

The Genomic and Genetic Toolbox of the Teleost Medaka (*Oryzias latipes*)

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ABSTRACT The Japanese medaka, *Oryzias latipes*, is a vertebrate teleost model with a long history of genetic research. A number of unique features and established resources distinguish medaka from other vertebrate model systems. A large number of laboratory strains from different locations are available. Due to a high tolerance to inbreeding, many highly inbred strains have been established, thus providing a rich resource for genetic studies. Furthermore, closely related species native to different habitats in Southeast Asia permit comparative evolutionary studies. The transparency of embryos, larvae, and juveniles allows a detailed *in vivo* analysis of development. New tools to study diverse aspects of medaka biology are constantly being generated. Thus, medaka has become an important vertebrate model organism to study development, behavior, and physiology. In this review, we provide a comprehensive overview of established genetic and molecular-genetic tools that render medaka fish a full-fledged vertebrate system.

KEYWORDS teleost; *Oryzias latipes*; medaka; genetics; genomics

MEDAKA (*Oryzias latipes*) is a small freshwater fish of the family Adrianichthyidae in the order Belontiiformes. It is closely related to other members of the super-order Acanthopterygii of ray-finned fish such as pufferfish (tetraodon and fugu), stickleback and killifish, while it is separated from zebrafish by ~150 million years (MY) of divergent evolution (Figure 1). A growing number of sequenced teleost genomes and several established genetic model systems (zebrafish, stickleback, medaka) thus provide a unique resource for comparative studies relating to vertebrate evolution. Medaka is native to Taiwan, Korea, China, and Japan. In Japan it is found in small rivers, creeks, and rice paddies on all main islands with the exception of Hokkaido. Medaka is a euryhaline species and can also live in brackish water (Figure 2, A and B) (Inoue and Takei 2002). As a resident of a temperate zone, medaka can tolerate a wide range of temperatures (4–40°) both as adult and

embryo (Sampetrean *et al.* 2009). Thus, in the laboratory, temperature can be used to control the developmental speed without adverse effects.

Medaka native to Japan and East Korea have a diploid karyotype of 48 chromosomes, but 46 chromosomes in medaka from West Korea and China (Uwa and Ojima 1981). The different chromosome number is due to a fusion of chromosome 11 and 13 by a Robertsonian translocation (Myosho *et al.* 2012b). The haploid genome size is ~800 Mb (Kasahara *et al.* 2007). Adults can reach a length of up to 4 cm and the wild-type pigmentation is greyish-brown (Figure 2D). However, mutant strains are available where body pigmentation is strongly reduced both at embryonic and adult stages (Figure 2C) (Wakamatsu *et al.* 2001; Kelsh *et al.* 2004). Interestingly, sex-specific pigmentation can be used to distinguish male from female embryos as early as 3 days postfertilization (dpf) (organogenesis stages) (Wada *et al.* 1998), since the *leucophore free* (*lf*, *slc2a15b*) locus that is required for pigmentation by leucophores is located on the sex chromosomes X and Y (Kimura *et al.* 2014). The XX–XY sex determination system (see below) allows the establishment of mutant strains with females (XX) that are homozygous for a loss-of-function *lf* allele and males (XY) that are heterozygous and will therefore exhibit wild-type leucophore pigmentation. The strains, Qurt and FLF2, have the wild allele

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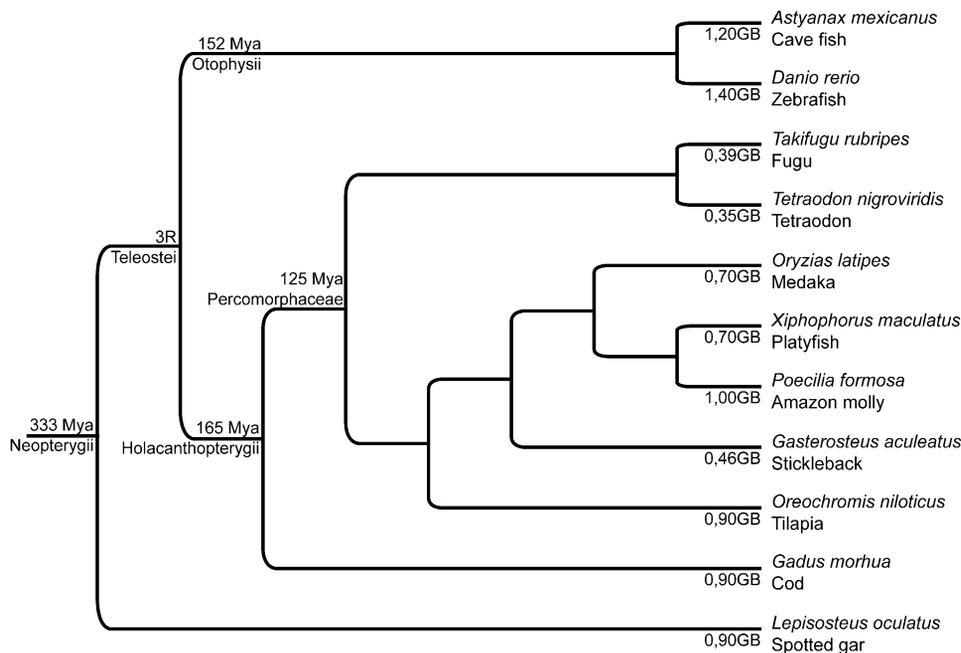


Figure 1 Evolutionary relationship of medaka and other teleost model systems. Only teleost model systems with publicly available genome sequence data on Ensembl are shown. The spotted gar is an outgroup of the teleost, which did not undergo a whole-genome duplication (3R). Medaka belongs to the Percormorphaceae, the clade with most sequenced teleost genomes, making it an ideal organism for comparative genomics. For each species, the Latin and the common name as well as the genome size is shown. The species tree was calculated using the Ensembl compara team (<http://www.ensembl.org/info/genome/compara/>). Taxonomy data are available at Ensembl and derived from the National Center for Biotechnology Information taxonomy browser.

on the Y chromosome and a mutant allele on the X chromosome, thus males have leucophores and female do not.

The life span of medaka under constant mating conditions (14 hr light/10 hr dark at 25–28°) in the laboratory is ~12 months. This can be extended to >2 years under conditions where the fish do not mate in combination with a reduced temperature (10 hr light/14 hr dark at 19°). Medaka originate from a temperate zone and show a seasonal mating behavior, requiring long light phases and short dark phases for reproduction, whereas temperature has little effect on fecundity (Koger *et al.* 1999). Medaka males and females can easily be distinguished due to a distinct sexual dimorphism. Most conspicuous is a slit in the male dorsal fin (Figure 2D). Medaka is oviparous with transparent eggs and embryos. Embryos hatch after 7–8 days at 28° as fully developed juvenile fish. The generation time of medaka is 8–12 weeks depending on strain and husbandry conditions.

