Ahsan B, Yamada T, Nagayasu Y, Doi K, Kasai Y, Jindo T, Kobayashi D, Shimada A, Toyoda A, Kuroki Y, Fujiyama A, Sasaki T, Shimizu A, Asakawa S, Shimizu N, Hashimoto SI, Yang J, Lee Y, Matsushima K, Sugano S, Sakaizumi M, Narita T, Ohishi K, Haga S, Ohta F, Nomoto H, Nogata K, Morishita T, Endo T, Shin-I T, Takeda H, Morishita S, Kohara Y (2007) The medaka draft genome and insights into vertebrate genome evolution. *Nature (Lond)* 447:714–719
- Kobayashi T, Matsuda M, Kajiura-Kobayashi H, Susuki A, Saito N, Nakamoto M, Shibata N, Nagahama Y (2004) Two DM domain genes, *DMY* and *DMRT1*, involved in testicular differentiation and development in the medaka, *Oryzias latipes*. *Dev Dyn* 231:518–526
- Kondo M, Nagao E, Mitani H, Shima A (2001) Differences in recombination frequencies during female and male meioses of the sex chromosomes of the medaka, *Oryzias latipes*. *Genet Res* 78:23–30
- Kondo M, Hornung U, Nanda I, Imai S, Sasaki T, Shimizu A, Asakawa S, Hori H, Schmid M, Shimizu N, Scharl M (2006) Genomic organization of the sex-determining and adjacent regions of the sex chromosomes of medaka. *Genome Res* 16:815–826
- Koopman P, Gubbay J, Vivian N, Goodfellow P, Lovell-Badge R (1991) Male development of chromosomally female mice transgenic for *Sry*. *Nature (Lond)* 351:117–121
- Kurokawa H, Saito D, Nakamura S, Katoh-Fukui Y, Ohta K, Baba T, Morohashi K, Tanaka M (2007) Germ cells are essential for sexual dimorphism in the medaka gonad. *Proc Natl Acad Sci USA* 104:16958–16963
- Matsuda M, Kusama T, Oshiro T, Kurihara Y, Hamaguchi S, Sakaizumi M (1997a) Isolation of a sex chromosome-specific DNA sequence in the medaka, *Oryzias latipes*. *Genes Genet Syst* 72:263–268
- Matsuda M, Yamagishi Y, Sakaizumi M, Joen S-R (1997b) Mitochondrial DNA variation in the Korean wild population of medaka, *Oryzias latipes*. *Korean J Limnol* 30:119–128
- Matsuda M, Yonekawa H, Hamaguchi S, Sakaizumi M (1997c) Geographic variation and diversity in the mitochondrial DNA of the medaka, *Oryzias latipes*, as determined by restriction endonuclease analysis. *Zool Sci* 14:517–526
- Matsuda M, Matsuda C, Hamaguchi S, Sakaizumi M (1998) Identification of the sex chromosomes of the medaka, *Oryzias latipes*, by fluorescence *in situ* hybridization. *Cytogenet Cell Genet* 82:257–262
- Matsuda M, Sotoyama S, Hamaguchi S, Sakaizumi M (1999) Male-specific restriction of recombination frequency in the sex chromosomes of the medaka, *Oryzias latipes*. *Genet Res* 73:225–231
- Matsuda M, Kawato N, Asakawa S, Shimizu N, Nagahama Y, Hamaguchi S, Sakaizumi M, Hori H (2001) Construction of a BAC library derived from the inbred Hd-rR strain of the teleost fish, *Oryzias latipes*. *Genes Genet Syst* 76:61–63
- Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Morrey CE, Shibata N, Asakawa S, Shimizu N, Hori H, Hamaguchi S, Sakaizumi M (2002) *DMY* is a Y-specific

- DM-domain gene required for male development in the medaka fish. *Nature (Lond)* 417:559–563
- Matsuda M, Shinomiya A, Kinoshita M, Suzuki A, Kobayashi T, Paul-Prasanth B, Lau EL, Hamaguchi S, Sakaizumi M, Nagahama Y (2007) *DMY* gene induces male development in genetically female (XX) medaka fish. *Proc Natl Acad Sci USA* 104:3865–3870
- Morinaga C, Saito D, Nakamura S, Sasaki T, Asakawa S, Shimizu N, Mitani H, Furutani-Seiki M, Tanaka M, Kondoh H (2007) The hotei mutation of medaka in the anti-Müllerian hormone receptor causes the dysregulation of germ cell and sexual development. *Proc Natl Acad Sci USA* 104:9691–9696
- Nanda I, Kondo M, Hornung U, Asakawa S, Winkler C, Shimizu A, Shan Z, Haaf T, Shimizu N, Shima A, Schmid M, Scharl M (2002) A duplicated copy of *DMRT1* in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. *Proc Natl Acad Sci USA* 99:11778–11783
- Otake H, Shinomiya A, Matsuda M, Hamaguchi S, Sakaizumi M (2006) Wild-derived XY sex-reversal mutants in the medaka, *Oryzias latipes*. *Genetics* 173:2083–2090
- Park SY, Jameson JL (2005) Minireview: transcriptional regulation of gonadal development and differentiation. *Endocrinology* 146:1035–1042
- Sakaizumi M, Moriwaki K, Egami N (1983) Allozymic variation and regional differentiation in wild populations of the fish *Oryzias latipes*. *Copeia* 1983:311–318
- Sato T, Yokomizo S, Matsuda M, Hamaguchi S, Sakaizumi M (2001) Gene-centromere mapping of medaka sex chromosomes using triploid hybrids between *Oryzias latipes* and *O. luzonensis*. *Genetica* 111:71–75
- Sato T, Endo T, Yamahira K, Hamaguchi S, Sakaizumi M (2005) Induction of female-to-male sex reversal by high temperature treatment in medaka, *Oryzias latipes*. *Zool Sci* 22:985–988
- Sato T, Suzuki A, Shibata N, Sakaizumi M, Hamaguchi S (2008) The novel mutant *scl* of the medaka fish, *Oryzias latipes*, shows no secondary sex characters. *Zoological Science* 25(3):299–306
- Scholz S, Rösler S, Schäffer M, Hornung U, Scharl M, Gutzeit HO (2003) Hormonal induction and stability of monosex populations in the medaka (*Oryzias latipes*): expression of sex-specific marker genes. *Biol Reprod* 69:673–678
- Shinomiya A, Otake H, Togashi KI, Hamaguchi S, Sakaizumi M (2004) Field survey of sex-reversals in the medaka, *Oryzias latipes*: genotypic sexing of wild populations. *Zool Sci* 21:613–619
- Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischauf AM, Lovell BR, Goodfellow PN (1990) A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature (Lond)* 346:240–244
- Suzuki A, Nakamoto M, Kato Y, Shibata N (2005) Effects of estradiol-17 β on germ cell proliferation and *DMY* expression during early sexual differentiation of the medaka *Oryzias latipes*. *Zool Sci* 22:791–796
- Takehana Y, Nagai N, Matsuda M, Tsuchiya K, Sakaizumi M (2003) Geographic variation and diversity of the cytochrome *b* gene in Japanese wild populations of medaka, *Oryzias latipes*. *Zool Sci* 20:1279–1291
- Takehana Y, Uchiyama S, Matsuda M, Jeon SR, Sakaizumi M (2004) Geographic variation and diversity of the cytochrome *b* gene in wild populations of medaka (*Oryzias latipes*) from Korea and China. *Zool Sci* 21:483–491
- Takehana Y, Naruse K, Sakaizumi M (2005) Molecular phylogeny of the medaka fishes genus *Oryzias* (Belontiiformes: Adrianichthyidae) based on nuclear and mitochondrial DNA sequences. *Mol Phylogenet Evol* 36:417–428
- Tilmann C, Capel B (2002) Cellular and molecular pathways regulating mammalian sex determination. *Recent Prog Horm Res* 57:1–18
- Uwa H, Ojima Y (1981) Detailed and banding karyotype analysis of the medaka, *Oryzias latipes*, in cultured cells. *Proc Jpn Acad* 57(B):39–43

- Volff JN, Kondo M, Schartl M (2003) Medaka dmY/dmrt1Y is not the universal primary sex-determining gene in fish. *Trends Genet* 19:196–199
- Yamamoto T (1953) Artificially induced sex-reversal in genotypic males of the medaka (*Oryzias latipes*). *J Exp Zool A Ecol Genet Physiol* 123:571–594
- Yamamoto T (1961) Progenies of sex-reversal females mated with sex-reversal males in the medaka, *Oryzias latipes*. *J Exp Zool A Ecol Genet Physiol* 146:163–179
- Yamamoto T (1975) Control of sex differentiation. In: Yamamoto T (ed) *Medaka (killifish) biology and strains*. Keigaku, Tokyo, pp 192–213

Chapter 18

Endocrine Regulation of Oogenesis in the Medaka, *Oryzias latipes*

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Abstract Regulation of ovarian activity is an integrated process encompassing both extraovarian signals and intrafollicular factors. The medaka, *Oryzias latipes*, has served as an excellent experimental model for studying the regulation of various processes of oogenesis such as oocyte growth, maturation, and ovulation. The advantage of the medaka model is the precise daily ovarian cyclicity and the ease of isolating large numbers of oocytes for in vitro studies. In addition, because of the identification of maturation-inducing hormones (steroids) in fish oocytes, the endocrine regulation of oocyte maturation has been investigated most extensively in fishes, including medaka. Here we provide a brief overview of the gene regulation of hormonally controlled processes of oocyte growth, maturation, and ovulation mainly in medaka, and present some novel data on the steroidogenic shift occurring in ovarian follicles during oocyte growth and maturation.

18.1 Introduction

Oogenesis in fish, as in other vertebrates, can be divided into two major parts: the prolonged growth phase, which is characterized by the enlargement of oocytes, and the maturation phase, during which fully grown oocytes become fertilizable. Oocytes of nonmammalian vertebrates grow while arrested in prophase I of meiosis.

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The principal events responsible for the enormous growth of fish oocytes occur predominantly during the phase of development termed vitellogenesis and involve the sequestration and packaging of a hepatically derived plasma precursor, vitellogenin, into yolk protein (Devlin and Nagahama 2002).

After the oocyte completes vitellogenesis, it becomes ready for the next phase of oogenesis, that is, the resumption of meiosis, which is accompanied by several maturational processes in the nucleus and cytoplasm of the oocyte. This process, called oocyte maturation, is a prerequisite for successful fertilization and consists of breakdown of the germinal vesicle (GVBD), chromosome condensation, and formation of the first polar body (Nagahama et al. 1994). Oocytes then progress through meiosis until metaphase II, when they again arrest until subsequent ovulation and fertilization. In simple terms, ovulation is the rupture of a follicle, releasing a fertilizable oocyte.

Oocyte growth, maturation, and ovulation in teleosts, similar to those in other vertebrates, are regulated by gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH). In most cases, however, gonadotropin action on oogenesis is not direct but is mediated through the follicular production of ovarian steroid hormones, which in turn mediate various stages of oogenesis. For example, FSH has been reported to increase follicular production of estradiol-17 β , which in turn promotes vitellogenesis by stimulating the synthesis and secretion of yolk protein in the liver (Nagahama et al. 1994). A number of in vivo and in vitro studies have also shown that LH acts on ovarian follicular cells, causing them to produce maturation-inducing hormone (MIH) or steroids (MIS) (in most fish, 17 α ,20 β -dihydroxy-4-pregnen-3-one, 17 α ,20 β -DP), which acts directly on the oocyte to initiate the process of oocyte maturation. Furthermore, ovulation is a complex process that is initiated by an LH surge.

The medaka, *Oryzias latipes*, provides an excellent model for the study of mechanisms involved in follicular development. This species usually spawns daily within 1 h of the onset of light when acclimated to a constant long photoperiod (14-h light/10-h dark) at 26°C. Under these conditions, vitellogenesis and oocyte maturation of individual follicles occur within 72 h. The largest follicles in the ovaries undergo GVBD at 6 h before daylight and are ovulated at 1 h before daylight (Fig. 18.1). The ovary contains numerous follicles of various sizes and developmental stages. Growth from 500–700 μ m to 850–950 μ m (1,200 μ m after ovulation) takes 48 h. Thus, the sequence of events associated with ovarian activity, such as vitellogenesis, oocyte maturation, and ovulation, can be accurately timed in this species (Sakai et al. 1987). Taking full advantage of this precise daily ovarian cyclicity in medaka, we have isolated ovarian follicles at 16 different developmental stages between 47 h before spawning (hbs) and 2 hbs. These isolated ovarian follicles can be used for in vitro studies of oocyte maturation and ovulation, and also to investigate the changes in follicular levels of mRNAs encoding steroidogenic enzymes, transcriptional factors, and steroid hormone receptors, etc.

This chapter briefly reviews the endocrine regulation of oocyte growth, maturation, and ovulation, mainly in medaka, and describes the data, some still unpublished, on the mechanisms by which gonadotropins exert their action on the production of two naturally occurring steroid hormones, estradiol-17 β and 17 α ,20 β -DP, by ovarian follicles.

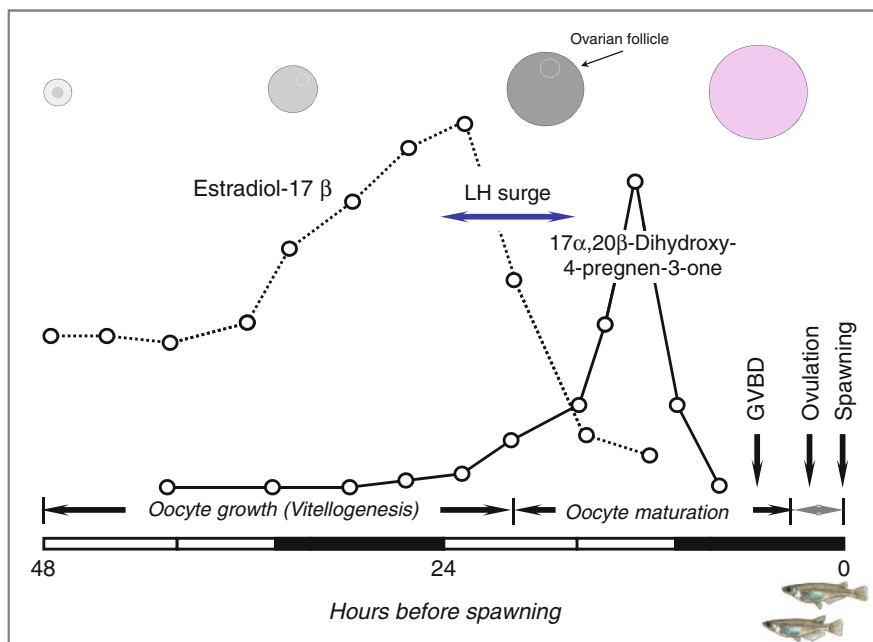


Fig. 18.1 Shift in follicular steroidogenesis from *estradiol-17β* during oocyte growth to *17α,20β-dihydroxy-4-pregnen-3-one* during oocyte maturation. *Ovarian follicles* (follicle-enclosed oocytes) of medaka were collected between 48 and 0 h before spawning and were incubated in the presence or absence of gonadotropins for 18 h. Steroids were measured by specific radioimmunoassays. GVBD, germinal vesicle breakdown; LH, luteinizing hormone (gonadotropin)

18.2 Ovarian Follicles

In adult medaka ovaries, ovarian follicles grossly divide into previtellogenic follicles, vitellogenic follicles, and postvitellogenic follicles. Postvitellogenic follicles were characterized by a well developed chorion layer compared with vitellogenic follicles. Ovarian follicles of medaka, similar to those of other vertebrates, are composed of two major cell layers, an outer thecal layer and an inner granulosa layer, which are separated by a substantial basal lamina (Nagahama 1983).

18.3 Oocyte Growth

18.3.1 FSH: *Estradiol-17β*

It is generally accepted that, in oviparous vertebrates, FSH has a dominant role in controlling vitellogenic growth of follicles, partly through stimulation of *estradiol-17β* biosynthesis by ovarian follicles. Although plasma levels of gonadotropins were not measured during the reproductive cycle of medaka because of their small body

size, we examined changes in the levels of FSH receptor (FSHR) and LH receptor (LHR) mRNAs in ovarian follicles during 48-h sampling periods (Shibata et al., unpublished data). An important role of FSH during the follicular growth phase of medaka was confirmed by high levels of FSHR mRNA in vitellogenic follicles collected between 47 and 35 hbs. Levels of FSHR transcripts then decreased significantly in vitellogenic follicles collected at 32 hbs and dropped to very low levels in late- to postvitellogenic follicles at 29 to 2 hbs. In situ hybridization analysis revealed that FSHR mRNA was expressed mainly in granulosa cells at the vitellogenic stage.

During the oocyte growth period, estradiol-17 β regulates ovarian development through its control of vitellogenin synthesis in the liver. The primary site of estradiol-17 β production in the medaka ovary is the follicle cells that surround vitellogenic oocytes. We have previously shown that the capacity of intact medaka follicles to produce estradiol-17 β in response to gonadotropin stimulation increases during oocyte growth but rapidly decreases in association with the ability of the oocyte to mature in response to gonadotropin (Sakai et al. 1987). A two-cell type model for the production of estradiol-17 β has been proposed in the salmonid ovarian follicle. In this model, the thecal cell layer, under the influence of gonadotropin, secretes an androgen substrate (testosterone), which diffuses into the granulosa cell layer where aromatase is located exclusively (Kagawa et al. 1982). However, the two-cell type model as described does not seem to be valid for medaka ovarian follicles (Onitake and Iwamatsu 1986).

18.3.2 Estradiol-17 β Precursor (Testosterone) Production

Estradiol-17 β is synthesized by its immediate precursor, testosterone, by the action of aromatizing enzymes (Fig. 18.2). Steroidogenic acute regulatory protein (StAR) and several steroidogenic enzymes such as cholesterol side-chain cleavage cytochrome P-450 (P450_{scc}), 3 β -hydroxysteroid dehydrogenase-isomerase (3 β -HSD), 17 α -hydroxylase/17,20-lyase cytochrome P-450 (P450_{c17}), and 17 β -hydroxysteroid dehydrogenase (17 β -HSD) are involved in the follicular production of testosterone. StAR is now established as a key rate-limiting mediator in the acute regulation of steroidogenesis by tropic hormones. StAR mediates biosynthesis of steroid hormones by controlling the transfer of cholesterol to mitochondria, where P450_{scc}, the enzyme that catalyzes the first and rate-limiting step in steroidogenesis, is located (Stocco 2000). In medaka, levels of transcripts encoding StAR, P450_{scc}, and 3 β -HSD were high in ovarian follicles during oocyte growth (Shibata et al., unpublished data), suggesting that the large increase in these transcripts during the growth phase is responsible for the production of testosterone.

Cytochrome P450_{c17} (steroid 17 α -hydroxylase/C17,20 lyase) is the single enzyme that mediates the 17 α -hydroxylase and 17,20 lyase activities and occupies the key position in the steroidogenic pathway for the production of C-18, -19, and -21 steroids in the gonads and head kidney of fishes (see Fig. 18.2). However, the

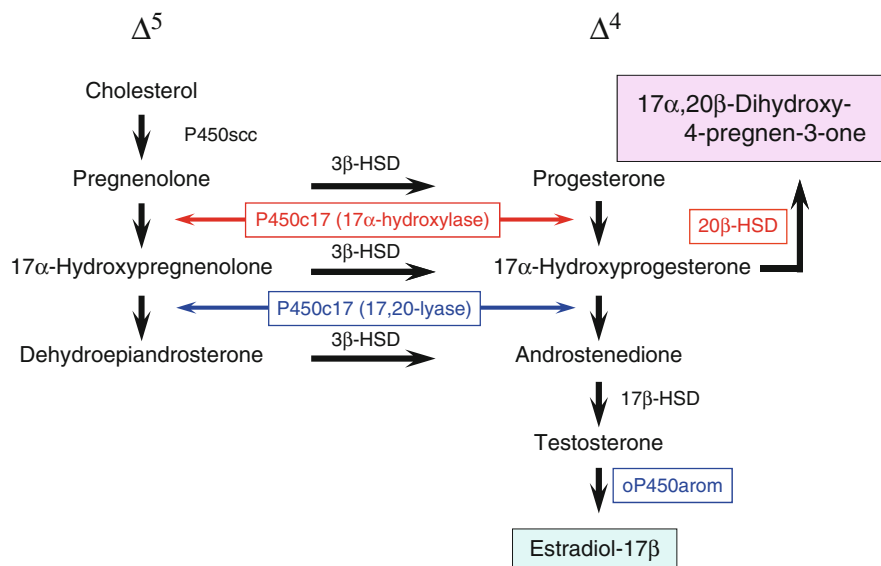


Fig. 18.2 Pathways of sex steroid biosynthesis in the fish ovary (Δ^4 and Δ^5 pathways). Cytochrome P450c17 with two enzymatic activities (17 α -hydroxylase and 17,20-lyase) takes the key position in the production of both estradiol-17 β and 17 α ,20 β -DP. For the production of estradiol-17 β , both the hydroxylase and lyase activities of P450c17 and cytochrome P450 aromatase (oP450arom) are needed. For the production of 17 α ,20 β -DP, only the hydroxylase activity of P450c17 and 20 β -hydroxysteroid dehydrogenase (20 β -HSD) is required. See text for more complete discussion

mechanism underlying its dual action continues to be controversial in the field of steroidogenesis in fish (Kobayashi et al. 1996), as well as mammals (Maller 2005). Recently, in addition to the conventional type of *P450c17* (*P450c17-I*), we identified a novel type of *P450c17* (*P450c17-II*) in the genomes of medaka, tilapia (*Oreochromis niloticus*), fugu (*Fugu rubripes*), and tetraodon (*Tetraodon nigroviridis*) (Zhou et al. 2007a). These two types of P450c17s are encoded by two different genes. Surprisingly, enzymatic assays revealed that P450c17-II possesses unique 17 α -hydroxylase activity, without any 17,20-lyase activity, in contrast to P450c17-I, which showed both 17 α -hydroxylase and 17,20-lyase activities (Fig. 18.3). Tissue distribution analyzed by reverse transcription-polymerase chain reaction (RT-PCR) clearly revealed that *P450c17-II* of medaka and tilapia was expressed not only in the gonads, but also in the head kidney (Fig. 18.3), although *P450c17-I* was exclusively expressed in the gonads. In situ hybridization showed that *P450c17-II*, but not *P450c17-I*, was expressed in the interrenal cells of the head kidney (the piscine counterpart of the mammalian adrenal) from 3 days after fertilization (medaka) and 5 days after hatching (tilapia) to the adult stage, whereas no expression of *P450c17-I* was observed in the head kidney at any stage of development (Fig. 18.3). These results suggest that only P450c17-II is responsible for cortisol production in the interrenal cells (Zhou et al. 2007a, b).

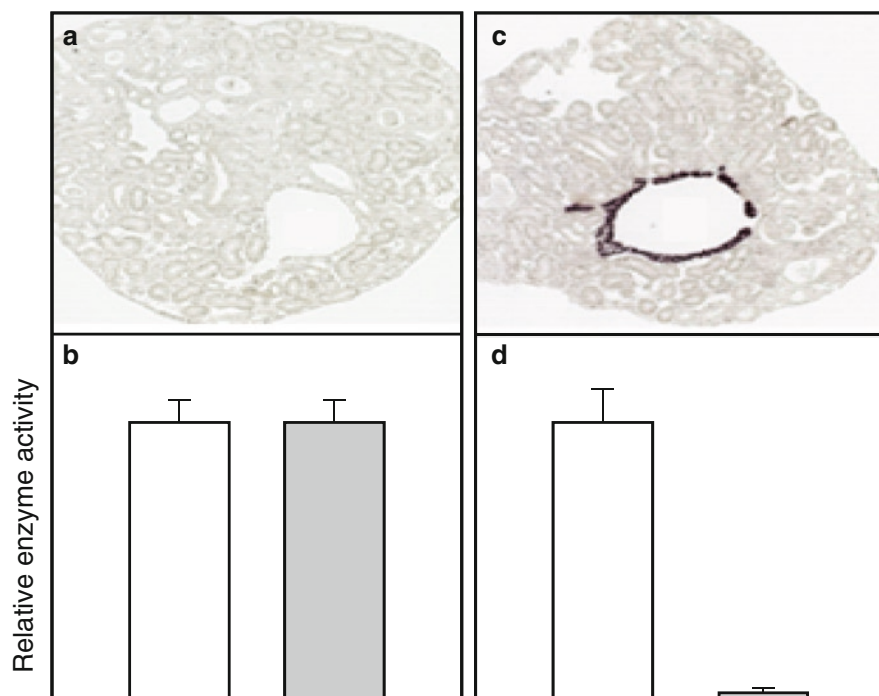


Fig. 18.3 Cellular localization and enzyme activity of medaka P450c17-I and P450c17-II. Expression (in situ hybridization) of *P450c17-II* (c), but not *P450c17-I* (a), is detected in the interrenal cells of the medaka head kidney. Medaka P450c17-I (b) possesses both 17 α -hydroxylase (open bar) and 17,20 lyase (closed bar) activity, whereas P450c17-II (d) possesses only 17 α -hydroxylase, but not 17,20 lyase, activity. Enzyme activity was determined by the conversion of radiolabeled steroid substrates using P450c17-I and P450c17-II recombinant proteins expressed in HEK 293 cells. Radiolabeled steroid metabolites were analyzed by thin-layer chromatography (TLC) and autoradiography

Real-time PCR analysis showed that the expression of *P450c17-I* was barely detectable in follicles at 47 to 35 hbs, rapidly increased from 32 hbs with a peak at 26 hbs, and then dropped from 20 hbs, reaching the basal levels by 11 hbs. These results strongly suggest that *P450c17-I* is needed mainly for testosterone production by vitellogenic follicles during oocyte growth (vitellogenesis).

18.3.3 Cytochrome P450 Aromatase (*oP450arom*, *Cyp19a1*)

In viviparous vertebrates, the ovarian form of cytochrome P450 aromatase (*oP450arom*, *cyp19a1*), which converts testosterone to estradiol-17 β , plays a crucial role in oocyte growth (vitellogenesis) (Nagahama et al. 1994; Devlin and Nagahama 2002). In medaka, a progressive increase in aromatase activity was observed in ovarian follicles during active vitellogenesis (Sakai et al. 1987, 1988).

Furthermore, in this species, aromatase activity was markedly enhanced by pregnant mare serum gonadotropin (PMSG) after 18 h of incubation of vitellogenic follicles (Nagahama et al. 1991). This action of PMSG was mimicked by forskolin and dbcAMP, which are known to raise the cellular level of cAMP. The enhancing effect of PMSG, forskolin, and dbcAMP on aromatization was completely inhibited by actinomycin D and cyclohexamide, suggesting that the action of PMSG is mediated through an adenylate cyclase-cAMP system and is dependent upon both transcriptional and translational processes.

Real-time PCR analysis showed that the expression of *oP450arom* was high at 47 hbs, rapidly decreased from 44 hbs with a lowest value at 38 hbs, then rapidly increased with a peak at 32 hbs, and then dropped from 29 hbs, finally reaching the basal levels by 26 hbs. The pattern of changes in *oP450arom* mRNA levels was broadly similar to the changes in the ability of isolated vitellogenic follicles to produce estradiol-17 β in response to gonadotropins and 17 α -hydroxyprogesterone (Sakai et al. 1987, 1988). In situ hybridization analysis revealed that *oP450arom* mRNA was expressed mainly in granulosa cells at the vitellogenic stage.

18.3.4 Upregulation of *oP450arom* by *Ad4BP/SF-1* and *Foxl2*

The promoter regions of medaka *oP450arom* genes contain Ad4 binding sites (Tanaka et al. 1995), and their Ad4 oligomeric sequences of the *oP450arom* gene form a complex with in vitro-translated Ad4BP/SF-1, an orphan member of the nuclear hormone receptor family, indicating that Ad4BP/SF-1 specifically binds to its DNA motif (Watanabe et al. 1999). We also demonstrated the natural presence of Ad4BP/SF-1-like protein in midvitellogenic ovarian follicles. In transient transfection studies, overexpression of Ad4BP/SF-1 resulted in an increased basal expression of *oP450arom* reporter gene constructs, whereas mutation of these sites resulted in decreased basal expression of *oP450arom* promoter activities. Thus, it is concluded that Ad4BP/SF-1 is necessary for the expression of the *oP450arom* gene in fish ovarian follicles (Watanabe et al. 1999).

In the medaka, the expression of both *oP450arom* and *Ad4BP/SF-1* increased in a parallel manner in ovarian follicles (granulosa cells) during vitellogenesis and declined sharply in follicles immediately before oocyte maturation (Fukada et al. 1996; Watanabe et al. 1999) (Fig. 18.4, left). As *Foxl2* is also co-localized with *Ad4BP/SF-1* and *oP450arom* in vitellogenic follicles, the effect of *Foxl2* on transcription of the *P450arom* promoter was also examined. By cotransfection of *Foxl2* with *oP450arom* promoter constructs, the *oP450arom* promoter was significantly activated about twofold compared with basal expression. Furthermore, with cotransfection of *Ad4BP/SF-1* and *Foxl2* simultaneously, the *oP450arom* promoter was significantly activated about 23-fold compared with controls. Thus, the coexistence of Ad4BP/SF-1 and *Foxl2* synergistically activated *oP450arom* transcription (Wang et al. 2007).

18.3.5 Downregulation of *oP450arom* by *Dax1*

Another orphan nuclear receptor *Dax1* (Dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X-chromosome, gene 1) has been shown to act as a negative regulator of many genes in the steroid biosynthetic pathway (Zazopoulos et al. 1997). To investigate whether *Dax1* is involved in the regulation of the steroidogenic pathway in medaka ovarian follicles, we analyzed its expression pattern in medaka ovaries during oocyte growth and maturation (Nakamoto et al. 2007; Zhou et al. 2007b). Although the expression of *Dax1* mRNA was low during the vitellogenic stage (47 to 17 hrs), there was a sharp increase between 17 and 8 hrs, immediately before spawning. In situ hybridization analysis revealed that in adult ovaries, *Dax1* mRNA was detected only in postvitellogenic follicles (granulosa cells) (Fig. 18.4, right) and was not detected in previtellogenic or vitellogenic follicles. In adult testes, *Dax1* mRNA was not detected. In contrast to *Dax1* expression, *Ad4BP/SF-1*, *Foxl2*, and *oP450arom* mRNAs were coexpressed in vitellogenic follicles, but not detected in postvitellogenic follicles. Thus, in medaka ovarian follicles, *Dax1* showed a complementary expression pattern against *Ad4BP/SF-1*, *Foxl2*, and *oP450arom*, and did not show any overlap. These results suggest that, in medaka, *Dax1* may repress transcription of *Ad4BP/SF-1* and *Foxl2* by binding to their promoters as found in mammals (Zazopoulos et al. 1997). Without *Ad4BP/SF-1* (and *Foxl2*), transcription of *oP450arom* is no longer stimulated, and consequently its expression ceases in these follicles.

It has been suggested that *Dax1* can repress *Ad4BP/SF-1*-mediated transactivation by direct protein–protein interaction with *Ad4BP/SF-1* in mammals (Ito et al. 1997). To investigate whether *Dax1* represses *Ad4BP/SF-1*-mediated transactivation of *oP450arom* via direct interaction with *Ad4BP/SF-1* in medaka, cotransfection experiments were performed using the luciferase reporter plasmid carrying 2.1 kb of

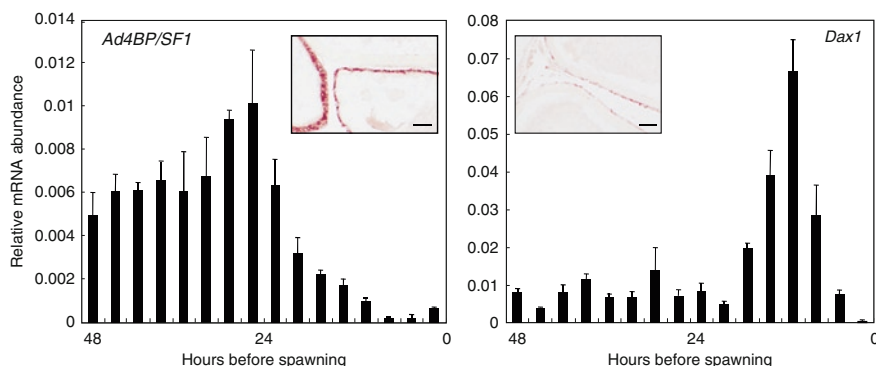


Fig. 18.4 Expression pattern of medaka *Ad4BP/SF-1* (left) and *Dax1* (right) during the 48-h sampling period by real-time polymerase chain reaction (PCR). In situ hybridization of *Ad4BP-SF1* in vitellogenic follicles and *Dax1* in postvitellogenic follicles is also shown (insets). Bars 100 μ m

the 5'-upstream region of the medaka *oP450arom* gene in human embryonic kidney 293 cells (HEK 293 cells). Cotransfection of *Ad4BP/SF-1* with *oP450arom* promoter constructs significantly increased luciferase activity fourfold over basal expression (Watanabe et al. 1999).

To investigate whether Dax1 could negatively regulate Ad4BP/SF-1- and Foxl2-induced *oP450arom* expression, *Dax1*, *Ad4BP/SF-1*, and *Foxl2* were cotransfected simultaneously (Nakamoto et al. 2007). As expected, addition of Dax1 caused a significant decrease in activation of the *oP450arom* promoter induced by Ad4BP/SF-1 and Foxl2 in a dose-dependent manner. Cotransfection experiments showed that Dax1 inhibited Ad4BP/SF-1- and Foxl2-mediated *oP450arom* expression in vitro. These results suggest that Dax1 inhibits functions of Ad4BP/SF-1 and Foxl2 via direct protein–protein interaction with Ad4BP/SF-1 and Foxl2 in medaka ovarian follicles. In conclusion, medaka Dax1 downregulates Ad4BP/SF-1- and Foxl2-mediated *oP450arom* expression in ovarian follicles. Suppression of *oP450arom* is probably a basic function of Dax1, and this function may have been conserved throughout evolution among vertebrates.

18.4 Oocyte Maturation

After the oocyte completes its growth, it is ready for the resumption of meiosis. In teleosts, as in other vertebrates, the fully grown oocyte possesses a large nucleus (germinal vesicle) in meiotic prophase. The germinal vesicle of this stage is generally located centrally or halfway between the center and the oocyte periphery. In general, the germinal vesicle cannot be seen by external observation because of the opaque cytoplasm. The first visible event associated with oocyte maturation is the migration of the germinal vesicle to the animal pole where the micropyle is situated.

Although oocyte maturation has been studied in a variety of vertebrates and invertebrates, including mammals (Mehlmann 2005), amphibians (Masui and Clarke 1979; Hammes 2004), fishes (Nagahama et al. 1994), and starfish (Kishimoto 1999), much is still unknown about the follicular signals that promote oocyte maturation in most vertebrates. Progesterone has been the assumed in vivo mediator of frog oocyte maturation for many decades. More recently, however, androgens, rather than progesterone, were shown to be the dominant mediators of *Xenopus laevis* oocyte maturation in vivo (Hammes 2004). In contrast to those of frogs and fish, mouse oocytes spontaneously mature when removed from the ovary. The role of steroids in triggering mammalian oocyte maturation has remained controversial.

Studies using well-characterized in vitro systems, as well as those of in vivo systems, have established that oocyte maturation in fish is regulated by three major mediators, gonadotropin (LH), maturation-inducing hormone or steroid (MIH or MIS), and maturation-promoting factor (MPF), that function sequentially at the level of the ovarian follicle cells, the oocyte surface, and oocyte cytoplasm, respectively (Nagahama and Yamashita 2008). Two progestogens have been

identified as the primary mediators of LH-induced oocyte maturation, $17\alpha,20\beta$ -DP (Nagahama and Adachi 1985) and $17,20\beta,21$ -trihydroxy-4-pregnen-3-one (20β -S) (Thomas and Trant 1989). A distinct family of G protein-coupled membrane-bound MIH receptors (mPR α) has been shown to mediate nongenomic actions of 20β -S and $17\alpha,20\beta$ -DP (Thomas 2008). The mPRs are directly coupled to G proteins and typically activate pertussis-sensitive inhibitory G proteins (Gi) to downregulate adenylyl cyclase activity. These MIH signals induce the de novo synthesis of cyclin B from the stored mRNA, which activates a preexisting 35-kDa cdc2 kinase via phosphorylation of its threonine 161 by cyclin-dependent kinase-activating kinase, thus producing the 34-kDa active cdc2 (active MPF) (Nagahama and Yamashita 2008). Upon egg activation, MPF is inactivated by degradation of cyclin B. This process is initiated by the 26S proteasome through the first cut in its NH₂ terminus at lysine 57 (Tokumoto et al. 1997).

18.4.1 Hormonal Control of Oocyte Maturation in Medaka

In medaka, as in other teleosts, the primary hormone involved in the induction of oocyte maturation is gonadotropin. An important role of LH during the follicular maturation phase of medaka was confirmed by our recent studies on the expression of *LHR* (Shibata and Hirai, unpublished data). Levels of *LHR* mRNA were relatively high in postvitellogenic follicles (collected until 11 hrs) and then dropped sharply in follicles collected between 8 and 2 hrs. In situ hybridization analysis revealed that *LHR* mRNA was expressed mainly in granulosa cells at the postvitellogenic stage. In medaka, removal of all follicular constituents, but not removal of the thecal layer alone, prevented gonadotropin-induced maturation of oocytes. Denuded oocytes underwent maturation in response to gonadotropin when cocultured with isolated granulosa cells (Iwamatsu 1980). These results suggest that medaka granulosa cells alone are capable of producing MIH in response to gonadotropin. This situation appears to differ from that in salmonids, in which both thecal and granulosa layers are necessary for the follicular production of MIH in response to gonadotropin (Young et al. 1986).

Using cell-free homogenates of medaka ovarian follicles isolated during oocyte maturation, 13 metabolites were detected by thin-layer chromatography (TLC). Among them, one particular metabolite exhibited very high maturation-inducing activity by an in vitro homologous GVBD bioassay. This metabolite was identified as $17\alpha,20\beta$ -DP by its chromatographic mobility in TLC and recrystallization. The timely synthesis of $17\alpha,20\beta$ -DP in medaka at the onset of oocyte maturation, together with the demonstration that this steroid is the most potent inducer of oocyte maturation in vitro, provide evidence that $17\alpha,20\beta$ -DP is the naturally occurring MIH in the medaka (Sakai et al. 1987; Fukada et al. 1994).

We also demonstrated that postvitellogenic immature oocytes of medaka expressed inhibitory guanine-nucleotide-binding-regulatory protein α -subunits

(Oba et al. 1997). More recently, we have shown, in collaboration with Dr. Peter Thomas, the presence of three types of membrane progesterone receptors (mPRs) in postvitellogenic oocytes of medaka (Shibata et al., unpublished data). Taken together, it is possible that $17\alpha,20\beta$ -DP acts on oocytes through mPRs resulting in activation of an inhibitory G protein pathway, thus lowering intracellular cyclic adenosine monophosphate (cAMP) levels, leading to the formation and activation of MPF, the final inducer of oocyte maturation.

18.4.2 $17\alpha,20\beta$ -DP Precursor (17α -Hydroxyprogesterone) Production

$17\alpha,20\beta$ -DP is synthesized by its immediate precursor, 17α -hydroxyprogesterone, by the action of 20β -hydroxysteroid dehydrogenase (20β -HSD) (see Fig. 18.2). StAR, P450scc, 3β -HSD, and P450c17 are involved in the follicular production of 17α -hydroxyprogesterone. Levels of both StAR and P450scc transcripts were abundant in late- to postvitellogenic follicles during the maturation phase. A surprising finding was low levels of 3β -HSD transcripts in postvitellogenic follicles throughout the maturation phase, a stage with an increased capacity of follicles to produce 17α -hydroxyprogesterone and $17\alpha,20\beta$ -DP. Phylogenetic analysis of 3β -HSD cDNA isolated from medaka follicles revealed that this type of 3β -HSD was classified as mammalian 3β -HSD2. Recently, we were able to isolate another form of 3β -HSD from medaka ovarian follicles (Shibata et al., unpublished data). It is thus possible that this form of 3β -HSD is responsible for the production of 17α -hydroxyprogesterone by postvitellogenic follicles during the maturation phase.

In medaka, there is a peak in *P450c17-II* expression during late- to postvitellogenesis, coinciding with the start of $17\alpha,20\beta$ -DP production (Zhou et al. 2007a, b). The decrease in *P450c17-I* and the increase in *P450c17-II* expression in postvitellogenic follicles just before the final oocyte maturation stage may reduce the C17,20-lyase activity in the follicles, leading to the accumulation of 17α -hydroxyprogesterone, favoring the shift from production of estradiol-17 β to $17\alpha, 20\beta$ -DP.

As stated earlier, the switching in the expression of *P450c17-I* to *P450c17-II* is responsible in part for the switching in the action of P450c17 during the steroidogenic shift in the fish ovary. The next important question is how the expression of the two P450c17 genes is regulated at the transcriptional level. We next investigated the regulatory mechanisms involved in the transcription of these two P450c17 genes. Medaka *P450c17-II* possesses two putative Ad4BP/SF-1 binding sites in its promoter region, whereas only a single Ad4BP/SF-1 binding site is present in *P450c17-I*, suggesting that the transcriptional regulation of these two P450c17 genes is somewhat different. High expression of *Ad4BP/SF-1* was observable in the ovarian follicles during vitellogenesis, in agreement with previous data on the role of Ad4BP/SF-1 in the regulation of aromatase transcription (Watanabe et al. 1999).

Substantial levels of *Foxl2* expression could be detected throughout the spawning cycle of medaka, but a peak was observed immediately before oocyte maturation

(Zhou et al. 2007b), coinciding with the peak in *P450c17-II* expression. We then used luciferase assays to investigate the possible involvement of two transcription factors (Ad4BP/SF-1 and Foxl2) in the expression of medaka *P450c17-I* and *-II*. Ad4BP/SF-1 alone activated the transcription of *P450c17-II*, and Foxl2 further enhanced the Ad4BP/SF-1-activated *P450c17-II* transcription. *P450c17-I* expression was also regulated by these transcription factors in a manner similar to that of *P450c17-II*, but with twofold lower efficiency. Similar enhancing effects of Foxl2 on Ad4BP/SF-1-activated *oP450arom* transcription have also been found in tilapia (Wang et al. 2007). Thus, it is highly possible that these two kinds of transcription factors play important roles in the differential expression of *P450c17-I* and *-II* by ovarian follicles during two different stages of ovarian development, oocyte growth and maturation.

18.4.3 20 β -Hydroxysteroid Dehydrogenase

20 β -HSD catalyzes the reduction of 20-carbonyl groups to 20 β -hydroxylated products during steroidogenesis. In fish, 20 β -HSD is the key enzyme involved in the conversion of 17 α -hydroxyprogesterone to MIHs (17 α ,20 β -DP and 20 β -S). A carbonyl reductase-like 20 β -HSD cDNA was cloned first from a rainbow trout ovarian cDNA library (Guan et al. 1999). Since then, the presence of carbonyl reductase-like 20 β -HSD has been reported in ovaries of several teleosts. In Nile tilapia, a species with an average ovulatory cycle of 14–18 days, carbonyl reductase-like 20 β -HSD was shown to increase in postvitellogenic follicles during oocyte maturation and after gonadotropin treatment (Senthilkumaran et al. 2002).