Medaka Wild Populations and Laboratory Strains

In Japan, medaka is divided into two geographically separated populations: the northern population of the northern region of Honshu Island and the southern population of southern Honshu, Kyushu, Shikoku, and Okinawa with a divergence time between these two groups of ~4 MYA (Figure 3) (Takehana *et al.* 2004a,b). Based on morphological criteria, it has recently been suggested that the northern population is an independent species (*O. sakaizumii*) (Asai *et al.* 2011). Under laboratory conditions, productive matings between these groups can be obtained and the resulting F₁ and F₂ male and female hybrids are fully fertile (Sakaizumi *et al.* 1992). The degree of polymorphism between northern and southern strains is in the range of 1% in coding and 4% in noncoding regions (Naruse *et al.*

2004a). Since the northern and southern populations are highly polymorphic and isogenic inbred lines have been established from both populations, these inbred lines are important tools for genetic mapping purposes (Naruse *et al.* 2004b).

Medaka is highly tolerant to inbreeding. This has been exploited decades ago to establish highly inbred strains from different wild populations (Hyodo-Taguchi 1980). In some cases these strains have been inbred for more than 100 generations by successive brother–sister crosses and can therefore be considered as isogenic. Highly inbred strains are predominantly homozygous with heterozygosity levels of $\sim 3 \times 10^{-5}$, whereas in wild catch, this level is ~100-fold higher (1.5×10^{-3}). The inbred strain Nilan has fewer cycles of inbreeding and consequently is 2×10^{-4} heterozygous (Spivakov *et al.* 2014). Currently >60 wild strains from both the northern and southern populations and ~14 derived inbred strains are available at the Japanese Medaka Stock Center (National BioResource Project Medaka, NBRP Medaka; <http://www.shigen.nig.ac.jp>). Apart from genomic polymorphisms, these inbred strains also exhibit strain-specific differences in behavior, body shape, brain morphology, and susceptibility to mutagens (Ishikawa *et al.* 1999; Kimura *et al.* 2007). Heritability of craniofacial traits has been demonstrated, indicating that the inbreeding of polymorphic populations in medaka reveals the genetic contribution to variability of these traits (Kimura *et al.* 2007). To further exploit the tolerance to inbreeding combined with genetic polymorphism, recently a detailed analysis of wild populations has been carried out with the aim to establish a panel of >100 inbred lines derived from a single polymorphic wild population (Spivakov *et al.* 2014). Such a panel of inbred lines with sequenced genomes will serve as a genetic source for genome-wide association studies (GWAS).

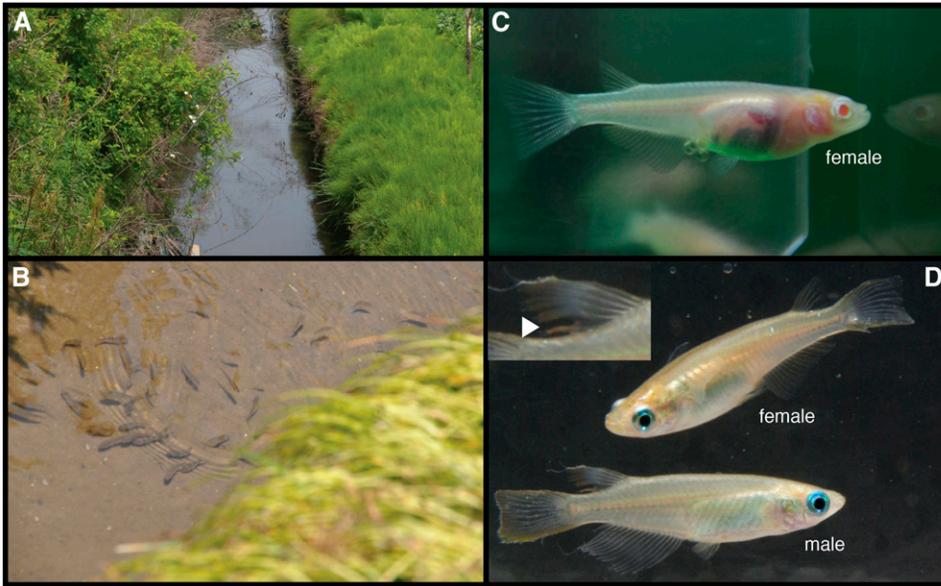


Figure 2 The Japanese medaka *Oryzias latipes*. (A and B) Medaka live in small rivers and creeks, such as irrigation systems of rice paddies. (C) The mutant QuiH strain completely lacks pigmentation. Internal organs are visible in adults. Due to the absence of body pigmentation, QuiH medaka are also free of autofluorescence. (D) The pigmentation of wild medakas is shown. Note also the sexual dimorphism of the dorsal fin. The male dorsal fin has a slit (arrowhead in inset).

Medaka Genomic Information

The medaka genome sequence project has been successfully completed (Kasahara *et al.* 2007) and the medaka draft genome is accessible through a number of genome browsers (http://www.ensembl.org/Oryzias_latipes/Info/Index, http://genome.ucsc.edu/cgi-bin/hgGateway?hgid=409674941_iubYP8aF1UOAsctCB3qI2E3t1Kff&clade=vertebrate&org=Medaka&db=0, and <http://viewer.shigen.info/medakavw/mapview/>). The medaka reference genome sequence is based on the Hd-rRII inbred line. In addition, the genome sequence of the HNI-II, Kaga, HSOK and Nilan and Kiyosu strains (Spivakov *et al.* 2014) are available (<http://www.ebi.ac.uk/birney-srv/medaka-ref-panel/data.html>). It is also possible to use blast searches against Hd-rR II and HNI-II scaffolds as well as raw shotgun reads of Hd-rRII (<http://dolphin.nig.ac.jp/medaka/index.php>). The NBRP genome browser also enables searches for SNPs in the HdrR, HNI, Nilan, HSOK, and Kaga strains (<http://medaka.lab.nig.ac.jp/cgi-bin/gb2/gbrowse/medaka/>).

Related Species

The genus *Oryzias* comprises >22 species that are widely distributed in South-Eastern and Western Asia and occupy a wide range of different habitats in tropical and temperate zones. This provides a rich source of species with a divergence time ranging from 4 to 30 MYA to study evolution and adaptation to different habitats. The most closely related species are *O. curvinotus* from Southern China and Vietnam and *O. luzonensis* from the Philippines. The divergence time of these sister species has been estimated to be ~5 MYA (Tanaka *et al.* 2007). A comparison of sex determination revealed an unexpected plasticity in the underlying molecular-genetic mechanism (Tanaka *et al.* 2007). *O. latipes*, *O. curvinotus*, and *O. luzonensis* possess an XX-XY sex determination system.

In *O. latipes* and *O. curvinotus* the sex determination genes DMY/DMRT1Yb, respectively, are on orthologous and syntenic male Y chromosomes (Matsuda *et al.* 2002, 2003; Nanda *et al.* 2002). On the other hand *O. luzonensis* has no DMY gene. There, *gsdf^Y* (gonadal soma derived growth factor on the Y chromosome) acts as the sex determination gene (Myosho *et al.* 2012a). Recently a third sex determination gene was identified in *O. dancena*. The sex chromosome of *O. dancena* is orthologous to the autosomal chromosome 10 of *O. latipes*. Positional cloning and loss-of-function experiments using zinc-finger nucleases (ZFNs) showed that the sex determination gene of *O. dancena* is *Sox3*, which is thought to be orthologous to the mammalian sex determination gene, *Sry* (Takehana *et al.* 2014). Thus, these closely related *Oryzias* species represent a unique paradigm to study this fast evolving trait. As these species live in different habitats, it is to be expected that also other traits, both physiological and behavioral have diverged. *O. latipes* crosses with either *O. curvinotus* or *O. luzonensis* result in sterile hybrids. However, *O. curvinotus* and *O. luzonensis* hybrids are fully fertile, thus a genetic segregation analysis of diverged traits between these sister species is possible (Tanaka *et al.* 2007).

Also external factors can influence sex determination in medaka. It has been shown that high temperatures during embryonic development can induce female-to-male sex reversal, whereas males are not affected (Sato *et al.* 2005; Hayashi *et al.* 2010). Also hypoxia can induce female-to-male sex reversal in medaka (Cheung *et al.* 2014).

In the following, we provide a detailed description of genetic and molecular-genetic tools that are available for medaka. Table 1 summarizes the described technologies.

Forward Genetics

Medaka has been used extensively as a genetic model for radiation biology (Shima and Shimada 1991, 2001).

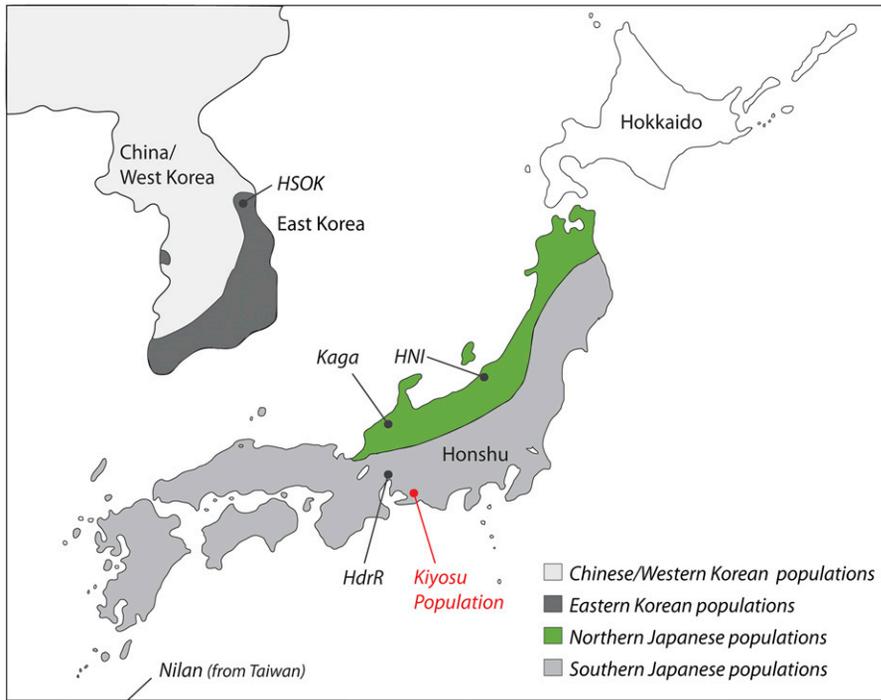


Figure 3 Geographical distribution of different *Oryzias latipes* populations in Japan, Korea, and China. Four main populations are distinguished based on genomic criteria such as allozyme variation and variation of mitochondrial DNA. The northern and southern populations are geographically separated and diverged about 4 MYA. The map was modified from that published in Spivakov *et al.* (2014).

Furthermore, systematic mutagenesis approaches, such as chemical mutagenesis, have been established for medaka (Shima and Shimada 1991, 2001; Ishikawa 2000; Loosli *et al.* 2000). The high fecundity, extra-uterine development, and transparency of eggs and embryos render medaka an excellent genetic system for unbiased forward genetic approaches using chemical mutagenesis. Furthermore, as outlined above, available highly inbred strains provide the uniform genetic background that is essential to both reduce genetic variance of a given phenotype and allow subsequent mapping of mutations. The mutagen of choice is ethylnitrosourea (ENU), which efficiently induces point mutations by base alkylation and thus results in genetically separable single gene mutations. Similar to ENU mutagenesis in zebrafish and mouse (Knapik 2000; Nguyen *et al.* 2011), a gene-specific mutation rate of $>1 \times 10^{-3}$ has been reported (Loosli *et al.* 2000; Furutani-Seiki *et al.* 2004). A number of small- and large-scale ENU mutagenesis screens have been carried out, where classical F_3 crossing schemes have been employed to identify recessive zygotic mutations affecting various processes (Loosli *et al.* 2000; Furutani-Seiki *et al.* 2004). Mutants from these screens are available at the NBRP Medaka Stock Center (<http://www.shigen.nig.ac.jp/medaka/strain/strainTop.jsp>).

Other forward genetic approaches rely on the mutagenic potential of transgene integrations. A major advantage of such approaches is that in most cases these mutations can be identified by locating the transgene insertion sites within the genome. In medaka, gene trap approaches with several transposons have been successfully employed (Sano *et al.* 2009; Froschauer *et al.* 2012). The transposase *Frog Prince* has been used to randomly integrate a GFP-based reporter

transgene with upstream splice acceptor sites (Sano *et al.* 2009). Insertion of this gene trap cassette results in GFP expression if the open reading frame is maintained in the spliced transcript. A total of 16% of the injected fish were transgenic founders that gave rise to GFP-expressing embryos. In addition to *Frog Prince*, the transposon system *Ac/Ds* has been used for gene trap approaches (Froschauer *et al.* 2012). The *Sleeping Beauty* transposon system was employed for enhancer trap screens (Grabher *et al.* 2003). In this case, a *Xenopus* cytoskeletal actin (*cska*) promoter was used to detect enhancer activities at the transgene insertion site. Thus, transgenic lines were generated with specific temporal and spatial reporter gene activity. It has recently been shown in zebrafish that such GFP reporter genes can be exchanged for a transactivator such as Gal4 using nonhomologous end joining induced by the Crispr/Cas9 system (Auer *et al.* 2014). Thus, once a line is obtained with a reporter expression pattern of interest, the enhancer trap can be repurposed to fit experimental needs.