In medaka, carbonyl reductase-like 20 β -HSD mRNA levels were relatively high and stable in early-, mid-, and late-vitellogenic follicles during the oocyte growth and maturation stage and dropped to very low levels in postvitellogenic follicles (Shibata et al., unpublished data). These changes in 20 β -HSD expression are partially reflected in the changes in the capacity of medaka postvitellogenic follicles to produce 17 α ,20 β -DP in response to gonadotropin or 17 α -hydroxyprogesterone (Sakai et al. 1987, 1988). Further studies are needed to determine whether this type of 20 β -HSD is the only enzyme that is responsible for the conversion of 17 α -hydroxyprogesterone to 17 α ,20 β -DP in medaka ovarian follicles during the oocyte maturation stage.

18.5 Ovulation

After the completion of the first meiotic division, mature oocytes (eggs) must be released from the surrounding follicle cells to be fertilized, an event called ovulation. The follicle layer must detach from the oocyte. Ultrastructural studies have revealed that both follicle cell microvilli and oocyte microvilli withdraw from

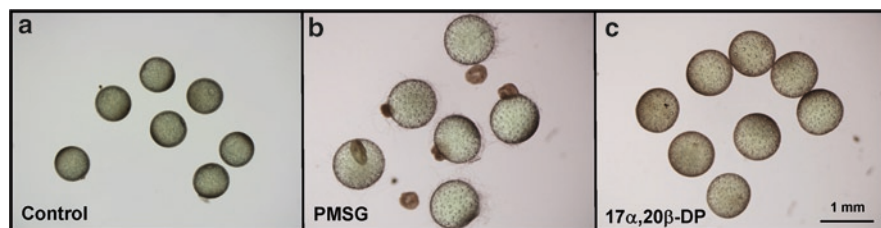


Fig. 18.5 Induction of oocyte maturation and ovulation in vitro by pregnant mare serum gonadotropin (PMSG) (b) and $17\alpha,20\beta$ -DP (c). Ovarian follicles were collected at 23 h before spawning and were incubated in the presence or absence (Control, a) of PMSG (b: 50 IU) or $17\alpha,20\beta$ -DP (c: 0.1 μ M) for 20 h

chorion near the time of ovulation, and a wide space is formed between the egg membrane and follicle near the time of ovulation. Ovulated eggs continue meiosis up to the second meiotic metaphase, the point at which fertilization first becomes possible, the entire process, or at least part, being hormone dependent. However, oocyte maturation and ovulation are not always associated because oocytes of most teleosts do not undergo ovulation following steroid stimulation in vitro.

Although ovulation is a physiologically well-characterized event, the underlying molecular pathways remain poorly understood. The ease of manipulating fish oocytes and ovarian fragments in vitro makes fish an excellent experimental model for investigating the regulatory mechanisms of ovulation. By using medaka, the process of follicle rupture and oocyte extrusion can be observed in vitro in isolated intact follicles (Fig. 18.5). To induce oocyte maturation and ovulation in medaka, ovarian follicles collected at 23 hbs were incubated with PMSG or $17\alpha,20\beta$ -DP for 20 h. PMSG induced both oocyte maturation and ovulation, although $17\alpha,20\beta$ -DP induced only oocyte maturation (Fig. 18.5). Importantly, none of the controls underwent oocyte maturation during the incubation period.

To investigate whether steroid nuclear receptors are involved in oocyte maturation and ovulation, we first used real-time PCR to examine the temporal expression profiles of transcripts encoding several steroid nuclear receptors such as estrogen receptor (ER)- α , - β 1, and - β 2, androgen receptor (AR)- α , glucocorticoids receptor (GR), and progesterone receptor (nPR), in ovarian follicles of medaka during the 48-h sampling period (Shibata et al., unpublished data). Interestingly, only nPR exhibited a distinct expression profile during the oocyte maturation phase. The greatest induction of nPR transcripts was found in follicles collected at 11 hbs. Our results also demonstrated relatively constant follicular expression levels of ER α and ER β 2 between 47 and 14 hbs. ER β 1 transcripts were constantly very low throughout the sampling period. Both AR- α and GR transcripts were relatively high in ovarian follicles during the growth phase and then decreased gradually.

The distinct expression pattern of nPR mRNA during the maturation phase prompted us to determine which factors (gonadotropins or $17\alpha,20\beta$ -DP) are responsible for this induction of nPR expression. When postvitellogenic follicles collected at 23 hbs were incubated with PMSG or $17\alpha,20\beta$ -DP, PMSG, but not

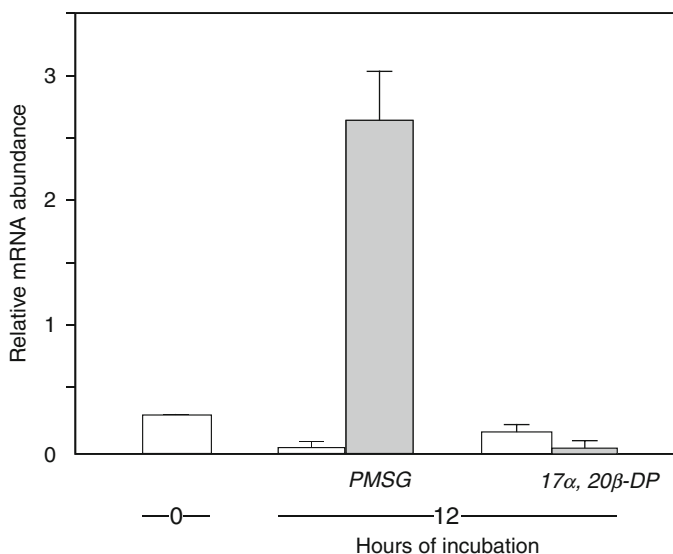


Fig. 18.6 Effects of *PMSG* and *17α,20β-DP* on the expression of progesterone receptor (nPR) mRNA in medaka ovarian follicles. Ovarian follicles were collected at 23 h before spawning and were incubated in the presence (*closed bars*) or absence (*open bars*) of *PMSG* (50 IU) or *17α,20β-DP* (0.1 μM) for 12 h. Real-time PCR was used to measure levels of mRNA

17α,20β-DP, was able to induce nPR mRNA expression in granulosa cells (Fig. 18.6). We also showed that the action of *PMSG* on the upregulation of nPR mRNA expression was mimicked by forskolin, an adenylate cyclase activator. These findings are consistent with the view that cAMP is the second messenger of *PMSG* action. Thus, the *in vitro* induction of ovulation and nPR mRNA expression by gonadotropin in postvitellogenic follicles of medaka suggest that *17α,20β-DP* is involved in the induction of ovulation through its binding to nPR. Similarly, progesterone plays important roles in the induction of ovulation in rats and mice. Inhibitors of progesterone synthesis have been shown to block LH-induced ovulation *in vivo* and *in vitro*. Failure of ovulation (follicle rupture) in media containing *17α,20β-DP* could be caused by insufficient expression of nPR in granulosa cells. The importance of nPR in ovulation is underscored by the infertility of nPR null mice. These mice failed to ovulate even after exogenous stimulation with gonadotropins, as indicated by the presence of unruptured preovulatory follicles in the ovary and the absence of oocytes in the oviduct and uterine horns (Lydon et al. 1995). Taken together, it is concluded that in medaka the processes of oocyte maturation and ovulation are both regulated by *17α,20β-DP*. Oocyte maturation is mediated by the *17α,20β-DP* membrane receptor (oocytes), whereas ovulation is regulated by the nuclear progesterone receptor (granulosa cells).

Another important advance has recently been made, in particular with the use of an in vitro ovulation system of medaka, in identifying hydrolytic enzymes essential for follicular rupture (Ogiwara et al. 2005). The follicular rupture in medaka is accomplished by the cooperation of at least three matrix metalloproteinases (gelatinase A and membrane-type metalloproteinases 1 and 2), together with the tissue inhibitor of metalloproteinase-2b protein. Identification of the hydrolytic enzymes responsible for follicle rupture will provide new perspectives for future studies on the proteolytic events that impact the ovulation process. Using medaka, it should be possible to determine the specific biochemical and molecular events by which gonadotropin (LH) and maturation-inducing hormone ($17\alpha,20\beta$ -DP) mediate the ovulatory process in fish.

18.6 Conclusion

Two major steroid hormones, estradiol- 17β and $17\alpha,20\beta$ -DP, play important roles in the regulation of oogenesis in fish. The impact of these hormones on oogenesis is determined by ligand availability, receptor expression, and the repression or induction of relevant regulatory genes. It is clear from this review that ovarian follicles collected at discrete stages of maturity from a sexually mature, un hormonally primed animal provide an excellent model for the study of the mechanisms involved in the regulation of oocyte growth, maturation and ovulation. These advantages collectively demonstrate the appropriateness of using the medaka as a valid model for the study of oogenesis, which has applicability and validity for other vertebrates including mammals.

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References

- Devlin RH, Nagahama Y (2002) Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture* 208:191–364
- Fukada S, Sakai N, Adachi S, Nagahama Y (1994) Steroidogenesis in the ovarian follicle of medaka (*Oryzias latipes*, a daily spanner) during oocyte maturation. *Dev Growth Differ* 36:81–88
- Fukada S, Tanaka M, Matsuyama M, Kobayashi D, Nagahama Y (1996) Isolation, characterization, and expression of cDNAs encoding the medaka (*Oryzias latipes*) ovarian follicle P-450 aromatase. *Mol Reprod Dev* 45:285–290

- Guan G, Tanaka M, Todo T, Young G, Yoshikuni M, Nagahama Y (1999) Cloning and expression of two carbonyl reductase-like 20 β -hydroxysteroid dehydrogenase cDNAs in ovarian follicles of rainbow trout (*Oncorhynchus mykiss*). *Biochem Biophys Res Commun* 255:123–128
- Hammes SR (2004) Steroids and oocyte maturation – a new look at an old story. *Mol Endocrinol* 18:769–775
- Ito M, Yu R, Jameson JL (1997) DAX-1 inhibits SF-1-mediated transactivation via a carboxy-terminal domain that is deleted in adrenal hypoplasia congenita. *Mol Cell Biol* 17:1476–1483
- Iwamatsu T (1980) Studies on oocyte maturation of the medaka *Oryzias latipes*. VIII. Role of follicular constituents in gonadotropin- and steroid-induced maturation of oocytes in vitro. *J Exp Zool* 211:231–239
- Kagawa H, Young G, Adachi S, Nagahama Y (1982) Estradiol-17 β production in amago salmon (*Oncorhynchus rhodurus*) ovarian follicles: role of the thecal and granulosa cells. *Gen Comp Endocrinol* 47:440–448
- Kishimoto T (1999) Activation of MPF at meiosis reinitiation in starfish oocytes. *Dev Biol* 214:1–8
- Kobayashi D, Tanaka M, Fukada S, Nagahama Y (1996) Steroidogenesis in the ovarian follicles of the medaka (*Oryzias latipes*) during vitellogenesis and oocyte maturation. *Zool Sci* 13:921–927
- Lydon JP, DeMayo FJ, Funk CR et al (1995) Mice lacking progesterone receptors exhibit pleiotropic reproductive abnormalities. *Genes Dev* 9:2266–2278
- Maller WL (2005) Minireview: regulation of steroidogenesis by electron transfer. *Endocrinology* 146:2544–2550
- Masui Y, Clarke HJ (1979) Oocyte maturation. *Int Rev Cytol* 57:185–282
- Mehlmann LM (2005) Stops and starts in mammalian oocytes: recent advances in understanding the regulation of meiotic arrest and oocyte maturation. *Reproduction* 130:791–799
- Nagahama Y (1983) The functional morphology of teleost gonads. In: Hoar WS, Randall DJ, Donaldson EM (eds) *Fish physiology*, vol IXA. Academic Press, New York, pp 223–275
- Nagahama Y, Adachi S (1985) Identification of a maturation-inducing steroid in a teleost, the amago salmon (*Oncorhynchus rhodurus*). *Dev Biol* 109:428–435
- Nagahama Y, Matsuhisa A, Iwamatsu T, Sakai N, Fukada S (1991) A mechanism for the action of pregnant mare serum gonadotropin on aromatase activity in the ovarian follicle of the medaka, *Oryzias latipes*. *J Exp Zool* 259:53–58
- Nagahama Y, Yamashita, M (2008) Regulation of oocyte maturation in fish. *Dev Growth Differ* 50:195–219
- Nagahama Y, Yamashita M, Tokumoto T (1994) Regulation of oocyte maturation in fish. *Curr Top Dev Biol* 30:103–145
- Nakamoto M, Wang DS, Suzuki A, Matsuda M, Nagahama Y, Shibata N (2007) *Dax1* suppresses P450arom expression in medaka ovarian follicles. *Mol Reprod Dev* 74:1239–1246
- Oba Y, Yoshikuni M, Tanaka M, Mita M, Nagahama Y (1997) Inhibitory guanine-nucleotide-binding regulatory protein α subunits in medaka (*Oryzias latipes*) oocytes. *Eur J Biochem* 249:846–853
- Ogiwara K, Takano N, Shinohara M, Murakami M, Takahashi T (2005) Gelatinase A and membrane-type matrix metalloproteinases 1 and 2 are responsible for follicle rupture during ovulation in the medaka. *Proc Natl Acad Sci USA* 102:8442–8447
- Onitake K, Iwamatsu T (1986) Immunocytochemical demonstration of steroid hormones in the granulosa cells of the medaka, *Oryzias latipes*. *J Exp Zool* 239:97–103
- Sakai N, Iwamatsu T, Yamauchi K, Nagahama Y (1987) Development of the steroidogenic capacity of medaka (*Oryzias latipes*) ovarian follicles during vitellogenesis and oocyte maturation. *Gen Comp Endocrinol* 66:333–342
- Sakai N, Iwamatsu T, Yamauchi K, Suzuki N, Nagahama Y (1988) Influence of follicular development of steroid production in the medaka (*Oryzias latipes*) ovarian follicles in response to exogenous substances. *Gen Comp Endocrinol* 71:516–523

- Senthilkumaran B, Sudhakumari CC, Chang XT, Kobayashi T, Oba Y, Guan GJ, Yoshiura Y, Yoshikuni M, Nagahama Y (2002) Ovarian carbonyl reductase-like 20 β -hydroxysteroid dehydrogenase shows distinct surge in messenger RNA expression during natural and gonadotropin-induced meiotic maturation in Nile tilapia. *Biol Reprod* 67:1080–1086
- Stocco DM (2000) The role of the StAR protein in steroidogenesis: challenges for the future. *J Endocrinol* 164:247–253
- Tanaka M, Fukada S, Matsuyama M, Nagahama Y (1995) Structure and promoter analysis of the cytochrome P-450 aromatase gene of the teleost fish, medaka (*Oryzias latipes*). *J Biochem (Tokyo)* 117:719–725
- Thomas P (2008) Characteristics of membrane progesterin receptor alpha (mPRa) and progesterone membrane receptor component one (PGMRC1) and their roles in mediating rapid progesterin actions. *Front Neuroendocrinol* 29:292–312
- Thomas P, Trant JM (1989) Evidence that 17, 20 β , 21-trihydroxy-4-pregnen-3-one is a maturation-inducing steroid in spotted seatrout. *Fish Physiol Biochem* 7:185–191
- Tokumoto T, Yamashita M, Tokumoto M et al (1997) Initiation of cyclin B degradation by the 26S proteasome upon egg activation. *J Cell Biol* 138:1313–1322
- Wang DS, Kobayashi T, Zhou LY, Paul-Prasanth B, Ijiri S, Sakai F, Okubo K, Morohashi K, Nagahama Y (2007) Foxl2 up-regulates aromatase gene transcription female-specifically by binding to the promoter as well as interacting with Ad4BP/SF-1. *Mol Endocrinol* 21:712–725
- Watanabe M, Tanaka M, Kobayashi D, Yoshiura Y, Oba Y, Nagahama Y (1999) Medaka (*Oryzias latipes*) FTZ-F1 potentially regulates the transcription of P450 aromatase in ovarian follicles: cDNA cloning and functional characterization. *Mol Cell Endocrinol* 149:221–228
- Young G, Adachi S, Nagahama Y (1986) Role of ovarian thecal and granulosa layers in gonadotropin-induced synthesis of a salmonid maturation-inducing substance (17 α , 20 β -dihydroxy-4-pregnen-3-one). *Dev Biol* 118:1–8
- Zazopoulos E, Lalli E, Stocco DM, Sassone-Corsi P (1997) DNA binding and transcriptional repression by DAX-1 blocks steroidogenesis. *Nature (Lond)* 390:311–315
- Zhou LY, Wang DS, Kobayashi T, Yano A, Paul-Prasanth B, Suzuki A, Sakai F, Nagahama Y (2007a) A novel type of P450c17 lacking the lyase activity is responsible for C21-steroid biosynthesis in the fish ovary and head kidney. *Endocrinology* 148:4288–4291
- Zhou LY, Wang DS, Shibata Y, Paul-Prasanth B, Suzuki A, Nagahama Y (2007b) Characterization, expression and transcriptional regulation of *P450c17-I* and *-II* in the medaka, *Oryzias latipes*. *Biochem Biophys Res Commun* 362:619–624

Chapter 19

Interspecific Medaka Hybrids as Experimental Models for Investigating Cell Division and Germ Cell Development

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Abstract Interspecific hybrids in the genus *Oryzias* (medaka) usually have abnormalities in reproduction and development. Analysis of the causes of the abnormalities at cellular and molecular levels will contribute to the understanding of basic mechanisms of cell division and gametogenesis. We describe the molecular and cellular mechanisms of embryonic lethality in a hybrid between *Oryzias latipes* and *Oryzias hubbsi* and of sex-specific abnormal gametogenesis in a hybrid between *O. latipes* and *Oryzias curvinotus*. Chromosomes derived from *O. hubbsi* are selectively eliminated in both *O. latipes* (♀)–*hubbsi* (♂) and *O. hubbsi* (♀)–*latipes* (♂) embryos. We propose a possible cellular mechanism of the abnormal mitosis. We also suggest that the hybrid M-phase-promoting factor (MPF; complex of Cdc2 and cyclin B, each of which is derived from different species) is a general molecule causing defective cell divisions in hybrids. In a hybrid between *O. latipes* and *O. curvinotus*, spermiogenesis proceeds and sperm-like cells each having 4C DNA are produced without cytokinesis. In hybrid females, the majority of oocytes stop meiosis at the zygotene stage, although a small number of oocytes give rise to diploid eggs. An incomplete synaptonemal complex devoid of SCP1 is likely to cause the aberrant gametogenesis in both sexes in common.

Abbreviations

CE	Central element
CGH	Comparative genomic hybridization

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CPC	Chromosomal passenger complex
DAPI	4',6-Diamidino-2-phenylindole
FISH	Fluorescence in situ hybridization
INCENP	Inner centromere protein
LEs	Lateral elements
MBT	Midblastula transition
MPF	M-phase-promoting factor
SC	Synaptonemal complex
TFs	Transverse filaments
WCP	Whole chromosome painting

19.1 Introduction

In many species, interspecific hybrids show various abnormalities, including embryonic lethal phenotypes by aberrant development and infertile phenotypes by production of abnormal gametes. This phenomenon is known as a fundamental mechanism of reproductive isolation (called the intrinsic postzygotic isolation mechanism), which is required for fixing and maintaining the species (Orr 2005). Although investigation of molecules that cause these abnormalities in hybrids provides information on how the species are fixed and maintained by reproductive isolation, hybrids can also be used as good tools for studying the regulatory mechanisms of embryogenesis and gametogenesis by regarding these hybrids as mutants, which are excellent experimental organisms used widely for biological research.

As with zebrafish, medaka fish (*Oryzias latipes*) have received much attention as experimental animals in various fields of biological science because their characteristics render them suitable for experiments (Naruse et al. 1994; Wittbrodt et al. 2002; Furutani-Seiki et al. 2004; Shima and Mitani 2004). The most remarkable characteristic that draws the line between medaka and zebrafish is that a hybrid between two closely related species among the genus *Oryzias* is easily produced and that the resulting hybrids exhibit various abnormalities in their embryogenesis and gametogenesis (Iwamatsu et al. 1984, 1986, 1994, 2003; Formacion and Uwa 1985; Hamaguchi and Sakaizumi 1992; Sakaizumi et al. 1992, 1993; Kurita et al., 1995; Hori and Iwamatsu 1996; Shimizu et al. 1997, 2000a,b), because the zebrafish lacks this characteristic. By understanding the cellular and molecular causes of abnormalities occurring in medaka hybrids, we can gain deeper insights into the mechanisms of regulation in embryogenesis and gametogenesis in normal individuals. In this chapter, we focus on two hybrids, a hybrid between *O. latipes* and *Oryzias hubbsi*¹ with defects in mitosis and a hybrid between *O. latipes* and *Oryzias*

¹*Oryzias hubbsi* was formerly included in *Oryzias javanicus*, but it has been reported as a new species (Roberts 1998). However, the nomenclature needs to be reconsidered, in accordance with the habitats of *O. hubbsi* and *O. javanicus*. *O. hubbsi* is an endemic species of Java and West Kalimantan (Iwamatsu et al. 1982), while *O. javanicus* inhabits Malaysia and Thailand. It is therefore appropriate that *O. hubbsi* be named *O. javanicus* (Iwamatsu, personal communication).

curvinotus with defects in meiosis, and describe cytological processes in the abnormal mitosis and meiosis, with suggestions of their causative mechanisms.

19.2 Abnormal Cell Division and Its Cause in Hybrids Between *O. latipes* and *O. hubbsi*

19.2.1 Loss of Chromosomes in the Hybrid Embryos

A hybrid medaka between *O. latipes* and *O. hubbsi* undergoes unusual embryogenesis, with a short and waved embryonic body, abnormal pectoral fins, microcephaly, retina pigmentation, and small eyes, and all embryos die within 9 days after fertilization. The abnormalities are not dependent on the direction of the cross; both *O. latipes* (♀)–*hubbsi* (♂) and *O. hubbsi* (♀)–*latipes* (♂) embryos show similar phenotypes (Iwamatsu et al. 1994; Hori and Iwamatsu 1996; Sakai et al. 2007). These phenotypes resemble those found in medaka carrying a haploid genome, so-called haploid syndrome (Araki et al. 2001). Moreover, it has been reported that chromosomes were improperly separated in the early cleavage of hybrid embryos (Iwamatsu et al. 2003), suggesting the occurrence of chromosome elimination during embryogenesis in the hybrid. We have confirmed this by chromosome analysis, which revealed that the hybrid cells have a significantly decreased number of chromosomes (Fig. 19.1a, b). At the morula stage, only about one-fourth of the hybrid cells were equipped with 48 chromosomes (the complete chromosome number in *O. latipes* and *O. hubbsi*), and the remainder showed aneuploidy. In 3-day embryos, which seemed normal in appearance, most of the cells showed aneuploidy with 18–24 chromosomes, and those with 24 chromosomes were predominant (Sakai et al. 2007). The hybrid cells had defects in chromosome behavior; some chromosomes lagged in motion toward the spindle poles at anaphase, being trapped at the metaphase plate (Fig. 19.1c–h). An aggregation of chromosomes at the metaphase plate was also observed frequently (Fig. 19.1e). It is likely that these lagging chromosomes fail to be delivered evenly to daughter cells and are eliminated from the cells via formation of micronuclei, as reported in a salmonid hybrid (Fujiwara et al. 1997). Although its frequency seemed higher in earlier stages, abnormal behavior of chromosomes was observed from the first cleavage throughout embryogenesis, even after the midblastula transition (MBT: 9–10 h after fertilization) (Iwamatsu 1994; Aizawa et al. 2003).

19.2.2 Preferential Elimination of *O. hubbsi* Chromosomes from the Hybrid Embryos

The finding that the chromosome number decreases to nearly half in the hybrid cells suggests that the chromosomes derived from one parent tend to be preferentially eliminated from the cells. This possibility was verified by fluorescence in situ hybridization (FISH) analyses using two different techniques, whole chromosome painting (WCP) and comparative genomic hybridization (CGH), which discriminate the fate of *O. hubbsi* chromosomes from *O. latipes* chromosomes during

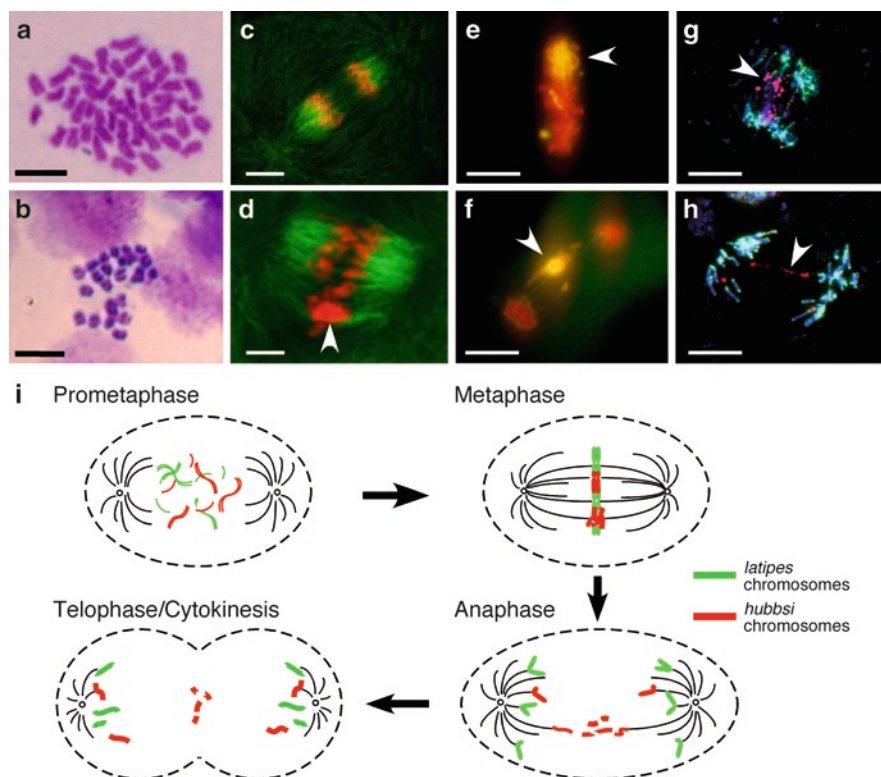


Fig. 19.1 Chromosomes in the wild-type medaka (*Oryzias latipes*; **a, c**) and the hybrid medaka (*Oryzias latipes* (♀)–*hubbsi* (♂); **b, d–h**). (The figure appears in color in the online version.) Samples were stained with Giemsa (**a, b**), anti-tubulin antibody (**c, d**), whole chromosome painting (WCP; **e, f**), or comparative genomic hybridization (CGH; **g, h**). The wild-type medaka embryo retains 48 chromosomes (**a**) with normal separation of chromosomes at anaphase (**c**), whereas the hybrid embryo contains a decreased number of chromosomes (**b**) with aggregated chromosomes at metaphase (arrowhead in **e**) and lagging chromosomes at anaphase (arrowheads in **d, f, g, h**). In WCP (**e, f**), the *O. hubbsi* chromosomes were stained with fluorescein isothiocyanate in green and both the *O. latipes* and the *O. hubbsi* chromosomes were stained with propidium iodide in red. The *O. hubbsi* chromosomes (shown in yellow) have aggregated at the metaphase plate (arrowhead in **e**) and remained at the equatorial region of the spindle at anaphase as lagging chromosomes (arrowhead in **f**). In CGH (**g, h**), the *O. latipes* chromosomes were stained with Alexa 488 in green and the *O. hubbsi* chromosomes were stained with Cy3 in red [DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) in blue]. The lagging chromosomes (shown by arrowheads) were stained reddish, indicating their *hubbsi* origin. The behavior of *latipes* and *hubbsi* chromosomes during mitosis of the hybrid cells is schematically shown (**i**). Bars **a–h** 5 μ m

embryogenesis (Sakai et al. 2007). FISH analyses revealed that the lagging chromosomes were derived from *O. hubbsi* (Fig. 19.1f–h) and that the *hubbsi* chromosomes were frequently aggregated at the equatorial plate in the hybrid cells at metaphase (Fig. 19.1e).

Similar to *O. latipes* (♀)–*hubbsi* (♂) hybrids, *O. hubbsi* (♀)–*latipes* (♂) hybrids are also nonviable because of the loss of chromosomes. Both WCP and CGH

revealed that the lagging chromosomes in *O. hubbsi* (♀)–*latipes* (♂) hybrids were originated from *hubbsi*, indicating that the *O. hubbsi* chromosomes always tend to be eliminated from the hybrid cells irrespective of the combination of parents (Sakai et al. 2007). Most *O. latipes* (♀)–*luzonensis* (♂) hybrids are also embryonically lethal, although *O. luzonensis* (♀)–*latipes* (♂) hybrids are viable (Formacion and Uwa 1985). We found that *O. luzonensis* chromosomes were preferentially eliminated in this case (Nakano et al., unpublished data).

19.2.3 Molecular Characteristics of Chromosomes Being Eliminated in the Hybrid Embryos

To gain an insight into the molecular mechanisms of abnormal chromosome behavior in the hybrid medaka, we examined expression patterns of several proteins that are thought to be involved in regulation of chromosome behavior: those include γ -tubulin, inner centromere protein (INCENP), Cdc20, Mad2, phospho-histone H3, and cohesin subunits (SMC1 α , SMC3, and Rad21) (Sakai et al. 2007). Whole-mount immunocytochemical analyses showed that, except for phospho-histone H3, there were no remarkable differences in the protein expression patterns between the wild-type and the hybrid medaka. In contrast, anomalous expression of phospho-histone H3 was observed in anaphase cells of the hybrid medaka. Both in the wild-type and hybrid medaka, phospho-histone H3 was present on chromosomes at metaphase and disappeared from the chromosomes that moved toward the spindle pole at anaphase. In the hybrid cells, however, phospho-histone H3 was still detectable on the lagging chromosomes at anaphase.

19.2.4 Cellular Mechanisms of Chromosome Elimination in the Hybrid Embryos

Oryzias hubbsi chromosomes are preferentially eliminated during the embryogenesis of reciprocal hybrids between *O. latipes* and *O. hubbsi*, through abnormal mitosis including impaired chromosome segregation at anaphase (Fig. 19.1i). Uniparental chromosome elimination has been reported in a salmonid hybrid, in which an incompatibility between the maternal cytoplasm and the paternal genome appears to cause the abnormal mitosis (Fujiwara et al. 1997), because the loss of chromosomes occurs mainly during the period from just after fertilization until the MBT, the time point when the zygotic genomes, including the paternal ones, begin to be expressed. In contrast to the salmonid hybrid, chromosome elimination in the hybrid medaka continues to occur even after the MBT (Sakai et al. 2007). Therefore, the incompatibility between the maternal cytoplasm and the paternal genome is unlikely to be a main cause of the abnormality in the hybrid medaka.

It has been reported that an *O. hubbsi* pronucleus undergoes chromosome condensation more slowly than does an *O. latipes* pronucleus during the fertilization process of an *O. latipes* (♀)–*hubbsi* (♂) hybrid (Iwamatsu et al. 2003), which provides a hint concerning the cellular mechanism of preferential elimination of *O. hubbsi* chromosomes in the hybrid. The timing of chromosome condensation should be tightly coupled to the timing of cell-cycle progression to guarantee the accurate delivery of sister chromatids to daughter cells. In the hybrid cells, however, the difference in the timing of chromosome condensation between two parents might perturb the coordination of nuclear (chromosomal) dynamics and cytoplasmic (spindle) dynamics. For example, if the cell-cycle progression is conducted by the timing of condensation of *latipes* chromosomes, then the slowly condensing *O. hubbsi* chromatins would fail to catch up with the cell-cycle progression. The finding that the lagging (*hubbsi*) chromosomes retain expression of the metaphase marker phospho-histone H3 at anaphase strongly suggests that they remain in the metaphase state. It is therefore proposed that the *O. hubbsi* chromosomes have a marked tendency to be left behind in cell-cycle progression to anaphase because of their intrinsic slow speed of chromatin condensation. A feasible scenario leading to the preferential elimination of *hubbsi* chromosomes in the hybrid cells is as follows: (1) at prophase, *hubbsi* chromatins tend to condense more slowly than *latipes* chromatins do; (2) at metaphase, some of the *hubbsi* chromosomes are late for accurate alignment at the equatorial plate of the spindle (the aggregation of *hubbsi* chromosomes at the metaphase plate (Fig. 19.1e) might relate to this scene); (3) these chromosomes can only interact incorrectly with spindle fibers, such as the case of merotelic kinetochore orientation (a single kinetochore becoming attached to microtubules from two spindle poles rather than just to one pole) reported for nocodazole-treated mammalian cultured cells (Cimini et al. 2001); and (4) they are left at the metaphase plate or are improperly separated at anaphase, thereby being eliminated from the cell.

The fact that 40% of cells have fewer than 24 chromosomes at the morula stage in the hybrid indicates that not only *O. hubbsi* chromosomes but also some *O. latipes* chromosomes are eliminated in the hybrid (Sakai et al. 2007). In addition, all the *hubbsi* chromosomes are not simultaneously eliminated in the hybrid cells. These events are not explainable by the aforementioned scenario, suggesting the involvement of plural mechanisms in the hybrid abnormalities. Identification of eliminated chromosomes, that is, whether certain chromosomes have a tendency toward preferential elimination or any chromosome is eliminated accidentally, might provide some clues to understanding the cellular mechanisms of chromosome elimination in the hybrid. To this end, we produced chromosome-specific painting probes by amplifying DNA sequences of medaka chromosomes that were singly sorted with fluorescence-activated cell sorter technology, and we found that at least nine chromosomes identified with the painting probes underwent various separation patterns, including lagging, during cell divisions, a finding that supports the notion of nonselective elimination (Konno et al., unpublished data). The random elimination of chromosomes at various stages of embryogenesis implies that the elimination is dependent on factors acting by chance in response to microenvironments in the hybrid cells.

19.2.5 A Universal Molecule Responsible for Impaired Mitosis in Hybrids

In general, hybrids cannot survive or reproduce themselves because of serious defects in their embryogenesis and gametogenesis, a fundamental mechanism of reproductive isolation. Several genes causative of reproductive isolation (known as speciation genes) have been identified, but the mechanisms by which these genes induce various defects in hybrids are poorly understood (Orr et al. 2004; Kulathinal and Singh 2008). Furthermore, the speciation genes have been discovered independently in each hybrid and, to our knowledge, there have been no proposals for a common molecule responsible for abnormalities occurring always in hybrids.

M-phase-promoting factor (MPF), a complex of Cdc2 and cyclin B, is a protein kinase acting as a key regulator of cell division by phosphorylating specific substrates at M phase (Loog and Morgan 2005). Besides wild-type MPF, hybrid cells after the MBT have MPF that consists of subunits derived from different species (called hybrid MPF in this chapter; Fig. 19.2a). We verified the possibility that hybrid MPF is a cause of abnormal cell divisions in hybrids. The identity in the amino acid sequence of *O. latipes* cyclin B is 94% to *O. curvinotus* cyclin B and 91% to *O. hubbsi* cyclin B, whereas the difference in Cdc2 is only 1 amino acid between *O. latipes* and *O. hubbsi* and 2 amino acids between *O. latipes* and *O. curvinotus*, of 303 amino acids. It is therefore likely that the characteristics of hybrid MPF chiefly depend on cyclin B.

Although a hybrid between *O. latipes* and *O. curvinotus* exhibits considerable defects in gametogenesis (Fig. 19.3), it undergoes normal embryogenesis. In contrast, a hybrid between *O. latipes* and *O. hubbsi* is embryonically lethal because aberrant cell divisions eliminate *O. hubbsi* chromosomes from the cells (Fig. 19.1). We examined the effects of hybrid MPF on cell division by artificially producing hybrid MPF molecules in *O. latipes* embryos through injection of mRNAs encoding *hubbsi* cyclin B as an experiment and *latipes* or *curvinotus* cyclin B as a control (Fig. 19.2b). In addition to cyclin B-bound Cdc2, oocytes contain a considerable amount of monomeric Cdc2. The oocytes also contain an activating enzyme for cyclin B-bound Cdc2 (Nagahama and Yamashita 2008). Therefore, cyclin B proteins produced from the injected mRNA immediately bind to the preexisting monomeric Cdc2, and the resulting cyclin B-bound Cdc2 is activated by the endogenous activating enzyme.

In agreement with normal development of *O. latipes* wild-type embryos and *O. latipes*–*O. curvinotus* hybrid embryos, *O. latipes* embryos injected with *latipes* or *curvinotus* cyclin B exhibit normal mitotic figures at M phase (Fig. 19.2c–f). In striking contrast to this, *O. latipes* embryos injected with *O. hubbsi* cyclin B show various types of abnormal cell divisions, including those found in the hybrid embryos, such as chromosome aggregation and elimination (Fig. 19.2g, h). Consequently, *latipes*–*curvinotus* hybrid MPF, as well as *latipes* wild-type MPF, has no detrimental effects on cell division, and *latipes*–*hubbsi* hybrid MPF induces abnormal cell divisions, in harmony with the patterns of embryonic development of each hybrid.

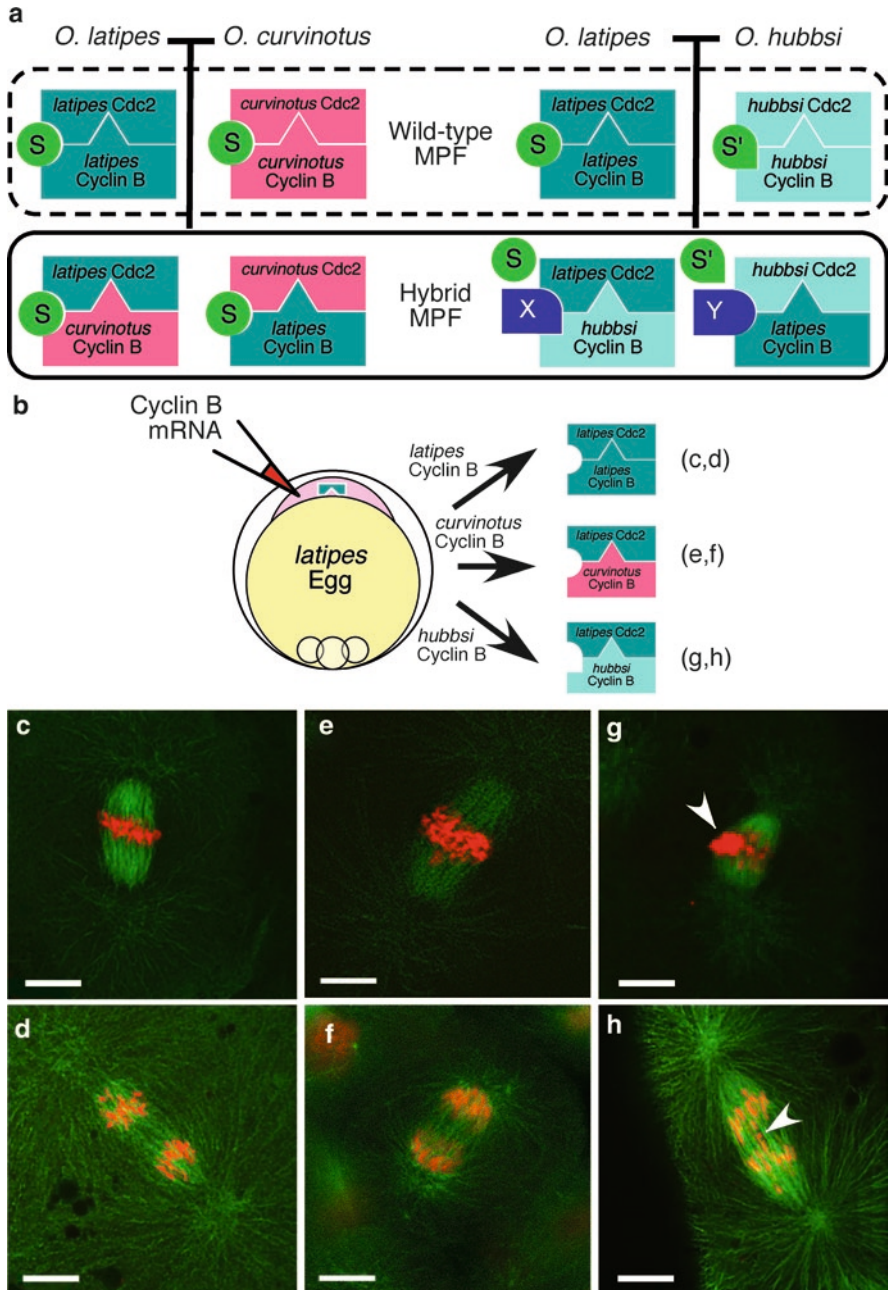


Fig. 19.2 Wild-type and hybrid M-phase-promoting factor (MPF) molecules. (The figure appears in color in the online version.) (a) MPF is a protein kinase consisting of two subunits, Cdc2 and cyclin B, and phosphorylates specific substrates (S, S') to promote the M phase. Hybrid MPF molecules between *O. latipes* and *O. hubbsi* cannot phosphorylate genuine substrate proteins (S, S') but phosphorylate proteins (X, Y) that must not be phosphorylated at M phase, thereby inducing abnormal cell divisions. Hybrid MPF molecules

Our findings provide the first experimental evidence that hybrid MPF is responsible for the abnormalities occurring in hybrid cells after the MBT. The hybrid MPF probably phosphorylates certain proteins that must not be phosphorylated in normal cells (Fig. 19.2a), inducing abnormal cell divisions dominantly. Because MPF is a ubiquitous protein kinase that controls cell division in all eukaryotes, our suggestion is generally applicable to hybrids in various species, although other factors should be accountable for abnormalities occurring in the early phase of embryogenesis before the MBT. Our suggestion also implies that many common enzymes consisting of two or more subunits can cause the abnormalities occurring in hybrids.

19.3 Abnormal Germ Cell Development and Its Cause in Hybrids Between *O. latipes* and *O. curvinotus*

19.3.1 Spermatogenesis in the Hybrids

F₁ individuals of *O. latipes* (♀)–*curvinotus* (♂) and *O. curvinotus* (♀)–*latipes* (♂) hybrids are viable, but their gametogenesis is irregular (Fig. 19.3). The males are sterile, and no haploid cells are produced in the testes (Hamaguchi and Sakaizumi 1992; Sakaizumi et al. 1992, 1993; Kurita et al. 1995; Shimizu et al. 1997, 2000a, b). The meiotic cell cycle of spermatocytes is arrested at around metaphase, but spermiogenesis proceeds on schedule despite the absence of normal meiosis. As a result, one spermatocyte differentiates into one sperm-like cell containing tetraploid DNA by unusual spermatogenesis without cytokinesis (Fig. 19.3a). The sperm-like cell has an abnormal flagellum and does not move in physiological saline (Shimizu et al. 1997). In addition, the nuclei of sperm-like cells are unstable in water, probably because of the lack of protamine, a protein specifically expressed in spermatis (Shimizu et al. 2000a).