Reverse Genetics

In fish model systems, antisense morpholinos are widely used to transiently interfere with gene function (Bill *et al.* 2009). These typically 25-bp-long oligos specifically interfere with gene function based on their complementarity to the target sequence either by blocking translation initiation or by interfering with splicing. The nonribose-based backbone renders morpholinos insensitive to enzymatic degradation. Recently, gene knockdown using peptide nucleic acids (PNAs) has been applied in medaka (Dorn *et al.* 2012). Both, morpholinos and PNAs are powerful antisense tools.

Table 1 Genetic and molecular-genetic tools established for medaka

Topic	Approach	Method
Forward genetics	Chemical mutagenesis Transposon mutagenesis	ENU mutagenesis (Loosli <i>et al.</i> 2000; Furutani-Seiki <i>et al.</i> 2004). Sleeping Beauty (Grabher <i>et al.</i> 2003; Grabher and Wittbrodt 2007) AcDs (Boon Ng and Gong 2011; Froschauer <i>et al.</i> 2012)
Reverse genetics	Antisense technologies miRNA studies Tilling Genome editing	Morpholinos (Thummel <i>et al.</i> 2006; Shestopalov <i>et al.</i> 2007; Thummel <i>et al.</i> 2008) PNA (Dorn <i>et al.</i> 2012) miRNA sponges miRNA knockdown miRNA mimics (Conte <i>et al.</i> 2010) ENU mutagenesis (Taniguchi <i>et al.</i> 2006) ZNF, TALEN, CRISPR/Cas9 (Ansay <i>et al.</i> 2012; 2013; Ansay and Kinoshita 2014; Ansay <i>et al.</i> 2014)
Transgenesis	Meganuclease Transposons	IScel (Grabher and Wittbrodt 2008) <i>Sleeping Beauty</i> , <i>AcDs</i> , <i>Frog Prince</i> , <i>Tol2</i> (Grabher <i>et al.</i> 2003; Grabher and Wittbrodt 2007; Sano <i>et al.</i> 2009; Boon Ng and Gong 2011; Froschauer <i>et al.</i> 2012; Kirchmaier <i>et al.</i> 2013)
Molecular-genetic tools	Site-specific recombinases Reporter lines Cell lineage tools Transactivation system	PhiC31 (Kirchmaier <i>et al.</i> 2013) Transgenic reporter cassettes with specific promoters Gaudi toolbox (Centanin <i>et al.</i> 2014) LexPR, Gal4, Tet system (Grabher and Wittbrodt 2004; Emelyanov and Parinov 2008; Knopf <i>et al.</i> 2010)
Gene expression studies	RNA detection Protein detection	<i>In situ</i> hybridization (ISH) (Ason <i>et al.</i> 2006; Inoue and Wittbrodt 2011) RNA seq (Schartl <i>et al.</i> 2012) Microarray (Kishi <i>et al.</i> 2006) Antibody staining (IHC) (Inoue and Wittbrodt 2011)

This is an overview of the vast toolbox that is available to tackle a wide range of biological questions.

PNAs have a higher affinity for RNA, yet they are less soluble and therefore the *in vivo* use is limited. Dorn *et al.* (2012) changed the chemical composition of the PNA backbone to increase solubility and showed efficient knockdown of the *six3* gene in medaka. In most cases these antisense morpholinos are injected into fertilized eggs at early cleavage stages to ensure a ubiquitous distribution to all cells of the developing embryo. If thus applied, they interfere with gene function during early development. To study gene function during later stages, morpholinos can be activated conditionally by light-induced uncaging (Shestopalov *et al.* 2007). Alternatively they can be applied to specific tissues by electroporation (Thummel *et al.* 2006, 2008). Also caging of mRNA has been used to conditionally express genes in fish embryos (Ando *et al.* 2001). However, recent results in zebrafish indicate that morpholino-based gene knockdown often results in unspecific off-target effects (Kok *et al.* 2014). Thus the use of small antisense molecules to study gene function also needs to be thoroughly evaluated in medaka.

To study the function of endogenous microRNAs (miRNAs), several tools have been applied in medaka (Conte *et al.* 2010). First, morpholinos directed against pre-miRNAs have been successfully used to knockdown mir204 and specific effects during retinal development have been observed. In the same study, commercially available miRNA mimetics (miRIDIAN microRNA Mimics) have been used to mimic mir204 activity. This was verified with dosage sensors, *i.e.*, fluorescent reporter genes that contain miRNA binding sites in the 3' UTR, such that binding of miRNAs to the reporter messenger RNA (mRNA) affects expression. In this way,

Meis2 has been verified as a target gene of mir204 by a target protector assay. Here, small RNAs occupy the putative miRNA binding site on the mRNA and thereby block miRNA-dependent inhibition of translation or mRNA degradation (Conte *et al.* 2010). So far no targeted approaches have been described in medaka, in which miRNA mimetics or knockdown tools were expressed in a tissue-specific manner. However, tissue-specific expression of short hairpin RNAs (shRNAs) has been described in several species, including mouse (Zuber *et al.* 2011) and zebrafish (Dong *et al.* 2009). Here, artificially constructed miRNAs based on the endogenous miRNA backbone of mir30e are inserted within a transgene and transcribed from a pol II promoter. However, conflicting results in zebrafish indicate that the use of shRNAs may not be applicable for all target genes and its functionality needs to be thoroughly tested on a case-by-case basis (Dong *et al.* 2009; Kelly and Hurlstone 2011; De Rienzo *et al.* 2012). Nevertheless, the use of shRNAs seems a promising approach to down-regulate gene expression levels in a controlled and tissue-specific manner in fish model systems (Dong *et al.* 2009).

Other genomic resources have been established to isolate mutations for specific genes in medaka. The targeted induced local lesions in genome (TILLING) method is one of the reverse genetic or gene-driven approaches based on random mutagenesis by ENU. A medaka TILLING library has been established that consists of 5760 ENU mutagenized F₁ fish (offspring from ENU-treated males crossed to wild-type females) and their genomic DNA archives. The average mutation rate of this medaka TILLING library is one mutation

per 350 kb. The resource center NBRP Medaka provides a screening system for the genomic DNA archives with high-resolution melting (HRM). Identified mutations can be recovered by *in vitro* fertilization of the corresponding sperm with wild-type eggs (see <http://www.shigen.nig.ac.jp/medaka/strain/aboutTilling.jsp>) (Taniguchi *et al.* 2006).