←
Fig. 19.2 (continued) between *O. latipes* and *O. curvinotus* can phosphorylate authentic substrates (S) because the differences in cyclin B in these species are not so significant for substrate recognition. (b) Artificial production of hybrid MPF by injecting mRNAs encoding *O. latipes*, *O. curvinotus*, or *O. hubbsi* cyclin B into *O. latipes* embryos. Oocytes contain a considerable amount of monomeric Cdc2 and an activating enzyme for cyclin B-bound Cdc2. Therefore, cyclin B proteins produced from the injected mRNA immediately bind to the preexisting monomeric Cdc2, and the resulting cyclin B-bound Cdc2 is activated by the endogenous activating enzyme. (c–h) Mitotic figures in medaka embryos injected with *O. latipes* (c, d), *O. curvinotus* (e, f), or *O. hubbsi* cyclin B. Embryos injected with *O. latipes* or *O. curvinotus* cyclin B show normal mitotic figures (c–f), whereas those injected with *O. hubbsi* cyclin B show abnormal mitotic figures, including chromosome aggregation (g) and lagging (h), as those found in the hybrid embryos (see Fig. 19.1). Bars (c–h) 10 μm

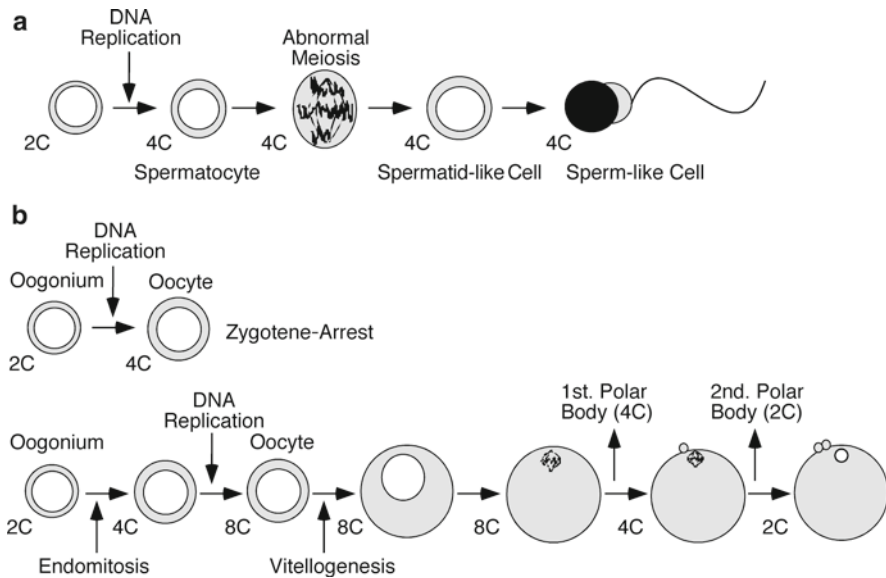


Fig. 19.3 Gametogenesis in the hybrid between *O. latipes* and *O. curvinotus*. **(a)** The hybrid male produces one sperm-like cell with 4C DNA content from one spermatocyte, in striking contrast to the wild-type male, which produces four haploid (1C) spermatozoa from one spermatocyte. **(b)** The hybrid female stops oogenesis at the zygotene stage, but some oocytes that have undergone endomitosis complete meiosis via formation of two polar bodies to become diploid (2C) eggs

19.3.2 Cohesin and Synaptonemal Complex in the Hybrid Male Germ Cells

In the hybrids, some chromosomes make a pair with their partner and align at the equator of the metaphase plate, whereas others fail to pair and locate near the spindle poles (Shimizu et al. 1997; see also Fig. 19.4j), indicating the occurrence of some defects in synaptonemal complex (SC) formation. Because cohesin subunits (SMC1 α , SMC1 β , SMC3, and Rad21) and SC proteins (SCP1 and SCP3, also known as SYCP1 and SYCP3, respectively) are critical for the formation of SC (Iwai et al. 2004, 2006), we examined their expression patterns in the hybrid testes, in comparison with those in the wild-type testes.

The expression patterns of all the cohesin subunits examined showed no difference between the wild-type and the hybrid testes. SCP1 was detected as a single 104-kDa band in both parents and reciprocal hybrids, and its protein content in the hybrids was about 15% of that in the parents (Fig. 19.4a). SCP3 was detected as 27- and 30-kDa bands in *O. latipes* and 28- and 31-kDa bands in *O. curvinotus*, because *O. curvinotus* SCP3 has three additional amino acids that are missing in *O. latipes* SCP3 and SCP3 is expressed as two forms that reflect different phosphorylation states (phosphorylated SCP3 exhibiting electrophoretic mobility slower

than the dephosphorylated form; Lammers et al. 1994, 1995). In the hybrid testes, SCP3 was expressed as four bands (Fig. 19.4a), indicating the expression of both *O. latipes* and *O. curvinotus* SCP3. In striking contrast to SCP1, the protein content of SCP3 in the hybrids was similar to that in the parents (Fig. 19.4a).

The SC consists of three elements: two lateral elements (LEs) adjacent to each homologous chromosome, transverse filaments (TFs) connecting the LEs, and a central element (CE) linking the TFs together (Fig. 19.4b). Previous electron microscopic observations of the hybrid gonads have demonstrated that germ cells possess asynaptic LEs (Hamaguchi and Sakaizumi 1992), suggesting a failure in organizing the CE and TFs during the formation of SC. We confirmed this by immunocytological examination of SCP3 for the LEs and SCP1 for the TFs.

There was no difference in localization patterns of SCP3 between the hybrid and the wild-type spermatocytes, but SCP1 showed remarkable expression patterns. In the wild-type spermatocytes at the pachytene stage, SCP1 was detected as distinct lines that represent the SC, whereas the lines in the hybrid spermatocytes were slender and shorter than those found in the wild-type spermatocytes. In addition, SCP1 and SCP3 colocalized to the whole region of each pachytene chromosome in the wild-type spermatocytes (Fig. 19.4c), whereas the colocalization was observed only on parts of some chromosomes in the hybrids (Fig. 19.4d), indicating incomplete formation of TFs in the hybrid spermatocytes. These findings strongly suggest that the reduced protein content and aberrant localization of SCP1 bring about the failure in pairing of homologous chromosomes in the hybrid spermatogenic cells. It remains to be elucidated whether the components of CE (such as SYCE1, SYCE2, and TEX12), which interact with SCP1 (Costa and Cooke 2007), are involved in irregular localization of SCP1 in the hybrid spermatocytes. The ultimate cause that promotes the defective expression and localization of SCP1 also remains a mystery. In this regard, it is notable that the expression patterns of SCP1 vary from cell to cell even in the samples derived from one individual (Iwai et al., unpublished data), implying that the difference in DNA sequence between paternal and maternal homologous chromosomes does not solely contribute to the mislocalization of SCP1 in the hybrid cell.

19.3.3 Cellular Mechanisms of Impaired Cytokinesis in the Hybrid Male Germ Cells

In addition to the failure in pairing of homologous chromosomes, the hybrid spermatocytes fail to undergo cytokinesis, resulting in the formation of sperm-like cells each having 4C DNA (Fig. 19.3a). To identify molecules responsible for the impaired cytokinesis, we examined the expression patterns of several proteins between wild-type and hybrid medaka. The expression patterns of α -tubulin and γ -tubulin suggest that almost all the hybrid spermatocytes form normal meiotic metaphase I spindles, although a multipolar spindle was observed in some spermatocytes. However, the hybrid spermatocytes have a peculiar chromosome

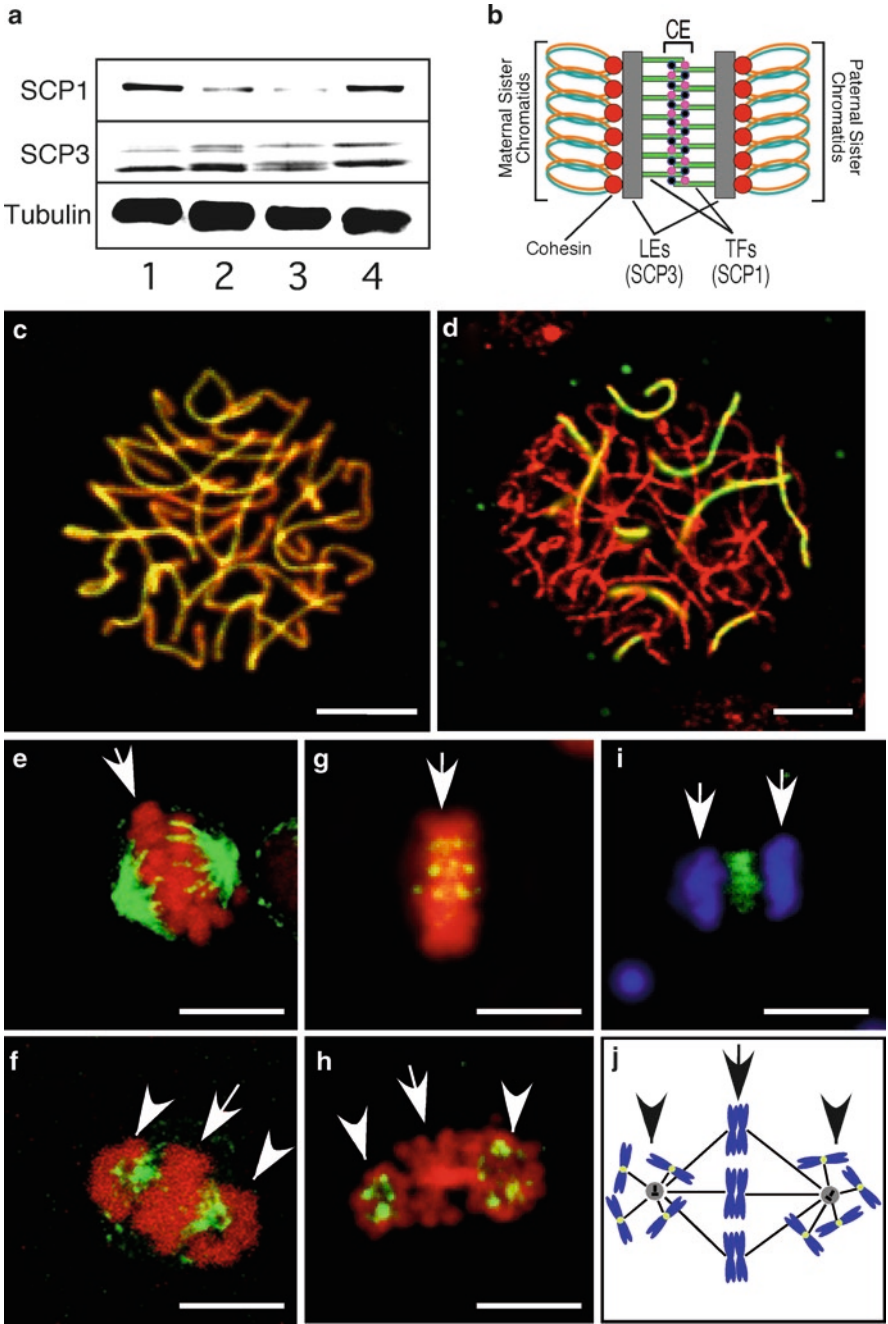


Fig. 19.4 SCP1, SCP3, and inner centromere protein (INCENP) in the hybrid between *O. latipes* and *O. curvinotus*. (The figure appears in color in the online version.) (a) Immunoblotting analyses of *SCP1*, *SCP3*, and *tubulin* (as a loading control) in the testes of parent medaka [1, *O. latipes*; 4, *O. curvinotus*) and hybrid medaka (2, *O. latipes* ♀ × *O. curvinotus* ♂);

arrangement; some chromosomes are aligned at the metaphase plate, whereas others aggregate near the spindle poles (Fig. 19.4e,f).

We also examined expression patterns of INCENP, a component of the chromosomal passenger complex (CPC), because CPC plays a pivotal role in regulating both chromosome arrangement and cytokinesis in cell division. CPC is composed of aurora B kinase and three nonenzymatic subunits, INCENP, survivin, and borealin, all of which are well conserved from yeast to humans. These components localize to the centromeres of chromosomes during prophase and metaphase, at which stages CPC functions in correcting erroneous kinetochore–microtubule attachment. Subsequently, the CPC components move from the centromeres to the central region of the spindle (the spindle midzone) at the metaphase–anaphase transition, and a subpopulation of them concentrates at the equatorial cortex, which is necessary to determine the position of the contractile ring, a chief structure executing cytokinesis (Hauf et al. 2007).

INCENP was present on chromosomes that came into line on the metaphase plate in the wild-type cells (Fig. 19.4g). In the hybrid cells, however, INCENP was present on chromosomes that were not aligned on the metaphase plate but absent on those aligned on the metaphase plate (Fig. 19.4h,j). Furthermore, INCENP relocated from the chromosomes to the spindle midzone at anaphase in the wild-type spermatocytes (Fig. 19.4i), while the hybrid spermatocytes did not proceed to anaphase, and consequently INCENP did not accumulate on the spindle midzone. Expression patterns of aurora B, another component of CPC, were similar to those of INCENP. These findings imply that the failure in localization of the CPC

Fig. 19.4 (continued) 3, *O. curvinotus* (♀)×*O. latipes* (♂)]. (b) A schematic drawing of the synaptonemal complex (SC). The cohesin complex links sister chromatids together. The lateral elements (LEs) consisting of SCP3 are present along homologous chromosomes (paternal and maternal sister chromatids). The transverse filaments (TFs) consisting of SCP1 and the central element (CE) connect the LEs. (c, d) Localization of SC proteins in *O. latipes* (c) and *O. latipes* (♀)–*curvinotus* (♂) (d) spermatocytes. Chromosome spreads were stained with a mixture of anti-SCP1 (in green) and anti-SCP3 (in red) antibodies. Note that SCP1 and SCP3 colocalize to the whole region of all chromosomes in the wild-type spermatocyte but that SCP1 localizes to a part of some chromosomes in the hybrid spermatocyte, although SCP3 exists on the whole region of all chromosomes. (e–j) Chromosome arrangement and expression patterns of inner centromere protein (INCENP) in *O. latipes* (e, g, i) and *O. latipes* (♀)–*curvinotus* (♂) (f, h, j) spermatocytes. Samples were stained with antitubulin antibody (e, f) or anti-medaka INCENP antibody in green and with DNA dye, propidium iodide (e–h) in red, or DAPI (i) in blue. A schematic diagram showing the localization of INCENP (in yellow) on chromosomes (in blue) is also shown (j). All chromosomes are aligned at the metaphase plate (arrows in e, g) and completely separated at anaphase (arrows in i) in the wild-type spermatocytes. In the hybrid spermatocytes, some chromosomes are precociously pulled toward the spindle pole at metaphase (arrowheads in f, h, j), although the remainder are aligned at the spindle equator (arrows in f, h, j). INCENP localizes to the centromeres of chromosomes at metaphase (g) and relocates to the spindle midzone at anaphase (i) in the wild-type spermatocytes. In contrast, INCENP exists on the centromeres of chromosomes gathered at the spindle poles (arrowheads in f, h, j) but not on those aligned at the metaphase plate (arrows in f, h, j) in the hybrid spermatocytes. Bars (c–i) 5 μm

components, INCENP and aurora B kinase, to the equatorial cortex via the spindle midzone inhibits the cell from determining the position where the contractile ring is formed, thereby resulting in defective cytokinesis in the hybrid cells.

Collectively, the most likely scenario leading to the formation of 4C sperm-like cells in the hybrid medaka is as follows: (1) although cohesin complex and SCP3 are expressed normally, decreased expression of SCP1 causes irregular formation of SC with discontinuous TFs along homologous chromosomes (Fig. 19.4d); (2) the incomplete SC fails to stabilize the pairing of homologous chromosomes, so that not all the chromosomes can line up on the metaphase plate (Fig. 19.4f); (3) a checkpoint mechanism that monitors the proper alignment of chromosomes at the metaphase plate stops meiosis to prevent the cell from entering anaphase; (4) even under the conditions of cell-cycle arrest, the CPC components, including INCENP and aurora B, are automatically released from the chromosomes aligned at the equator of the metaphase plate, but subsequent accumulation of CPC to the spindle midzone and then to the equatorial cortex, which is a prerequisite for accurate cytokinesis, does not occur; and (5) spermiogenesis proceeds irrespective of cytokinesis, resulting in the formation of sperm-like cells each having 4C DNA.

19.3.4 Oogenesis in the Hybrids

In hybrid females (Fig. 19.3b), most oocytes stop meiosis at the zygotene stage (Hamaguchi and Sakaizumi 1992; Sakaizumi et al. 1992, 1993; Kurita et al. 1995). This characteristic is useful for producing maternal-zygotic mutants (Shimada et al. 2008). Similar to hybrid spermatogenic cells, the expression patterns of SCP3 in hybrid oocytes were similar to those in wild-type oocytes, but SCP1 was expressed irregularly in the hybrid oocytes (Iwai et al., unpublished data). These findings strongly suggest that the defects in SC formation and chromosome pairing are closely related to the impaired expression and localization of SCP1 in both male and female hybrid medaka. The differences in phenotypes between the female and the male appear to depend on when the checkpoint mechanism acts in meiosis and to what extent gametogenesis is independent of meiosis. In the females, there must be a checkpoint mechanism monitoring the completion of chromosomal pairing at the zygotene stage of prophase I, and oogenesis never proceeds when meiosis is arrested. In contrast, a checkpoint mechanism is not active at prophase I but is active at metaphase in the males, and spermatogenesis proceeds automatically regardless of meiosis. The biological significance of the sex-dependent differences in checkpoint regulation of meiosis remains to be elucidated.

Although many of the oocytes stop their meiosis at the zygotene stage, a small number of oocytes undergo meiosis and become diploid eggs with the ability of fertilization in the hybrid medaka (Fig. 19.3b). Because the two sets of “homologous” chromosomes come from different species, most of them do not pair at meiotic metaphase I in the hybrid spermatocytes (Shimizu et al. 1997). However, the hybrid oocytes are equipped with twice the number of chromosomes (48 vs. 24

in normal oocytes) at the first meiotic prophase, and both first and second meiotic divisions proceed normally. In addition, all chromosomes in the germinal vesicles (nuclei) of the hybrid oocytes are bivalent with chiasmata, indicating that “homologous” chromosomes are present even in the hybrid females (Shimizu et al. 2000b). Taken together, the process of diploid egg production in the hybrid medaka is explainable as follows (Fig. 19.3b): (1) oogonial chromosomes are doubled by endomitosis, mitosis without cytokinesis, immediately before entering meiosis, resulting in oogonia with pairs of identical chromosomes; (2) the tetraploid (4C) oogonia undergo premeiotic DNA replication and enter meiosis, producing oocytes with 8C DNA content; and (3) normal meiotic division with extrusion of two polar bodies produces diploid (2C) eggs. A similar mechanism of unreduced egg formation has been found in triploid fish (Cimino 1972; Zhang et al. 1998), except for the triploid crucian carp *Carassius auratus langsdorfii*, in which triploid eggs are produced by a defect in meiosis itself (Yamashita et al. 1993). Further studies are needed to verify whether endomitosis is a phenomenon specific to the hybrid or is an intrinsic characteristic of the normal medaka. In any case, analyzing this issue will provide insights into the regulatory mechanisms of transition from mitosis to meiosis, a big decision during gametogenesis.

19.4 Conclusions

In this chapter, we have summarized abnormal mitosis and meiosis that occur in medaka hybrids. We believe that analyses of the causative mechanisms of abnormality in the hybrids at cellular and molecular levels provide deeper insights into the regulatory mechanisms of mitosis and meiosis in normal organisms. Although mutants are powerful tools for studying various subjects in biological sciences, their production and maintenance is laborious, especially when they carry mutations responsible for fundamental biological processes including cell division and gametogenesis. In contrast, a medaka hybrid between two closely related species is easily produced, and the resulting hybrids exhibit various abnormalities in embryonic development and gametogenesis as demonstrated here, thereby providing an excellent experimental system for investigating regulatory mechanisms of mitosis, meiosis, and germ cell development. It is certain that we will be able to learn much more from medaka hybrids by the advancement of analytical technology that enables us to manipulate gene expression in medaka cells, especially in germ cells, such as induction or suppression of arbitrary gene expression under cell culture conditions and knockout of specific genes by the application of homologous recombination.

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References

- Aizawa K, Shimada A, Naruse K, Mitani H, Shima A (2003) The medaka midblastula transition as revealed by the expression of the paternal genome. *Gene Expr Patterns* 3:43–47
- Araki K, Okamoto H, Graveson AC, Nakayama I, Nagoya H (2001) Analysis of haploid development based on expression patterns of developmental genes in the medaka *Oryzias latipes*. *Dev Growth Differ* 43:591–599
- Cimini D, Howell B, Maddox P, Khodjakov A, Degross F, Salmon ED (2001) Merotelic kinetochore orientation is a major mechanism of aneuploidy in mitotic mammalian tissue cells. *J Cell Biol* 153:517–527
- Cimino MC (1972) The nuclear DNA content of diploid and triploid *Poeciliopsis* and other poeciliid fishes with reference to the evolution of unisexual forms. *Chromosoma (Berl)* 47:297–307
- Costa Y, Cooke HJ (2007) Dissecting the mammalian synaptonemal complex using targeted mutations. *Chromosome Res* 15:579–589
- Formacion MJ, Uwa H (1985) Cytogenetic studies on the origin and species differentiation of the Philippine medaka, *Oryzias luzonensis*. *J Fish Biol* 27:285–291
- Fujiwara A, Abe S, Yamaha E, Yamazaki F, Yoshida MC (1997) Uniparental chromosome elimination in the early embryogenesis of the inviable salmonid hybrids between masu salmon female and rainbow trout male. *Chromosoma (Berl)* 106:44–52
- Furutani-Seiki M, Sasado T, Morinaga C et al (2004) A systematic genome-wide screen for mutations affecting organogenesis in medaka, *Oryzias latipes*. *Mech Dev* 121:647–658
- Hamaguchi S, Sakaizumi M (1992) Sexually differentiated mechanisms of sterility in interspecific hybrids between *Oryzias latipes* and *O. curvinotus*. *J Exp Zool* 263:323–329
- Hauf S, Biswas A, Langegger M, Kawashima SA, Tsukahara T, Watanabe Y (2007) Aurora controls sister kinetochore mono-orientation and homolog bi-orientation in meiosis-I. *EMBO J* 26:4475–4486
- Hori R, Iwamatsu T (1996) Experiments on interspecific hybridization among *Oryzias melastigma*, *Oryzias javanicus* and *Oryzias latipes*. *Bull Ogaki Women's Coll* 37:1–6
- Iwai T, Lee J, Yoshii A, Yokota T, Mita K, Yamashita M (2004) Changes in the expression and localization of cohesin subunits during meiosis in a non-mammalian vertebrate, the medaka fish. *Gene Expr Patterns* 4:495–504
- Iwai T, Yoshii A, Yokota T, Sakai C, Hori H, Kanamori A, Yamashita M (2006) Structural components of the synaptonemal complex, SYCP1 and SYCP3, in the medaka fish *Oryzias latipes*. *Exp Cell Res* 312:2528–2537
- Iwamatsu T (1994) Stages of normal development in the medaka *Oryzias latipes*. *Zool Sci* 11:825–839
- Iwamatsu T, Imai A, Kawamoto A, Inden A (1982) On *Oryzias javanicus* collected at Jakarta, Singapore and West Kalimantan. *Annot Zool Jpn* 55:190–198
- Iwamatsu T, Uwa H, Inden A, Hirata K (1984) Experiments on interspecific hybridization between *Oryzias latipes* and *Oryzias celebensis*. *Zool Sci* 1:653–663
- Iwamatsu T, Watanabe T, Hori R, Lam TJ, Saxena OP (1986) Experiments on interspecific hybridization between *Oryzias melastigma* and *Oryzias javanicus*. *Zool Sci* 3:287–293
- Iwamatsu T, Mori T, Hori R (1994) Experimental hybridization among *Oryzias* species. I. *O. celebensis*, *O. javanicus*, *O. latipes*, *O. luzonensis* and *O. melastigma*. *Bull Aichi Univ Educ* 43:103–112
- Iwamatsu T, Kobayashi H, Yamashita M, Shibata Y, Yusa A (2003) Experimental hybridization among *Oryzias* species. II. Karyogamy and abnormality of chromosome separation in the cleavage of interspecific hybrid between *Oryzias latipes* and *O. javanicus*. *Zool Sci* 20:1381–1387
- Kulathinal RJ, Singh RS (2008) The molecular basis of speciation: from patterns to processes, rules to mechanisms. *J Genet* 87:327–338
- Kurita J, Oshiro T, Takashima F, Sakaizumi M (1995) Cytogenetic studies on diploid and triploid oogenesis in interspecific hybrid fish between *Oryzias latipes* and *O. curvinotus*. *J Exp Zool* 273:234–241

- Lammers JHM, Offenberg HH, van Aalderen M, Vink ACG, Dietrich AJJ, Heyting C (1994) The gene encoding a major component of the lateral elements of synaptonemal complexes of the rat is related to X-linked lymphocyte-regulated genes. *Mol Cell Biol* 14:1137–1146
- Lammers JHM, van Aalderen M, Peters AHFM, van Pelt AAM, Gaemers IC, de Rooij DG, de Boer P, Offenberg HH, Dietrich AJJ, Heyting C (1995) A change in the phosphorylation pattern of the 30000–33000 M_r synaptonemal complex proteins of the rat between early and mid-pachytene. *Chromosoma (Berl)* 104:154–163
- Loog M, Morgan DO (2005) Cyclin specificity in the phosphorylation of cyclin-dependent kinase substrates. *Nature (Lond)* 434:104–108
- Nagahama Y, Yamashita M (2008) Regulation of oocyte maturation in fish. *Dev Growth Differ* 50:S195–S219
- Naruse K, Sakaizumi M, Shima A (1994) Medaka as a model organism for research in experimental biology. *Fish Biol J Medaka* 6:47–52
- Orr HA (2005) The genetic basis of reproductive isolation: insights from *Drosophila*. *Proc Natl Acad Sci USA* 102:6522–6526
- Orr HA, Masly JP, Presgraves DC (2004) Speciation genes. *Curr Opin Genet Dev* 14:675–679
- Roberts TR (1998) Systematic observations on tropical Asian medakas or ricefishes of the genus *Oryzias*, with descriptions of four new species. *Ichthyol Res* 45:213–224
- Sakai C, Konno F, Nakano O, Iwai T, Yokota T, Lee J, Nishida-Umehara C, Kuroiwa A, Matsuda Y, Yamashita M (2007) Chromosome elimination in the interspecific hybrid medaka between *Oryzias latipes* and *O. hubbsi*. *Chromosome Res* 15:697–709
- Sakaizumi M, Shimizu Y, Hamaguchi S (1992) Electrophoretic studies of meiotic segregation in inter- and intraspecific hybrids among East Asian species of the genus *Oryzias* (Pisces: Oryziatidae). *J Exp Zool* 264:85–92
- Sakaizumi M, Shimizu Y, Matsuzaki T, Hamaguchi S (1993) Unreduced diploid eggs produced by interspecific hybrids between *Oryzias latipes* and *O. curvinotus*. *J Exp Zool* 266:312–318
- Shima A, Mitani H (2004) Medaka as a research organism: past, present and future. *Mech Dev* 121:599–604
- Shimada A, Yabusaki M, Niwa H, Yokoi H, Hatta Y, Kobayashi D, Takeda H (2008) Maternal-zygotic medaka mutants for *fgfr1* reveal its essential role in the migration of the axial mesoderm but not the lateral mesoderm. *Development (Camb)* 125:281–290
- Shimizu Y-H, Shibata N, Yamashita M (1997) Spermatogenesis without preceding meiosis in the hybrid medaka between *Oryzias latipes* and *O. curvinotus*. *J Exp Zool* 279:102–112
- Shimizu Y-H, Mita K, Tamura M, Onitake K, Yamashita M (2000a) Requirement of protamine for maintaining nuclear condensation of medaka (*Oryzias latipes*) spermatozoa shed into water but not for promoting nuclear condensation during spermatogenesis. *Int J Dev Biol* 44:195–199
- Shimizu Y-H, Shibata N, Sakaizumi M, Yamashita M (2000b) Production of diploid eggs through premeiotic endomitosis in the hybrid medaka between *Oryzias latipes* and *O. curvinotus*. *Zool Sci* 17:951–958
- Wittbrodt J, Shima A, Scharlt M (2002) Medaka – a model organism from the far East. *Nat Rev Genet* 3:53–64
- Yamashita M, Jiang J, Onozato H, Nakanishi T, Nagahama Y (1993) A tripolar spindle formed at meiosis I assures the retention of the original ploidy in the gynogenetic triploid crucian carp, *Carassius auratus langsdorfii*. *Dev Growth Differ* 35:631–636
- Zhang Q, Arai K, Yamashita M (1998) Cytogenetic mechanisms for triploid and haploid egg formation in the triploid loach *Misgurnus anguillicaudatus*. *J Exp Zool* 281:608–619

Part IV

Evolution

Chapter 20

Reconstruction of the Vertebrate Ancestral Genome Reveals Dynamic Genome Reorganization in Early Vertebrates*

Yoichiro Nakatani, Hiroyuki Takeda, Yuji Kohara, and Shinichi Morishita

Abstract Although several vertebrate genomes have been sequenced, little is known about the genome evolution of early vertebrates and how large-scale genomic changes such as the two rounds of whole genome duplications (2R WGD) affected evolutionary complexity and novelty in vertebrates. Reconstructing the ancestral vertebrate genome is highly nontrivial because of the difficulty in identifying traces originating from the 2R WGD. To resolve this problem, we developed a novel method capable of pinning down remains of the 2R WGD in human and medaka fish genomes using invertebrate tunicate and sea urchin genes to define ohnologs, that is, paralogs produced by the 2R WGD. We validated the reconstruction using the chicken genome, which was not considered in the reconstruction step, and observed that many ancestral proto-chromosomes were retained in the chicken genome and had one-to-one correspondence to chicken microchromosomes, thereby confirming the reconstructed ancestral genomes. Our reconstruction revealed a contrast between

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the slow karyotype evolution after the second WGD and the rapid, lineage-specific genome reorganizations that occurred in the ancestral lineages of major taxonomic groups such as teleost fishes, amphibians, reptiles, and marsupials.

20.1 Introduction

Early vertebrate genome evolution has long been in need of clarification, and it is now of particular interest because several distantly related vertebrate genomes were recently sequenced. The 2R hypothesis postulates that two rounds of whole genome duplication (2R WGD) occurred at the base of the vertebrate lineage (Ohno 1970; Holland et al. 1994) because of the observation that invertebrates have one *Hox* gene cluster, whereas lobe-finned fishes and land vertebrates have four clusters. However, the 2R hypothesis has been considered quite controversial until recently (Skrabanek and Wolfe 1998; Gibson and Spring 2000; Friedman and Hughes 2001; Wolfe 2001; Abi-Rached et al. 2002; Furlong and Holland 2002; Gu et al. 2002; McLysaght et al. 2002; Durand 2003; Panopoulou et al. 2003; Seoighe 2003; Vandepoele et al. 2004; Panopoulou and Poustka 2005) because it leaves open the possibility of one round of WGD followed by large-scale duplications such as segmental and chromosomal duplications. Recently, Dehal and Boore showed that a large part of the human genome contains four-way paralogous chromosomal regions, which are traces of the 2R WGD (Dehal and Boore 2005).

The task of reconstructing ancestral vertebrate proto-chromosomes before the 2R WGD is very different from ordinary synteny analysis using orthologs because the effect of the 2R WGD must be carefully examined. Moreover, it is necessary to determine human chromosome regions originating from the same ancestral chromosome at the second WGD and integrate these regions to rebuild the ancestral karyotype. Thus, we first identified groups of human genes, called “ohnologs” (Wolfe 2001), duplicated by the 2R WGD by ensuring that individual genes in a group were most similar to the identical orthologous deuterostome gene of the sea urchin (Sea Urchin Genome Sequencing Consortium 2006) or tunicate (Dehal et al. 2002; Shoguchi et al. 2006). The identification of ohnologs is a difficult task because all ohnologs and their corresponding *Ciona* genes are rarely conserved as a consequence of numerous losses and duplications of *Ciona*, human, and medaka genes. Given these difficulties, ohnologs were grouped based on the method of Dehal and Boore (Dehal and Boore 2005). These ohnologs typically occur consecutively in paralogous chromosomal regions in the human genome and are likely to represent a remaining block derived from a single gnathostome (jawed vertebrate; see phylogenetic tree of vertebrates in Fig. 20.6) proto-chromosome. To test if they are really remaining blocks of the gnathostome proto-chromosomes, their syntenies in the medaka genome (Kasahara et al. 2007) were investigated (Nakatani et al. 2007). The final step in our novel analysis combined qualified remaining blocks into vertebrate and gnathostome proto-chromosomes using information on ohnolog distribution among the blocks. This series of steps was newly developed for this reconstruction (Nakatani et al. 2007).

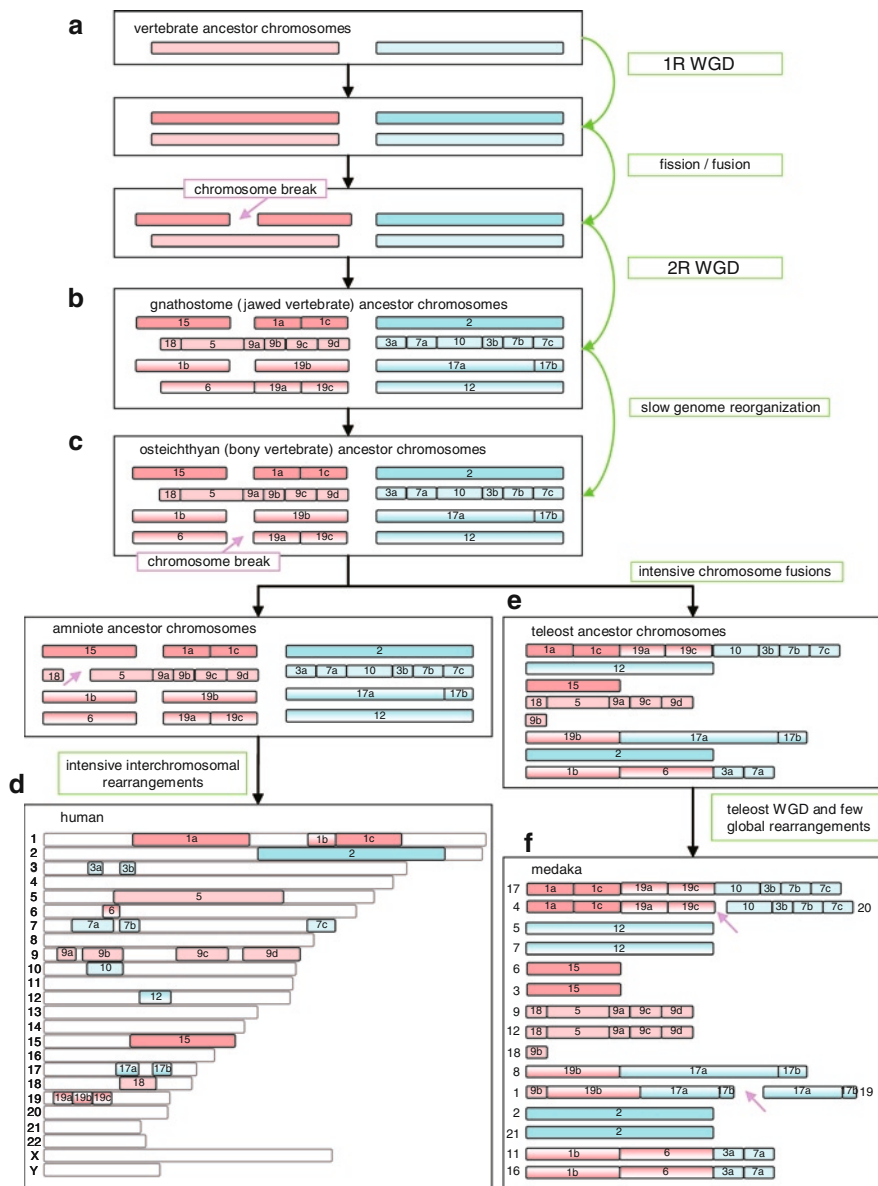


Fig. 20.1 Vertebrate chromosome evolution scenario. **(a)** For simplicity, we illustrate two proto-chromosomes duplicated by the first round (1R) of whole genome duplication (WGD). Subsequently, fission divided one of the duplicated chromosomes. **(b)** The second round of WGD (2R WGD) doubled the proto-chromosomes. Blocks in chromosomes are labeled with their respective chromosome positions in the human genome. **(c)** After the second WGD, early vertebrates underwent slow changes in karyotype over a long evolutionary process. **(d)** In the ancestral mammalian lineage, intensive interchromosomal rearrangements occurred and the ancestral chromosomes were broken into smaller segments that were distributed across many human chromosomes. **(e)** In the ancient ray-finned fish lineage, intensive chromosome fusions merged the ancestral chromosomal segments into ancestral teleost chromosomes. **(f)** Another round of WGD in the ancestral teleost doubled proto-chromosomes, but subsequently few global rearrangements shaped the present medaka genome

Next, we attempted to validate the reconstructed gnathostome proto-chromosomes. If extant genomes have experienced intensive interchromosomal rearrangements, they are hardly syntenic to the reconstructed ancestral genome and are not useful in the validation. Among sequenced vertebrate genomes, we searched for a genome that was not used in the reconstruction step but preserved the protokaryotype. The chicken genome was most promising, given the hypothesis that avian microchromosomes represent archaic linkage groups of ancestral vertebrates (Burt 2002). Indeed, we observed that many ancestral gnathostome proto-chromosomes in our reconstruction had one-to-one correspondence with microchromosomes in the chicken genome, thus providing a strong validation of our reconstruction.

We reconstructed the gnathostome (jawed vertebrate), osteichthyan (bony vertebrate), and amniote (the group including reptiles, birds, dinosaurs, and mammals) ancestral genomes, which are located at key phylogenetic positions, leading to a novel scenario of genome evolution in early vertebrates. The two rounds of WGD events duplicated 10–13 proto-chromosomes in the vertebrate ancestor, producing the gnathostome (jawed vertebrate) ancestor ($n \approx 40$). Subsequent chromosome fusions reduced the number of chromosomes in the osteichthyan protokaryotype ($n \approx 31$) and in the amniote protokaryotype ($n \approx 26$). These estimates of chromosome number are considerably larger than previous estimates and contradict the widely held hypothesis that the osteichthyan protokaryotype was $n \approx 12$ (Postlethwait et al. 2000; Jaillon et al. 2004; Naruse et al. 2004; Woods et al. 2005; Kohn et al. 2006) and that land vertebrate genomes were shaped by lineage-specific chromosome “fissions” (Postlethwait et al. 2000). In contrast, our results demonstrate that many lineage-specific chromosome “fusions” shaped the ancestral karyotypes of major taxonomic groups, such as teleost fishes, amphibians, reptiles, and marsupials.

20.2 Results

20.2.1 *Evolution of Vertebrate Chromosomes*

Figure 20.1 presents our scenario of vertebrate chromosome evolution. Although the ancestral genome would have more than two proto-chromosomes before the two rounds of WGD, for simplicity we display two of the reconstructed ancestral vertebrate chromosomes (see Fig. 20.1a). The first round of WGD doubled these two chromosomes, and fission occurred in one of the duplicated copies of the red chromosome. After the second round of WGD (Fig. 20.1b), sister chromosomes were gradually disrupted by genome rearrangements and broken into smaller blocks of chromosomal segments during early vertebrate evolution (Fig. 20.1c). Eventually, these blocks were distributed over several human chromosomes (Fig. 20.1d) because of intensive interchromosomal rearrangements in the mammalian lineage (Burt et al. 1999). However, in the teleost fish lineage, intensive chromosome fusions and another WGD occurred after the divergence from the osteichthyan ancestor.

As illustrated in Fig. 20.1, conserved vertebrate linkage (CVL) groups, which are groups of genes located on a single chromosome after the second round of WGD, constitute chromosomal blocks in the human genome. These blocks can also be identified by doubly conserved synteny (DCS) analysis with the medaka genome (Jaillon et al. 2004; Kellis et al. 2004). For example, although blocks 19a, 19b, and 19c are located consecutively in the human genome, both 19a and 19c are syntenic to each of the duplicated medaka chromosomes 4 and 17, whereas duplicates of 19b are found in two duplicated medaka chromosomes (1 and 8). Other neighboring blocks, that is, 1b–1c, 3a–3b, 7a–7b, and 9a–9b, are located on distinct medaka chromosomes. This finding implies that deciding the boundaries of such neighboring blocks is extremely important; however, the task was difficult when genes on the human chromosomes were compared with *Ciona* and sea urchin genes as an outgroup because of the intensive rearrangements in the mammalian lineage. We therefore needed additional information. Thus, we fully used the medaka chromosomes as an outgroup of mammals to clarify the boundaries [see details in Nakatani et al. (2007)]. The CVL blocks were essential in reconstructing ancestral vertebrate proto-chromosomes.

20.2.2 Reconstruction of the Vertebrate Ancestral Genome

Here, we assumed a simplified model in which no major genome rearrangements occurred between the 2R WGD events, although we present a more elaborate model later in this chapter. The expected signature of two rounds of WGD is illustrated in Fig. 20.2a–d. The 2R WGD quadruplicated the gray chromosomes, producing sister chromosomes each having the same set of genes arranged in the same order. In reality, gene losses and chromosome rearrangements must have taken place between the 2R WGD, but for simplicity these details are omitted from Fig. 20.2a. Wolfe proposed calling these duplicated gene pairs “ohnologs” after Susumu Ohno (Wolfe 2001). One ohnolog is represented by a dot in a triangle whose X- and Y-axis coordinates represent the positions of duplicated genes in the sister chromosomes produced by the 2R WGD. Because ohnologs are produced by chromosome-wide duplication and not by smaller-scale gene duplication, they are observed between pairs of distinct sister chromosomes, as illustrated by dots in the gray region. In contrast, no ohnologs would be found within one sister chromosome, which is shown by the absence of ohnolog dots in the green regions. After the 2R WGD, chromosome breaks (fissions and translocations), fusions, or inversions must have altered some of the sister chromosomes (Fig. 20.2b). The accumulation of rearrangements gradually disrupted the ancestral gene order and scattered the ohnolog dots (Fig. 20.2c). Furthermore, intensive interchromosomal rearrangements in the mammalian lineage (Burt et al. 1999) must have distributed blocks across the human genome (Fig. 20.2d). Reconstruction of ancestral proto-chromosomes is a task of reordering the CVL blocks in Fig. 20.2d to estimate the orderings in Fig. 20.2b,c.