Several tools that allow specific manipulations of the genome have been developed recently that are applicable to many organisms, including medaka. These tools are based on sequence-specific binding of a nuclease that cleaves the genomic double-stranded DNA (dsDNA), thereby inducing a double strand break (DSB) (Gaj *et al.* 2013). Subsequently, the DSB repair by nonhomologous end joining (NHEJ) introduces small insertion/deletions (indels) at high frequency and thus introduces mutations. In addition to introducing mutations, exogenous DNA can be inserted into the site of the DSB via NHEJ (Auer *et al.* 2014) or homology-directed repair (HDR) (Bedell *et al.* 2012). Two types of sequence-specific nucleases are used for these purposes. ZFNs as well as transcription activator-like effector nucleases (TALENs) have a bipartite structure with a flexible and modular DNA binding domain fused to the effector FokI nuclease. A modular set of ZFNs or TALENs can be designed to bind a specific sequence in the genome and induce a DSB at that site (Ansai *et al.* 2012, 2013). Recently, it was shown that sequence-specific binding of a guide RNA coupled to a nuclease can very efficiently induce DSBs (Cong *et al.* 2013; Mali *et al.* 2013). This CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9-based system uses a single guide RNA (sgRNA) that targets the nuclease Cas9 in a sequence-specific manner to generate a DSB. Importantly, the use of TALENs and the CRISPR/Cas9 system has been greatly simplified. Highly efficient cloning methods for TALENs have been developed (Cermak *et al.* 2011; Sanjana *et al.* 2012; Ma *et al.* 2013; Sakuma *et al.* 2013; Schmid-Burgk *et al.* 2013). For the CRISPR/Cas9 system, it suffices to adjust the short target site within the guide RNA according to the genomic target sequence.

Initial reports of targeted mutagenesis in medaka demonstrated the ZFN- and TALEN-mediated knockout of an EGFP transgene (Ansai *et al.* 2012) as well as endogenous genes (Ansai *et al.* 2013, 2014). Also site-directed mutagenesis using the CRISPR/Cas9 system has been successfully employed in medaka (Ansai and Kinoshita 2014). The authors showed that genome modifications by CRISPR/Cas9 were transmitted to the F₁ with frequencies ranging from 42.9 to 100%. Recently, a medaka target site prediction tool for sgRNAs has been established (CCTop: Medaka CRISPR target predictor, <http://crispr.cos.uni-heidelberg.de/index.html>). This tool assists in the design of sgRNAs for any target sequence. The target site as well as potential off-target sites in a genome are predicted and the best target site can be chosen based on the likelihood for off-site targeting. Importantly, potential target and off-target sites in the medaka genome are matched with gene annotations that were refined with RNAseq data (T. Thumberger, J. L. Mateo

and J. Wittbrodt, unpublished data). CRISPR sgRNA evaluation and microhomology prediction tools are available at NBRP Medaka (<http://viewer.shigen.info/cgi-bin/crispr/crispr.cgi>).

Transgenesis Toolbox

Transgenic medaka fish can be generated using several established protocols. To introduce the required DNA, RNA, and proteins into zygotes, microinjection is the method of choice, albeit electroporation (Inoue *et al.* 1990), intracytoplasmic sperm injection (Liu *et al.* 2011), as well as the use of a particle gun (Kinoshita *et al.* 2003) has been reported. A detailed protocol for microinjection into fertilized medaka eggs is available (Rembold *et al.* 2006a).

Microinjection of plasmid DNA leads to the integration of multiple copies of the entire plasmid. Often these insertions are concatemers with a high-copy number. Furthermore the generation of transgenic fish using plasmid DNA is inefficient (1–10%) due to a low genomic integration rate and highly mosaic distribution in the injected animal (Thermes *et al.* 2002). Meganuclease ISceI-mediated transgenesis is more efficient (25–50%). Plasmid DNA containing two ISceI recognition sites is co-injected with meganuclease ISceI protein (Grabher and Wittbrodt 2008). The transgenesis vector is cut by the meganuclease that contains nuclear localization signals, which are thought to increase nuclear import of the injected DNA. The resulting linear DNA is efficiently integrated into the genome via unknown mechanisms. Meganuclease-based transgenesis results in the insertion of low-copy number concatemers at multiple sites. Additionally, single-copy transgene integrations are possible and can be screened for (Thermes *et al.* 2002).

Transgenes can be integrated into the genome via the use of transposons. The *Sleeping Beauty* (SB) transposon belongs to the Tc1/mariner transposon family. It has been established by reconstituting a functional transposon from silent transposon fragments identified in the genomes of rainbow trout and Atlantic salmon (Ivics *et al.* 1997). Further optimization of the system allowed to generate hyperactive versions of SB, most notably SB100x (Mátés *et al.* 2009). The original as well as hyperactive versions have been used for the efficient generation of transgenic medaka fish (Grabher *et al.* 2003; Grabher and Wittbrodt 2007; Kirchmaier *et al.* 2013). SB leads to integrations with single-copy insertions as well as low-copy tandem arrays (Grabher *et al.* 2003). Other transposases that have been successfully used in medaka are *Frog Prince* (Sano *et al.* 2009), another Tc1/mariner transposase, and *Ac/Ds* (Boon Ng and Gong 2011; Froschauer *et al.* 2012), a transposase belonging to the hAT family like *Tol2*. *Tol2* is an autonomous transposon that has been identified in the genome of medaka (Transposon *oryzias latipes*) (Abe *et al.* 2011). This transposon is widely used to mediate genomic insertion of exogenous DNA in zebrafish. Thus, a plethora of functionally established *Tol2*-based constructs are available. Interestingly, *Tol2* can

also be used as a transgenesis vector in medaka, since expression of *Tol2* transposase has no or very few deleterious effects on the medaka genome (Takehana *et al.* 2014). It is thus possible to use established *Tol2*-based constructs from zebrafish also in medaka. It is of course necessary to test in each case the promoter activity, as species-specific differences may result in different temporal and spatial expression patterns.

To select potential transgenic founder fish, a coselection marker is often used. For example, two transgenesis vectors can be co-injected and often the transgenes on both vectors are co-inserted (Rembold *et al.* 2006a). Alternatively, a selection reporter cassette is included in the transgene. For that, strong tissue-specific promoters such as the lens *crystallin* promoter (Vopalensky *et al.* 2010) or the heart muscle-specific *cmlc2* promoter (Auman *et al.* 2007) are routinely used. This allows assessing the distribution of the injected DNA in the injected embryos (mosaicism) and thereby the preselection of potential transgenic founder fish. However, such linked reporter cassettes can also interfere with the transgene proper (Kirchmaier *et al.* 2013).