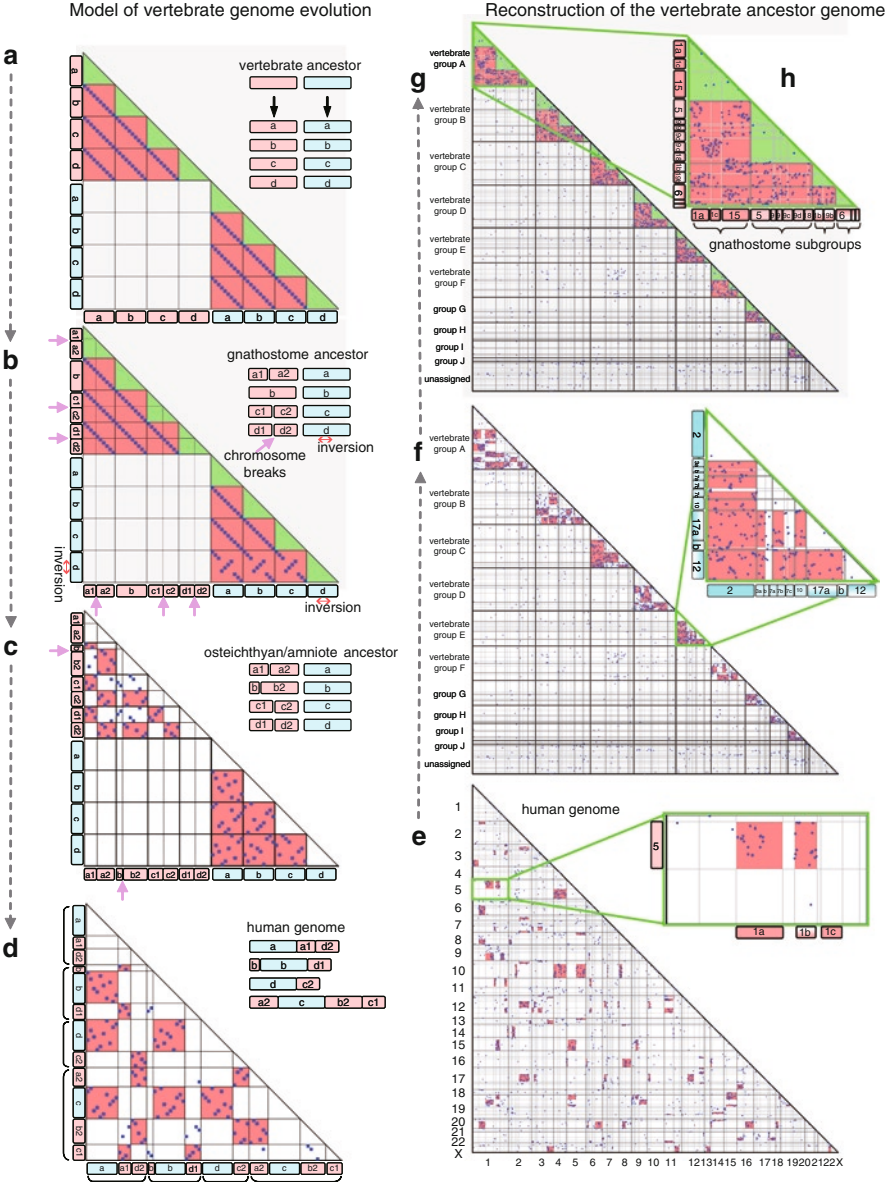


Fig. 20.2 Model of vertebrate genome evolution and reconstruction of the ancestral genome. (a) For simplicity, suppose that the ancestral chromosome had ten genes. The 2R WGD produced ohnologs (dots along the diagonal line in the triangular dot plot) in the duplicated chromosomes. (b) Chromosome breaks and inversions may have altered the order of ohnologs on the sister chromosomes. (c) In the course of early vertebrate genome evolution, the ancestral gene order was disrupted by many inversions, resulting in scattered ohnolog dots. (d) Eventually, conserved vertebrate linkage (CVL) blocks were distributed across several human chromosomes by intensive interchromosomal rearrangements. (a)–(d) A typical model of genome evolution involving the 2R WGD. In the next step, we handle real human genome data.

Figure 20.2e presents the actual distribution of ohnologs in the human genome, in which CVL blocks are placed in order from human chromosome 1 to X. The reconstruction step started with categorizing the blocks into groups that were derived from a single ancestral vertebrate chromosome before the 2R WGD. For this purpose, any two CVL blocks sharing several ohnologs were placed in the same “vertebrate” group (see Fig. 20.2f). The subsequent step divided the blocks within each group into subgroups that represented duplicated proto-chromosomes in the gnathostome ancestor. To this end, we performed an exhaustive search for the optimal subgrouping so that significantly more ohnologs were shared between distinct subgroups, as illustrated by the red regions in Fig. 20.2g, whereas few ohnologs were observed within any single subgroup, as indicated by the gray regions. The details of the reconstruction procedure are described in the Methods. Our reconstruction in Fig. 20.2g shows that in five of ten ancestral vertebrate chromosomes, estimating four sister chromosomes was more significant than inferring two, three, or five sister chromosomes. CVL blocks with a small number of ohnologs fail to be recognized as sister chromosomes because of their low statistical significance, thereby yielding a reconstructed vertebrate group with fewer than four duplicated chromosomes. In contrast, five ancestral vertebrate chromosomes (A–E) had fairly large groups of CVL blocks with more than 250 ohnolog gene pairs, providing strong support for the 2R hypothesis.

20.2.3 Assuming Fusions and Fissions Between the 2R WGD

For simplicity, we have thus far assumed no major chromosome rearrangements between the 2R WGD events. Here, we extend the simplified model to consider the possibility of chromosome fusions and fissions between the 2R WGD events, although how to detect remains of these events is a nontrivial question. Figure 20.3a presents vertebrate groups A, B, and F in Fig. 20.2f, and it is remarkable to observe pairs of light gray boxes with few ohnologs in individual triangles because these boxes are expected to contain many ohnologs according to the simplified model



Fig. 20.2 (continued) (e) This figure part is a real instance of the dot plot in (d). CVL blocks were ordered from the human chromosomes 1 to X, and ohnologs shared among these CVL blocks were plotted. *Gray*, regions representing pairs of paralogous CVL blocks with a great number of ohnologs ($P < 10^{-4}$). (f) This view corresponds to the state in (c). CVL blocks were reordered in such a way that paralogous CVL blocks were grouped so that each group represented one ancestral vertebrate chromosome. (g) This state corresponds to that in (b). CVL blocks within individual vertebrate groups were further reordered to obtain ancestral gnathostome subgroups (namely, chromosomes), which were duplicated from a single ancestral vertebrate chromosome by the 2R WGD events. The partition of subgroups optimizes the significance defined by Nakatani et al. (Nakatani et al. 2007). (h) The vertebrate group A was decomposed into four gnathostome subgroups by statistical analysis, indicating that the ancestral chromosome underwent 2R WGD

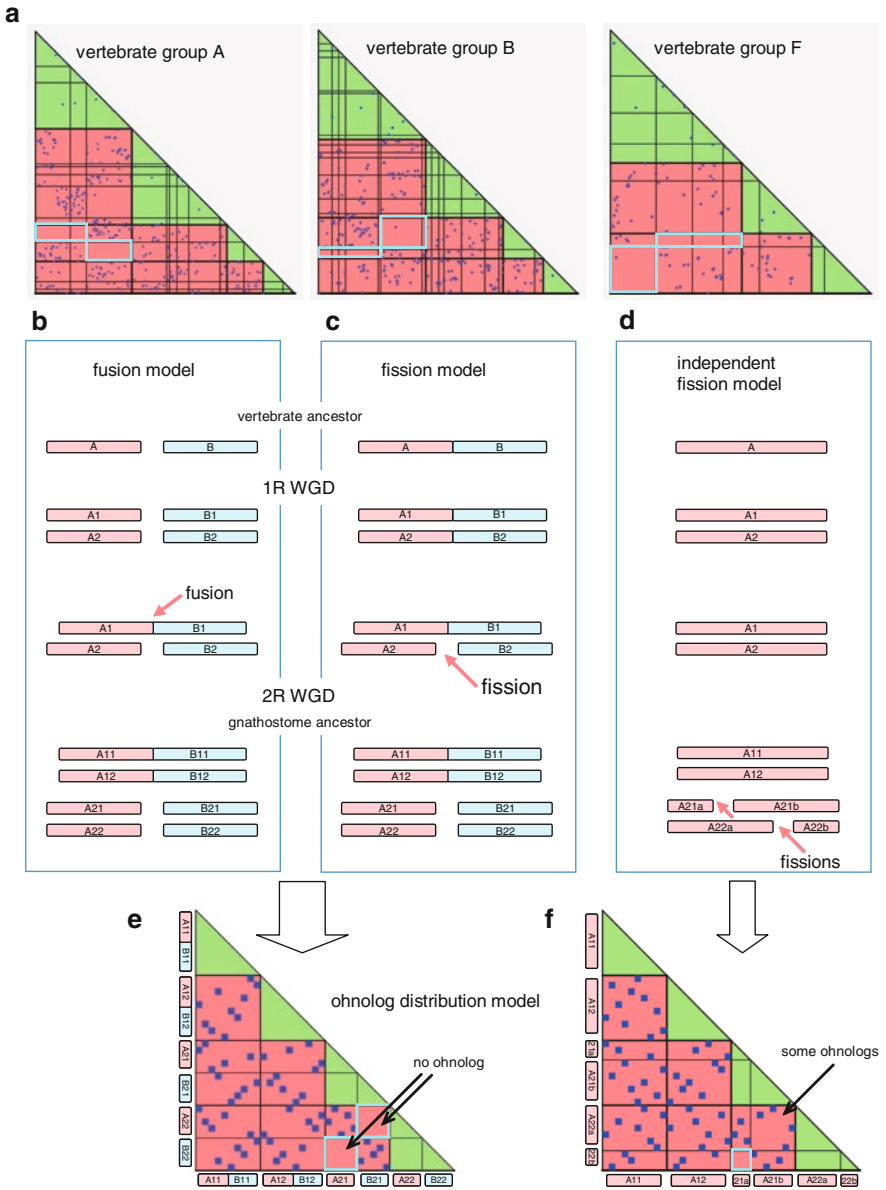


Fig. 20.3 Models of chromosome evolution during 2R WGD. (a) Vertebrate groups A, B, and E from Fig. 20.2f. Pairs of light gray boxes with few ohnologs may be the remains of genome rearrangements between the two WGD events. (b) Two distinct ancestor chromosomes (A and B) were duplicated by the first WGD, and duplicated chromosomes A1 and B1 underwent a chromosome fusion. (c) One ancestor chromosome consisting of two parts (A and B) was duplicated by the first WGD, and a copy of the duplicated chromosomes was split into two chromosomes (A1 and B1) by a chromosome fission event. (d) After the second round of WGD, two of the four sister chromosomes underwent two independent chromosome fissions. Because the two fissions split two

assumed so far. We think that these phenomena provide clues to the problem of inferring genome rearrangements between the 2R WGD; here, we show three alternative scenarios that may produce these phenomena.

Figure 20.3b shows the first scenario in which duplicates of two ancestral chromosomes are fused, whereas Fig. 20.3c shows the second scenario, in which one duplicate of a single ancestral chromosome is divided by a fission event. Both scenarios generate the same ohnolog distribution pattern among the six descendent chromosomes as illustrated in Fig. 20.3e; note that pairs A21–B22 and B21–A22 have no ohnologs. The third scenario in Fig. 20.3d indicates that neither fissions nor fusions take place between the 2R WGD events, but independent fission events occur for duplicated sister chromosomes after the 2R WGD. As illustrated in Fig. 20.3f, the pair A21a–A22b is free of ohnologs; however, some ohnologs are observed in the pair A21b–A22a. Both pairs would have no ohnologs if the two independent fission events took place at the identical chromosomal positions, but this case is less likely. Among the three scenarios, the former two scenarios of one fusion or fission event are more parsimonious than the third scenario of two independent fissions, and they better explain pairs of light blue boxes with few ohnologs in Fig. 20.3a. Thus, we estimated that among ten vertebrate groups, A, B, and F had fusions or fissions, whereas the other seven groups were free of these chromosome rearrangements.

However, it remains difficult to judge whether a fusion or fission happened between the 2R WGD; each of the vertebrate groups A, B, and F may have had two ancestral chromosomes before the 2R WGD according to the fusion scenario, or one proto-chromosome if fission took place. This argument may be settled by using the genomes of some invertebrates as an outgroup. However, the draft genome of the sea urchin (Sea Urchin Genome Sequencing Consortium 2006) consists of very small genomic fragments, and the *Ciona* draft genome (Shoguchi et al. 2006), with a nearly complete chromosome map, fails to preserve proto-chromosomes before the divergence from vertebrates, presumably because of numerous chromosome rearrangements. Therefore, at this stage, we estimated that the ancestral vertebrate had 10 to 13 proto-chromosomes before the 2R WGD. We assert that the ancestral gnathostome had 40 proto-chromosomes and was not affected by the selection of a fusion or fission scenario.

20.2.4 Validation of Reconstructed Proto-Chromosomes

To check the validity of the reconstructed proto-chromosomes, we searched for a genome that preserved the ancestral chromosomes among available deuterostome,

Fig. 20.3 (continued) chromosomes (A11 and A12) at different chromosome positions, blocks A21b and A22a have some ohnologs in common. (c) Both the fusion and fission models in (b) and C produce the same distribution of ohnologs, especially pairs of light gray boxes without ohnologs. (f) The distribution originating from independent fission events in (d) is distinguishable from that in (e), as indicated by one light gray box

chordate, and vertebrate genomes. The sea urchin draft genome (Sea Urchin Genome Sequencing Consortium 2006) is largely fragmented into very small pieces, whereas the *Ciona* genome (Shoguchi et al. 2006) experienced numerous genome rearrangements after the divergence from vertebrates more than 500 million years ago; for example, even *Hox* genes are separated onto multiple chromosomes (Ikuta et al. 2004). Thus, these two genomes fail to preserve proto-chromosomes. We also examined vertebrate genomes and found that rat, mouse, and dog genomes did not preserve the protokaryotype because of several interchromosomal rearrangements in the mammalian lineage (Burt et al. 1999). Among tested genomes, the chicken genome significantly preserved the reconstructed ancestral gnathostome chromosomes. More precisely, a series of CVL blocks that occur both in one ancestral gnathostome chromosome and in one chicken chromosome presents strong evidence for the reconstruction; in contrast, occurrences of CVL blocks of one chicken chromosome in different sister chromosomes of the gnathostome ancestor suggest intensive interchromosomal rearrangements in the avian lineage or possible mistakes in the reconstruction. We observed that CVL blocks located in the same chicken microchromosomes belonged primarily to the same ancestral gnathostome chromosomes (Fig. 20.4). To be precise, chicken chromosomes 7, 8, 11, 15, 19, 20, 21, 22, 23, 24, 27, and 28 had one-to-one correspondence to E0, A2, B4, H0, H1, B1, F0, C3, B3, J0, E2, and A1, respectively (Fig. 20.5). Thus, this clear correspondence between chicken and gnathostome ancestor chromosomes provides a firm grounding for our reconstruction.

20.2.5 *Reconstruction of Ancestral Osteichthyan and Amniote Proto-Chromosomes*

The reconstruction of proto-chromosomes in the gnathostome ancestor allowed us to infer the karyotypes of evolutionarily important ancestors such as the bony vertebrate (osteichthyan) ancestor and the amniote ancestor, leading to novel insights into genome evolution after the 2R WGD. Previous studies have suggested that the osteichthyan protokaryotype was $n \approx 12$ by treating the teleost ancestor of medaka, *Tetraodon*, and zebrafish before teleost WGD as the osteichthyan ancestor (Postlethwait et al. 2000; Jaillon et al. 2004; Naruse et al. 2004; Woods et al. 2005) or by removing the possibility of chromosome fusions from consideration (Kohn et al. 2006). However, this hypothesis ($n \approx 12$) appears to disagree with our observations because many chromosomes in the chicken genome ($n = 39$) have one-to-one corresponding proto-chromosomes in the gnathostome ancestor ($n \approx 40$), as shown in Fig. 20.5, whereas most chromosomes in the teleost ancestor ($n \approx 13$) have synteny blocks with multiple chromosomes in the gnathostome ancestor. These observations led us to postulate that the osteichthyan protokaryotype had more chromosomes than the previous estimate ($n \approx 12$), and that many genome rearrangements took place from the osteichthyan protokaryotype to the teleost ancestor.

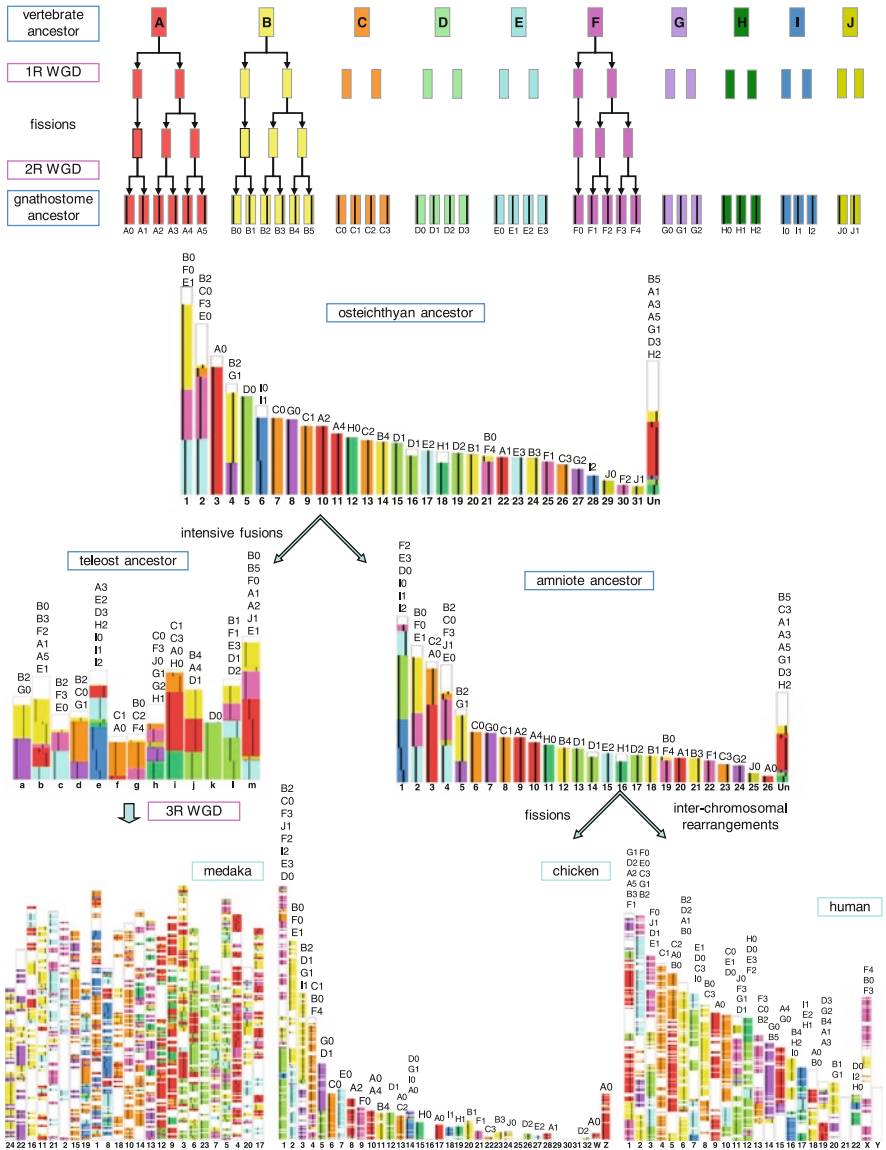


Fig. 20.4 Reconstructed ancestral chromosomes. Ancestral vertebrate chromosomes A, B, and F had two alternative scenarios, fusions or fissions, between the 2R WGD events (as shown in Fig. 20.3). Thus, the number of proto-chromosomes ranges from 10 to 13 depending on the choice of two alternatives. The figure illustrates the scenario in which only fissions took place. Ten reconstructed proto-chromosomes in the vertebrate ancestor shown at the *top* are assigned distinct identifiers, and their daughter chromosomes in the gnathostome ancestor are distinguished by their respective *vertical bars*. In the genomes of the osteichthyan, teleost, and amniote ancestors, and human, chicken, and medaka genomes, genomic regions are assigned identifiers and *vertical bars* that represent correspondences of individual regions to the proto-chromosomes in the gnathostome ancestor from which respective regions originated. Unassigned blocks are shown in the *rightmost* chromosome (*Un*) in the osteichthyan and amniote ancestors

		G pa1	G pa2	G pa3	G pa4	G pa5	G pa6	G pa7	G pa8	G pa9	G pa10	G pa11	G pa12	G pa13	G pa14	G pa15	G pa16	G pa17	G pa18	G pa19	G pa20	G pa21	G pa22	G pa23	G pa24	G pa25	G pa26	G pa27	G pa28	G pa29	G pa30	G pa31	Un
A	A0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12
	A1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	26
	A2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
	A3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	A4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	A5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	17
	B0	0	315	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	B1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	B2	8	0	303	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6
	B3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C	B4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	B5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C0	37	0	0	0	0	244	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C1	0	0	0	255	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D	C3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	36
	D0	399	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	D1	0	20	0	165	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	D2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	D3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E	E0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	E1	0	230	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	E2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	E3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10
	F0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F	F1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
	F2	26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	F3	234	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	F4	0	0	0	64	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	G0	0	0	0	0	332	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G	G1	0	77	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	G2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	H0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	H1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	H2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
I	I0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	I1	0	0	52	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	I2	80	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	J0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	J1	26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Un	Un	119	73	22	0	0	0	24	0	0	0	0	29	0	23	0	0	21	18	0	0	0	0	0	0	0	0	0	0	0	0	19	4

Fig. 20.5 Syntenic chromosome correspondence between the chicken and gnathostome ancestor chromosomes. In the table, the *number in a cell* indicates the number of orthologous genes in syntenic regions between the chicken chromosome in the *column* and the reconstructed gnathostome proto-chromosome in the *row*. To emphasize chicken chromosomes that were primarily derived from a single ancestral gnathostome chromosome, cells with the maximum number of orthologs are colored *light gray* or *gray*. In particular, *gray cells* imply chicken chromosomes that have one-to-one correspondence to their ancestral gnathostome chromosomes

To test this hypothesis, we attempted to reconstruct the osteichthyan protokaryotype by comparing the chicken and ancestral teleost karyotypes with that of the gnathostome ancestor. This approach is somewhat different from the traditional method of using an outgroup of the chicken and teleost ancestor because we instead used the gnathostome ancestor, which is an ancestor of the osteichthyan ancestor. In the reconstruction, two CVL blocks were expected to occur in the same osteichthyan proto-chromosome if the two CVL blocks were located on the identical chromosome of at least two of the three karyotypes: chicken, teleost ancestor, and gnathostome ancestor. We call this rule the 2-of-3 rule, and we describe the details and rationale of the rule in our earlier paper (Nakatani et al. 2007). The resulting osteichthyan protokaryotype was $n \approx 31$, which confirms our postulate that the number of osteichthyan proto-chromosomes is more than the previous estimation of $n \approx 12$.

Regarding the amniote ancestor, Kohn et al. (2006) estimated $n \approx 18$ using the teleost ancestor as an outgroup of the human and chicken genomes. However, in our reconstruction, the teleost ancestor genome underwent several rearrangements, especially fusions, after divergence from the osteichthyan ancestor. Thus, we used the reconstructed osteichthyan ancestor, in place of the teleost ancestor, together with the human and chicken genomes to reconstruct the amniote ancestor chromosomes. Using the 2-of-3 rule, we estimated that the number of amniote proto-chromosomes was $n \approx 26$, which is significantly larger than the previous estimate of

$n \approx 18$. Overall, we conclude that chromosome numbers in early vertebrates are considerably larger than previous estimates (Jaillon et al. 2004; Naruse et al. 2004; Woods et al. 2005; Kohn et al. 2006).

20.2.6 Karyotype Evolution and Taxon-Specific Fusion Model

The reconstructed ancestral karyotypes in Fig. 20.4 provide a scenario for karyotype evolution in vertebrates, as depicted in Fig. 20.6. Before the first round of WGD, the vertebrate ancestor karyotype was $n \approx 10$ –13, and the subsequent 2R WGD and some genome rearrangements yielded the gnathostome ancestor of $n \approx 40$. After the divergence of bony vertebrates (Osteichthyes) and cartilaginous fishes (Chondrichthyes), genome rearrangements reduced the number of chromosomes in the osteichthyan ancestor to $n \approx 31$. In the meantime, modern cartilaginous fishes have genomes comprising a variety of karyotypes; the number of chromosomes in the haploid set, n , ranges from 25 to 50. We speculate that some extant cartilaginous fishes may retain the gnathostome ancestral karyotype ($n \approx 40$), assuming no genome duplication events took place in the cartilaginous lineage. After the divergence of ray-finned and lobe-finned fishes, in the lineage of ray-finned fishes (Actinopterygii), chromosome fusions reduced the number of chromosomes and produced the teleost ancestor with $n \approx 13$. Our fusion model made it necessary to revise the $n \approx 12$ ancestral osteichthyan protokaryotype hypothesis, which treats the teleost ancestor of approximately ~350 million years ago (MYA) as the representative of the osteichthyan ancestor of ~450 MYA (Postlethwait et al. 2000; Jaillon et al. 2004; Naruse et al. 2004; Woods et al. 2005; Kohn et al. 2006). Subsequently, the whole genome duplication in the teleost ancestor doubled the number of chromosomes to $n \approx 26$. The number of chromosomes in the teleost lineage has remained nearly unchanged during evolution, and the chromosome numbers of extant teleost species peak at $n = 24$ or 25.

In the lineage of lobe-finned fishes (Sarcopterygii), the distribution of chromosome number differs largely across vertebrate taxa (Fig. 20.6). In particular, an interesting observation in our model is that the ancestors of major vertebrate groups such as teleosts, amphibians, squamates, and metatherians underwent numerous chromosome fusions (except for birds), leading to slow changes in karyotype, as indicated by the distribution of chromosome number. Similar speculations have been made regarding amphibians and reptiles; Morescalchi postulated that the number of chromosomes decreased in the ancestral amphibian based on the large number of chromosomes in some primitive amphibians (Green and Sessions 1991). With respect to reptiles, Olmo indicated that ancestral reptiles had a higher rate of karyotypic changes (Olmo 2005). Our karyotype evolution model is consistent with these hypotheses.

In contrast, a large increase in the number of chromosomes was found in the avian lineage. One explanation is that fewer repeat sequences in avian genomes were likely to result in chromosome fissions rather than fusions and interchromosomal

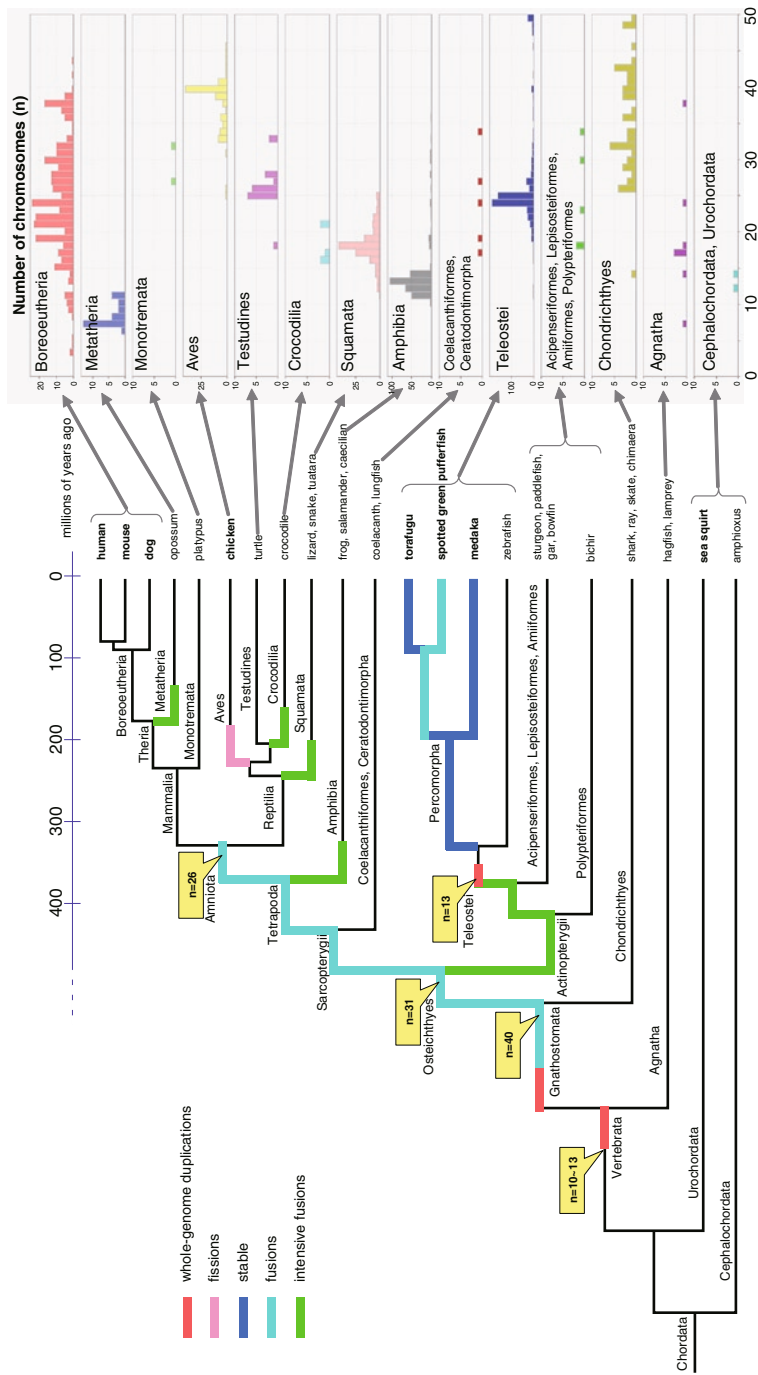


Fig. 20.6 Changes in chromosome number during vertebrate karyotype evolution. The reconstructed proto-chromosomes in Fig. 20.4 allow us to discuss how the number of chromosomes has changed in individual vertebrate lineages. *Left*, the phylogenetic tree of vertebrates; *right*, the distribution of chromosome number in individual lineages (from Gregory et al. Animal Genome Size Database, <http://www.genomesize.com>). Considering the numbers of proto-chromosomes in Fig. 20.4, two ancient whole genome duplication events almost quadrupled the number of chromosomes; subsequently, chromosome numbers in individual lineages tended to decrease in many lineages, although not in the avian lineage

rearrangements (Burt et al. 1999; Burt 2002). Another interesting finding involved microchromosomes in the avian lineage. Burt argued that many avian microchromosomes were already present in the common ancestor of birds and other terrestrial vertebrates and were conserved for more than 400 million years (Burt 2002). Our analysis extends this idea considerably by showing that many of the microchromosomes had one-to-one correspondence with proto-chromosomes in the gnathostome ancestor.

We conclude that, in early vertebrate genome evolution, two ancient whole genome duplication events increased the number of chromosomes, but subsequent chromosome fusions reduced the number of chromosomes in individual lineages, with the exception of the avian lineage.

20.3 Conclusions

We present the first study to reconstruct the vertebrate protokaryotype before the 2R-WGD events. The reconstruction was made possible by the newly developed computational method outlined in Figs. 20.2 and 20.3. The reconstructed genome organization of the vertebrate ancestor provides new insights into early vertebrate genome evolution. First, some rearrangements occurred during the 2R WGD in the ancestral vertebrate. Second, after the second WGD, the gnathostome, osteichthyan, and amniote ancestors underwent slow karyotype evolution. Third, rapid, lineage-specific chromosome fusions shaped the ancestral genomes of major vertebrate taxa such as teleosts, amphibians, reptiles, and marsupials.

Our reconstruction will also integrate newly sequenced genomes such as the pipid frog (*Xenopus tropicalis*), gray short-tailed opossum (*Monodelphis domestica*), and amphioxus (*Branchiostoma floridae*) into the global picture of vertebrate and chordate genome evolution. Our presumption is that the *X. tropicalis* genome evolved from the osteichthyan ancestor genome after undergoing numerous chromosome fusions. Because those fusions took place specifically in the ancestral amphibian lineage, they would be largely different from fusions that took place from the osteichthyan ancestor to the teleost ancestor, which will make it difficult to identify one-to-one correspondence between chromosomes of *X. tropicalis* and the teleost ancestor.

References

- Abi-Rached L, Gilles A et al (2002) Evidence of en bloc duplication in vertebrate genomes. *Nat Genet* 31(1):100–105
- Burt DW (2002) Origin and evolution of avian microchromosomes. *Cytogenet Genome Res* 96(1–4):97–112
- Burt DW, Bruley C et al (1999) The dynamics of chromosome evolution in birds and mammals. *Nature (Lond)* 402(6760):411–413
- Dehal P, Boore JL (2005) Two rounds of whole genome duplication in the ancestral vertebrate. *PLoS Biol* 3(10):e314

- Dehal P, Satou Y et al (2002) The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. *Science* 298(5601):2157–2167
- Durand D (2003) Vertebrate evolution: doubling and shuffling with a full deck. *Trends Genet* 19(1):2–5
- Friedman R, Hughes AL (2001) Pattern and timing of gene duplication in animal genomes. *Genome Res* 11(11):1842–1847
- Furlong RF, Holland PW (2002) Were vertebrates octoploid? *Philos Trans R Soc Lond B Biol Sci* 357(1420):531–544
- Gibson TJ, Spring J (2000) Evidence in favour of ancient octaploidy in the vertebrate genome. *Biochem Soc Trans* 28(2):259–264
- Green DM, Sessions SK (1991) Amphibian cytogenetics and evolution. Academic, San Diego
- Gu X, Wang YF et al (2002) Age distribution of human gene families shows significant roles of both large- and small-scale duplications in vertebrate evolution. *Nat Genet* 31(2):205–209
- Holland PW, Garcia-Fernandez J et al (1994) Gene duplications and the origins of vertebrate development. *Development (Suppl)* (Camb):125–133
- Ikuta T, Yoshida N et al (2004) *Ciona intestinalis* Hox gene cluster: its dispersed structure and residual colinear expression in development. *Proc Natl Acad Sci USA* 101(42):15118–15123
- Jaillon O, Aury JM et al (2004) Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature (Lond)* 431(7011):946–957
- Kasahara M, Naruse K et al (2007) The medaka draft genome and insights into vertebrate genome evolution. *Nature (Lond)* 447(7145):714–719
- Kellis M, Birren BW et al (2004) Proof and evolutionary analysis of ancient genome duplication in the yeast *Saccharomyces cerevisiae*. *Nature (Lond)* 428(6983):617–624
- Kohn M, Hogel J et al (2006) Reconstruction of a 450-My-old ancestral vertebrate protokaryotype. *Trends Genet* 22(4):203–210
- McLysaght A, Hokamp K et al (2002) Extensive genomic duplication during early chordate evolution. *Nat Genet* 31(2):200–204
- Nakatani Y, Takeda H et al (2007) Reconstruction of the vertebrate ancestral genome reveals dynamic genome reorganization in early vertebrates. *Genome Res* 17(9):1254–1265
- Naruse K, Tanaka M et al (2004) A medaka gene map: the trace of ancestral vertebrate proto-chromosomes revealed by comparative gene mapping. *Genome Res* 14(5):820–828
- Ohno S (1970) Evolution by gene duplication. Springer, New York
- Olmo E (2005) Rate of chromosome changes and speciation in reptiles. *Genetica* 125(2–3):185–203
- Panopoulou G, Poustka AJ (2005) Timing and mechanism of ancient vertebrate genome duplications – the adventure of a hypothesis. *Trends Genet* 21(10):559–567
- Panopoulou G, Hennig S et al (2003) New evidence for genome-wide duplications at the origin of vertebrates using an amphioxus gene set and completed animal genomes. *Genome Res* 13(6):1056–1066
- Postlethwait JH, Woods IG et al (2000) Zebrafish comparative genomics and the origins of vertebrate chromosomes. *Genome Res* 10(12):1890–1902
- Sea Urchin Genome Sequencing Consortium (2006) The genome of the sea urchin *Strongylocentrotus purpuratus*. *Science* 314(5801):941–952
- Seoighe C (2003) Turning the clock back on ancient genome duplication. *Curr Opin Genet Dev* 13(6):636–643
- Shoguchi E, Kawashima T et al (2006) Chromosomal mapping of 170 BAC clones in the ascidian *Ciona intestinalis*. *Genome Res* 16(2):297–303
- Skrabanek L, Wolfe KH (1998) Eukaryote genome duplication – where's the evidence? *Curr Opin Genet Dev* 8(6):694–700
- Vandepoele K, De Vos W et al (2004) Major events in the genome evolution of vertebrates: parane age and size differ considerably between ray-finned fishes and land vertebrates. *Proc Natl Acad Sci USA* 101(6):1638–1643
- Wolfe KH (2001) Yesterday's polyploids and the mystery of diploidization. *Nat Rev Genet* 2(5):333–341
- Woods IG, Wilson C et al (2005) The zebrafish gene map defines ancestral vertebrate chromosomes. *Genome Res* 15(9):1307–1314

Chapter 21

Genome Duplication and Subfunction Partitioning: *Sox9* in Medaka and Other Vertebrates

Hayato Yokoi and John H. Postlethwait

Abstract Teleost fish experienced a round of whole genome duplication (WGD) after their lineage segregated from the tetrapod lineage. The resulting duplicate genes underwent lineage-specific diversification, and in some cases, as in *Sox9*, both duplicates, *sox9a* and *sox9b*, persisted and partitioned ancestral regulatory and structural functions between them. Because mutations and genome rearrangements accumulate within a genome as lineages diverge, partitioning of gene subfunctions can differ in different species. Here, as a case study, we show partitioning of ancestral subfunctions between *sox9a* and *sox9b* in medaka, zebrafish, and other teleosts that highlights lineage-specific divergence of gene functions. These differences shed light on evolutionary mechanisms after genome duplication and emphasize general principles regarding gene orthologies when using animals with duplicated genomes as models for human disease.

21.1 Introduction

Genome duplication is implicated as a major source of genetic complexity (Conant and Wolfe 2008). Two rounds of whole genome duplication (WGD) (R1 and R2) occurred in stem vertebrates (Holland et al. 1994; Spring 1997; Wolfe 2001; Aparicio et al. 2002; Dehal and Boore 2005; Putnam et al. 2008), and an extra round of WGD took place in the teleost lineage [R3, teleost genome duplication (TGD)] after it separated from the tetrapod lineage (Amores et al. 1998; Postlethwait et al. 1998; Taylor et al. 2003; Jaillon et al. 2004; Kasahara et al. 2007; Nakatani

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et al. 2007). Additional WGD as well as the differential loss of gene duplicates in different lineages can disturb a one-to-one correspondence between teleost and tetrapod genes (Wittbrodt et al. 1998; Meyer and Schartl 1999; Postlethwait 2007). Thus, we must use caution in assigning gene orthologies when using teleosts as models for the discovery of common and general features comparing diverse species (Postlethwait 2007; Cañestro et al. 2007; Catchen et al. 2009).

After WGD, mutations can accumulate in coding and regulatory sequences, leading to several possible evolutionary scenarios. (1) One of the duplicates could maintain original functions and the other copy could become nonfunctional through a pseudogenization (nonfunctionalization) event. This course appears to be the most frequent outcome (Brunet et al. 2006). (2) One of the duplicates could retain original functions and the other copy could acquire a new adaptive function (neofunctionalization). Nonfunctionalization and neofunctionalization were hypothesized by Susumu Ohno 40 years ago (Ohno 1970). (3) In a third possibility, both duplicates could be preserved by the complementary partitioning of ancestral structural and regulatory subfunctions (Force et al. 1999; Hughes 1999; Stoltzfus 1999; Postlethwait et al. 2004; Seiler et al. 2005). Partitioning of subfunctions between duplicated genes loosens functional constraints and thus increases evolvability (Kirschner and Gerhart 1998; Wagner 2005). For this reason, WGD is thought to provide a force for evolutionary diversification of vertebrates, especially teleost fish (Force et al. 1999; Postlethwait et al. 2004; Ravi and Venkatesh 2008).

Subfunctionalization of duplicated genes can occur during evolution. As a consequence, partitioning of some subfunctions might have occurred before speciation events in teleost evolution and are thus shared by all descendent species, such as subfunction e1 in Fig. 21.1. Other subfunctions, such as e2 in Fig. 21.1, might be preserved in both duplicates in some species and differentially partitioned in others. Still other subfunctions, such as e3, might have gone to single copy in all descendent

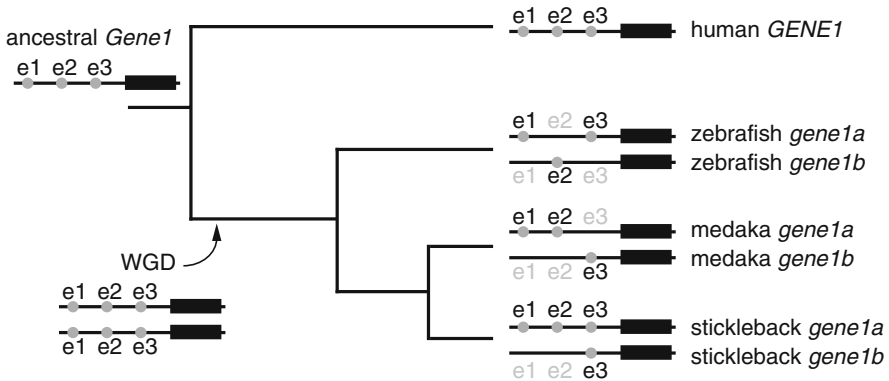


Fig. 21.1 A model for lineage-specific subfunction partitioning. A whole genome duplication (WGD) occurred in stem teleost fish producing two gene duplicates, *gene1a* and *gene1b*. Because these duplicates accumulated mutations after speciation, subfunction partitioning could be lineage specific for subfunctions retained in duplicate by the last common ancestor of any two species. Some subfunctions can be shared among all teleosts (e1), and others might be partitioned differently among species (e2 and e3)

lineages but have been differentially partitioned (see Fig. 21.1). Understanding the pattern of subfunction partitioning and its evolutionary consequences is important because the connectivity of teleost and human genomes is crucial for translating research on teleost models to human biology.

In this chapter, we use *Sox9* to describe general mechanisms of duplicated gene evolution following WGD, and ask how similar or how different the partitioning of subfunctions has been in the lineages of medaka, zebrafish, and stickleback.

21.2 *Sox9* and Its Teleost Co-Orthologs, *sox9a* and *sox9b*

Sox9 provides a convenient case study to learn about evolutionary change in co-orthologs arising in a WGD event followed by lineage-specific gene evolution. *Sox9* is a transcription factor belonging to the *Sry*-related HMG box (*Sox*) gene family (Wright et al. 1993; Bowles et al. 2000). Haploinsufficiency of human *SOX9* results in campomelic dysplasia (CD, OMIM: #114290), a disease characterized by defects in skeletal development and male-to-female sex reversal (Wagner et al. 1994; Foster et al. 1994). CD patients also show various other clinical features including cleft palate, hypoplastic lungs, and heart and renal malformations, and most patients die in infancy (Houston et al. 1983; Mansour et al. 1995, 2002). Consistent with the wide variety of human phenotypes, analyses of mice bearing conditional *Sox9* knockout mutations revealed multiple functions, including action in the development of neural crest cells, glial cells, and gonads (reviewed in Kiefer 2007; Guth and Wegner 2008). The variety of *Sox9* functions makes it a good subject for investigating duplicate gene evolution.

In teleost fish, *sox9a* and *sox9b* were both preserved after the TGD, as shown by a phylogenetic tree including *Sox9* and the other *SoxE* family members, *Sox8* and *Sox10* (Fig. 21.2) (Chiang et al. 2001; Yokoi et al. 2002; Cresko et al. 2003; Zhou et al. 2003; Koopman et al. 2004; Nakamoto et al. 2005; Kluver et al. 2005). This tree shows that (1) in medaka and ricefield eel, *sox9* genes have been called *sox9*, *sox9a1*, and *sox9a2* (Yokoi et al. 2002; Zhou et al. 2003; Nakamoto et al. 2005), but more appropriate nomenclature should probably be *sox9a* and *sox9b* (Kluver et al. 2005) because they are co-orthologs of tetrapod *Sox9*; and that (2) the nomenclature of pufferfish *sox9* duplicates (Koopman et al. 2004) is reversed (AAQ18508 is the ortholog of *Sox9a* and AAQ18507 is the ortholog of *Sox9b*).

Here we describe in more detail representative subfunctions of *Sox9* in cartilage development, retinal differentiation, and gonad differentiation, and their partitioning patterns in teleost fish.

21.2.1 *Sox9* Functions in Cartilage and Subfunction Partitioning in Zebrafish

The role of *Sox9* in cartilage development has been investigated deeply. Human CD patients show abnormalities in the skeletal system (Houston et al. 1983; Mansour

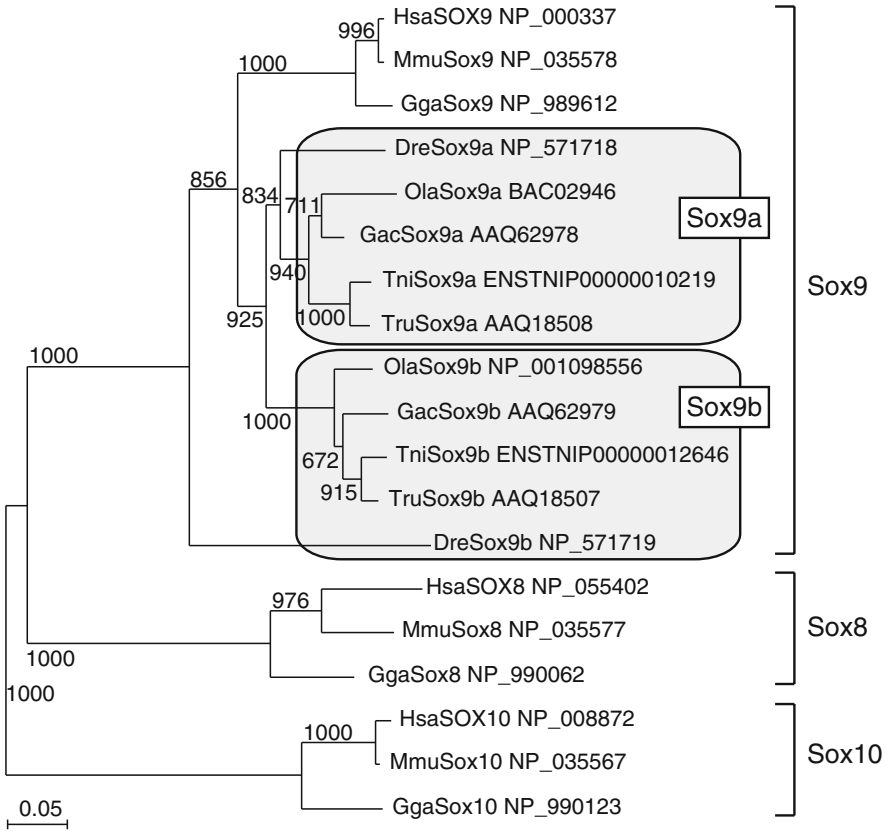


Fig. 21.2 A phylogenetic tree for SoxE group proteins. Amino acid sequences of Sox9a and Sox9b from five teleost fish (zebrafish, stickleback, medaka, tetraodon, and fugu) and Sox8, Sox9, and Sox10 from human, mouse, and chicken were used. Sox9a and Sox9b clades branched as co-orthologs of tetrapod Sox9. Note that zebrafish Sox9b shows an unusually rapid evolution, but synteny conservation confirmed that it is the zebrafish ortholog of *sox9b* (Chiang et al. 2001). Dre, *Danio rerio*, zebrafish; Gac, *Gasterosteus aculeatus*, stickleback; Gga, *Gallus gallus*, chicken; Hsa, *Homo sapiens*, human; Mmu, *Mus musculus*, mouse; Ola, *Oryzias latipes*, medaka; Tni, *Tetraodon nigroviridis*, tetraodon; Tru, *Takifugu rubripes*, pufferfish. NCBI accession numbers or Ensembl protein IDs are indicated

et al. 1995, 2002), and in mouse and chicken, *Sox9* is expressed in chondrogenic tissues (Wright et al. 1995; Ng et al. 1997; Healy et al. 1999), suggesting a conserved role of *Sox9* in cartilage development among tetrapods. Most expression domains of the mammalian *Sox9* gene partitioned into two *sox9* co-orthologs in teleosts. In zebrafish, for example, the ethmoid plate of the palate expresses only *sox9a*, whereas the lower jaw elements, mandibular and ceratohyal, express both *sox9a* and *sox9b* (Fig. 21.3a,b) (Yan et al. 2005; Yokoi et al. 2009). Expression of *sox9a* is high in cartilage precursors in pharyngeal arches, but expression of *sox9b* is high in the epithelial sheath surrounding the *sox9a*-positive perichondrium and

cartilage (Yan et al. 2005). The subfunction partitioning pattern of *sox9a* and *sox9b* is different among zebrafish, medaka, and stickleback, but the overall sum of the expression domains is comparable to that of zebrafish and tetrapods (Yokoi et al. 2002; Cresko et al. 2003; Kluver et al. 2005).

In the mouse as in the human, *Sox9* haploinsufficiency causes skeletal defects (Bi et al. 2001), and so conditional knockout strategies are essential to investigate functions (Kist et al. 2002). In contrast to mouse and human, zebrafish that are singly or doubly heterozygous for mutations in *sox9a* and *sox9b* all develop normally into fertile adults (Piotrowski et al. 1996; Yan et al. 2002, 2005). Using these mutants, we compared the expression profiles of wild-type and *sox9a*; *sox9b* double-mutant embryos using microarray transcriptome analysis to identify candidate *Sox9* target genes. Analysis revealed that *col2a1a* and *col11a2* are downregulated in *sox9* mutants (Yokoi et al. 2009). Mutant studies showed that *col11a2* expression in the ethmoid plate is *sox9a* dependent and that *col11a2* expression in mandibular and ceratohyal cartilages requires both *sox9a* and *sox9b* function (Yokoi et al. 2009) (Fig. 21.3c–f). These *collagen* expression patterns are consistent with the partitioned expression pattern of *sox9a* and *sox9b* in craniofacial skeletogenesis (Fig. 21.3a, b). These results revealed that the proteins encoded by *sox9a* and *sox9b* have both retained ancestral functions essential for regulating *col11a2* expression, but because of the partitioning of *sox9* regulatory subfunctions, different *sox9* co-orthologs control *col11a2* in different pharyngeal cartilages. *Col2a1* and *Col11a2* are well characterized as direct targets of *Sox9* in mouse and human (Bell et al. 1997; Lefebvre et al. 1997; Ng et al. 1997; Bridgewater et al. 1998; Bi et al. 1999; Liu et al. 2000), so our results suggest that *Sox9* regulation of *Collagen* genes is conserved between teleosts and tetrapods.

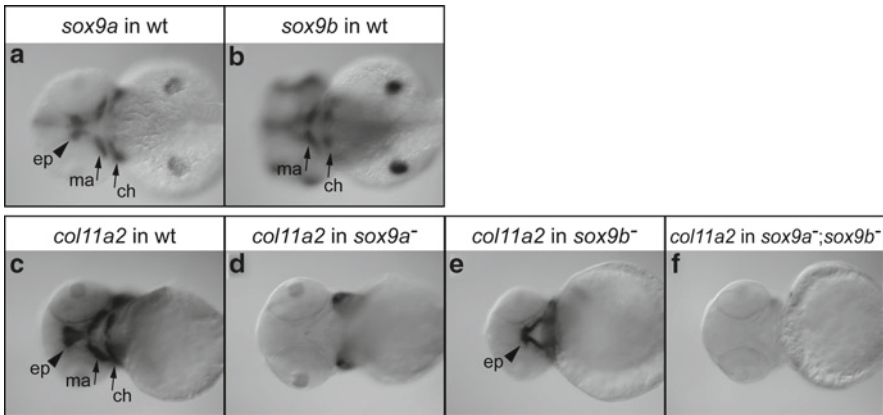


Fig. 21.3 Partitioning of functions of *Sox9*. Zebrafish *sox9a* is expressed in the ethmoid plate, mandibular, and ceratohyal (a), whereas *sox9b* is expressed in the mandibular and ceratohyal cartilages, but not in the ethmoid plate (b). Consistent with these partitioned expression patterns, expression of *col11a2* (c), one of the candidate targets, is lost in the *sox9a* mutant (d), *sox9b* mutant (e), and *sox9a;sox9b* double mutant (f) embryos. This result shows increasing genomic complexity with conserved protein functions after WGD. *ch* ceratohyal, *ep* ethmoid plate, *ma* mandibular

Additional candidate Sox9 target genes from our microarray experiments include a group of genes expressed in retina, which we discuss next.

21.2.2 *Sox9 Function in Retinal Differentiation Partitioned to *sox9b* in Teleosts*

Microarray analysis comparing wild-type and *sox9a*; *sox9b* double mutants identified a group of genes expressed in retina that were downregulated in mutants, thereby uncovering a new Sox9 function (Yokoi et al. 2009). Candidate target genes revealed by the microarray experiments included *calbindin 2a* (*calb2a*), *calbindin 2b* (*calb2b*), *cone rod homeobox* (*crx*), *neuroligin*, *retinoschisis* (X-linked, juvenile) 1 (*rs1*), *sox4a*, and *visual system homeobox 1* (*vsx1*). In the developing eye at 2 days post fertilization (dpf), *sox9b* is expressed but *sox9a* is not (Chiang et al. 2001; Yan et al. 2005; Yokoi et al. 2009). Consistent with *sox9* gene expression patterns, the retinal expression of candidate target genes depended on *sox9b* but not *sox9a* (Yokoi et al. 2009). A study using conditional knockout independently revealed that *Sox9* is required for Müller glial cell development in the mouse (Poché et al. 2008), suggesting that the function of *Sox9* in retinal differentiation is ancestral among vertebrates and that it partitioned to *sox9b* in the zebrafish lineage. Expression of *sox9b* in the developing eye is also reported in stickleback and medaka (Cresko et al. 2003; Kluver et al. 2005); thus, this partitioning is likely to have occurred before the divergence of these three species.

21.2.3 *Sox9 Function in Gonads*

Sox9 is a key regulator in mammalian sex determination pathways. Human CD patients show sex reversal, resulting in XY females (Foster et al. 1994; Wagner et al. 1994). In the mouse, *Sox9* is initially expressed in both sexes before the onset of *Sry* expression (Kent et al. 1996; Morais da Silva et al. 1996), then Sf1/Ad4BP and *Sry* upregulate *Sox9* expression in male gonads; subsequently, *Sox9* itself feeds back to maintain *Sox9* transcription (Sekido and Lovell-Badge 2008). Chromosomally XX mice expressing *Sox9* ectopically in the gonads develop testes (Vidal et al. 2001), and complementarily, chromosomally XY *Sox9* knockout mice develop as females (Chaboissier et al. 2004; Barrionuevo et al. 2006). These results show that *Sox9* is necessary and sufficient for male sex differentiation in mammals. Male gonad expression of *Sox9* is also reported in nonmammalian vertebrates, including chicken, turtle, and alligator (Kent et al. 1996; Morais da Silva et al. 1996; Spotila et al. 1998; Western et al. 1999), suggesting that the function of *Sox9* in testis differentiation is conserved among tetrapods.

Expression of *sox9* co-orthologs is complicated in teleost fish. Medaka *sox9b* starts to be expressed in somatic cells in developing gonads in both sexes, but only the developing testis maintains *sox9b* expression (Nakamoto et al. 2005), which is comparable with the expression pattern of mouse *Sox9*. In contrast, in medaka, *sox9a* is expressed in developing oocytes (Yokoi et al. 2002), clearly different from the transient expression of *Sox9* in the somatic portion of mouse ovaries. In zebrafish, the reverse is true, with *sox9a* expressed more strongly in the developing testis, but *sox9b* expressed more strongly in oocytes (Rodríguez-Marí et al. 2005). The reciprocal partitioning of gonadal functions in medaka and zebrafish lineages demonstrates lineage-specific subfunction partitioning, but its functional significance is a question for future research.

21.3 Divergent Partitioning of *Sox9* Functions Among Zebrafish, Medaka, and Stickleback

Although functional studies have been conducted only in zebrafish, embryonic expression patterns of *sox9* co-orthologs have been analyzed in other teleosts, including medaka and stickleback. Functional analysis using mutants and/or morpholino antisense knockdown would provide direct evidence of gene function, but identification of mutants is not an easy task. Expression analysis is immediately convenient and shows when and where the gene might function. As already mentioned, subfunction partitioning can occur reciprocally after speciation events for subfunctions retained in duplicate by the last common ancestor of two species, such as e2 or e3 in Fig. 21.1.

To compare expression patterns of gene duplicates across species at a single developmental stage, we examined the expression patterns of *sox9a* and *sox9b* at the 12-somite stage in zebrafish, medaka, and stickleback. Results revealed divergent partitioning patterns (Fig. 21.4). Somite expression of *sox9a* is evident in zebrafish, but not in medaka or stickleback. Expression of *sox9b* in neural crest is conserved among all three species. Although detailed comparison of expression patterns will be published elsewhere, the patterns revealed several key differences in expression patterns among these three species. Considering the timing of lineage segregation, comparative analysis using different teleosts highlights how subfunctions partitioned and subsequently evolved over phylogenetic time.

Divergent partitioning of an upstream regulator of *sox9* has also been demonstrated. Fgf signaling upregulates *Sox9* expression in a mouse chondrocyte line, and *Fgfr1* can mediate this signaling (Murakami et al. 2000). Because *Fgfr1* mutants are lethal in mammals (Deng et al. 1994; Yamaguchi et al. 1994), it is difficult to investigate its role in late developmental events. Teleosts have duplicated *fgfr1* genes, and mutation in *fgfr1a* causes a scaleless phenotype in zebrafish and domesticated carp (Rohner et al. 2009). The partially redundant second copy of *fgfr1* allows zebrafish and carp to bypass the embryonic lethality of the *fgfr1* mutation,

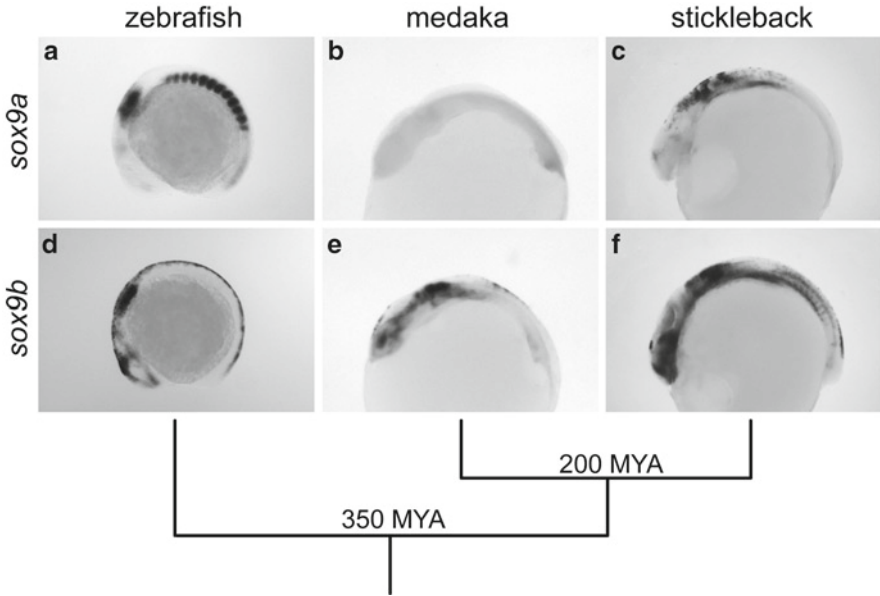


Fig. 21.4 Divergent partitioning of *sox9* expression patterns among teleosts. Expression of *sox9a* (a–c) and *sox9b* (d–f) at the 12-somite stage in zebrafish (a, d), medaka (b, e), and stickleback (c, f). In these species, *sox9a* and *sox9b* are expressed in different but overlapping sets of tissues showing partitioning of expression domains between the co-orthologs. The expression of *sox9b* (d–f) is similar among three species, expressed in retina, otic vesicle, and neural crest cells in head and trunk. Expression of *sox9a* is more divergent among these species, in otic vesicle and somite in zebrafish (a), no expression in medaka at this stage (b), and in neural crest cells around eye and otic vesicle in stickleback (c). Lines at the bottom of the figure show phylogenetic relationships among these species. The zebrafish lineage separated from other two lineages around 350 MYA, and medaka and stickleback lineages separated around 200 MYA (Yamanoue et al. 2006)

showing that genome duplication relaxed the pleiotropic effect of the single copy mammalian *Fgfr1* gene. Importantly, the scaleless phenotype of *fgfr1a* mutants in zebrafish and carp contrasts to the trunkless, tailless phenotype of the medaka *fgfr1a* (*headfish*) mutant (Yokoi et al. 2007; Shimada et al. 2008). These results suggest that subfunction partitioning in *fgfr1* occurred after the divergence of the medaka lineage from the lineage of zebrafish and carp, providing a good example of models discussed in this section.

21.4 Closing Remarks and Perspectives

Genome duplication followed by subfunction partitioning can result in reduced pleiotropic constraint (Force et al. 1999; Postlethwait et al. 2004; Conant and Wolfe 2008). Analyses of subfunction partitioning have so far focused mainly on expression patterns of co-orthologs with respect to patterns inferred for the preduplication

ancestral gene. Research focus should now shift to the characterization of cis-regulatory elements that underlie partitioned expression patterns. As described above, the partitioning of regulatory subfunctions (as judged by expression patterns) between co-orthologs is often different among species, so cis-acting regulatory elements should be lost in a lineage-specific manner and the distribution of cis-elements should be different among species. Full genome sequences are available for two pufferfish, medaka, stickleback, and zebrafish (Aparicio et al. 2002; Jaillon et al. 2004; Kasahara et al. 2007), and genome sequences of tetrapods can serve as unduplicated outgroups for the TGD. Comparative genomics of teleosts and tetrapods can facilitate the identification of conserved noncoding elements (CNEs) (Woolfe et al. 2005; Cañestro et al. 2007) as shown by the comparison of human and fugu genomes, which identified several regulatory elements that conserved and partitioned into fugu *sox9b* (Bagheri-Fam et al. 2001, 2006). However, tetrapods are distantly diverged from the last unduplicated ray fin genome, so we need a better outgroup genome, such as a basal ray fin fish, for example, amia, gar, paddlefish, or bichir. Although many CNEs are shown to serve as cis-regulatory elements (Woolfe et al. 2005; Vavouri et al. 2006), some CNEs are dispensable for embryonic development (Ahituv et al. 2007) and some elements are functionally conserved without sequence similarities (Fisher et al. 2006). Thus, thorough transgenic analyses to test the functions of CNEs and other potential regulatory sequences are indispensable to understand the regulatory mechanisms of partitioned expression and the molecular basis of subfunction partitioning.

Another important aspect of subfunctionalization is the functional divergence of the duplicates after the WGD. Sox9 is a transcriptional activator, and the transactivation domain is located in the C-terminal portion of the protein (Südbeck et al. 1996). Functional comparisons of zebrafish Sox9a and Sox9b to mouse Sox9 showed, first, that all three proteins bind the HMG consensus sequence in vitro, suggesting very similar sequence preferences, and second, that the transactivation potential of mouse Sox9 is relatively high and zebrafish Sox9b is less active in COS-1 cells (Chiang et al. 2001). Because Sox9 functions both as a monomer and as a dimer (Bernard et al. 2003; Sock et al. 2003; Genzer and Bridgewater 2007), gene duplication provides increased possibilities for complexity. Some zebrafish cell types contain only Sox9a or Sox9b but others contain both proteins, and so different cell types can form different monomers and dimers. With recent advances in BAC modification and transgenic methods (Lee et al. 2001; Yang et al. 2006), direct comparison between zebrafish *sox9a* and *sox9b*, and between medaka *sox9a* and zebrafish *sox9a*, become possible in vivo. For example, we may ask whether medaka *sox9a* and zebrafish *sox9b* can rescue zebrafish *sox9a* mutants if expressed in the correct tissue.

Here, we have discussed the evolution of *Sox9* in teleosts following the TGD. Vertebrates experienced two earlier rounds of WGD (R1 and R2) before the tetrapod–teleost split (Holland et al. 1994; Spring 1997; Wolfe 2001; Dehal and Boore 2005; Putnam et al. 2008). The *SoxE* gene family, including *Sox8*, *Sox9*, and *Sox10*, evolved from a common ancestor in these early WGD events (Bowles et al. 2000; Guth and Wegner 2008), and likely partitioned the functions of a single ancestral

SoxE gene. Although *Sox9* regulates development of the cranial cartilage component of neural crest derivatives (Yan et al. 2002, 2005; Mori-Akiyama et al. 2003), *Sox10* regulates development of other neural crest derivatives, including pigment cells and enteric nervous system cells (Southard-Smith et al. 1998; Kelsh and Eisen 2000; Dutton et al. 2001), showing partitioning of functions in neural crest development between *Sox9* and *Sox10*. *Sox8* and *Sox9* also share redundant functions in testis development (Chaboissier et al. 2004; Barrionuevo et al. 2009). The amphioxus *SoxE* gene serves as an ancestral outgroup for vertebrates *SoxE* genes (Meulemans and Bronner-Fraser 2004, 2007). The duplication of *Sox9* in the TGD provides a model for the general principles underlying the evolution of gene functions after WGD events, and the integration of that information into the understanding of gene evolution in the R1 and R2 WGD events, which are shared by humans and teleost fish, promises to shed light on the evolution of genes and genomes in the invention of the vertebrates.

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References

- Ahituv N, Zhu Y, Visel A, Holt A, Afzal V, Pennacchio LA, Rubin EM (2007) Deletion of ultra-conserved elements yields viable mice. *PLoS Biol* 5:e234
- Amores A, Force A, Yan YL, Joly L, Amemiya C, Fritz A, Ho RK, Langeland J, Prince V, Wang YL, Westerfield M, Ekker M, Postlethwait JH (1998) Zebrafish *hox* clusters and vertebrate genome evolution. *Science* 282:1711–1714
- Aparicio S, Chapman J, Stupka E, Putnam N, Chia JM, Dehal P, Christoffels A, Rash S, Hoon S, Smit A, Gelpke MD, Roach J, Oh T, Ho IY, Wong M, Detter C, Verhoef F, Predki P, Tay A, Lucas S, Richardson P, Smith SF, Clark MS, Edwards YJ, Doggett N, Zharkikh A, Tavtigian SV, Pruss D, Barnstead M, Evans C, Baden H, Powell J, Glusman G, Rowen L, Hood L, Tan YH, Elgar G, Hawkins T, Venkatesh B, Rokhsar D, Brenner S (2002) Whole-genome shotgun assembly and analysis of the genome of *Fugu rubripes*. *Science* 297:1301–1310
- Bagheri-Fam S, Ferraz C, Demaille J, Scherer G, Pfeifer D (2001) Comparative genomics of the *SOX9* region in human and *Fugu rubripes*: conservation of short regulatory sequence elements within large intergenic regions. *Genomics* 78:73–82
- Bagheri-Fam S, Barrionuevo F, Dohrmann U, Gunther T, Schule R, Kemler R, Mallo M, Kanzler B, Scherer G (2006) *Long-range* upstream and downstream enhancers control distinct subsets of the complex spatiotemporal *Sox9* expression pattern. *Dev Biol* 291:382–397
- Barrionuevo F, Bagheri-Fam S, Klattig J, Kist R, Taketo MM, Englert C, Scherer G (2006) Homozygous inactivation of *Sox9* causes complete XY sex reversal in mice. *Biol Reprod* 74:195–201
- Barrionuevo F, Georg I, Scherthan H, Lecureuil C, Guillou F, Wegner M, Scherer G (2009) Testis cord differentiation after the sex determination stage is independent of *Sox9* but fails in the combined absence of *Sox9* and *Sox8*. *Dev Biol* 327:301–312
- Bell DM, Leung KK, Wheatley SC, Ng LJ, Zhou S, Ling KW, Sham MH, Koopman P, Tam PP, Cheah KS (1997) SOX9 directly regulates the type-II collagen gene. *Nat Genet* 16:174–178
- Bernard P, Tang P, Liu S, Dewing P, Harley VR, Vilain E (2003) Dimerization of SOX9 is required for chondrogenesis, but not for sex determination. *Hum Mol Genet* 12:1755–1765
- Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrughe B (1999) *Sox9* is required for cartilage formation. *Nat Genet* 22:85–89

- Bi W, Huang W, Whitworth DJ, Deng JM, Zhang Z, Behringer RR, de Crombrughe B (2001) Haploinsufficiency of *Sox9* results in defective cartilage primordia and premature skeletal mineralization. *Proc Natl Acad Sci USA* 98:6698–6703
- Bowles J, Schepers G, Koopman P (2000) Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. *Dev Biol* 227:239–255
- Bridgewater LC, Lefebvre V, de Crombrughe B (1998) Chondrocyte-specific enhancer elements in the *Col11a2* gene resemble the *Col2a1* tissue-specific enhancer. *J Biol Chem* 273:14998–15006
- Brunet FG, Crollius HR, Paris M, Aury JM, Gibert P, Jaillon O, Laudet V, Robinson-Rechavi M (2006) Gene loss and evolutionary rates following whole-genome duplication in teleost fishes. *Mol Biol Evol* 23:1808–1816
- Cañestro C, Yokoi H, Postlethwait JH (2007) Evolutionary developmental biology and genomics. *Nat Rev Genet* 8:932–942
- Catchen JM, Conery JS, Postlethwait JH (2009) Automated identification of conserved synteny after whole-genome duplication. *Genome Res* 19:1497–1505
- Chaboissier MC, Kobayashi A, Vidal VI, Lützkendorf S, van de Kant HJ, Wegner M, de Rooij DG, Behringer RR, Schedl A (2004) Functional analysis of *Sox8* and *Sox9* during sex determination in the mouse. *Development (Camb)* 131:1891–1901
- Chiang EF, Pai CI, Wyatt M, Yan YL, Postlethwait J, Chung B (2001) Two *Sox9* genes on duplicated zebrafish chromosomes: expression of similar transcription activators in distinct sites. *Dev Biol* 231:149–163
- Conant GC, Wolfe KH (2008) Turning a hobby into a job: how duplicated genes find new functions. *Nat Rev Genet* 9:938–950
- Cresko WA, Yan YL, Baltrus DA, Amores A, Singer A, Rodríguez-Marí A, Postlethwait JH (2003) Genome duplication, subfunction partitioning, and lineage divergence: *Sox9* in stickleback and zebrafish. *Dev Dyn* 228:480–489
- Dehal P, Boore JL (2005) Two rounds of whole genome duplication in the ancestral vertebrate. *PLoS Biol* 3:e314
- Deng CX, Wynshaw-Boris A, Shen MM, Daugherty C, Ornitz DM, Leder P (1994) Murine FGFR-1 is required for early postimplantation growth and axial organization. *Genes Dev* 8:3045–3057
- Dutton KA, Pauliny A, Lopes SS, Elworthy S, Carney TJ, Rauch J, Geisler R, Haffter P, Kelsh RN (2001) Zebrafish *colourless* encodes *sox10* and specifies non-ectomesenchymal neural crest fates. *Development (Camb)* 128:4113–4125
- Fisher S, Grice EA, Vinton RM, Bessling SL, McCallion AS (2006) Conservation of *RET* regulatory function from human to zebrafish without sequence similarity. *Science* 312:276–279
- Force A, Lynch M, Pickett FB, Amores A, Yan YL, Postlethwait J (1999) Preservation of duplicate genes by complementary, degenerative mutations. *Genetics* 151:1531–1545
- Foster JW, Dominguez-Steglich MA, Guioli S, Kwok C, Weller PA, Stevanović M, Weissenbach J, Mansour S, Young ID, Goodfellow PN, Brook JD, Schafer AJ (1994) Campomelic dysplasia and autosomal sex reversal caused by mutations in an *SRY*-related gene. *Nature (Lond)* 372:525–530
- Genzer MA, Bridgewater LC (2007) A *Col9a1* enhancer element activated by two interdependent SOX9 dimers. *Nucleic Acids Res* 35:1178–1186
- Guth SI, Wegner M (2008) Having it both ways: Sox protein function between conservation and innovation. *Cell Mol Life Sci* 65:3000–3018
- Healy C, Uwanogho D, Sharpe PT (1999) Regulation and role of Sox9 in cartilage formation. *Dev Dyn* 215:69–78
- Holland PW, Garcia-Fernandez J, Williams NA, Sidow A (1994) Gene duplications and the origins of vertebrate development. *Dev Suppl* 1994:125–133
- Houston CS, Opitz JM, Spranger JW, Macpherson RI, Reed MH, Gilbert EF, Herrmann J, Schinzel A (1983) The campomelic syndrome: review, report of 17 cases, and follow-up on the currently 17-year-old boy first reported by Maroteaux et al in 1971. *Am J Med Genet* 15:3–28
- Hughes AL (1999) Adaptive evolution of genes and genomes. Oxford University Press, New York

- Jaillon O, Aury JM, Brunet F, Petit JL, Stange-Thomann N, Mauceli E, Bouneau L, Fischer C, Ozouf-Costaz C, Bernot A, Nicaud S, Jaffe D, Fisher S, Lutfalla G, Dossat C, Segurens B, Dasilva C, Salanoubat M, Levy M, Boudet N, Castellano S, Anthouard V, Jubin C, Castelli V, Katinka M, Vacherie B, Biemont C, Skalli Z, Cattolico L, Poulain J, De Berardinis V, Cruaud C, Duprat S, Brottier P, Coutanceau JP, Gouzy J, Parra G, Lardier G, Chapple C, McKernan KJ, McEwan P, Bosak S, Kellis M, Volff JN, Guigo R, Zody MC, Mesirov J, Lindblad-Toh K, Birren B, Nusbaum C, Kahn D, Robinson-Rechavi M, Laudet V, Schachter V, Quetier F, Saurin W, Scarpelli C, Wincker P, Lander ES, Weissenbach J, Roest Crollius H (2004) Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature (Lond)* 431:946–957
- Kasahara M, Naruse K, Sasaki S, Nakatani Y, Qu W, Ahsan B, Yamada T, Nagayasu Y, Doi K, Kasai Y, Jindo T, Kobayashi D, Shimada A, Toyoda A, Kuroki Y, Fujiyama A, Sasaki T, Shimizu A, Asakawa S, Shimizu N, Hashimoto S, Yang J, Lee Y, Matsushima K, Sugano S, Sakaizumi M, Narita T, Ohishi K, Haga S, Ohta F, Nomoto H, Nogata K, Morishita T, Endo T, Shin IT, Takeda H, Morishita S, Kohara Y (2007) The medaka draft genome and insights into vertebrate genome evolution. *Nature (Lond)* 447:714–719
- Kelsh RN, Eisen JS (2000) The zebrafish colourless gene regulates development of non-ectomesenchymal neural crest derivatives. *Development (Camb)* 127:515–525
- Kent J, Wheatley SC, Andrews JE, Sinclair AH, Koopman P (1996) A male-specific role for *SOX9* in vertebrate sex determination. *Development (Camb)* 122:2813–2822
- Kiefer JC (2007) Back to basics: *Sox* genes. *Dev Dyn* 236:2356–2366
- Kirschner M, Gerhart J (1998) Evolvability. *Proc Natl Acad Sci USA* 95:8420–8427
- Kist R, Schrewe H, Balling R, Scherer G (2002) Conditional inactivation of *Sox9*: a mouse model for campomelic dysplasia. *Genesis* 32:121–123
- Kluver N, Kondo M, Herpin A, Mitani H, Scharl M (2005) Divergent expression patterns of *Sox9* duplicates in teleosts indicate a lineage specific subfunctionalization. *Dev Genes Evol* 215:297–305
- Koopman P, Schepers G, Brenner S, Venkatesh B (2004) Origin and diversity of the *Sox* transcription factor gene family: genome-wide analysis in *Fugu rubripes*. *Gene (Amst)* 328:177–186
- Lee EC, Yu D, Martinez de Velasco J, Tassarollo L, Swing DA, Court DL, Jenkins NA, Copeland NG (2001) A highly efficient *Escherichia coli*-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* 73:56–65
- Lefebvre V, Huang W, Harley VR, Goodfellow PN, de Crombrughe B (1997) *SOX9* is a potent activator of the chondrocyte-specific enhancer of the pro $\alpha 1(\text{II})$ collagen gene. *Mol Cell Biol* 17:2336–2346
- Liu Y, Li H, Tanaka K, Tsumaki N, Yamada Y (2000) Identification of an enhancer sequence within the first intron required for cartilage-specific transcription of the $\alpha 2(\text{XI})$ collagen gene. *J Biol Chem* 275:12712–12718
- Mansour S, Hall CM, Pembrey ME, Young ID (1995) A clinical and genetic study of campomelic dysplasia. *J Med Genet* 32:415–420
- Mansour S, Offiah AC, McDowall S, Sim P, Tolmie J, Hall C (2002) The phenotype of survivors of campomelic dysplasia. *J Med Genet* 39:597–602
- Meulemans D, Bronner-Fraser M (2004) Gene-regulatory interactions in neural crest evolution and development. *Dev Cell* 7:291–299
- Meulemans D, Bronner-Fraser M (2007) Insights from amphioxus into the evolution of vertebrate cartilage. *PLoS One* 2:e787
- Meyer A, Scharl M (1999) Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. *Curr Opin Cell Biol* 11:699–704
- Morais da Silva S, Hacker A, Harley V, Goodfellow P, Swain A, Lovell-Badge R (1996) *Sox9* expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. *Nat Genet* 14:62–68
- Mori-Akiyama Y, Akiyama H, Rowitch DH, de Crombrughe B (2003) *Sox9* is required for determination of the chondrogenic cell lineage in the cranial neural crest. *Proc Natl Acad Sci USA* 100:9360–9365

- Murakami S, Kan M, McKeehan WL, de Crombrughe B (2000) Up-regulation of the chondrogenic *Sox9* gene by fibroblast growth factors is mediated by the mitogen-activated protein kinase pathway. *Proc Natl Acad Sci USA* 97:1113–1118
- Nakamoto M, Suzuki A, Matsuda M, Nagahama Y, Shibata N (2005) Testicular type *Sox9* is not involved in sex determination but might be in the development of testicular structures in the medaka, *Oryzias latipes*. *Biochem Biophys Res Commun* 333:729–736
- Nakatani Y, Takeda H, Kohara Y, Morishita S (2007) Reconstruction of the vertebrate ancestral genome reveals dynamic genome reorganization in early vertebrates. *Genome Res* 17:1254–1265
- Ng LJ, Wheatley S, Muscat GE, Conway-Campbell J, Bowles J, Wright E, Bell DM, Tam PP, Cheah KS, Koopman P (1997) SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. *Dev Biol* 183:108–121
- Ohno S (1970) *Evolution by gene duplication*. Springer, New York
- Piotrowski T, Schilling TF, Brand M, Jiang YJ, Heisenberg CP, Beuchle D, Grandel H, van Eeden FJ, Furutani-Seiki M, Granato M, Haffter P, Hammerschmidt M, Kane DA, Kelsh RN, Mullins MC, Odenthal J, Warga RM, Nusslein-Volhard C (1996) Jaw and branchial arch mutants in zebrafish II: anterior arches and cartilage differentiation. *Development (Camb)* 123:345–356
- Poché RA, Furuta Y, Chaboissier MC, Schedl A, Behringer RR (2008) Sox9 is expressed in mouse multipotent retinal progenitor cells and functions in Muller glial cell development. *J Comp Neurol* 510:237–250
- Postlethwait JH (2007) The zebrafish genome in context: ohnologs gone missing. *J Exp Zool B Mol Dev Evol* 308:563–577
- Postlethwait JH, Yan YL, Gates MA, Horne S, Amores A, Brownlie A, Donovan A, Egan ES, Force A, Gong Z, Goutel C, Fritz A, Kelsh R, Knapik E, Liao E, Paw B, Ransom D, Singer A, Thomson M, Abduljabbar TS, Yelick P, Beier D, Joly JS, Larhammar D, Rosa F, Westerfield M, Zon LI, Johnson SL, Talbot WS (1998) Vertebrate genome evolution and the zebrafish gene map. *Nat Genet* 18:345–349
- Postlethwait J, Amores A, Cresko W, Singer A, Yan YL (2004) Subfunction partitioning, the teleost radiation and the annotation of the human genome. *Trends Genet* 20:481–490
- Putnam NH, Butts T, Ferrier DE, Furlong RF, Hellsten U, Kawashima T, Robinson-Rechavi M, Shoguchi E, Terry A, Yu JK, Benito-Gutierrez EL, Dubchak I, Garcia-Fernandez J, Gibson-Brown JJ, Grigoriev IV, Horton AC, de Jong PJ, Jurka J, Kapitonov VV, Kohara Y, Kuroki Y, Lindquist E, Lucas S, Osoegawa K, Pennacchio LA, Salamov AA, Satou Y, Sauka-Spengler T, Schmutz J, Shin IT, Toyoda A, Bronner-Fraser M, Fujiyama A, Holland LZ, Holland PW, Satoh N, Rokhsar DS (2008) The amphioxus genome and the evolution of the chordate karyotype. *Nature (Lond)* 453:1064–1071
- Ravi V, Venkatesh B (2008) Rapidly evolving fish genomes and teleost diversity. *Curr Opin Genet Dev* 18:544–550
- Rodríguez-Marí A, Yan YL, Bremiller RA, Wilson C, Cañestro C, Postlethwait JH (2005) Characterization and expression pattern of zebrafish *anti-Müllerian hormone (amh)* relative to *sox9a*, *sox9b*, and *cyp19a1a*, during gonad development. *Gene Expr Patterns* 5:655–667
- Rohner N, Bercsenyi M, Orban L, Kolanczyk ME, Linke D, Brand M, Nusslein-Volhard C, Harris MP (2009) Duplication of *fgfr1* permits Fgf signaling to serve as a target for selection during domestication. *Curr Biol* 19:1642–1647
- Seiler C, Finger-Baier KC, Rinner O, Makhankov YV, Schwarz H, Neuhauss SC, Nicolson T (2005) Duplicated genes with split functions: independent roles of *protocadherin15* orthologues in zebrafish hearing and vision. *Development (Camb)* 132:615–623
- Sekido R, Lovell-Badge R (2008) Sex determination involves synergistic action of SRY and SF1 on a specific *Sox9* enhancer. *Nature (Lond)* 453:930–934
- Shimada A, Yabusaki M, Niwa H, Yokoi H, Hatta K, Kobayashi D, Takeda H (2008) Maternal-zygotic medaka mutants for *fgfr1* reveal its essential role in the migration of the axial mesoderm but not the lateral mesoderm. *Development (Camb)* 135:281–290
- Sock E, Pagon RA, Keymolen K, Lissens W, Wegner M, Scherer G (2003) Loss of DNA-dependent dimerization of the transcription factor SOX9 as a cause for campomelic dysplasia. *Hum Mol Genet* 12:1439–1447

- Southard-Smith EM, Kos L, Pavan WJ (1998) *Sox10* mutation disrupts neural crest development in *Dom* Hirschsprung mouse model. *Nat Genet* 18:60–64
- Spotila LD, Spotila JR, Hall SE (1998) Sequence and expression analysis of WT1 and Sox9 in the red-eared slider turtle, *Trachemys scripta*. *J Exp Zool* 281:417–427
- Spring J (1997) Vertebrate evolution by interspecific hybridisation: are we polyploid? *FEBS Lett* 400:2–8
- Stoltzfus A (1999) On the possibility of constructive neutral evolution. *J Mol Evol* 49:169–181
- Südbeck P, Schmitz ML, Baeuerle PA, Scherer G (1996) Sex reversal by loss of the C-terminal transactivation domain of human SOX9. *Nat Genet* 13:230–232
- Taylor JS, Braasch I, Frickey T, Meyer A, Van de Peer Y (2003) Genome duplication, a trait shared by 22000 species of ray-finned fish. *Genome Res* 13:382–390
- Vavouri T, McEwen GK, Woolfe A, Gilks WR, Elgar G (2006) Defining a genomic radius for long-range enhancer action: duplicated conserved non-coding elements hold the key. *Trends Genet* 22:5–10
- Vidal VP, Chaboissier MC, de Rooij DG, Schedl A (2001) *Sox9* induces testis development in XX transgenic mice. *Nat Genet* 28:216–217
- Wagner A (2005) Robustness, evolvability, and neutrality. *FEBS Lett* 579:1772–1778
- Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, Pasantes J, Bricarelli FD, Keutel J, Hustert E, Wolf U, Tommerup N, Schempp W, Scherer G (1994) Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the *SRY*-related gene *SOX9*. *Cell* 79:1111–1120
- Western PS, Harry JL, Graves JA, Sinclair AH (1999) Temperature-dependent sex determination: upregulation of *SOX9* expression after commitment to male development. *Dev Dyn* 214:171–177
- Wittbrodt J, Meyer A, Scharl M (1998) More genes in fish? *BioEssays* 20:511–515
- Wolfe KH (2001) Yesterday's polyploids and the mystery of diploidization. *Nat Rev Genet* 2:333–341
- Woolfe A, Goodson M, Goode DK, Snell P, McEwen GK, Vavouri T, Smith SF, North P, Callaway H, Kelly K, Walter K, Abnizova I, Gilks W, Edwards YJ, Cooke JE, Elgar G (2005) Highly conserved non-coding sequences are associated with vertebrate development. *PLoS Biol* 3:e7
- Wright EM, Snopek B, Koopman P (1993) Seven new members of the *Sox* gene family expressed during mouse development. *Nucleic Acids Res* 21:744
- Wright E, Hargrave MR, Christiansen J, Cooper L, Kun J, Evans T, Gangadharan U, Greenfield A, Koopman P (1995) The *Sry*-related gene *Sox9* is expressed during chondrogenesis in mouse embryos. *Nat Genet* 9:15–20
- Yamaguchi TP, Harpal K, Henkemeyer M, Rossant J (1994) *fgfr-1* is required for embryonic growth and mesodermal patterning during mouse gastrulation. *Genes Dev* 8:3032–3044
- Yamanoue Y, Miya M, Inoue JG, Matsuura K, Nishida M (2006) The mitochondrial genome of spotted green pufferfish *Tetraodon nigroviridis* (Teleostei: Tetraodontiformes) and divergence time estimation among model organisms in fishes. *Genes Genet Syst* 81:29–39
- Yan YL, Miller CT, Nissen RM, Singer A, Liu D, Kirn A, Draper B, Willoughby J, Morcos PA, Amsterdam A, Chung BC, Westerfield M, Haffter P, Hopkins N, Kimmel C, Postlethwait JH (2002) A zebrafish *sox9* gene required for cartilage morphogenesis. *Development (Camb)* 129:5065–5079
- Yan YL, Willoughby J, Liu D, Crump JG, Wilson C, Miller CT, Singer A, Kimmel C, Westerfield M, Postlethwait JH (2005) A pair of Sox: distinct and overlapping functions of zebrafish *sox9* co-orthologs in craniofacial and pectoral fin development. *Development (Camb)* 132:1069–1083
- Yang Z, Jiang H, Chachainasakul T, Gong S, Yang XW, Heintz N, Lin S (2006) Modified bacterial artificial chromosomes for zebrafish transgenesis. *Methods* 39:183–188
- Yokoi H, Kobayashi T, Tanaka M, Nagahama Y, Wakamatsu Y, Takeda H, Araki K, Morohashi K, Ozato K (2002) *Sox9* in a teleost fish, medaka (*Oryzias latipes*): evidence for diversified function of *Sox9* in gonad differentiation. *Mol Reprod Dev* 63:5–16

- Yokoi H, Shimada A, Carl M, Takashima S, Kobayashi D, Narita T, Jindo T, Kimura T, Kitagawa T, Kage T, Sawada A, Naruse K, Asakawa S, Shimizu N, Mitani H, Shima A, Tsutsumi M, Hori H, Wittbrodt J, Saga Y, Ishikawa Y, Araki K, Takeda H (2007) Mutant analyses reveal different functions of *fgfr1* in medaka and zebrafish despite conserved ligand–receptor relationships. *Dev Biol* 304:326–337
- Yokoi H, Yan YL, Miller MR, BreMiller RA, Catchen JM, Johnson EA, Postlethwait JH (2009) Expression profiling of zebrafish *sox9* mutants reveals that *Sox9* is required for retinal differentiation. *Dev Biol* 329:1–15
- Zhou R, Liu L, Guo Y, Yu H, Cheng H, Huang X, Tiersch TR, Berta P (2003) Similar gene structure of two *Sox9a* genes and their expression patterns during gonadal differentiation in a teleost fish, rice field eel (*Monopterus albus*). *Mol Reprod Dev* 66:211–217

Chapter 22

Human Population Genetics Meets Medaka

Hiroki Oota and Hiroshi Mitani

Abstract A significant feature of Medaka (*Oryzias latipes*) is its well-known genetic, morphological and physiological diversity. Population studies of medaka have shown that it is a promising candidate for a “vertebrate” model system of population genetics. The study of genome diversity in humans is one of the key post-genome sequencing projects, accomplished by analyzing genetic polymorphisms in human populations, although in most cases little is known as to what functional differences are induced by polymorphisms and what effect they have on human adaptation. We expect that Medaka populations can be a useful model to examine the relationship between allelic functional differences and habitat environment as an analogy of human populations. Herein we review the potential usefulness of Medaka in the study of human population genetics.