These protocols discussed above result in transgene insertions at random genomic loci. Since position effects may affect transgene function and insertional mutagenesis may occur, several independent transgenic lines of a given transgene have to be assessed to verify an observed trait, either expression or phenotype. Recently, a site-specific transgenesis toolbox for medaka has been established that relies on the serine integrase derived from the bacteriophage PhiC31 (Kirchmaier *et al.* 2013). The integrase PhiC31 mediates the recombination between an attP site in the genome and an attB site in a targeting vector. Transgenic lines harboring single intergenic insertion sites containing the attP sequence in gene deserts were established (Figure 4B). These insertion sites serve as neutral integration sites for all transgenes of interest containing the attB site. Importantly, the genomic loci have been characterized using the Hsp70 promoter, indicating that position effects in the targeted loci are negligible. Since the same integration site can be used for different transgenes, the resulting transgenic lines are directly comparable. The landing site is designed such that correct transgene integration results in a specific change of fluorescence, allowing to screen for correct genomic integration and therefore to preselect for transgenic founder fish. Additionally, a Cre-mediated locus cleanup step removes most parts of the initial integration site, reducing interference from linked coselection markers.

Molecular-Genetic Toolbox

Reporter transgenes based on the expression of fluorescent proteins are often used to study gene function. The transparency of embryonic tissue including the brain allows the analysis of such reporter genes *in vivo*. Furthermore, pigmentation mutants that are almost completely transparent also allow reporter gene analysis in adults (Wakamatsu *et al.*

2001). Thus, medaka is ideally suited to analyze regulatory elements *in vivo* (Mongin *et al.* 2011; ENCODE Project Consortium *et al.* 2012; Kirchmaier *et al.* 2013). In addition, reporter gene expression can be used to label specific tissues and cell types (Tanaka *et al.* 2001; Rembold *et al.* 2006b; Martinez-Morales *et al.* 2009; Willems *et al.* 2012). In Figure 4B, the result from an enhancer test assay using PhiC31 integrase is depicted. In this case, the integration of regulatory DNA elements by PhiC31-mediated recombination results in specific expression of the GFP reporter gene that is present in the landing site on chromosome 18. Importantly, GFP expression originates from the targeted locus only and high somatic activity of PhiC31 integrase allows the analysis of reporter gene expression directly in the injected embryos. Centanin *et al.* (2011) showed that all cells in a transgenic line can be permanently labeled using a ubiquitous enhancer/promotor to drive GFP expression. Medaka has also been used to study the higher order neuronal circuits of specific taste bud cells using transgenic reporter lines (Ieki *et al.* 2013). In this case, the *wheat germ agglutinin* (WGA) gene was expressed under the *mfplc-β2* promoter. WGA protein is a *trans*-synaptic tracer that is used to study neuronal networks (Yoshihara 2002). Recently, osteoblasts have been conditionally ablated using a fusion of CFP with the *nitroreductase* gene (Willems *et al.* 2012). This CFP-NTR fusion gene was specifically expressed in osteoblasts using the osterix promoter. Prolonged treatment with the drug metronidazole (Mtz) induced apoptosis specifically in CFP-NTR-expressing cells. Also, medaka has been used to study environmental influences using transgenic reporter lines as bioindicators for the hormone estrogen, androgen activity, toxic compounds such as 2,3,7,8-Tetrachlordibenzodioxin and heat stress (Zeng *et al.* 2005; Boon Ng and Gong 2011; Kawaguchi *et al.* 2012; Ng and Gong 2013; Sébillot *et al.* 2014).

Several tools have been generated to achieve temporal as well as spatial control of transgene expression. Ubiquitous gene expression for a given time period can be achieved using heat shock promoters. The endogenous hsp70 promoter from zebrafish (Blechinger *et al.* 2002) as well as artificially constructed heatshock promoters (Bajoghli *et al.* 2004) provide temporal control to transgene expression. For example, a heatshock by elevating the ambient temperature from 28° to 37° for 20 min results in a strong activation of the heatshock promoter in medaka embryos (Oda *et al.* 2010; Herder *et al.* 2013). Additionally, heatshocks can be applied locally using a hot soldering iron (Hardy *et al.* 2007) or infrared (IR) laser light with the IR-laser-evoked gene operator (LEGO) system (Kamei *et al.* 2008; Deguchi *et al.* 2009; Okuyama *et al.* 2013). Given the transparency of the medaka model, the temperature can be increased locally using infrared laser light with single-cell resolution, thereby activating a heatshock promoter only in that cell. This allows precise fate-mapping studies (Shimada *et al.* 2013). In this study, the expression of Cre recombinase is initiated via IR-LEGO in cells of the somites. These cells are permanently labeled by Cre-mediated induction of GFP expression and ultimately reveal the mesodermal origin of the trunk

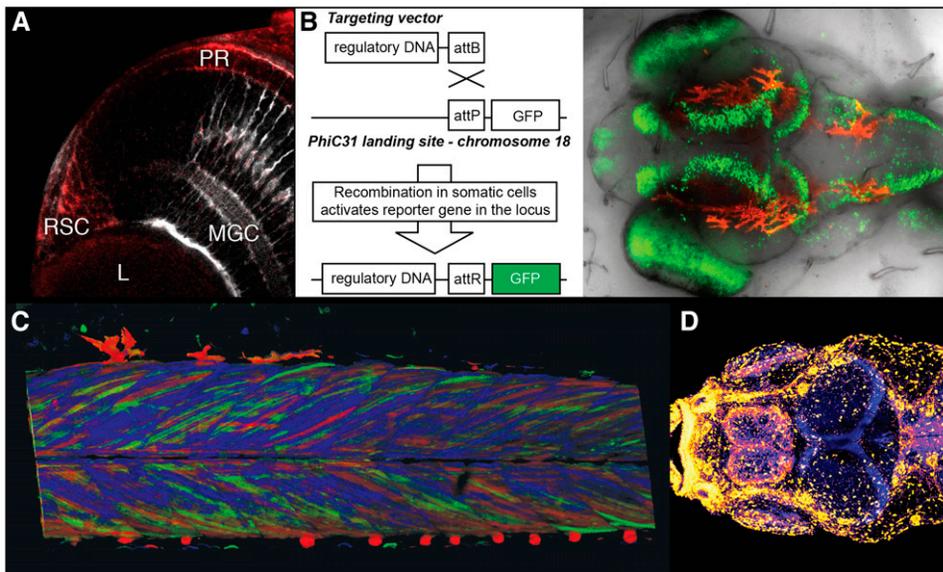


Figure 4 Gene expression and cell lineage analysis in medaka. (A) Whole-mount *in situ* hybridization to specifically detect retinal homeobox gene *Rx2* mRNA in the retina. *Rx2* (red) is expressed in photoreceptor cells (PR), stem cells (RSC) in the ciliary marginal zone of the retina, and Mueller glia cells (MGC). L, lens. Glutamine synthase protein in Mueller glia cells is codetected by antibody staining (white), indicating *Rx2* expression in Mueller glia cells (red dots inside white MGCs). (B) Recombination scheme: the GFP reporter gene is present in the PhiC31 landing site. The regulatory DNA to drive GFP reporter gene expression is provided with a targeting vector. PhiC31 integrase activity leads to the recombination of the regulatory DNA into the GFP reporter gene cassette in the genome. *In vivo* imaging of neuroblal GFP expression from a

brain-specific enhancer within a PhiC31 landing site. Note autofluorescent pigment cells (red). (C) *In vivo* analysis of 8-dpf Gaudi fish. Stochastic recombination of a brainbow cassette by heatshock-activatable Cre recombinase results in a differential labeling of cells in the entire body. A 3D representation of the somites is shown. (D) A whole-mount BrdU incorporation assay in a 10-dpf hatchling reveals proliferating cells (yellow) in the body. Nuclei are counterstained with Dapi (blue).