22.1 Introduction

Although animal models exist for studying human biology, it is much more difficult to find a suitable animal model for human population genetics. The posthuman genome sequencing project has extended to comparative genomics with other primates, particularly chimpanzees and macaques, and to genome-wide studies of human populations. The former allow us mainly to provide genomic information of experimental model animals, and the latter exclusively intends to promote association studies of disease and to pharmacogenomics (Frazer et al. 2007; Gibbs

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et al. 2007; The_Chimpanzee_Sequencing_and_Analysis_Consortium 2005; The_international_HapMap_consortium 2005). The international HapMap and the human diversity projects are representative of genome-wide studies of human populations based on single nucleotide polymorphisms (SNPs), which have identified many disease-causative genes (Altshuler et al. 2008; Bodmer and Bonilla 2008; Burton et al. 2007; Hancock et al. 2008; Miyagawa et al. 2008; Young et al. 2005) and detected the signals of natural selection by population/evolutionary genetic tests (Barreiro et al. 2008; Bustamante et al. 2005; Nielsen et al. 2009; Pickrell et al. 2009; Sabeti et al. 2006, 2007). Thus, humans (*Homo sapiens*) are the best studied species for disease and evolution based on genome-wide data. In many cases, however, the functional difference caused by SNP alleles is unclear. These differences and the selection signals shown in the population data are detected based on the allele frequency spectrum. To analyze such subtle differences it is therefore necessary to use a suitable model animal to which population analysis can be applied. Currently, the collaboration team (human population geneticist, Hiroki Oota, and molecular geneticist, Hiroshi Mitani) is focusing on Medaka (*Oryzias latipes*) to examine differences between alleles. In this chapter, we introduce our approaches for assessing medaka as a relevant model animal for population studies.

22.2 The Search for a “Vertebrate” Animal Model for Population Genetics

The recent and enormous accumulation of SNP data gives us a chance to detect natural selection signals in humans. However, we have no relevant experimental model animals to examine how the alleles at the SNPs affect environmental adaptation in the human population where a selection signal has been detected. When considering an appropriate animal model, an important factor is the evolutionary relatedness of the organism to humans. Figure 22.1 illustrates the divergence time between human and selected model animals.

In primates, the quality of the whole genome sequences is relatively high for the chimpanzee (*Pan troglodytes*) and macaque (*Macaca mulatta*), which diverged from humans approximately 7 million and 35 million years ago, respectively. However, for both species it is quite difficult to collect population samples from their wild habitat (although for the macaque it is easier than for the chimpanzee), and it is necessary to provide special facilities to house the animals, limiting the popularization of the primate model in general laboratories.

Currently, the mouse (*Mus musculus*) is the most popular model animal for molecular and developmental biology studies. Mice can be cared for and reared easily, compared to the difficulties and expense associated with chimpanzees and macaques. The quality of the mouse whole genome sequence is also quite high, but as they are basically inbred strains and it is somewhat difficult to collect wild mice, the mouse is not suitable for population studies. Classically, the fruit fly

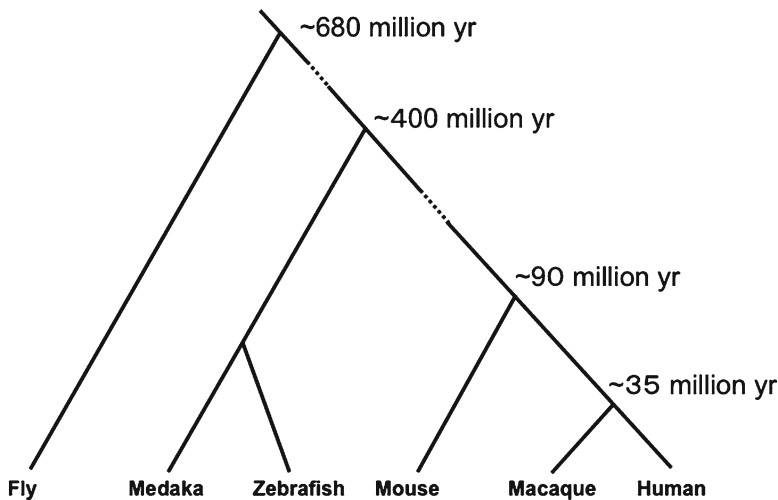


Fig. 22.1 A cladogram showing approximate divergent times between human and model animals

(*Drosophila*) has been the best model system for population genetics. Two attractive features of the fruit fly are that the complete genome sequence is available for 12 species (Clark et al. 2007; Stark et al. 2007) and, compared to humans, the genome is relatively small in size. Unfortunately, they are invertebrate and diverged from humans more than 680 million years (see Fig. 22.1). One of the negative features of an invertebrate model system is that their codon usage differs from humans (Tamura et al. 2004). In addition, the effective population size of *Drosophila* is thought to be much larger than that of humans. Although *Drosophila* fits the assumption of random mating, humans do not fit this assumption in many cases, which limits the usefulness of *Drosophila* as a population model.

In the search for a relevant vertebrate model animal for studying human SNP functional differences, small fish are a promising candidate for such a model system. Zebrafish (*Danio rerio*) is the most popular and well established model fish in the world. Effective transgenesis and mutagenesis protocols have been developed for zebrafish, but unfortunately it is not well known how zebrafish genetically diverged, meaning that it is not a very suitable model for human population studies. Herein we direct our attention to Medaka (*O. latipes*), as there are many studies on Medaka phenotypic diversity (Egami 1971a; Shima and Mitani 2004; Wittbrodt et al. 2002). Since the early 20th century, Medaka has a long history as an experimental animal, and many inbred strains have been established (Shima and Mitani 2004; Wittbrodt et al. 2002). Most of the experimental techniques (transgenesis, mutagenesis, etc.) developed for zebrafish can also be applied to Medaka (Mitani et al. 2006). The whole genome sequence (Kasahara et al. 2009) and the complete cDNA sequences (<http://www.shigen.nig.ac.jp/medaka/est/est.jsp>) are also readily available. Hence, we selected Medaka as a candidate for a vertebrate model animal for the study of human population genetics.

22.3 Medaka Shows High Genetic Polymorphisms with Morphological and Physiological Diversity

Actinopterygii, constituting the class of ray-finned fish, represents the most diversified group of vertebrates, currently comprising more than 25,000 species, which is more than half of all vertebrate species (Nelson 1994). Atherinomorpha, which includes the genus *Oryzias* and its plentiful number of species and highly diversified ecological and morphological characteristics, is the most successful fish group at the surface layer of the ocean and in many freshwater habitats. *Oryzias* species have been divided into three major monophyletic groups, namely the *latipes*, *javanicus*, and *celebensis* groups, which correspond to the three chromosomal groups (biarmed, monoarmed, and fused chromosome groups) previously proposed from karyological analyses (Magtoon et al. 1992).

Each medaka species in the genus *Oryzias* shows a specific pattern of geographic distribution; some species are endemic to freshwater lakes of an Indonesian island, while others are widely distributed in freshwater, brackish water, and seawater of Asia (Roberts 1998). Egami (1953) first reported a significant difference in the mean number of anal fin rays among Medaka specimens collected in different areas. Cross-breeding experiments between fish obtained at different localities have demonstrated that the character is controlled by the actions of polygenic genes (Egami 1954). After the pioneering work of Egami, the biochemical characteristics of these populations have been confirmed by allozymes and mitochondria sequencing analysis (Sakaizumi et al. 1983, 1984). Meanwhile, several inbred strains have been established from the Northern and Southern and the East Korean populations (Hyodo-Taguchi 1996; Hyodo-Taguchi 1980). The aligned genomes of the inbred strains Hd-rR and HNI from the Southern and Northern populations, respectively, showed that the genome-wide SNP rate was 3.42%, and the average K_A/K_S ratio of 8,889 qualified predicted genes was significantly higher than that for the human–chimpanzee lineage (Kasahara et al. 2007).

Morphological diversities that may be attributable to genetic polymorphism were frequently found using inbred strains and stocks from wild populations (as described below). A statistically significant difference in the total brain size and the relative sizes of subdivisions among brains from genetically distant inbred strains, which had been bred and raised under the same conditions, was reported (Ishikawa et al. 1999). Kimura et al. (2007) quantified craniofacial traits in the adult fish of two inbred strains, HNI and Hd-rR, and found that many craniofacial traits are controlled by genetic factors, some of which could be assigned on a linkage map. Also, the number of Medaka vertebrae tends to increase with latitude, which results from an increase in the number of abdominal vertebrae, even if they were kept at a same temperature experimentally (Yamahira and Nishida 2009; Yamahira et al. 2009).

The physiological diversities are also shown among wild populations of Medaka. Medaka from higher latitudes have more rapid growth rates than those from lower-latitude populations (Yamahira and Takeshi 2008). The juvenile from higher-latitude

populations grow faster between 20 and 32°C, and the thermal growth optimum does not differ greatly among populations. Differences in heat and cold resistance were found among inbred strains depending on the developmental stage of the embryo (Kubota and Shima 1991; Sekiya et al., personal communication). Fish cells are able to survive and propagate at a wide range of temperatures. These “eurythermic” characteristics of cell growth are retained even when cells are transferred to in vitro culture, using media and growth conditions similar to those used for mammalian cell culture. It has been proposed that the thermosensitivity of goldfish cell culture lines with different permissive temperatures is cytogenetically determined (Mitani et al. 1989). Hirayama et al. (2006) examined the temperature acclimation response of various Medaka cell lines derived from different species and populations. The permissive growth temperature in all cell lines from the Northern population and East Korean population was 15°C, but the cell lines derived from the Southern population and *Oryzias celebensis*, which inhabits a tropical zone, could not grow at this temperature.

Little is known about the thermal sensitivity of mitochondrial regulatory properties. The number of tandemly repeated, 11-nucleotide units in the Medaka mtDNA D-loop region varies greatly among local populations. The number of repeats is more variable in the Northern Japanese population than in the Southern one, whereas two other *Oryzias* species inhabiting tropical regions have no repeats. Comprehensive comparisons between the number of repeat units and meteorological data indicated that the number of repeats correlated to colder environments and higher seasonal climatic changes. These phenotypes in thermal adaptability within the medaka population are considered as physiological polymorphism with genetic diversity that was acquired during their expansion into the temperate zone. For these reasons, medaka appears to be an ideal model species for revealing the mechanisms involved in adaptive population divergence.

22.4 First Screening Showed Medaka Was Polymorphic in Genes That Were Polymorphic in Humans

To use medaka species as a model for population genetics, it is important to examine the polymorphic nature of medaka genes in relationship to those of humans. We expect that orthologous genes can be detected in both medaka and humans easily, although we do not know if the genes have the same functions in both species. Because medaka and humans diverged more than 400 million years ago (see Fig. 22.1), the distribution of accumulated mutations should also differ in the respective genomes. A recent study of SNP screening in medaka has provided insight into the polymorphism nature of loci in both species (Matsumoto et al. 2009).

Matsumoto et al. (2009) selected genes that included SNPs highly differentiated among human populations. The differentiation was detected by a large difference in the allele frequencies between the populations compared, which is a signal of positive selection within a species. The average pairwise synonymous/nonsynonymous

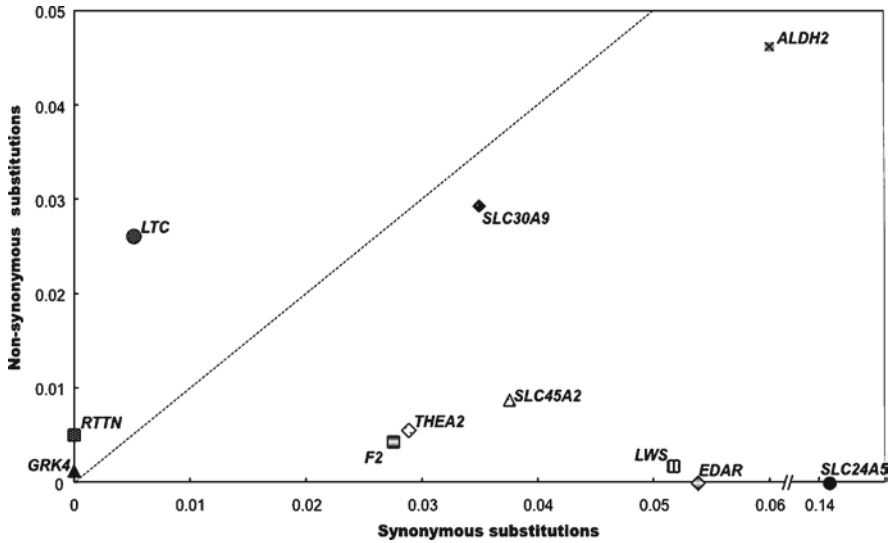


Fig. 22.2 Synonymous (X-axis) and nonsynonymous (Y-axis) substitution ratios estimated by the Nei Gojobori method in 11 medaka genes (Matsumoto et al. 2009)

substitution ratios for the 11 orthologs in 27 medaka strains were calculated and compared (Fig. 22.2). Overall, the 11 genes were substantially polymorphic in medaka as well as humans. The *ALDH2* (acetaldehyde dehydrogenase 2) and *SLC30A9* (solute carrier family 30 zinc transporter, member 9) genes displayed a high synonymous/nonsynonymous substitution ratio, suggesting that these genes are relaxed from genetic constraint (the annotations of the genes are those used in humans). The *SLC24A5* (solute carrier family 45, member 2) and *EDAR* (ectodysplasin A receptor) genes showed many synonymous substitutions but lacked nonsynonymous substitutions, indicating that these genes are under strong negative selection. *THEA2* is a known temperature response gene that is expressed in brown adipose tissue in response to cold stress in mice (Adams et al. 2001). Medaka *THEA2* contains a nonsynonymous SNP at exactly the same position as in Philippine medaka (*Oryzias luzonensis*), and where a highly differentiated SNP was also observed in humans. It also showed a high synonymous to nonsynonymous ratio, suggesting that this gene is under genetic constraint. The average pairwise synonymous/nonsynonymous substitution ratios of three genes, *LTC* (lactase), *RTTN* (rotatin), and *GRK4* (G protein-coupled receptor kinase 4), were greater than 1, although any one of the individual pairs was not statistically significant in either the *LTC* or *GRK4* genes. *RTTN* showed a strong signal of positive selection that was statistically significant. Rotatin, the product of *RTTN*, is required for axial rotation and left–right specification in mouse embryos (Faisst et al. 2002). Thus, the first, simple screening did discover genes plausibly related to positive selection in both medaka and human populations.

22.5 Medaka Genetic Diversity Compared with Human Genetic Diversity

It is well known that medaka have very high phenotypic and genetic diversity (Egami 1971b; Shima and Mitani 2004; Wittbrodt et al. 2002), although there has never been a study of inter- or intra-population diversity. Natural selection operating within a species is usually detected based on the allele frequency spectrum. We are interested in the genetic structure of medaka because the allele frequency also fluctuates with random genetic drift, population subdivision, demography, and migration.

Recently, a comprehensive investigation of medaka genetic diversity was conducted based on nucleotide sequences in the D-loop region and cytochrome *b* gene of mitochondrial DNA (mtDNA) (Katsumura et al. 2009). To begin, we should define the term “population” as it relates to Medaka. Previous studies have reported the existence of Northern, Southern, East Korean, and China-West Korean populations of medaka based on the clustering pattern of phylogenetic trees (Sakaizumi 1986; Sakaizumi and Joen 1987; Sakaizumi et al. 1983; Takehana et al. 2003). However, Katsumura et al. (2009) tentatively dismissed the nomenclature to redefine “population” based on the Medaka mating networks. Normally, the mating networks reflect the clustering patterns of phylogenetic trees, and until recently it was not precisely known how the area and artificial and/or natural migration patterns related to the mating network of wild Medaka. To determine this, Katsumura et al. (2009) studied inter- and intra-population diversity using two sampling methods: one was a grid-based sampling method using one individual from each local site, and the other was a deme-based sampling method using approximately 100 individuals from each wild, local site. Katsumura et al. (2009) thus regarded a school of medaka as a “deme” (a wild, local population), which is the smallest mating unit of wild Medaka. Three local-wild populations, Chiba-K, Ibaraki-K, and Kanagawa-O, were investigated and compared with the northern Japanese (N.JPN) and southern Japanese (S.JPN) Medaka “groups” that corresponded to the Northern and the Southern “populations” in previous studies. Each group is the combined “grid samples” collected from local populations and are maintained as lab stocks in the Graduate School of Frontier Sciences, University of Tokyo (Shima et al. 1985).

Tables 22.1 and 22.2 compare the inter- and intra-population genetic diversity based on the mtDNA D-loop region in Medaka and humans as reported in previous studies (Katsumura et al. 2009; Oota et al. 2002). The intrapopulation diversity, mean pairwise difference (MPD), and values of deme samples were two- to three-fold smaller than those of the N.JPN and S.JPN groups (grid samples) (Table 22.1), suggesting that local populations in each group are highly differentiated from one another. In fact, the F_{st} value between the Ibaraki-K and Chiba-N populations was 0.217 (Table 22.2), which indicates that 21.7% of the total genetic diversity results from interpopulation diversity and suggests substantial subdivision of the population. In humans, the average F_{st} value among 15 African populations is 0.201

Table 22.1 Intra-population genetic diversity in Medaka and humans

Groups	Area		Number of sites (509)	Mean pairwise difference (MPD)
Medaka				
		No. individuals		Intra-population MPD
	N.JPN	14	491	10.53±5.11
	S.JPN	17	501	15.77±7.41
	Chiba-N	124	467	7.36±3.47
	Ibaraki-K	90	467	8.02±3.76
	Kanagawa-O	159	472	4.91±2.40
Human				
		No. populations		Intra-population average MPD (Oota et al. 2002)
	Africa	15		7.99±2.72
	Europe	12		4.63±0.94
	Asia	12		7.12±0.91
	Eurasia	27		5.95±1.51

The MPD were calculated using the Tamura and Nei substitution model with $\alpha=0.26$ for both medaka and humans
N.JPN northern Japanese Medaka group, *S.JPN* southern Japanese Medaka group

Table 22.2 Inter-population genetic diversity in Medaka and humans

Medaka			Interpopulation F_{st}
	Ibaraki-K <-> Chiba-N		0.2171
	N.JPN (14) <-> S.JPN (17)		0.9050
Human			Interpopulation average F_{st} (Oota et al. 2002)
	Africa (15)		0.2010
	Europe (12)		0.0660
	Asia (12)		0.0330
	Eurasia (27)		0.0860

Note: The numbers in *parentheses* represent the number of grid-sampled individuals in Medaka and the population number in humans. The F_{st} values were calculated using the Tamura and Nei substitution model with $\alpha=0.26$ for both Medaka and humans

(Table 22.2), indicating that almost the same level of interpopulation genetic diversity observed in Africa exists between the two Medaka local-wild populations (demes). The intrapopulation MPD of Chiba-N (7.36±3.47) is almost equal to the intrapopulation average MPD of Africans (7.99±2.72), which is greater than those of Europeans, Asians, and Eurasians (Table 22.1), and suggests a very high intrapopulation diversity of wild, local Medaka populations. Thus, a comparison of genetic diversity between two species can give us a hint what kind of human populations (hunters-gatherers or farmers) correspond to Medaka local-wild populations in genetic diversity, although the mutation rates of Medaka mtDNA D-loop region would be required with accuracy.

22.6 Conclusion

The following facts suggest that medaka represents a promising vertebrate model animal for human population genetics. First, the habitat areas of medaka species (genus *Oryzias*), including Medaka, expand to various geographic regions, and stocks from local population show great physiological diversity. Second, there have been found genes showing signals of positive selection in both human and medaka. Third, we can discuss the meaning of the functional differences of the polymorphic medaka genes in connection with habitat environment and the size effect of local populations.

References

- Adams SH, Chui C, Schilbach SL, Yu XX, Goddard AD, Grimaldi JC, Lee J, Dowd P, Colman S, Lewin DA (2001) BFIT, a unique acyl-CoA thioesterase induced in thermogenic brown adipose tissue: cloning, organization of the human gene and assessment of a potential link to obesity. *Biochem J* 360:135–142.
- Altshuler D, Daly MJ, Lander ES (2008) Genetic mapping in human disease. *Science* 322:881–888.
- Barreiro LB, Laval G, Quach H, Patin E, Quintana-Murci L (2008) Natural selection has driven population differentiation in modern humans. *Nat Genet* 40:340–345.
- Bodmer W, Bonilla C (2008) Common and rare variants in multifactorial susceptibility to common diseases. *Nat Genet* 40:695–701.
- Burton PR, Clayton DG, Cardon LR, Craddock N, Deloukas P, Duncanson A, Kwiatkowski DP, McCarthy MI, Ouwehand WH, Samani NJ, Todd JA, Donnelly P, Barrett JC, Davison D, Easton D, Evans DM, Leung HT, Marchini JL, Morris AP, Spencer CC, Tobin MD, Attwood AP, Boorman JP, Cant B, Everson U, Hussey JM, Jolley JD, Knight AS, Koch K, Meech E, Nutland S, Prowse CV, Stevens HE, Taylor NC, Walters GR, Walker NM, Watkins NA, Winzer T, Jones RW, McArdle WL, Ring SM, Strachan DP, Pembrey M, Breen G, St Clair D, Caesar S, Gordon-Smith K, Jones L, Fraser C, Green EK, Grozeva D, Hamshire ML, Holmans PA, Jones IR, Kirov G, Moskvina V, Nikolov I, O'Donovan MC, Owen MJ, Collier DA, Elkin A, Farmer A, Williamson R, McGuffin P, Young AH, Ferrier IN, Ball SG, Balmforth AJ, Barrett JH, Bishop TD, Iles MM, Maqbool A, Yuldasheva N, Hall AS, Braund PS, Dixon RJ, Mangino M, Stevens S, Thompson JR, Bredin F, Tremelling M, Parkes M, Drummond H, Lees CW, Nimmo ER, Satsangi J, Fisher SA, Forbes A, Lewis CM, Onnie CM, Prescott NJ, Sanderson J, Matthew CG, Barbour J, Mohiuddin MK, Todhunter CE, Mansfield JC, Ahmad T, Cummings FR, Jewell DP, et al. (2007) Association scan of 14, 500 nonsynonymous SNPs in four diseases identifies autoimmunity variants. *Nat Genet* 39:1329–1337.
- Bustamante CD, Fledel-Alon A, Williamson S, Nielsen R, Hubisz MT, Gnanowski S, Tanenbaum DM, White TJ, Sninsky JJ, Hernandez RD, Civello D, Adams MD, Cargill M, Clark AG (2005) Natural selection on protein-coding genes in the human genome. *Nature* 437:1153–1157.
- Clark AG, Eisen MB, Smith DR, Bergman CM, Oliver B, Markow TA, Kaufman TC, Kellis M, Gelbart W, Iyer VN, Pollard DA, Sackton TB, Larracuente AM, Singh ND, Abad JP, Abt DN, Adryan B, Aguade M, Akashi H, Anderson WW, Aquadro CF, Ardell DH, Arguello R, Artieri CG, Barbash DA, Barker D, Barsanti P, Batterham P, Batzoglou S, Begun D, Bhutkar A, Blanco E, Bosak SA, Bradley RK, Brand AD, Brent MR, Brooks AN, Brown RH, Butlin RK, Caggese C, Calvi BR, Bernardo de Carvalho A, Caspi A, Castrezana S, Celniker SE, Chang JL, Chapple C, Chatterji S, Chinwalla A, Civetta A, Clifton SW, Comeron JM, Costello JC,

- Coyne JA, Daub J, David RG, Delcher AL, Delehaunty K, Do CB, Ebling H, Edwards K, Eickbush T, Evans JD, Filipowski A, Findeiss S, Freyhult E, Fulton L, Fulton R, Garcia AC, Gardiner A, Garfield DA, Garvin BE, Gibson G, Gilbert D, Gnerre S, Godfrey J, Good R, Gotea V, Gravely B, Greenberg AJ, Griffiths-Jones S, Gross S, Guigo R, Gustafson EA, Haerty W, Hahn MW, Halligan DL, Halpern AL, Halter GM, Han MV, Heger A, Hillier L, Hinrichs AS, Holmes I, Hoskins RA, Hubisz MJ, Hultmark D, Huntley MA, Jaffe DB, Jagadeeshan S, et al. (2007) Evolution of genes and genomes on the *Drosophila* phylogeny. *Nature* 450:203–218.
- Egami N (1953) Studies on the variation of the number of the anal fin rays in *Oryzias latipes*. I. Geographical variation in wild populations. (In Japanese, with English abstract). *Jpn J Ichthyol* 3:87–89.
- Egami N (1954) Geographical variation in the male characters of the fish *Oryzias latipes*. *Annot Zool Jpn* 27:7–12.
- Egami N (1971a) Further notes on the life span of the teleost, *Oryzias latipes*. *Exp Gerontol* 6:379–382.
- Egami N (1971b) The medaka, *Oryzias latipes* (teleost fish) as a laboratory animal. The ICLA Asian Pacific Meeting of Laboratory Animals, Tokyo and Inuyama, 20–25 September, 1971.
- Faisst AM, Alvarez-Bolado G, Treichel D, Gruss P (2002) Rotatin is a novel gene required for axial rotation and left-right specification in mouse embryos. *Mech Dev* 113:15–28.
- Frazer KA, Ballinger DG, Cox DR, Hinds DA, Stuve LL, Gibbs RA, Belmont JW, Boudreau A, Hardenbol P, Leal SM, Pasternak S, Wheeler DA, Willis TD, Yu F, Yang H, Zeng C, Gao Y, Hu H, Hu W, Li C, Lin W, Liu S, Pan H, Tang X, Wang J, Wang W, Yu J, Zhang B, Zhang Q, Zhao H, Zhou J, Gabriel SB, Barry R, Blumenstiel B, Camargo A, Defelice M, Faggart M, Goyette M, Gupta S, Moore J, Nguyen H, Onofrio RC, Parkin M, Roy J, Stahl E, Winchester E, Ziaugra L, Altshuler D, Shen Y, Yao Z, Huang W, Chu X, He Y, Jin L, Liu Y, Sun W, Wang H, Wang Y, Xiong X, Xu L, Wayne MM, Tsui SK, Xue H, Wong JT, Galver LM, Fan JB, Gunderson K, Murray SS, Oliphant AR, Chee MS, Montpetit A, Chagnon F, Ferretti V, Leboeuf M, Olivier JF, Phillips MS, Roumy S, Sallee C, Verner A, Hudson TJ, Kwok PY, Cai D, Koboldt DC, Miller RD, Pawlikowska L, Taillon-Miller P, Xiao M, Tsui LC, Mak W, Song YQ, Tam PK, Nakamura Y, Kawaguchi T, Kitamoto T, Morizono T, Nagashima A, Ohnishi Y, Sekine A, Tanaka T, Tsunoda T, et al. (2007) A second generation human haplotype map of over 3.1 million SNPs. *Nature* 449:851–861.
- Gibbs RA, Rogers J, Katze MG, Bumgarner R, Weinstock GM, Mardis ER, Remington KA, Strausberg RL, Venter JC, Wilson RK, Batzer MA, Bustamante CD, Eichler EE, Hahn MW, Hardison RC, Makova KD, Miller W, Milosavljevic A, Palermo RE, Siepel A, Sikela JM, Attaway T, Bell S, Bernard KE, Buhay CJ, Chandrasekhar MN, Dao M, Davis C, Delehaunty KD, Ding Y, Dinh HH, Dugan-Rocha S, Fulton LA, Gabisi RA, Garner TT, Godfrey J, Hawes AC, Hernandez J, Hines S, Holder M, Hume J, Jhangiani SN, Joshi V, Khan ZM, Kirkness EF, Cree A, Fowler RG, Lee S, Lewis LR, Li Z, Liu YS, Moore SM, Muzny D, Nazareth LV, Ngo DN, Okwuonu GO, Pai G, Parker D, Paul HA, Pfannkuch C, Pohl CS, Rogers YH, Ruiz SJ, Sabo A, Santibanez J, Schneider BW, Smith SM, Sodergren E, Svatek AF, Utterback TR, Vattathil S, Warren W, White CS, Chinwalla AT, Feng Y, Halpern AL, Hillier LW, Huang X, Minx P, Nelson JO, Pepin KH, Qin X, Sutton GG, Venter E, Walenz BP, Wallis JW, Worley KC, Yang SP, Jones SM, Marra MA, Rocchi M, Schein JE, Baertsch R, Clarke L, Csuros M, Glasscock J, Harris RA, Havlak P, Jackson AR, Jiang H, et al. (2007) Evolutionary and biomedical insights from the rhesus macaque genome. *Science* 316:222–234.
- Hancock AM, Witonsky DB, Gordon AS, Eshel G, Pritchard JK, Coop G, Di Rienzo A (2008) Adaptations to climate in candidate genes for common metabolic disorders. *PLoS Genet* 4:e32.
- Hirayama M, Mitani H, Watabe S (2006) Temperature-dependent growth rates and gene expression patterns of various medaka *Oryzias latipes* cell lines derived from different populations. *J Comp Physiol [B]* 176:311–320.
- Hyodo-Taguchi Y (1996) Inbred strains of the medaka, *Oryzias latipes*. *Fish Biol J Medaka* 8:11–14.

- Hyodo-Taguchi Y (1980) Establishment of inbred strains of the teleost, *Oryzias latipes* (in Japanese with English abstract). Zool Mag 89:283–301.
- Ishikawa Y, Yoshimoto M, Yamamoto N, Ito H (1999) Different brain morphologies from different genotypes in a single teleost species, the medaka (*Oryzias latipes*). Brain Behav Evol 53:2–9.
- Kasahara M, Naruse K, Sasaki S, Nakatani Y, Qu W, Ahsan B, Yamada T, Nagayasu Y, Doi K, Kasai Y, Jindo T, Kobayashi D, Shimada A, Toyoda A, Kuroki Y, Fujiyama A, Sasaki T, Shimizu A, Asakawa S, Shimizu N, Hashimoto S, Yang J, Lee Y, Matsushima K, Sugano S, Sakaizumi M, Narita T, Ohishi K, Haga S, Ohta F, Nomoto H, Nogata K, Morishita T, Endo T, Shin IT, Takeda H, Morishita S, Kohara Y (2007) The medaka draft genome and insights into vertebrate genome evolution. Nature 447:714–719.
- Katsumura T, Oda S, Mano S, Suguro N, Watanabe K, Mitani H, Oota H, Kawamura S (2009) Genetic differentiation among local populations of medaka fish (*Oryzias latipes*) evaluated through grid- and deme-based sampling. Gene 443(1–2):170–177.
- Kimura T, Shimada A, Sakai N, Mitani H, Naruse K, Takeda H, Inoko H, Tamiya G, Shinya M (2007) Genetic analysis of craniofacial traits in the medaka. Genetics 177:2379–2388.
- Magtoon W, Nadee N, Higsdhitani T, Takata K, Uwa H (1992) Karyotype evolution and geographical distribution of the Thai-medaka, *Oryzias minutillus*, in Thailand. J Fish Biol 41:489–497.
- Matsumoto Y, Oota H, Asaoka Y, Nishina H, Watanabe K, Bujnicki JM, Oda S, Kawamura S, Mitani H (2009) Medaka: a promising model animal for comparative population genomics. BMC Res Notes 2:88.
- Mitani H, Naruse K, Shima A (1989) Eurythermic and stenothermic growth of cultured fish cells and their thermosensitivity. J Cell Sci 93 (Pt 4):731–737.
- Miyagawa T, Kawashima M, Nishida N, Ohashi J, Kimura R, Fujimoto A, Shimada M, Morishita S, Shigeta T, Lin L, Hong SC, Faraco J, Shin YK, Jeong JH, Okazaki Y, Tsuji S, Honda M, Honda Y, Mignot E, Tokunaga K (2008) Variant between CPT1B and CHKB associated with susceptibility to narcolepsy. Nat Genet 40:1324–1328.
- Naruse K, Hori H, Shimizu N, Kohara Y, Takeda H (2004) Medaka genomics: a bridge between mutant phenotype and gene function. Mech Dev 121:619–628.
- Nelson (1994) Fishes of the world. Wiley, New York.
- Nielsen R, Hubisz MJ, Hellmann I, Torgerson D, Andres AM, Albrechtsen A, Gutenkunst R, Adams MD, Cargill M, Boyko A, Indap A, Bustamante CD, Clark AG (2009) Darwinian and demographic forces affecting human protein coding genes. Genome Res 19:838–849.
- Oota H, Kitano T, Jin F, Yuasa I, Wang L, Ueda S, Saitou N, Stoneking M (2002) Extreme mtDNA homogeneity in continental Asian populations. Am J Phys Anthropol 118:146–153.
- Pickrell JK, Coop G, Novembre J, Kudaravalli S, Li JZ, Absher D, Srinivasan BS, Barsh GS, Myers RM, Feldman MW, Pritchard JK (2009) Signals of recent positive selection in a world-wide sample of human populations. Genome Res 19:826–837.
- Roberts TR (1998) Systematic observations on tropical Asian medakas or rice fishes of the genus *Oryzias*, with descriptions of four new species. Ichthyol Res 45:213–224.
- Sabeti PC, Schaffner SF, Fry B, Lohmueller J, Varilly P, Shamovsky O, Palma A, Mikkelsen TS, Altshuler D, Lander ES (2006) Positive natural selection in the human lineage. Science 312:1614–1620.
- Sabeti PC, Varilly P, Fry B, Lohmueller J, Hostetter E, Cotsapas C, Xie X, Byrne EH, McCarroll SA, Gaudet R, Schaffner SF, Lander ES, Frazer KA, Ballinger DG, Cox DR, Hinds DA, Stuve LL, Gibbs RA, Belmont JW, Boudreau A, Hardenbol P, Leal SM, Pasternak S, Wheeler DA, Willis TD, Yu F, Yang H, Zeng C, Gao Y, Hu H, Hu W, Li C, Lin W, Liu S, Pan H, Tang X, Wang J, Wang W, Yu J, Zhang B, Zhang Q, Zhao H, Zhou J, Gabriel SB, Barry R, Blumenstiel B, Camargo A, Defelice M, Faggart M, Goyette M, Gupta S, Moore J, Nguyen H, Onofrio RC, Parkin M, Roy J, Stahl E, Winchester E, Ziaugra L, Altshuler D, Shen Y, Yao Z, Huang W, Chu X, He Y, Jin L, Liu Y, Sun W, Wang H, Wang Y, Xiong X, Xu L, Wayne MM, Tsui SK, Xue H, Wong JT, Galver LM, Fan JB, Gunderson K, Murray SS, Oliphant AR, Chee MS, Montpetit A, Chagnon F, Ferretti V, Leboeuf M, Olivier JF, Phillips MS, Roumy S, Sallee C,

- Verner A, Hudson TJ, Kwok PY, Cai D, Koboldt DC, Miller RD, Pawlikowska L, Taillon-Miller P, Xiao M, Tsui LC, et al. (2007) Genome-wide detection and characterization of positive selection in human populations. *Nature* 449:913–918.
- Sakaizumi M (1986) Genetic-divergence in wild populations of medaka, *Oryzias latipes* (Pisces, *Oryziatidae*) from Japan and China. *Genetica* 69:119–125.
- Sakaizumi M, Joen S (1987) Two divergent groups in the wild populations of medaka *Oryzias latipes* (Pisces: *Oryziatidae*) in Korea. *Korean J Limnol* 20:13–20.
- Sakaizumi M, Moriwaki K, Egami N (1983) Allozymic variation and regional differentiation in wild populations of the fish *Oryzias latipes*. *Copeia* 2:311–318.
- Sakaizumi M, Suzuki H, Sakaizumi M, Suzuki H, Moriwaki K, Kominami R, Muramastu M (1984). Geographic variation of ribosomal-RNA gene structure in wild populations of the medaka *Oryzias latipes*. *Jpn J Genet* 59(6):651–651.
- Shima A, Mitani H (2004) Medaka as a research organism: past, present and future. *Mech Dev* 121:599–604.
- Shima A, Shimada A, Komura J, Isa K, Naruse K, Sakaizumi M, Egami N (1985) The preservation and utilization of wild populations of the medaka, *Oryzias latipes*. *Medaka* 3:1–4.
- Stark A, Lin MF, Kheradpour P, Pedersen JS, Parts L, Carlson JW, Crosby MA, Rasmussen MD, Roy S, Deoras AN, Ruby JG, Brennecke J, Hodges E, Hinrichs AS, Caspi A, Paten B, Park SW, Han MV, Maeder ML, Polansky BJ, Robson BE, Aerts S, van Helden J, Hassan B, Gilbert DG, Eastman DA, Rice M, Weir M, Hahn MW, Park Y, Dewey CN, Pachter L, Kent WJ, Haussler D, Lai EC, Bartel DP, Hannon GJ, Kaufman TC, Eisen MB, Clark AG, Smith D, Celniker SE, Gelbart WM, Kellis M (2007) Discovery of functional elements in 12 *Drosophila* genomes using evolutionary signatures. *Nature* 450:219–232.
- Takehana Y, Nagai N, Matsuda M, Tsuchiya K, Sakaizumi M (2003) Geographic variation and diversity of the cytochrome b gene in Japanese wild populations of Medaka, *Oryzias latipes*. *Zool Sci* 20:1279–1291.
- Tamura K, Subramanian S, Kumar S (2004) Temporal patterns of fruit fly (*Drosophila*) evolution revealed by mutation clocks. *Mol Biol Evol* 21:36–44.
- The Chimpanzee Sequencing and Analysis Consortium (2005) Initial sequence of the chimpanzee genome and comparison with the human genome. *Nature* 437:69–87.
- The International HapMap Consortium (2005) A haplotype map of the human genome. *Nature* 437:1299–1320.
- Wittbrodt J, Shima A, Scharl M (2002) Medaka—a model organism from the far east. *Nat Rev Genet* 3:53–64.
- Yamahira K, Nishida T (2009) Latitudinal variation in axial patterning of the medaka (Actinopterygii: Adrianichthyidae): Jordan's rule is substantiated by genetic variation in abdominal vertebral number. *Biol J Linnean Society* 96:856–866.
- Yamahira K, Nishida T, Arakawa A, Iwasaki H (2009) Heritability and genetic correlation of abdominal versus caudal vertebral number in the medaka (Actinopterygii: Adrianichthyidae): genetic constraints on evolution of axial patterning? *Biol J Linnean Society* 96:867–874.
- Yamahira K, Takeshi K (2008) Variation in juvenile growth rates among and within latitudinal populations of the medaka. *Populat Ecol* 50:3–8.
- Young JH, Chang YP, Kim JD, Chretien JP, Klag MJ, Levine MA, Ruff CB, Wang NY, Chakravarti A (2005) Differential susceptibility to hypertension is due to selection during the out-of-Africa expansion. *PLoS Genet* 1:e82.

Chapter 23

Evolution of the Major Histocompatibility Complex: A Lesson from the *Oryzias* Species

Masaru Nonaka and Kentaro Tsukamoto

Abstract The genomic organization of the teleost major histocompatibility complex (MHC) shows a significant deviation from that of the “standard” MHC of the eutherian mammals in that the class IA genes are not linked to the class IIA and B genes. However, progress in the phylogenetic analysis of the jawed vertebrate MHC reveals that the “standard” mammalian MHC is also highly derived because the tight linkage between the class IA genes and the genes directly involved in the class I antigen processing/presentation process is disrupted. In the medaka MHC class I region, these genes form a tight and uninterrupted cluster, probably reflecting the ancestral genomic organization of the MHC. One of these genes, *PSMB8* (proteasome subunit beta type 8), which is responsible for the generation of the peptides presented by the MHC class I molecules, shows a marked dimorphism in medaka. The same dimorphic alleles are present in other *Oryzias* species, indicating that they are under balancing selection and have been transmitted from species to species. Although the physiological or evolutionary meaning of this balancing selection is still to be clarified, similar dimorphisms of *PSMB8* are widely recognized among non-eutherian vertebrates, suggesting that the presence of the *PSMB8* dimorphism is associated with the tight linkage between the class IA genes and the genes directly involved in the class I antigen processing/presentation process.

23.1 Comparative Genomics of the Jawed Vertebrate Major Histocompatibility Complex

The major histocompatibility complex (MHC) was originally identified as a major determinant locus for the success of cancer transplantation in mice. Later, MHC compatibility was shown to be required for successful transplantation of not

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only tumor cells but also normal cells, tissues, and organs. However, because transplantation is a highly artificial situation, the physiological function of the MHC remained puzzling. Finally, the MHC molecules (MHC classes I and II) were shown to play a pivotal role in antigen presentation to T lymphocytes, and their involvement in allotransplant rejection was understood in the context of this physiological function (Zinkernagel and Doherty 1979). With the progress of the human genome project, the entire nucleotide sequence of the human MHC genomic region was determined in 1999 (Consortium 1999). The human MHC spans more than 4 Mb, containing more than 200 genes, and is one of the most gene-dense regions of the human genome. One curious feature of the human MHC is the accumulation of genes involved in the immune functions. In addition to the MHC class IA and IIA/B molecules that present antigen peptides to T cells, the molecules directly involved in MHC class I antigen processing/presentation, immunoproteasome beta subunits (PSMB8 and PSMB9), TAP transporters (ABCB2 and ABCB3), and TAP-binding protein (TAPBP), are also encoded in the human MHC. In the MHC class I antigen presentation pathway, immunoproteasomes proteolytically degrade endogenous proteins with specific residues at their C-termini, and the resultant peptides are transported into the endoplasmic reticulum (ER) lumen by TAP transporters to bind to the MHC class I molecules (Rock and Goldberg 1999). Therefore, the immunoproteasomes are believed to be responsible for the determination of the C-terminus of the antigenic peptides presented by the MHC class I molecules (Rock et al. 2004). Of the seven alpha and seven beta subunits of the 20S proteasome, only three beta subunits have proteolytic activity. These active beta subunits of the constitutive proteasome are PSMB5 (X), PSMB6 (Y), and PSMB7 (Z), with chymotrypsin-like, caspase-like, and trypsin-like peptidase activities, respectively. They are replaced by the interferon γ (IFN- γ)-inducible beta subunits, PSMB8 (LMP7), PSMB9 (LMP2), and PSMB10 (MECL-1), respectively, to form the immunoproteasome (Tanaka and Kasahara 1998). These changes in subunit composition are believed to increase the chymotrypsin-like activity of the proteasomes, to generate peptides with hydrophobic C-terminal residues suitable for binding to the clefts of MHC class I molecules. Although the products of the MHC class IA, *PSMB8*, *PSMB9*, *ABCB2*, *ABCB3*, and *TAPBP* genes show an intimate functional linkage, as just described, there is almost no structural similarity among these genes. Therefore, the curious linkage found in the human MHC region is among functionally linked but structurally unrelated genes.