exoskeleton. Heat-inducible Cre transgenic lines and Cre-mediated GFP expression lines (TG918 and TG891, respectively) are available from NBRP (<http://www.shigen.nig.ac.jp/medaka/>). Alternatively, inducible transactivation systems can be used. However, the use of such systems in medaka is still quite limited. A heatshock-inducible Gal4/upstream activating sequence (UAS) transactivation system has been published (Grabher and Wittbrodt 2004). Other inducible systems that are used in zebrafish, such as mifepristone inducible hybrid transcription factor LexPR systems (Emelyanov and Parinov 2008) or the Tet (tetracycline-controlled transcriptional regulation) On/Off system (Knopf *et al.* 2010) are functional in medaka (J. Wittbrodt, unpublished data). Thus, several driver lines for the Tet transactivation system are available at the Medaka Stock Center of NBRP (<http://www.shigen.nig.ac.jp/medaka/>).

Temporal control of transgene activation can also be achieved using site-specific recombinases. Transgene activation can be achieved by recombination-based removal of a stop cassette or fluorescence reporter gene that separates the gene of interest from a promoter. These cassettes are flanked by the recognition sites for recombinases such as loxP (Matsuzaki *et al.* 2013) or FRT (Itou *et al.* 2009). Recombinase activity can be induced using specific promoters, heatshock (Shimada *et al.* 2013; Kobayashi *et al.* 2013; Okuyama *et al.* 2013; Centanin *et al.* 2014), or drug admission (Yoshinari *et al.* 2012). In contrast to the tools described above, in this case transgene activation is irreversible after the activation of recombinase. In most cases Cre or Flp recombinase have been used for this purpose. These site-specific recombinases recognize specific DNA sequences, the recognition target sites (RTSs). For Cre and Flp, recombinases from the A1 family, specific RTSs exist. They have

a defined structure with two inverted repeat regions around a central core. Heterologous sites exist in which the sequence of the central core differs. Recombination can only occur between sites with identical central core sequences. This allows the combinatorial use of heterologous RTSs. Depending on the orientation to each other, recombination between two RTSs either leads to deletion or inversion of the sequence in between (reviewed in Branda and Dymecki 2004).

Site-specific recombinases are also used to mediate stochastic activation of transgene expression. A prominent example that relies on this approach is the brainbow system (Livet *et al.* 2007; Pan *et al.* 2013). Here, recombinase activity leads to a rearrangement of the transgene in a stochastic manner such that different fluorescent proteins are expressed from the transgene in different cells (Figure 4C). Multiple copies of the cassette in the genome lead to the stochastic combination of expressed fluorescent proteins enabling color barcoding of cells (Höckendorf *et al.* 2012; Pan *et al.* 2013). So far, the functionality of stochastic cell labeling using recombinases in teleosts has been demonstrated in zebrafish (Gupta and Poss 2012; Pan *et al.* 2013) and in medaka (Centanin *et al.* 2014). This medaka toolbox, Gaudí, allows a lineage analysis of individual cells by stochastic labeling of cells at embryonic or adult stages. The toolkit consists of several ubiquitously expressing brainbow lines with heatshock- or tamoxifen-inducible Cre driver lines.

In many cases, it is important to label cells that express an untagged transgene. To achieve this, vectors harboring reporter gene constructs can be co-injected with other transgene-containing vectors using a meganuclease- or transposase-based approach (Rembold *et al.* 2006a). The different vectors mostly cosegregate in the progeny of the

injected cell, thereby leading to coexpression. Alternatively, internal ribosome entry sites (IRESs) have been used successfully (Fukamachi *et al.* 2009; Matsuzaki *et al.* 2013). However, IRESs appear to have context-dependent limitations (Tanaka and Kinoshita 2001). Pseudopolycistronic open reading frames separated by short viral sequences (such as T2A) allow a more robust coexpression of several proteins from a single transgene in equal amounts (Provost *et al.* 2007). The conformation of these short viral peptides induce a cotranslational staggering of the ribosome and resulting lack of a peptide bond formation in the nascent polypeptide chain. This results in a physical separation of the polypeptide chains. The use of T2A constructs circumvents the need to use fusion proteins and thereby precludes potential artifacts resulting from the fusion. Recently, this technique has been used to label medaka retinal cells that ectopically express variants of small GTPases (Herder *et al.* 2013). Also, this technique has been utilized in medaka to visualize Cre expression by mCherry fluorescence (Yoshinari *et al.* 2012).

Gene Expression Studies

Established protocols are available for gene expression studies either in whole mount embryos or sections. These *in situ* hybridization methods use antisense RNA probes labeled with either fluorescein or digoxigenin to visualize complementary RNA molecules in fixed tissues. Detection relies on color reactions using either alkaline phosphatase- or horseradish peroxidase-coupled antibodies (Henrich *et al.* 2005). To analyze coexpression of genes at high resolution with confocal microscopy, peroxidase-dependent tyramide signal amplification (PerkinElmer) linked to fluorescent dyes can be used. Antisense locked nucleic acid probes have been successfully used to visualize the expression of miRNAs in medaka (Ason *et al.* 2006). Detection of mRNAs by *in situ* hybridization can also be coupled with detection of proteins by antibodies using immunocytochemistry (Figure 4A) (Inoue and Wittbrodt 2011).

The number of available antibodies that are specific for medaka proteins is still limited. Nevertheless, a large number of antibodies against mouse or human epitopes has been shown to be functional for immunocytochemistry in medaka. Furthermore, a heatshock-based epitope retrieval step has been established (Inoue and Wittbrodt 2011). This protocol improves the immunostaining for several antibodies by a simple heatshock-based epitope retrieval step. While the aforementioned methods are useful to study a limited number of genes, genome-wide analysis of gene expression has been performed in medaka using microarrays as well as RNAseq. Especially, DNA oligonucleotide microarrays have been used to study the impact of toxic compounds on gene expression (Kishi *et al.* 2006). RNAseq has been used to study the transcriptome of melanoma in a medaka tumor model. The authors were able to identify alternatively spliced transcripts between different melanoma subtypes (Schartl *et al.* 2012). RNAseq in combination

with the analysis of epigenetic marks (histone modifications) was successfully applied for a comparative study to analyze gene expression during the phylotypic period of medaka and zebrafish development (Tena *et al.* 2014). The authors found that similarities in gene expression correlate with the phylotypic period and the existence of key constrained nodes in the gene networks governing the vertebrate body plan has been postulated.