Comparative analyses of the MHC genomic structure have been performed in various jawed vertebrates, first by classical linkage analysis, and then by genome sequence analysis. Figure 23.1 summarizes the MHC genomic organization of the various jawed vertebrates studied so far, which suggests that the tight linkage among the MHC class IA genes, the class IIA/B genes, and the other genes directly involved in class I antigen presentation was established in the common ancestor of the jawed vertebrates (Kelley et al. 2005). This basic MHC configuration seems to have been conserved in the shark, although this result was obtained only by linkage analysis (Ohta et al. 2000), and the detailed gene order and orientations are still to be determined by genome sequence analysis. Similarly, the genomic organization

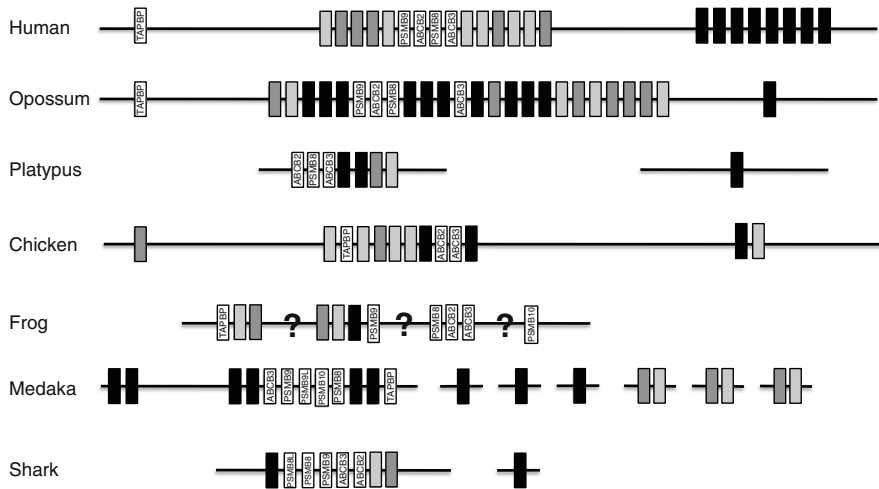


Fig. 23.1 Schematic representation of the major histocompatibility complex (MHC) genomic organization of human (*Homo sapiens*), opossum (*Monodelphis domestica*), platypus (*Ornithorhynchus anatinus*), chicken (*Gallus gallus*), frog (*Xenopus tropicalis*), medaka (*Oryzias latipes*), and shark (*Ginglymostoma cirratum*). Only class IA genes (black), class IIA genes (dark gray), class IIB genes (light gray), and the genes directly involved in class I antigen presentation/processing (white) are shown. Horizontal lines represent chromosomes. Question marks in “Frog” indicate that the evidence for physical linkage is still missing as a result of incomplete genome assembly, although previous linkage analysis suggested their continuity. Sizes of the genes and the intergenic regions are not to scale, and the genes are depicted next to each other. Gaps among genes indicate that the size of the intergenic region is more than 1 Mb

of the *Xenopus* MHC, determined by linkage analysis and draft sequence analysis (Ohta et al. 2006), indicates the conservation of the basic structure of the ancestral MHC. In contrast, the teleost MHC genome shows a uniquely derived configuration, in that the teleost orthologs of the mammalian MHC-encoded genes are dispersed on several chromosomes (Bingulac-Popovic et al. 1997; Naruse et al. 2000). However, an extremely tight linkage is observed among the MHC class IA genes and the genes involved in class I antigen processing/presentation: *ABC3*, *PSMB8*, *PSMB9*, *PSMB9-like*, *PSMB10*, and *TAPBP* (Clark et al. 2001; Matsuo et al. 2002; Michalova et al. 2000). Figure 23.1 shows that in the MHC of the teleost medaka the MHC class IA genes are distributed on four chromosomes and the class IIA/B genes on three chromosomes.

The absence of a tight linkage between the MHC class IA genes and the genes involved in MHC class I antigen processing/presentation in some mammalian MHCs is a further example of the evolutionary deviations in the genomic organization of the MHC. The tight linkage among these genes is conserved in not only nonmammalian jawed vertebrates but also in the marsupial opossum (Belov et al. 2006) and the monotreme platypus (Dohm et al. 2007). Because marsupials and monotremes occupy the basal branches of the mammal phylogeny, the tight linkage among the genes involved in the MHC class I antigen presentation pathway was

probably lost in a common ancestor of the placental mammals. Therefore, although the MHC polymorphisms have been studied predominantly in placental mammals thus far, it is doubtful how far the results obtained can be generalized to all jawed vertebrates because of the highly derived genomic organization of the eutherian MHC. The medaka (*Oryzias latipes*) is one of the best organisms in which to study the polymorphisms and evolution of the MHC, because genetically divergent wild populations (Takehana et al. 2003) and several inbred strains are available. There are also about 20 *Oryzias* species distributed in southeastern Asia (Takehana et al. 2005). Furthermore, the gene organization of the medaka MHC, as described below, suggests that it represents the canonical structure of the vertebrate MHC class I region.

23.2 Gene Organization of the Medaka MHC Class I Region

The first nucleotide sequence analysis of the medaka MHC class I region was performed in the Hd-rR inbred strain derived from the southern population (SP; Matsuo et al. 2002). A 425,862-bp continuous sequence was obtained by the shotgun sequencing of two bacterial artificial chromosome (BAC) clones, and the genes were mainly identified by cDNA sequencing and with the gene prediction program GENSCAN. Twenty-two expressed genes and three pseudogenes were identified in this region (Fig. 23.2). The most striking feature of the medaka MHC revealed by this analysis was the tight linkage of the functionally related genes involved in the class I antigen presentation pathway, without the intervention of

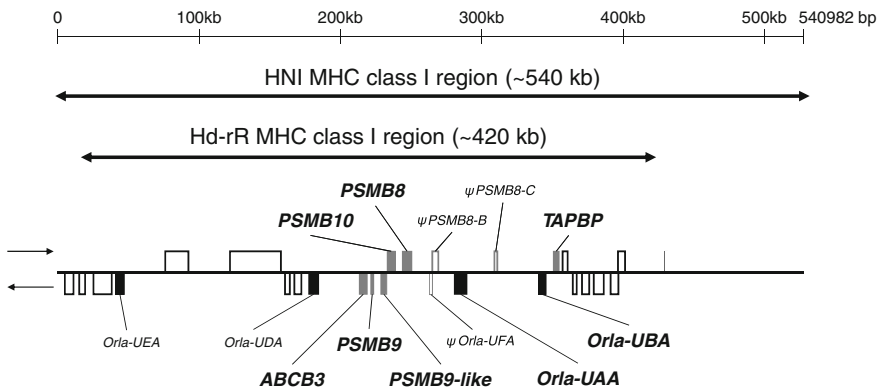


Fig. 23.2 Gene map of the HNI MHC class I region. The *black and gray boxes* represent MHC class IA genes and genes involved in antigen processing and presentation, respectively. *Black and gray rectangles* indicate immuno-related genes or others and pseudogenes, respectively. Genes shown *above the horizontal line* have the transcriptional orientation from left to right, and those *below the line* from right to left. The *double-headed arrows* over the gene map indicate the region covered by Hd-rR and HNI sequences

other genes. There are two classical MHC class IA genes, *Orla-UAA* and *Orla-UBA*, and six genes encoding proteins directly involved in their antigen processing and presentation: *ABCB3*, *PSMB9*, *PSMB9-like*, *PSMB10*, *PSMB8*, and *TAPBP*. Comparative studies of the genomic structures of the teleost MHC class I region have indicated a high degree of structural conservation among the teleost species (Clark et al. 2001; Lukacs et al. 2007; Michalova et al. 2000; Shiina et al. 2005).

23.3 Intraspecific Diversity in the MHC Class I Region Between Two Inbred Strains

The “proper” MHC genes of the placental mammals display unique polymorphisms, characterized by the presence of a large number of alleles occurring at similar frequencies and the large genetic distances between some of these alleles (Klein et al. 1993). However, there has been no extensive analysis of the allelic differences in the MHC region as a whole at the nucleotide sequence level in non-mammalian species. Therefore, to determine the intraspecific diversity in the medaka MHC class I region, the nucleotide sequence of the MHC class I region of the HNI inbred strain derived from the northern population (NP) was determined and compared with that of the Hd-rR strain. A 540,982-bp continuous sequence was obtained by complete nucleotide sequence analysis of four BAC clones (Tsukamoto et al. 2005). All the genes identified in the Hd-rR MHC class I region sequence had counterparts in this HNI sequence, in the same order and with the same transcriptional orientation (see Fig. 23.2).

A dot plot comparison of HNI and Hd-rR with more than 400 kb is shown in Fig. 23.3. This region was divided into three subregions (1, 2, and 3) based on the degree of sequence conservation between HNI and Hd-rR. Subregions 1, 2, and 3 contain the genes from *GNBP* to *PSMB9-like*, from *PSMB10* to *Orla-UBA*, and from *TAPBP* to *TUBB*, respectively. A single diagonal line is observed, with some interruptions, in subregions 1 and 3, and these subregions show a gap-free nucleotide identity of about 96%, representing the average value of the nucleotide identity between HNI and Hd-rR (Kasahara et al. 2007). In contrast, subregion 2 shows no obvious diagonal line. Instead, there are many short, nondiagonal lines. This subregion contains *PSMB10*, *PSMB8*, and two MHC class IA genes (*Orla-UAA* and *Orla-UBA*). In the protein-coding regions, almost all the genes located in subregions 1 and 3 show more than 97% identity, at both the nucleotide and amino acid levels, between the two strains. In contrast, all four genes in subregion 2, *PSMB10*, *PSMB8*, *Orla-UAA*, and *Orla-UBA*, show extremely low identities in the exons and almost no conservation within the introns. The allelic divergence of the *PSMB8* and *PSMB10* genes between these two strains is striking, and the nucleotide identities of the coding regions were only 80.3% and 92.0%, respectively (Tsukamoto et al. 2005). Because the average nucleotide sequence divergence between these strains, including both the coding and noncoding sequences, is about 3.4% (Kasahara et al. 2007), the coding sequence divergence of the *PSMB8* gene is extraordinary.

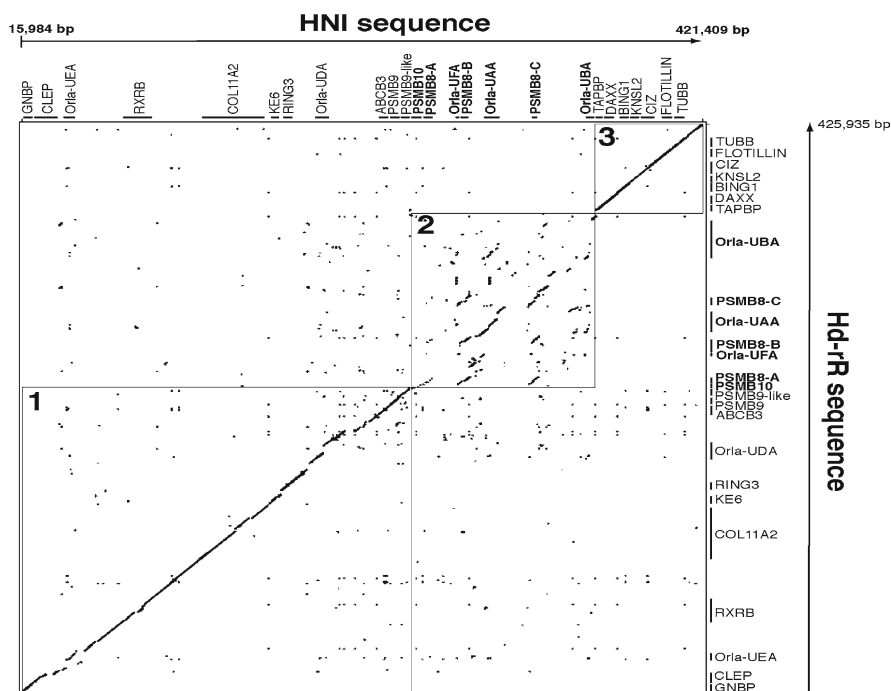


Fig. 23.3 Dot plot comparison of the sequences of the HNI and Hd-rR MHC class I regions. The horizontal and vertical axes represent the HNI and Hd-rR sequences, respectively. Each aligning gap-free segment with more than 50% identity is plotted. Subregions 1, 2, and 3 are enclosed by rectangles 1, 2, and 3, respectively. The PSMB8-B, PSMB8-C, and Orla-UFA are pseudogenes

23.4 Dichotomous Haplotypic Lineages of *PSMB8* and *PSMB10* in the MHC Class I Region of the Genus *Oryzias*

Because the HNI and Hd-rR strains were established from NP and SP, respectively, it is possible that the observed extreme divergence of the *PSMB8* and *PSMB10* genes between these two strains reflects the genetic differences between NP and SP. To clarify this point, we analyzed the *PSMB10* and *PSMB8* genes of 1,245 wild individuals collected from ten localities representing the three genetically divergent populations: the China–West Korean population (CWKP), NP, and SP. The region encompassing *PSMB10* and *PSMB8* was amplified from genomic DNA by primers designed to the coding sequences conserved between the HNI and Hd-rR strains. A total of 45 haplotypes were identified among the wild individuals, and their complete nucleotide sequences were determined. On a phylogenetic tree constructed with the neighbor-joining method from the amino acid sequences of the coding regions, the *PSMB8* and *PSMB10* sequences were divided into two clades, supported by high bootstrap values (Fig. 23.4). These two clades were designated the

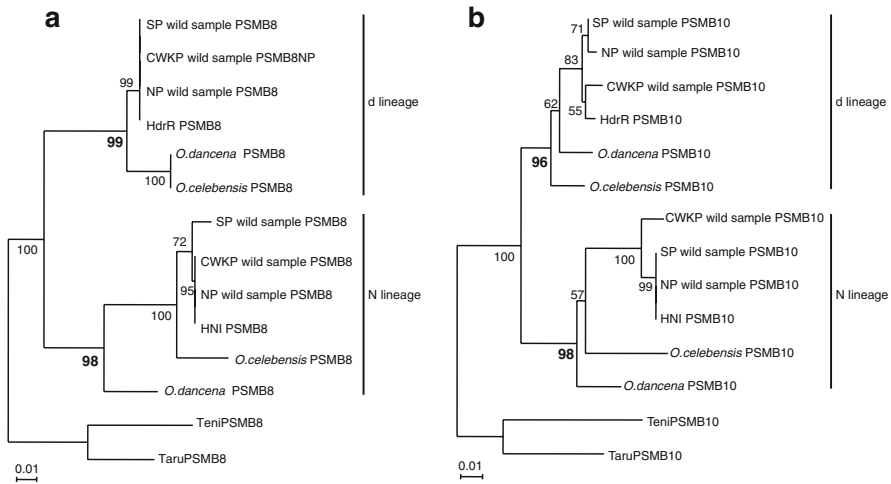


Fig. 23.4 Phylogenetic trees of *PSMB8* (left) and *PSMB10* (right) alleles in the genus *Oryzias*. The deduced amino acid sequences of mature peptides were aligned by ClustalX 2.0, and the phylogenetic tree was constructed by the neighbor-joining method. The numbers on each branch represent bootstrap probabilities (>50%) based on 1,000 bootstrap trials. **(a)** Phylogenetic tree of the *PSMB8* genes based on mature peptides of 181 amino acid residues. The sequences of TaruPSMB8 (*Takifugu rubripes*, accession no. CAC13117) and TeniPSMB8 (*Tetraodon nigroviridis*, accession no. CAG11683) were used as outgroup. **(b)** Phylogenetic tree of the *PSMB10* genes based on mature amino acid sequences of 234 amino acid residues. The sequences of TaruPSMB10 (*Takifugu rubripes*, accession no. CAC13118) and TeniPSMB10 (*Tetraodon nigroviridis*, accession no. CAG11682) were used as outgroup. *O.*, *Oryzias*

N lineage (fixed in the HNI strain) and the *d* lineage (fixed in the Hd-rR strain), and the two allelic lineages of these genes segregated together in all individuals analyzed, constituting dichotomous haplotypic lineages (*N* and *d* haplotypes) (Tsukamoto et al. 2009). To visualize the nucleotide sequence diversity between the haplotypic lineages, a *d* haplotype and an *N* haplotype of the Niigata population were compared using PipMaker (<http://bio.cse.psu.edu>). In a dot plot analysis (Fig. 23.5a), diagonal lines were observed in all exonic regions (shown by shading), indicating that the exons are conserved between the two haplotypic lineages. In contrast, the diagonal lines were missing from most intronic regions, except for introns 1, 2, 3, and 5 of the *PSMB10* gene. In the region between *PSMB10* and *PSMB8*, a diagonal line (~330 bp) was detected upstream from exon 1 of the *PSMB8* gene, probably corresponding to the promoter region of this gene. A percentage identity plot (Fig. 23.5b) indicated that exons 1, 2, 3, 4, and 5 of the *PSMB10* gene showed high identities (97–100%), whereas exons 6, 7, and 8 of the *PSMB10* gene and all the exons of the *PSMB8* gene had lower identities (73–93%). These results indicate that the divergent region between these haplotypic lineages encompasses the 3'-half of the *PSMB10* gene, starting from exon 6, and the entire *PSMB8* gene. Moreover, both haplotypic lineages were identified in all three groups, although the frequency of the *d* haplotypic lineage (73–100%) was much

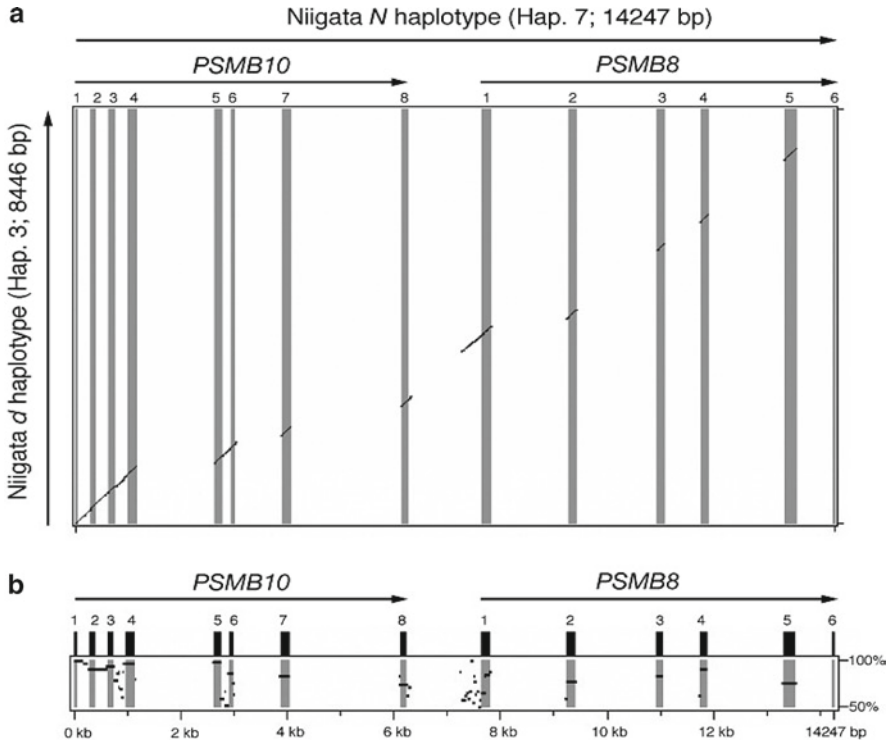


Fig. 23.5 Dot plot and percent identity plot between *N* and *d* haplotypic lineages. The nucleotide sequence of the Niigata *N* haplotype (14,247 bp) was compared with that of the Niigata *d* haplotype (8,446 bp). **(a)** The *horizontal and vertical axes* represent the sequences of Niigata *N* and *d* haplotypes, respectively. Each aligning gap-free segment with more than 50% identity is plotted. The exon positions of the Niigata *N* haplotype are *shaded* in the plot, and the *arrows* represent length and transcriptional orientation of the genes. **(b)** The *horizontal axis* represents the sequence of Niigata *N* haplotype; the *vertical axis* is the percent nucleotide identity. Each gap-free segment aligned with Niigata *d* haplotype is shown by *horizontal lines* and plotted according to their percent identities. The *black boxes* represent exon position shaded in the plots

higher than that of the *N* haplotypic lineage (0–27%) in all the wild populations analyzed. These findings indicate that dichotomous haplotypic lineages were established before the divergence of the three groups of *O. latipes*.

To clarify the origin of the dichotomous haplotypic lineages within the genus *Oryzias*, the polymorphisms of the *PSMB8* and *PSMB10* genes were analyzed using field samples of medaka-related species (*Oryzias celebensis* collected from Sulawesi Island, Indonesia, and laboratory stocks of *Oryzias dancena* from India and Thailand), with the same strategy used for *O. latipes*. The *Oryzias* species are classified into three species groups: *latipes*, *celebensis*, and *javanicus* species groups. The *celebensis* and *javanicus* groups are considered to be sister groups (Takehana et al. 2005). *O. latipes*, *O. celebensis*, and *O. dancena* are included in the *latipes*, *celebensis*, and *javanicus* species groups, respectively. The two haplotypic

lineages recognized in *O. latipes* were identified in both species (see Fig. 23.4) (Mehta et al. 2009), and the frequency of the *d* haplotype (60%) was higher than that of the *N* haplotype (40%) in a wild population of *O. celebensis*. The higher frequency of the *d* haplotype in medaka relatives is similar to the trend observed in wild populations of *O. latipes* (73–100%) (Tsukamoto et al., unpublished data). Therefore, these dichotomous haplotypic lineages have been retained for at least 29 million years (MY), being transferred from an ancestral species to descendant species. It is unclear how these highly divergent lineages were established. However, the mechanism for maintaining the dichotomous haplotypic lineages seems to be the suppression of recombination because the sequence divergence between the lineages is very great.

23.5 Predicted Functional Differences Between the Two Lineages of the *PSMB8* Gene

Immunoproteasomes are believed to be responsible for determining the C-terminal residues of the antigenic peptides to be presented by the MHC class I molecules, and PSMB8, PSMB9, and PSMB10 are the proteolytically active subunits of the immunoproteasome. The *d* and *N* allelic lineages of *PSMB8* could encode the PSMB8 molecules with different cleavage specificities, because substitutions were observed at residues 31 and 53, which may be involved in the formation of the S1 pocket (see Fig. 23.6a) (Groll et al. 1997; Unno et al. 2002). At residue 31, the *N* lineage has Tyr, with a bulky aromatic side chain, whereas the *d* lineage has Val/Ala, with a smaller hydrophobic side chain (Fig. 23.6a). Moreover, Gln53 of the *d* lineage is substituted with Lys, which has a positively charged side chain, in the *N* lineage. Therefore, *d* may be more suitable for the chymotrypsin-like activity postulated for mammalian PSMB8, the S1 pocket of which is mainly composed of hydrophobic residues (Groll et al. 1997; Unno et al. 2002), than for PSMB8N. The C-terminal anchor residues of the peptides derived from MHC class I molecules are mainly hydrophobic in mice and humans (Rammensee et al. 1995), and so are probably produced by PSMB8, with its chymotrypsin-like activity. Therefore, it is conceivable that PSMB8d would be more suitable than PSMB8N for the production of antigenic peptides from most proteins. The long-lasting persistence of the *d* and *N* haplotypic lineages in wild populations of medaka and in other *Oryzias* species, combined with the possible functional differences between PSMB8d and PSMB8N, suggest that these haplotypic lineages are under balancing selection. Because the frequency of the *d* haplotype was much higher than that of the *N* haplotype in all the wild medaka populations analyzed, we hypothesize that PSMB8d is more efficient than PSMB8N in handling most pathogens, although there are certain pathogens for which PSMB8N is more effective than PSMB8d. If this is actually the case, the form of balancing selection that maintains these two haplotypic lineages is selection that varies in space and time (Hedrick 2006), rather than overdominance or frequency-dependent selection. Conversely, the *PSMB10N* and *PSMB10d* lineages show no indication of functional

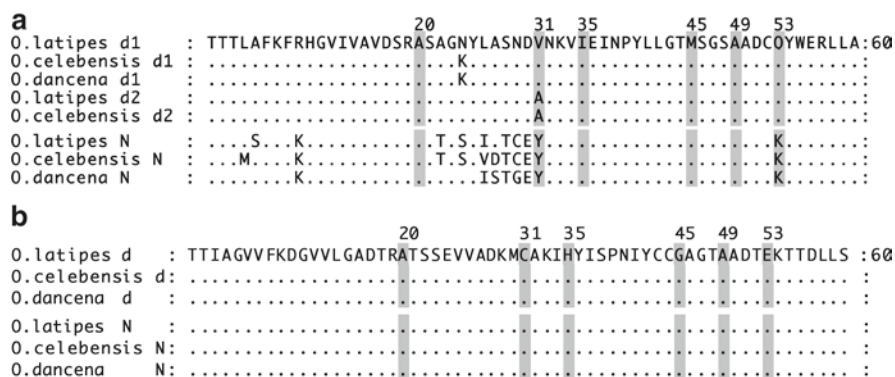


Fig. 23.6 Multiple amino acid sequence alignments of mature peptide of *Oryzias* (*O.*) PSMB8 and PSMB10. Multiple sequence alignment of *Oryzias* PSMB8 or PSMB10 was performed using the amino acid sequences with the ClustalX. Dots indicate the identity to the residues in the uppermost sequence. The gray-shaded residues indicate the 20th, 31st, 35th, 45th, 49th, and 53rd residues probably located in the S1 pocket of PSMB8 and PSMB10 (Groll et al. 1997; Unno et al. 2002). (a) A part of amino acid sequence alignment of mature PSMB8. (b) A part of amino acid sequence alignment of mature PSMB10

differentiation, because all the residues involved in the S1 pocket of PSMB10 are perfectly conserved (Fig. 23.6b). Thus, the 3'-half of the *PSMB10* gene, which constitutes the haplotype with the *PSMB8* gene, may just be a hitchhiker.

23.6 Diversity of the MHC Class I Region Among *Oryzias* Species

To clarify the evolution and diversity of the MHC class I region in *Oryzias* species, the nucleotide sequences of the MHC class I region containing the *d* haplotypic lineage of *O. luzonensis* (*latipes* group) and the *d* and *N* haplotypic lineages of *O. dancena* (*javanicus* group) were determined. A continuous sequence of *O. luzonensis* (*Orlu-d*) of about 193 kb was obtained from two BAC clones, and continuous *O. dancena* sequences of *Orda-d* (about 142 kb) and *Orda-N* (about 341 kb) were obtained from one and two BAC clones, respectively (Fig. 23.7) (Mehta et al. 2009). The orders and transcriptional orientations of the predicted genes in these two species were basically the same as those of *O. latipes* (see Fig. 23.2). However, the MHC class IA genes showed apparently species-specific variations in copy number. There are no *PSMB8* pseudogene loci in the MHC class I regions of these species, although *O. latipes* has two pseudoloci. In dot plot analyses of the *Oryzias* MHC class I regions, the central region encompassing the *PSMB10*, *PSMB8*, and MHC class IA genes showed a high degree of sequence diversity at both the intra- and interspecies levels, whereas the flanking regions on both sides of the central divergent region showed a normal level of sequence conservation, as in most of the non-MHC

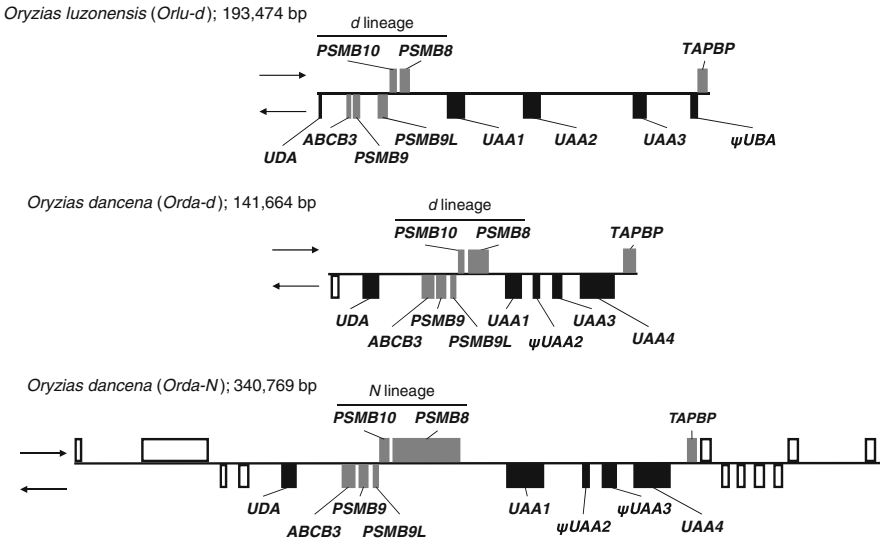


Fig. 23.7 Gene map of the MHC class I region of *O. luzonensis* (Orlu-d) and *O. dancena* (Orda-d and Orda-N). Black, gray, and white boxes represent the same genes mentioned in Fig. 23.1. Pseudogenes are shown as “ψ”

regions of the genome. The central divergent region is further divided into two subregions. The first subregion spans from *PSMB10* to *PSMB8*, and the dichotomous lineages of this subregion showed a transspecies dimorphism, as described earlier. The second subregion consists of the MHC class IA genes and shows conspicuous interspecies variation. Thus, three independent evolutionary patterns under distinctive selective pressures have been identified in the *Oryzias* MHC class I region.

23.7 Evolutionary Meaning of the MHC Gene Organization

The haplotypic lineages containing the MHC class IA genes and some antigen processing/presentation genes have been reported in other vertebrate species. In *Xenopus*, the MHC class IA, *PSMB8*, *ABCB2*, and *ABCB3* genes form two haplotypic lineages, which have been retained for 80–100 MY among *Xenopus* species (Ohta et al. 2003). The rat *ABCB3* gene has two allelic lineages and segregates as haplotypes with the MHC class IA allele *RT1-A* in certain combinations (Joly et al. 1998). In both cases, the functional incompatibility of the proteins encoded in the different haplotypes is implied by the presence of the haplotypic lineages. The *PSMB8* and *PSMB10* alleles detected in the *Oryzias* species can also be classified into the *N* and *d* lineages, defining dichotomous haplotypic lineages. The dimorphic lineages are conserved in all three *Oryzias* species groups, the *latipes*, *celebensis*,

and *javanicus* groups, indicating that the origin of the dimorphic lineages predated speciation within the genus *Oryzias*. Therefore, these dimorphic lineages are probably under strong balancing selection, and are thus transmitted from species to species. In contrast, the *Oryzias* MHC class IA genes have evolved in a species-specific manner, resulting in variable copy numbers among the *Oryzias* species. Moreover, *Oryzias* class IA genes are polymorphic rather than dimorphic, and there is no correlation between the polymorphic class IA alleles and the dimorphic *PSMB8* and *PSMB10* lineages (Tsukamoto et al., unpublished data).

As described here, in the human MHC, the MHC class IA genes and the genes involved in class I antigen processing/presentation – *ABCB2*, *ABCB3*, *PSMB8*, *PSMB9*, and *TAPBP* – are separated from each other by more than 1 Mb. Moreover, the *PSMB10* gene is located outside the MHC region. In contrast, the MHC class IA genes are closely linked to their antigen processing/presentation genes, *ABCB*, *PSMB*, and *TAPBP*, in all the nonmammalian jawed vertebrates analyzed (Flajnik and Kasahara 2001), except for chicken and quail, in which the IFN- γ -inducible *PSMB* gene has not been identified (Kaufman et al. 1999; Shiina et al. 2004). Therefore, it is tempting to speculate a causal relationship between the intimate linkage of the MHC class IA genes and the genes directly involved in MHC class I antigen processing/presentation, and the presence of dichotomous haplotypes of the MHC class I antigen processing/presentation genes. Although the polymorphism of the medaka MHC class IA genes is yet to be analyzed thoroughly in wild populations, dichotomous MHC haplotypes appear to be the rule rather than the exception in the nonmammalian jawed vertebrate MHC. Therefore, the apparent correlation between the intimate physical linkage of the MHC class IA genes and the antigen processing/presentation genes, and the dimorphism of the antigen processing/presentation genes implies that the physical linkage guarantees the coevolution of these genes. The MHC class I antigen presentation system genes of the eutherian mammals that have secondarily lost this linkage may also have lost the possibility for coevolution.

References

- Belov K, Deakin JE, Papenfuss AT, Baker ML, Melman SD, Siddle HV, Gouin N, Goode DL, Sargeant TJ, Robinson MD, Wakefield MJ, Mahony S, Cross JG, Benos PV, Samollow PB, Speed TP, Graves JA, Miller RD (2006) Reconstructing an ancestral mammalian immune supercomplex from a marsupial major histocompatibility complex. *PLoS Biol* 4:e46
- Bingulac-Popovic J, Figueroa F, Sato A, Talbot WS, Johnson SL, Gates M, Postlethwait JH, Klein J (1997) Mapping of mhc class I and class II regions to different linkage groups in the zebrafish, *Danio rerio*. *Immunogenetics* 46:129–134
- Clark MS, Shaw L, Kelly A, Snell P, Elgar G (2001) Characterization of the MHC class I region of the Japanese pufferfish (*Fugu rubripes*). *Immunogenetics* 52:174–185
- Consortium TM (1999) Complete sequence and gene map of a human major histocompatibility complex. The MHC sequencing consortium. *Nature (Lond)* 401:921–923
- Dohm JC, Tsend-Ayush E, Reinhardt R, Grutzner F, Himmelbauer H (2007) Disruption and pseudoautosomal localization of the major histocompatibility complex in monotremes. *Genome Biol* 8:R175

- Flajnik MF, Kasahara M (2001) Comparative genomics of the MHC: glimpses into the evolution of the adaptive immune system. *Immunity* 15:351–362
- Groll M, Ditzel L, Lowe J, Stock D, Bochtler M, Bartunik HD, Huber R (1997) Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature (Lond)* 386:463–471
- Hedrick PW (2006) Genetic polymorphism in heterogeneous environments: the age of genomics. *Annu Rev Ecol Evol Syst* 37:67–93
- Joly E, Le Rolle AF, Gonzalez AL, Mehling B, Stevens J, Coadwell WJ, Hunig T, Howard JC, Butcher GW (1998) Co-evolution of rat TAP transporters and MHC class I RT1-A molecules. *Curr Biol* 8:169–172
- Kasahara M, Naruse K, Sasaki S, Nakatani Y, Qu W, Ahsan B, Yamada T, Nagayasu Y, Doi K, Kasai Y, Jindo T, Kobayashi D, Shimada A, Toyoda A, Kuroki Y, Fujiyama A, Sasaki T, Shimizu A, Asakawa S, Shimizu N, Hashimoto S, Yang J, Lee Y, Matsushima K, Sugano S, Sakaizumi M, Narita T, Ohishi K, Haga S, Ohta F, Nomoto H, Nogata K, Morishita T, Endo T, Shin IT, Takeda H, Morishita S, Kohara Y (2007) The medaka draft genome and insights into vertebrate genome evolution. *Nature (Lond)* 447:714–719
- Kaufman J, Milne S, Gobel TW, Walker BA, Jacob JP, Auffray C, Zoorob R, Beck S (1999) The chicken B locus is a minimal essential major histocompatibility complex. *Nature (Lond)* 401:923–925
- Kelley J, Walter L, Trowsdale J (2005) Comparative genomics of major histocompatibility complexes. *Immunogenetics* 56:683–695
- Klein J, Satta Y, O’Huigin C, Takahata N (1993) The molecular descent of the major histocompatibility complex. *Annu Rev Immunol* 11:269–295
- Lukacs MF, Harstad H, Grimholt U, Beetz-Sargent M, Cooper GA, Reid L, Bakke HG, Phillips RB, Miller KM, Davidson WS, Koop BF (2007) Genomic organization of duplicated major histocompatibility complex class I regions in Atlantic salmon (*Salmo salar*). *BMC Genomics* 8:251
- Matsuo MY, Asakawa S, Shimizu N, Kimura H, Nonaka M (2002) Nucleotide sequence of the MHC class I genomic region of a teleost, the medaka (*Oryzias latipes*). *Immunogenetics* 53:930–940
- Mehta RB, Nonaka MI, Nonaka M (2009) Comparative genomic analysis of the major histocompatibility complex class I region in the teleost genus *Oryzias*. *Immunogenetics* 61:385–399
- Michalova V, Murray BW, Sultmann H, Klein J (2000) A contig map of the Mhc class I genomic region in the zebrafish reveals ancient synteny. *J Immunol* 164:5296–5305
- Naruse K, Fukamachi S, Mitani H, Kondo M, Matsuoka T, Kondo S, Hanamura N, Morita Y, Hasegawa K, Nishigaki R, Shimada A, Wada H, Kusakabe T, Suzuki N, Kinoshita M, Kanamori A, Terado T, Kimura H, Nonaka M, Shima A (2000) A detailed linkage map of medaka, *Oryzias latipes*: comparative genomics and genome evolution. *Genetics* 154:1773–1784
- Ohta Y, Okamura K, McKinney EC, Bartl S, Hashimoto K, Flajnik MF (2000) Primitive synteny of vertebrate major histocompatibility complex class I and class II genes. *Proc Natl Acad Sci USA* 97:4712–4717
- Ohta Y, Powis SJ, Lohr RL, Nonaka M, Pasquier LD, Flajnik MF (2003) Two highly divergent ancient allelic lineages of the transporter associated with antigen processing (TAP) gene in *Xenopus*: further evidence for co-evolution among MHC class I region genes. *Eur J Immunol* 33:3017–3027
- Ohta Y, Goetz W, Hossain MZ, Nonaka M, Flajnik MF (2006) Ancestral organization of the MHC revealed in the amphibian *Xenopus*. *J Immunol* 176:3674–3685
- Rammensee HG, Friede T, Stevanović S (1995) MHC ligands and peptide motifs: first listing. *Immunogenetics* 41:178–228
- Rock KL, Goldberg AL (1999) Degradation of cell proteins and the generation of MHC class I-presented peptides. *Annu Rev Immunol* 17:739–779
- Rock KL, York IA, Goldberg AL (2004) Post-proteasomal antigen processing for major histocompatibility complex class I presentation. *Nat Immunol* 5:670–677

- Shiina T, Shimizu S, Hosomichi K, Kohara S, Watanabe S, Hanzawa K, Beck S, Kulski JK, Inoko H (2004) Comparative genomic analysis of two avian (quail and chicken) MHC regions. *J Immunol* 172:6751–6763
- Shiina T, Dijkstra JM, Shimizu S, Watanabe A, Yanagiya K, Kiryu I, Fujiwara A, Nishida-Umehara C, Kaba Y, Hirono I, Yoshiura Y, Aoki T, Inoko H, Kulski JK, Ototake M (2005) Interchromosomal duplication of major histocompatibility complex class I regions in rainbow trout (*Oncorhynchus mykiss*), a species with a presumably recent tetraploid ancestry. *Immunogenetics* 56:878–893
- Takehana Y, Nagai N, Matsuda M, Tsuchiya K, Sakaizumi M (2003) Geographic variation and diversity of the cytochrome b gene in Japanese wild populations of medaka, *Oryzias latipes*. *Zool Sci* 20:1279–1291
- Takehana Y, Naruse K, Sakaizumi M (2005) Molecular phylogeny of the medaka fishes genus *Oryzias* (Belontiiformes: Adrianichthyidae) based on nuclear and mitochondrial DNA sequences. *Mol Phylogenet Evol* 36:417–428
- Tanaka K, Kasahara M (1998) The MHC class I ligand-generating system: roles of immunoproteasomes and the interferon-gamma-inducible proteasome activator PA28. *Immunol Rev* 163:161–176
- Tsukamoto K, Hayashi S, Matsuo MY, Nonaka MI, Kondo M, Shima A, Asakawa S, Shimizu N, Nonaka M (2005) Unprecedented intraspecific diversity of the MHC class I region of a teleost medaka, *Oryzias latipes*. *Immunogenetics* 57:420–431
- Tsukamoto K, Sakaizumi M, Hata M, Sawara Y, Eah J, Kim CB, Nonaka M (2009) Dichotomous haplotypic lineages of the immunoproteasome subunit genes, PSMB8 and PSMB10, in the MHC class I region of a teleost medaka, *Oryzias latipes*. *Mol Biol Evol* 26:769–781
- Unno M, Mizushima T, Morimoto Y, Tomisugi Y, Tanaka K, Yasuoka N, Tsukihara T (2002) The structure of the mammalian 20S proteasome at 2.75 Å resolution. *Structure* 10:609–618
- Zinkernagel RM, Doherty PC (1979) MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. *Adv Immunol* 27:51–177

Chapter 24

Molecular Evolution of Teleostean Hatching Enzymes and Their Egg Envelope Digestion Mechanism: An Aspect of Co-Evolution of Protease and Substrate

Shigeki Yasumasu, Kaori Sano, and Mari Kawaguchi

Abstract At the hatching of medaka embryos, the egg envelope is completely solubilized by two proteases, high choriolytic enzyme (HCE, or choriolysin H) and low choriolytic enzyme (LCE, or choriolysin L). HCE causes the egg envelope to swell, and LCE solubilizes the swollen envelope. Molecular phylogenetic analysis has shown that the hatching enzyme was originally composed of a single enzyme, and during evolution, the hatching system consisting of two types of enzymes was established by duplication and diversification of the genes. We compared the egg envelope digestion mechanism between zebrafish having the single enzyme, ZHE1, and medaka having the two enzymes, HCE and LCE. The digestion manner of ZHE1 was highly homologous to that of HCE with respect to swelling of the egg envelope. The cross-species digestion experiment using enzymes and substrates of both zebrafish and medaka revealed that the substrate specificity of ZHE1 is quite similar to that of HCE, whereas the specificity of LCE is different from those of ZHE1 and HCE. Further analysis showed that ZHE1 and HCE maintain the character of an ancestral hatching enzyme, swelling of the egg envelope, and that LCE acquires a new function, the complete digestion of the HCE-swollen egg envelope. Considering several factors such as the origin of egg envelope protein and the spawning environment of eggs, we discuss a co-evolutionary aspect of the hatching enzyme and egg envelope.

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24.1 History of Studies on the Medaka Hatching Enzyme and Its Substrate

Hatching enzymes are the proteolytic enzymes that digest the egg envelope (Fig. 24.1a). Among them, the enzyme of the medaka, *Oryzias latipes*, has been extensively studied, and this study has a long history (Yamagami et al. 1992). In 1944, the medaka enzyme was first studied by Ishida at the University of Tokyo (Ishida 1944a,b). Ishida identified hatching gland cells that synthesize the hatching enzyme in the prehatching embryos of medaka and showed that the extract from the zymogen granules accumulated in the cells had activity to digest the egg envelope. After that, the hatching enzyme was purified from hatching liquid as a single band on starch gel electrophoresis (Yamagami 1972) and revealed to be a metalloprotease based on inhibitor analyses (Yamagami 1973). However, agarose gel electrophoresis showed that the extract from hatching gland cells was separated into several active fractions: the fractions having an egg envelope-digestion activity and the fraction having an egg envelope-swelling activity (Ohi and Ogawa 1970). One of the problems of this study was whether the hatching enzyme was composed of a single enzyme or multiple enzymes. Shoots et al. (1983) argued that the swelling of the envelope is one of the intermediate changes of the envelope leading to its complete digestion. The problem was settled by a series of experiments showing that two proteases were separately purified from hatching liquid and characterized (Yasumasu et al. 1988, 1989a,b). The two enzymes are called high choriolytic enzyme (HCE, choriolysin H; EC 3.4.24.67) and low choriolytic enzyme (LCE, choriolysin L; EC 3.4.24.66). HCE and LCE cooperatively digest the egg envelope: HCE swells the egg envelope, and LCE completely solubilizes the HCE-swollen envelope (Fig. 24.1b–d). LCE alone is not able to digest or affect the intact egg envelope.

The cDNA cloning analysis revealed that both enzymes belonged to the astacin protease family, and each of them consisted of about 200 amino acids with 55% identity (Yasumasu et al. 1992, 1994; Bond and Beynon 1995). Their genes were first expressed at the pillow, a precursor cell mass of hatching gland cells, which is located at the anterior end of the body axis of late gastrulae. As development progressed, the cells expressing hatching enzyme genes migrated to the pharyngeal cavity of embryos, and were finally located in the inner wall of mouth cavity and gill of prehatching embryos (Inohaya et al. 1995).

Gross morphological study showed that the egg envelope was composed of a thick inner layer and a thin outer layer. The hatching enzyme mainly digests the inner layer. The subunit proteins consisting of the inner layer were well characterized at the protein level. The inner layer comprised two groups of subunit glycoproteins, ZI-1,2 and ZI-3. The precursors of ZI-1,2 and ZI-3, called choriogenin, were synthesized in the liver of spawning female fish under the influence of estrogen, transported to the surface of the vitellogenic oocytes through the bloodstream, and assembled into the inner layer of the envelope (Hamazaki et al. 1989). It is the first demonstration that the protein constituents of the fish egg envelope are of liver origin but not of oocyte origin. Three cDNAs for the precursors of the three subunits

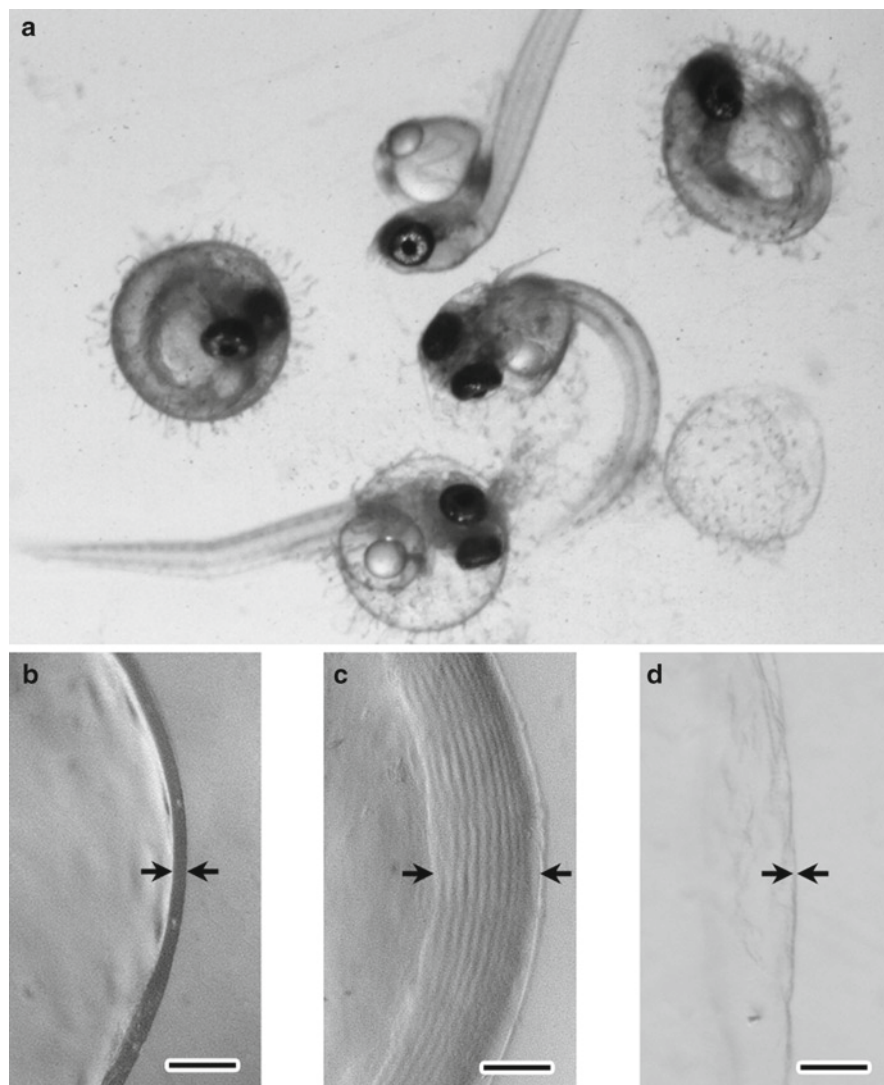


Fig. 24.1 (a) Hatching of medaka embryos. The egg envelope was completely solubilized by cooperative digestion of high choriolytic enzyme (HCE, or choriolysin H) and low choriolytic enzyme (LCE, or choriolysin L) (b–d, arrows). The isolated egg envelope (b) was incubated with only HCE, and the inner layer was markedly swollen (c). The HCE-swollen egg envelope was incubated with LCE; the inner layer was completely solubilized and only the thin sheet-like outer layer remained undigested (d). Bars 100 μ m

were cloned. Choriogenin H (ChgH) and choriogenin H minor (ChgHm) were the precursors of heterogeneous ZI-1,2, whereas choriogenin L (ChgL) was the precursor of homogeneous ZI-3 (Murata et al. 1995, 1997; Sugiyama et al. 1998). ZI-1,2 and ZI-3 contained the zona pellucida (ZP) domain, which is common in the egg

envelope subunit proteins of all vertebrates (Rankin and Dean 2000). In general, the subunit proteins of vertebrate egg envelopes are categorized into three groups: ZPA, ZPB, and ZPC. ChgL (the precursor of ZI-3) is grouped into ZPC and ChgH and ChgHm (the precursors of ZI-1,2) are in ZPB. ZI-1,2 and ZI-3 are constructed by an N-terminal region and a ZP domain (Fig. 24.2a). ZI-1,2 has a short structural motif, called the trefoil domain, between the N-terminal region and ZP domain. In addition, the N-terminal region of ZI-1,2 is much longer than that of ZI-3, comprising characteristic repetitive sequences called the Pro-X-Y repeat.

The envelope of an unfertilized egg (unfertilized egg envelope, UFE) is soft and fragile. After fertilization, the envelope turns into a tough, hard structure (fertilized egg envelope, FE). This phenomenon is called “egg envelope hardening.” Such egg envelope hardening is suggested to be the result of the formation of ϵ -(γ -glutamyl) lysine isopeptide cross-links by transglutaminase (Sugiyama and Iuchi 2000). The inner layer of FE was completely solubilized by the cooperative action of HCE and LCE, whereas the inner layer of UFE was easily solubilized by either HCE or LCE.

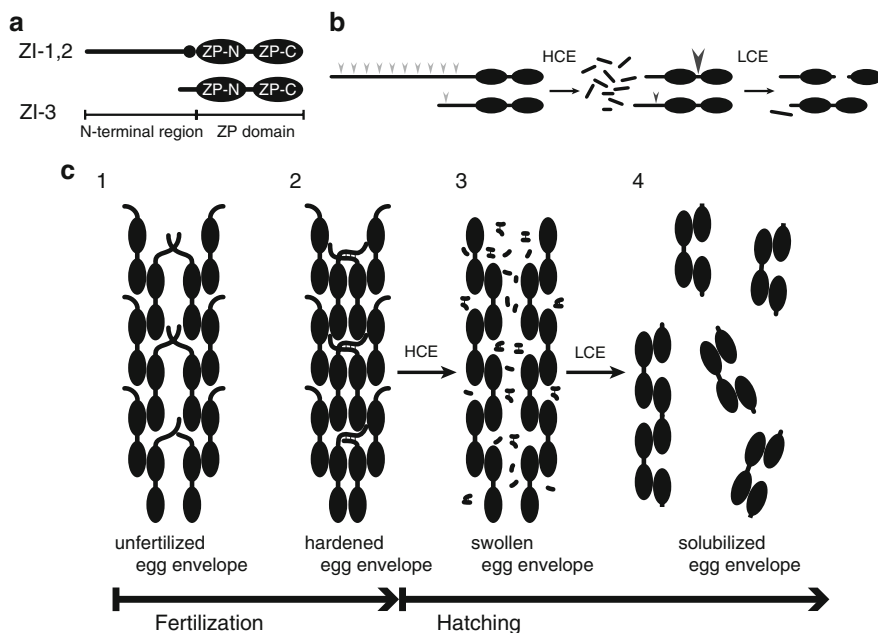


Fig. 24.2 (a) Schematic representation of structure of the subunit proteins of egg envelope. Black circle and black ovals indicate trefoil domain in ZI-1,2 and ZP subdomains, respectively. (b) Sites on egg envelope proteins cleaved by HCE (gray arrowheads) and LCE (black arrowheads). (c) Predicted macromolecular structure of the egg envelope and its structural changes at fertilization, and at hatching. 1, unfertilized egg envelope. 2, hardened egg envelope. After fertilization, the ϵ -(γ -glutamyl) lysine isopeptide cross-links are formed between the N-terminal regions of the subunit proteins, and the egg envelope becomes compact and hardens. 3, HCE swells and loosens the egg envelope by cleaving the N-terminal regions of egg envelope subunits into fine fragments. 4, LCE digests and solubilizes the HCE-swollen envelope by disruption of the filamentous structure formed by polymerization of ZP domains

24.2 Egg Envelope Digestion Mechanism

To examine the molecular mechanism of egg envelope digestion, the sites on egg envelope subunit proteins cleaved by HCE or LCE were determined using UFE and/or FE as substrate. HCE and LCE cleaved different target sites on the proteins (Yasumasu et al. 2010).

HCE selectively cleaved the N-terminal regions of ZI-1,2 and ZI-3, especially the Pro-X-Y repeat sequences in ZI-1,2 into fine peptide fragments, the majority of which was the 6-amino-acid peptide Y-Pro-X-Y-Pro-X (Fig. 24.2b). These polypeptide fragments contain most of the ϵ -(γ -glutamyl) lysine isopeptide cross-links (Lee et al. 1994) (Fig. 24.2c). Therefore, it is reasonable to say that the molecular structural change of the egg envelope leading to swelling results from the removal of such cross-links from the hardened egg envelope by HCE cleaving the Pro-X-Y sequences into fine fragments. LCE is able to digest UFE but hardly digests FE, probably because of the difference of accessibility of LCE to the LCE-cleaving sites on the envelope; that is, the structure of UFE is loosened enough for LCE to gain access to LCE sites, but the hard structure of the intact FE prevents access of LCE to the sites. At hatching, the digestion of FE by HCE makes LCE accessible to LCE sites.

The swollen envelope, in defect of the N-terminal regions as already mentioned, is considered to be mainly or exclusively composed of the ZP-domains of ZI-1,2 and ZI-3. In mouse and salmon, recent studies have reported that the urea-extracted subunit proteins from UFE, when urea is removed, can be polymerized into long filaments through their noncovalent interactions (Darie et al. 2008; Litscher et al. 2008). Such self-polymerization depends on the nature of ZP domains; that is, the ZP domain itself is suggested to be responsible for their polymerization into filament (Jovine et al. 2002; Qi et al. 2002). Therefore, it is possible to say that the architecture of the swollen egg envelope is constructed by a filamentous structure formed by self-polymerization of ZP domains. In addition, the ZP domain is known to be composed of two subdomains, the N-terminal subdomain (ZP-N) and the C-terminal subdomain (ZP-C) (Llorca et al. 2007; Monné et al. 2008). The two subdomains are connected by a protease-sensitive intervening sequence (Jovine et al. 2005) (see Fig. 24.2a). We applied such information to our study. The LCE-cleaving site was located at the middle of ZI-1,2 at the protease-sensitive region between ZP-N and ZP-C (Fig. 24.2b). Therefore, LCE cleaving its site results in the disruption of the filamentous structure (Yasumasu et al. 2010) and leads to complete solubilization of the swollen egg envelope (Fig. 24.2c). Thus, HCE and LCE are suggested to play different roles in egg envelope digestion.

24.3 Molecular Evolution of Hatching Enzyme Genes

Recently, we have extended our work to molecular evolution of hatching enzymes in teleostean fishes and the molecular mechanism of their egg envelope digestion, which led to a study on co-evolution of the hatching enzyme and egg envelope.

First, we constructed a phylogenetic tree of hatching enzyme genes to elucidate the molecular evolution of hatching enzyme (HE) genes in Teleostei (Kawaguchi et al. 2006, 2007, 2009, 2010). We cloned the cDNAs or genes of 27 fish species to cover almost all categories of Teleostei. Using the nucleotide sequences for mature enzyme portions, we constructed a phylogenetic tree by the maximum-likelihood method (Fig. 24.3) (Kawaguchi et al. 2010). According to the phylogeny based on the mitochondrial DNA of Teleostei, Osteoglossomorpha first branched off from an ancestor, followed by Elopomorpha, and then Otocephala and Euteleostei branched paraphyletically (Fig. 24.4a). The branching patterns of HE genes basically agreed with this teleostean phylogenetic relationship, as supported by high bootstrap values (see Fig. 24.3). The tree shows an interesting aspect in the evolution of fish HE genes. Each of the elopomorph fishes, the basal group in Teleostei, possessed several HE genes. However, these multiple genes formed a monophyletic clade, and their branch lengths in the clade were shorter than those of other HE genes. Thus, elopomorph HE genes are similar to each other and are composed of a single type of genes. After that, the ancestor of Otocephala and Euteleostei gained two types of hatching enzyme genes by duplication, i.e., the otocephalan and euteleostean genes are divided into two clades named clade I and clade II (Fig. 24.3). Euteleostean fishes possess two types of genes coding clade I/HCE and clade II/LCE that were originally found in medaka (*Belontiiformes*) and were characterized at the protein level. The orthologs of both genes were also cloned from all euteleostean fishes examined. On the other hand, the early branching group of Otocephala, that is, *Clupeiformes* and *Gonorynchiformes* also had clade I and clade II genes. However, the fishes of *Cypriniformes*, *Characiformes*, *Gymnotiformes*, and *Siluriformes* (collectively referred to as *Otophysi*) had only clade I genes. Thus, the diversification into clade I and clade II is suggested to have occurred in the common ancestor of Otocephala and Euteleostei (*Clupeocephala*), and clade II genes have been lost in *Otophysi*. In other words, the results are briefly summarized as follows. Teleostean HE was originally composed of a single enzyme, and then two types of enzymes were produced by the duplication and diversification of the HE gene in the lineage to *Clupeocephala*. In terms of the number and variety of enzymes, the hatching system in the living fishes are classified into four groups: that is, the single enzyme system in Elopomorpha, the clade I/HCE–clade II/LCE system in Euteleostei, the clade I and clade II system in *Clupeiformes* and *Gonorynchiformes*, and the single enzyme system in *Otophysi* that is composed of clade I enzymes (Fig. 24.4b).

24.4 Evolution of Egg Envelope Digestion Mechanism

According to molecular phylogenetic analysis, the HCE–LCE system common in euteleostean fishes is a system developed late in evolution, because elopomorph fishes have the single enzyme system. We compared the egg envelope digestion mechanism between the HCE–LCE system in medaka and the single enzyme system in zebrafish *Danio rerio*, belonging to *Cypriniformes*. First, we purified only one clade I enzyme (ZHE1) from zebrafish and confirmed that zebrafish has

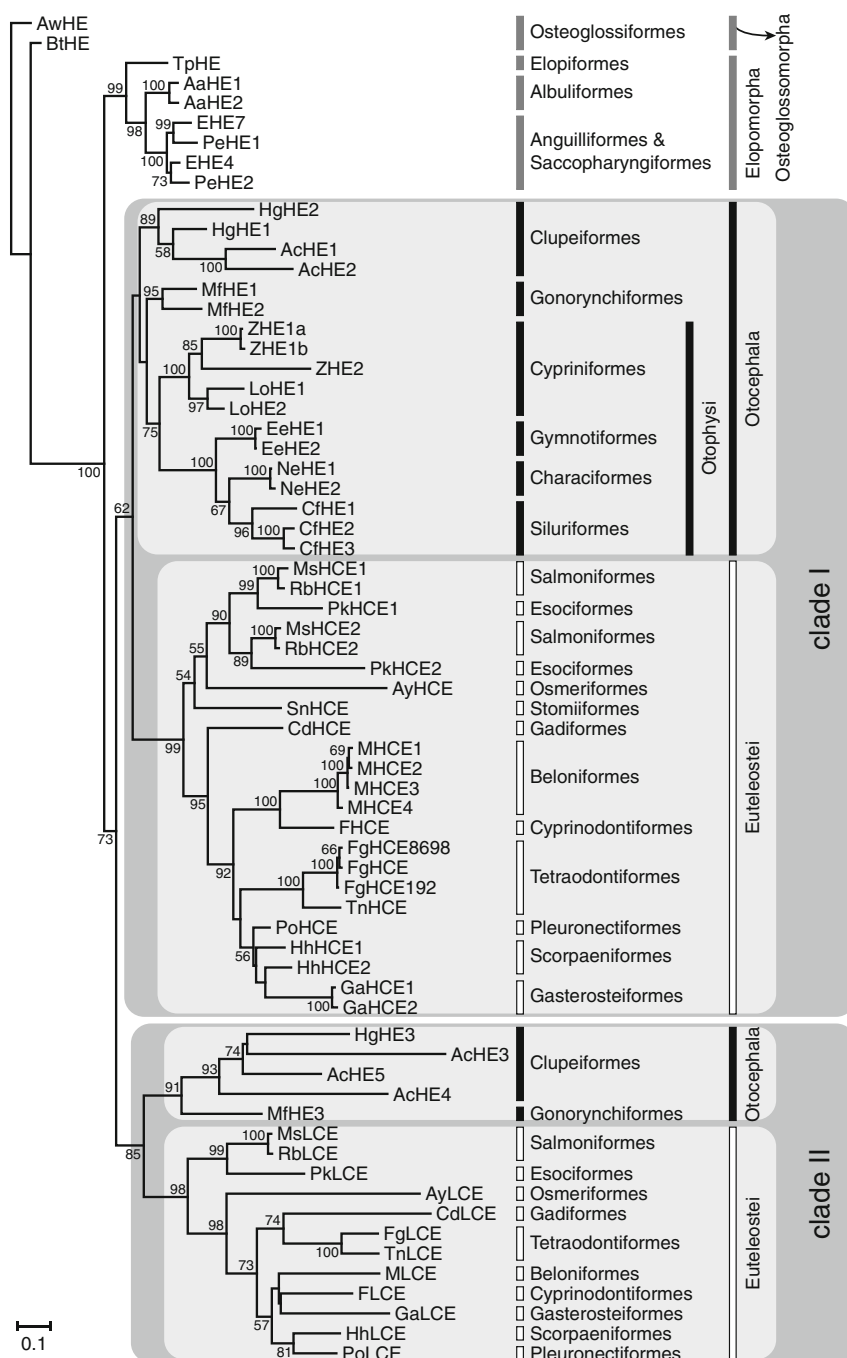


Fig. 24.3 A maximum-likelihood tree of nucleotide sequences of hatching enzyme genes of Teleostei. Arowana hatching enzyme (*AwHE*) gene was used as an outgroup. *Numbers at the nodes* indicate bootstrap values shown as percentages. Order names for the fishes examined are shown at the *right* of gene names

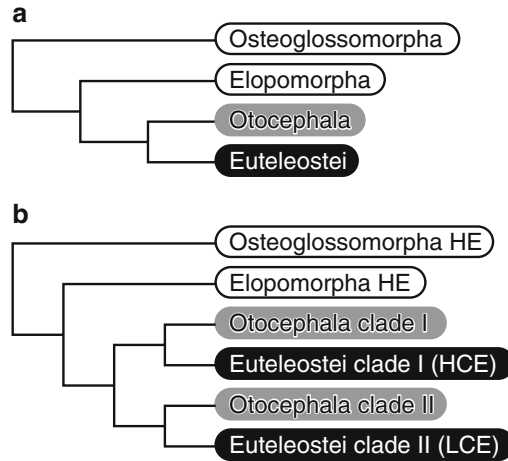


Fig. 24.4 Molecular phylogenetic relation of teleostean fishes or hatching enzyme genes. (a) Teleostean fishes have been divided into four subdivisions, based on whole mitogenome (mitochondrial DNA) sequences: Osteoglossomorpha, Elopomorpha, Otocephala, and Euteleostei. (b) Summary of the phylogenetic tree of hatching enzyme genes. Elopomorph fishes possess a single type of hatching enzyme gene. Otocephalan and euteleostean fishes possess two types of hatching enzyme (*HE*) genes, called clade I and clade II genes

the single enzyme system at the protein level (Sano et al. 2008). At the natural hatching of the zebrafish embryo, the egg envelope was not completely solubilized but was softened and ruptured by the movement of the embryo. In electron microscopic observations, the purified ZHE1 swelled and softened the isolated envelope, similar to that in natural hatching. Determination of cleaving sites on egg envelope revealed that ZHE1 also cleaved the N-terminal regions of egg envelope subunits where many ϵ -(γ -glutamyl) lysine cross-links were considered to be located (see Fig. 24.5b). Therefore, the manner of egg envelope digestion of ZHE1 is homologous to that of medaka HCE. The result corresponds well with the phylogenetic analysis showing that both ZHE1 and HCE belong to clade I.

24.5 Comparison of the Substrate Specificity of HCE, LCE, and ZHE1

To evaluate substrate specificity, we changed the substrate–enzyme combination between zebrafish and medaka and performed the cross-species digestion experiment using UFE as substrate. Either the zebrafish or medaka egg envelope was digested by purified HCE, LCE, or ZHE1 and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Fig. 24.5a). When the medaka egg envelope was used as substrate, the pattern of ZHE1 digestion was the same as that of HCE digestion but quite different from that of LCE digestion. In the case of the

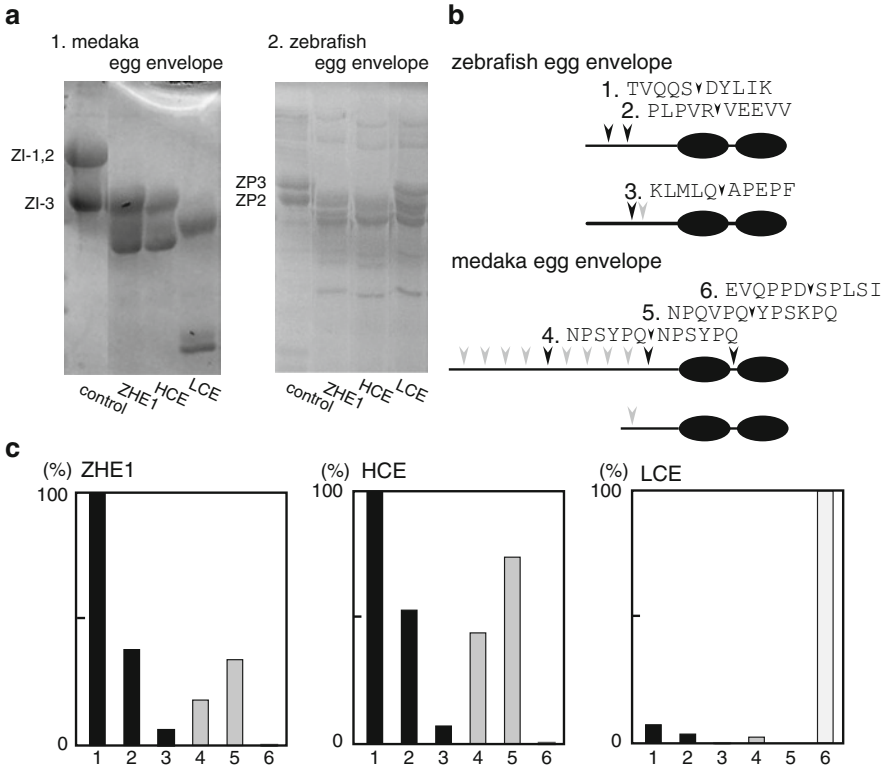


Fig. 24.5 Substrate specificity of the enzymes. **(a)** Cross-species digestion experiment. 1, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of medaka unfertilized egg envelope (UFE) digests by ZHE1, HCE, or LCE for 15 min. 2, SDS-PAGE of the digests of zebrafish UFE by ZHE1, HCE, or LCE for 10 min. The names of major egg envelope proteins are shown on the left of each panel. **(b)** Positions of cleaving sites and the amino acid sequences of synthetic peptide substrates. Gray and black arrowheads show the cleaving sites and the cleaving sites used for the peptide substrates, respectively. **(c)** Comparison of the specific activities of ZHE1, HCE, and LCE toward synthetic peptide substrates. The synthetic peptides were designed from amino acid sequences of each cleaving sites for ZHE1 (1–3), HCE (4, 5), or LCE (6), as shown in (b). The substrate showing the highest activity of each enzyme is expressed as 100%

zebrafish egg envelope, the pattern of HCE digest was similar to that of ZHE1 digest. The pattern of LCE digest was also similar to those of ZHE1 and HCE; however, its digestion efficiency was much lower than those of ZHE1 and HCE (see Fig. 24.5a–2). The sequence analyses from the N-terminals of the digests revealed that both HCE and ZHE1 cleaved the same sites of egg envelope proteins of two species.

Next, the cleaving efficiency of ZHE1, HCE, or LCE was quantitatively estimated using synthetic peptide substrates. The total six peptides designed from three ZHE1-cleaving sites (1, 2, and 3 in Fig. 24.5b), two HCE sites (Pro-X-Y sequence, 4 and 5 in Fig. 24.5b), and one LCE site (middle of ZP domain, 6 in Fig. 24.5b), were employed. The results revealed that ZHE1 and HCE cleaved the same peptides

in similar efficiency (Fig. 24.5c). On the other hand, the cleaving efficiency of LCE toward the peptides for ZHE1- and HCE-cleaving sites was much lower than those of HCE and ZHE1. Furthermore, LCE had another efficient cleaving site, the middle of the ZP domain, where ZHE1 and HCE hardly cleaved. Therefore, the substrate specificity of ZHE1 and HCE was quite similar to each other, but that of LCE was different from the other two. The two enzymes ZHE1 and HCE, belonging to clade I, were tentatively named “swelling enzyme” and LCE, belonging to clade II was called “solubilizing enzyme.”

We investigated the hatching enzyme of the Japanese eel *Anguilla japonica*, belonging to Elopomorpha that is sister to the common ancestor of zebrafish and medaka. As suggested by the phylogenetic analysis, only a single enzyme (EHE) was purified from eel hatching liquid. The purified EHE swells and softens the egg envelope by cleaving the N-terminal regions of the egg envelope subunit proteins, but does not cleave the inside of ZP domain (Sano et al. unpublished results). Therefore, a single enzyme-dependent swelling of egg envelope is an ancestral form of egg envelope digestion. From an evolutionary aspect of egg envelope digestion, “swelling enzymes” such as ZHE1 and HCE keep their substrate specificity during evolution. LCE, the “solubilizing enzyme,” would be produced by changing the substrate specificity of the ancestral enzyme and could cleave the middle of the ZP domain. The results suggested that LCE acquired a new function, the solubilization of the swollen egg envelope.

24.6 Molecular Co-Evolution of Hatching Enzyme and Egg Envelope Subunit Protein

During evolution, mutations would be independently accumulated in both the genes for hatching enzyme and egg envelope. Some mutations of the two genes would be selected under a common pressure with respect to egg envelope digestion. Such evolution of protease and substrate is one of the typical phenomena called molecular co-evolution. Comparing amino acid sequences between egg envelope subunits, we can see that the overall identity of ZP domains was approximately 60%. However, there is no similarity in the N-terminal regions between zebrafish and medaka, on which the cleaving sites for “swelling enzyme” are located. In addition, we cannot find any common amino acid residue important in substrate recognition, because no conserved sequence is found around the cleaving sites of two species. Enzymological study has suggested that ZHE1 and HCE have broad substrate specificity (Yasumasu et al. 1989a; Sano et al. 2008). Therefore, the broad substrate specificities of the “swelling enzymes” could permit a relatively high substitution rate in the N-terminal regions, and the manner of egg envelope digestion was conserved in the “swelling enzymes”. The cross-species digestion experiments revealed that the substrate specificity was similar in “swelling enzymes.” The phylogenetic analysis revealed that ZHE1 and HCE belong to clade I. From an evolutionary view point, the sequences of N-terminal regions have changed within the range of the substrate specificity of clade I enzymes which slowly changed. Therefore, we think

a substrate recognition mechanism common in “swelling enzymes” must have existed. Further molecular-level information on the interaction of enzyme to substrate should be required to find the common substrate-recognition rule of “swelling enzymes.”

24.7 An Aspect of the Advantages of Evolution into a Two-Enzyme Hatching System

What advantage has the two-enzyme system brought to fish species? The morphology of the egg envelope and the environment of hatching should be considered, because both hatching enzyme and egg envelope are considered to have co-evolved under the influence of the environment. As one of the examples, we have studied the hatching of an ovoviviparous fish, the black rockfish *Sebastes schlegelii*, the embryos of which grow and hatch in the maternal body. As compared with oviparous fishes, the egg envelope of ovoviviparous fish is in general thin and fragile, which may be the result of maternal protection from mechanical stress. The black rockfish egg envelope is not an exception. Investigation showed that the expression of the HCE gene was suppressed at a lower level, and in addition, the LCE gene was pseudogenized (Kawaguchi et al. 2008). The result implies that thinning of the egg envelope and inefficiency of the digestion activity of hatching enzyme would have occurred synchronously during the evolution of black rockfish.

We focused on the thickness of the egg envelope. Euteleostean fishes such as salmon and medaka have a thick and tough egg envelope (approximately 30–40 μm and 20 μm , respectively), whereas many elopomorph and otophysan fishes have a thin envelope (zebrafish, approximately 4 μm ; Japanese eel, several micrometers). Therefore, the egg envelopes seem to have become thicker accompanying evolution into the HCE–LCE system. The thickness of the egg envelope may be related to the place at which the egg envelope subunit proteins are synthesized. Many euteleostean fishes synthesize egg envelope protein in the liver (Hamazaki et al. 1989; Lyons et al. 1993), whereas elopomorph and otophysan fishes synthesize it in the oocyte (Wang and Gong 1999; Chang et al. 1996; Sano et al. 2010). Because the liver is one of the largest organs in the body, it is conceivable that the liver has an advantage in synthesizing a large amount of protein. Thus, euteleostean fishes possess the HCE and LCE genes and tend to have a thick and tough egg envelope. On the other hand, in otocephalan fishes, which are of oocyte origin, the early branching group possesses two types of hatching enzymes, whereas the late diverging group, Otophysi, has lost the clade II gene (see Figs. 24.3 and 24.4b). It will be interesting to know the function of the otocephalan clade II hatching enzymes, which form a clade together with that of LCE, the “solubilizing enzyme,” and have not yet been characterized.

The egg envelope of demersal eggs is generally known to be thick, whereas that of pelagic eggs is thin (Kunz 2004; Stehr and Hawkes 1979). This difference in egg envelope seems to be dependent on adaptation to the prehatching environment of each embryo. Demersal eggs are spawned in seaweed beds or the bottom of a river and sea,

and pelagic eggs are spawned in the open fields of a gulf or open sea. Thus, the envelope of demersal eggs is considered to be more protective than that of pelagic eggs. In addition, such an egg envelope of demersal eggs would permit the embryos to hatch at a later stage of development than those of pelagic eggs. In fact, some euteleostean fishes, such as medaka and rainbow trout, which spawn demersal eggs, hatched out at a later stage of development (10 days at 25°C and 36 days at 10°C, respectively) than elopomorph and otophysan fishes, which spawn pelagic eggs (Japanese eel, 30 h at 24°C; anchovy, 35 h at 18°C). As a result, demersal embryos develop more fully grown bodies before hatching. Such a developmental characteristic may form a body sufficient for successfully transferring from a self-nutrient life to a free-living and food-eating life. However, the zebrafish, which spawn demersal eggs, hatches at the earlier or more juvenile stage of development (48 h at 28.5°C). Zebrafish, having the single enzyme system, did not acquire the thick envelope during evolution, probably because of the oocyte origin of the envelope proteins. The enzyme system and the origin of the egg envelope protein seem to be related to spawning environment and the length of prehatching development in each fish species.

24.8 Concluding Remarks

In this chapter, the molecular evolution of the fish hatching enzyme and its co-evolution with egg envelope protein was discussed by referring to the experimental results we obtained recently. The co-evolution is well explained when the relationship of the enzyme system to the thickness of the egg envelope is taken into account, as well as the relationship of the environment of hatching to the length of the prehatching developmental period. The next work to be done is to elucidate the mechanism of the molecular co-evolution of hatching enzyme and egg envelope proteins. How is substrate specificity (the substrate recognition mechanism) conserved in the “swelling enzyme” and changed from the ancestral “swelling enzyme” into the “solubilizing enzyme”? Recently, the three-dimensional structure of HCE and ZHE1 has been elucidated (Kudo et al. 2004; Okada et al. 2009). The goal of our molecular-level understanding of the co-evolutionary process is to find and discriminate conserved or changeable amino acid residues that are important or unimportant in the substrate specificity of the hatching enzymes.

References

- Bond JS, Beynon RJ (1995) The astacin family of metalloendopeptidases. *Protein Sci* 4:1247–1261
- Chang YS, Wang SC, Tsao CC, Huang FL (1996) Molecular cloning, structural analysis, and expression of carp ZP3 gene. *Mol Reprod Dev* 44:295–304
- Darie CC, Janssen WG, Litscher ES, Wassarman PM (2008) Purified trout egg vitelline envelope proteins VE β and VE γ polymerize into homomeric fibrils from dimers in vitro. *Biochim Biophysica Acta* 1784:385–392

- Hamazaki TS, Nagahama Y, Iuchi I, Yamagami K (1989) A glycoprotein from the liver constitutes the inner layer of the egg envelope (zona pellucida interna) of the fish, *Oryzias latipes*. *Dev Biol* 133:101–110
- Inohaya K, Yasumasu S, Ishimaru M, Ohyama A, Iuchi I, Yamagami K (1995) Temporal and spatial patterns of gene expression for the hatching enzyme in the teleost embryo, *Oryzias latipes*. *Dev Biol* 171:374–385
- Ishida J (1944a) Hatching enzyme in the fresh-water fish, *Oryzias latipes*. *Annot Zool Jpn* 22:137–154
- Ishida J (1944b) Further studies on the hatching enzyme of the fresh-water fish, *Oryzias latipes*. *Annot Zool Jpn* 22:155–164
- Jovine L, Qi H, Williams Z, Litscher E, Wassarman PM (2002) The ZP domain is a conserved module for polymerization of extracellular proteins. *Nat Cell Biol* 4:457–461
- Jovine L, Darie CC, Litscher ES, Wassarman PM (2005) Zona pellucida domain proteins. *Annu Rev Biochem* 74:83–114
- Kawaguchi M, Yasumasu S, Hiroi J, Naruse K, Inoue M, Iuchi I (2006) Evolution of teleostean hatching enzyme genes and their paralogous genes. *Dev Genes Evol* 216:769–784
- Kawaguchi M, Yasumasu S, Hiroi J, Naruse K, Suzuki T, Iuchi I (2007) Analysis of the exon–intron structures of fish, amphibian, bird and mammalian hatching enzyme genes, with special reference to the intron loss evolution of hatching enzyme genes in Teleostei. *Gene (Amst)* 392:77–88
- Kawaguchi M, Nakagawa M, Noda T, Yoshizaki N, Hiroi J, Nishida M, Iuchi I, Yasumasu S (2008) Hatching enzyme of ovoviparous black rockfish *Sebastes schlegelii*: environmental adaptation of hatching enzyme and evolutionary aspects of formation of pseudogene. *FEBS J* 275:2884–2898
- Kawaguchi M, Fujita H, Yoshizaki N, Hiroi J, Okouchi H, Nagakura Y, Noda T, Watanabe S, Katayama S, Iwamuro S, Nishida M, Iuchi I, Yasumasu S (2009) Different hatching strategies in embryos of two species, pacific herring *Clupea pallasii* and Japanese anchovy *Engraulis japonicus*, that belong to the same order Clupeiformes, and their environmental adaptation. *J Exp Zool B312*:95–107
- Kawaguchi M, Hiroi J, Miya M, Nishida M, Iuchi I, Yasumasu S (2010) Phylogeny-dependent intron–loss evolution of hatching enzyme genes in teleostei. *BMC Evol Biol* 10:260
- Kudo N, Yasumasu S, Iuchi I, Tanokura M (2004) Crystallization and preliminary X-ray analysis of HCE-1, a hatching enzyme of medaka fish, *Oryzias latipes*. *Acta Crystallogr D60*:725–726
- Kunz YW (2004) Developmental biology of teleost fishes. Springer Netherlands, Amsterdam
- Lee KS, Yasumasu S, Nomura K, Iuchi I (1994) HCE, a constituent of the hatching enzymes of *Oryzias latipes* embryos, releases unique proline-rich polypeptides from its natural substrate, the hardened chorion. *FEBS Lett* 339:281–284
- Litscher ES, Janssen WG, Darie CC, Wassarman PM (2008) Purified mouse egg zona pellucida glycoproteins polymerize into homomeric fibrils under non-denaturing conditions. *J Cell Physiol* 214:153–157
- Llorca O, Trujillo A, Blanco FJ, Bernabeu C (2007) Structural model of human endoglin, a trans-membrane receptor responsible for hereditary hemorrhagic telangiectasia. *J Mol Biol* 365: 694–705
- Lyons CE, Payette KL, Price JL, Huang RCC (1993) Expression and structural analysis of a teleost homologue of a mammalian zona pellucida gene. *J Biol Chem* 268:21351–21358
- Monné M, Han L, Schwend T, Burendahl S, Jovine L (2008) Crystal structure of the ZP-N domain of ZP3 reveals the core fold of animal egg coats. *Nature (Lond)* 456:653–657
- Murata K, Sasaki T, Yasumasu S, Iuchi I, Enami J, Yasumasu I, Yamagami K (1995) Cloning of cDNAs for the precursor protein of a low-molecular-weight subunit of the inner layer of the egg envelope (chorion) of the fish *Oryzias latipes*. *Dev Biol* 167:9–17
- Murata K, Sugiyama H, Yasumasu S, Iuchi I, Yasumasu I, Yamagami K (1997) Cloning of cDNA and estrogen-induced hepatic gene expression for choriogenin H, a precursor protein of the fish egg envelope (chorion). *Proc Natl Acad Sci USA* 94:2050–2055

- Ohi Y, Ogawa N (1970) Electrophoretic fractionation of the hatching enzyme of medaka, *Oryzias latipes*. Zool Mag (Tokyo) 79:17–18
- Okada A, Nagata K, Sano K, Yasumasu S, Kubota K, Ohtsuka J, Iuchi I, Tanokura M (2009) Crystallization and preliminary X-ray analysis of ZHE1, a hatching enzyme from the zebrafish *Danio rerio*. Acta Crystallogr F65:1018–1020
- Qi H, Williams Z, Wassarman PM (2002) Secretion and assembly of zona pellucida glycoproteins by growing mouse oocytes microinjected with epitope-tagged cDNAs for mZP2 and mZP3. Mol Biol Cell 13:530–541
- Rankin T, Dean J (2000) The zona pellucida: using molecular genetics to study the mammalian egg coat. Rev Reprod 5:114–121
- Sano K, Inohaya K, Kawaguchi K, Yoshizaki N, Iuchi N, Yasumasu S (2008) Purification and characterization of zebrafish hatching enzyme: an evolutionary aspect of the mechanism of egg envelope digestion. FEBS J 275:5934–5946
- Sano K, Kawaguchi M, Yoshikawa M, Iuchi I, Yasumasu S (2010) Evolution of the teleostean zona pellucida gene inferred from the egg envelope protein genes of the Japanese eel, *Anguilla japonica*. FEBS J 277:4674–4684
- Shoots AFM, Sackers MRJ, Overkamp PSG, Denucé JM (1983) Hatching in the teleost *Oryzias latipes*: limited proteolysis causes egg envelope swelling. J Exp Zool 226:93–100
- Stehr CM, Hawkes JM (1979) The comparative ultrastructure of egg membrane and associated pore structures in the starry flounder, *Platichthys stellatus* (Pallas), and pink salmon, *Oncorhynchus gorbuscha* (Wallbaum). Cell Tissue Res 202:347–356
- Sugiyama H, Iuchi I (2000) Molecular structure and hardening of egg envelope in fish. Recent Res Dev Comp Biochem Physiol 1:139–161
- Sugiyama H, Yasumasu S, Murata K, Iuchi I, Yamagami K (1998) The third egg envelope subunit in fish: cDNA cloning and analysis, and gene expression. Dev Growth Differ 40:35–45
- Wang H, Gong Z (1999) Characterization of two zebrafish cDNA clones encoding egg envelope protein ZP2 and ZP3. Biochim Biophys Acta 1446:156–160
- Yamagami K (1972) Isolation of a choriolytic enzyme (hatching enzyme) of the teleost, *Oryzias latipes*. Dev Biol 29:343–348
- Yamagami K (1973) Some enzymological properties of a hatching enzyme (chorionase) isolated from the fresh-water teleost, *Oryzias latipes*. Comp Biochem Physiol 46B:603–616
- Yamagami K, Hamazaki TS, Yasumasu S, Masuda K, Iuchi I (1992) Molecular and cellular basis of formation, hardening, and breakdown of the egg envelope in fish. Int Rev Cytol 136:51–92
- Yasumasu S, Iuchi I, Yamagami K (1988) Medaka hatching enzyme consists of two kinds of proteases which act cooperatively. Zool Sci 5:191–195
- Yasumasu S, Iuchi I, Yamagami K (1989a) Purification and partial characterization of high choriolytic enzyme (HCE), a component of the hatching enzyme of the teleost, *Oryzias latipes*. J Biochem 105:204–211
- Yasumasu S, Iuchi I, Yamagami K (1989b) Isolation and some properties of low choriolytic enzyme (LCE), a component of the hatching enzyme of the teleost, *Oryzias latipes*. J Biochem 105:212–218
- Yasumasu S, Yamada K, Akasaka K, Mitsunaga K, Iuchi I, Shimada H, Yamagami K (1992) Isolation of cDNAs for LCE and HCE, two constituent proteases of the hatching enzyme of *Oryzias latipes*, and concurrent expression of their mRNAs during development. Dev Biol 153:250–258
- Yasumasu S, Iuchi I, Yamagami K (1994) cDNAs and the genes of HCE and LCE, two constituents of the medaka hatching enzyme. Dev Growth Differ 36:241–250
- Yasumasu S, Kawaguchi M, Ouchi S, Sano S, Murata K, Sugiyama H, Akema T, Iuchi I (2010) Mechanism of egg envelope digestion by hatching enzymes, HCE and LCE in medaka, *Oryzias latipes*. J Biochem 148:439–448

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