Cell Transplantation

Cell transplantation is used to efficiently generate chimeric individuals (Ho and Kane 1990; Hong *et al.* 1998). This approach is frequently used to study cell lineages and to address cell autonomy *vs.* nonautonomy of a given process (Winkler *et al.* 2000). Fish model systems such as medaka and zebrafish are especially well suited for transplantation assays due to the extrauterine development of the relatively large eggs. To generate chimeric embryos, cells are transplanted at the blastula stage from a donor to a host embryo using glass needles. The extent of chimeric tissue can be manipulated by the number of transplanted cells. Furthermore, the developmental potential and determination of cells can be tested by heterotopic transplantation where cells are transplanted to an ectopic position. Transplanted cells are labeled by either stable inert compounds such as fluorescein-dextran or by genetic means. Frequently ubiquitous or specific promoters driving expression of fluorescent proteins are used to label donor- and host-derived cells (Rembold *et al.* 2006b; Centanin *et al.* 2011).

By cell transplantation into adult fish, a melanoma model was established in medaka (Hasegawa *et al.* 2009). Here, GFP-labeled melanoma cells were introduced into adult fish and thus tumor development was visualized. This allowed the study of tumor cell behavior such as proliferation or metastasis *in vivo*.

Furthermore, cell transplantation can be used to obtain donor-derived gonads in sterile hosts. For example, cells of fertile donors can be transplanted into sterile *Oryzias latipes* × *Oryzias curvinotus* hybrids. The transplanted cells can then give rise to gonads in the otherwise sterile host. Shimada *et al.* (2007) used this approach to obtain maternal-zygotic mutant embryos that allowed the study of maternal contribution to *fgfr1* function.

Resources

An excellent source of information is Kinoshita *et al.* 2009.

Researchers working with medaka can rely on a wide range of resources that are coordinated and provided by the NBRP Medaka. NBRP Medaka, which was initiated in 2002, is a central repository and archive for medaka resources (Sasado *et al.* 2010). The core facilities of NBRP Medaka are the National Institute for Basic Biology (NIBB, Okazaki, Japan), Niigata University, Riken, and Miyazaki University. NBRP Medaka provides three different resources, namely,

medaka strains, genomic resources, and hatching enzymes (<http://www.shigen.nig.ac.jp/medaka/>). As of 2014, NBRP Medaka provides 548 medaka strains including 373 mutants, 74 transgenic lines, 11 inbred lines, 6 consomic lines (chromosome replacement between two different inbred lines), 65 wild stocks, and 20 medaka-related species (<http://www.shigen.nig.ac.jp/medaka/strain/strainTop.jsp;jsessionid=A637DE046C1536C0251485E216900ECF>). NBRP Medaka also provides medaka complementary DNA (cDNA) clones and transcriptome data (<http://www.shigen.nig.ac.jp/medaka/est/est.jsp>). At this website, 730,259 sequences from 33 cDNA libraries, including 11 full-length cDNA libraries are available. Furthermore, BAC and fosmid libraries of *O. latipes* and BAC libraries of *O. dancena*, *O. luzonensis*, *O. celebenesis*, *O. hubbsi*, and *O. javanicus* are available. End sequence data of *O. latipes* BAC and fosmid clones allow *in silico* identification of the clones of interest by blast similarity search (<http://www.shigen.nig.ac.jp/medaka/est/blast.jsp>) as well as using a genome browser (<http://viewer.shigen.info/medakavw/mapview/>). In addition, 3D PCR pools of all BAC libraries are available. Three-dimensional screening requests for libraries should be submitted to NBRP Medaka (mbrc@nibb.ac.jp).

The NBRP Medaka website provides several databases: the *Medaka Book* is an online lab-manual with basic techniques for medaka maintenance and husbandry as well as basic and advanced laboratory protocols; the *Medaka Atlas* describes the brain anatomy and blood vascular system; the *Medaka Tree* depicts phylogenetic relationships of medaka wild population and medaka related species based on mitochondrial DNA sequences. Furthermore NBRP Medaka hosts genome browsers. *Medaka Map (beta)* (<http://viewer.shigen.info/medakavw/mapview/>) is a genome browser with several useful tools: physical maps, alignment view, blast search, *in silico* PCR, sequence cutter, pattern match-ZFN, pattern match CRISPR, and search for CRISPR target sites. TILLING provides the basic information of the medaka TILLING library and information for its screening (<http://www.shigen.nig.ac.jp/medaka/strain/strainTillingPlateAction.do>).

The medaka chorion is very hard and thus has to be removed for many experimental manipulations of embryos. The most effective way to remove the chorion is using medaka hatching enzyme. Medaka hatching enzyme is not commercially available; however NBRP Medaka is providing hatching enzyme as a crude hatching solution (supernatant of hatching embryos).

Cryopreservation of Sperm and Primordial Germ Cells

Medaka sperm cryopreservation is well established and routinely used for long-term preservation of medaka fish strains. Sperm that has been cryopreserved for >10 years shows no decrease of sperm motility and fertilization rate. A detailed protocol for sperm cryopreservation is described in Kinoshita *et al.* 2009.

Medaka eggs, as many other teleost eggs, are very large and yolk rich compared to mammalian eggs. Thus, cryopreservation of mature eggs has not been successful so far. However, primordial germ cells (PGCs) of fish can produce sperm and eggs after germ cell transplantation. In rainbow trout, PGCs were successfully cryopreserved and transplanted to hosts, from which donor-derived offspring were obtained (Kobayashi *et al.* 2007). Recently, it was shown that eggs and sperm could be generated from cryopreserved whole testes of rainbow trout (Lee *et al.* 2013). Seki and colleagues found that this protocol is applicable to medaka (S. Seki, personal communication). Thus cryopreservation of whole testes and subsequent transplantation of these cryopreserved to host gonads will allow the cryopreservation of medaka strains. This will allow the long-term storage of live inbred strains.

Future and Perspectives

Medaka offers a number of unique features and resources that in combination with established state-of-the art molecular-genetic tools make it an important vertebrate model. A century of genetic studies has provided a rich resource of strains and genomic data that serve as a basis for genetic studies such as interaction with environment and dissection of complex traits. A wide range of live and genomic resources as well as data repositories have been centralized and are available through NBRP Medaka. Newly established tools enable targeted manipulation of gene function. Specific genome manipulations are now possible with the newly established CRISPR/Cas9 as well as ZNF and TALEN systems. Importantly, these novel approaches enable homologous recombination as a means for precise genomic manipulations in medaka. Closely related species from a wide range of habitats provide the resources for comparative studies addressing evolution and speciation. This will be complemented with comparative studies using other established teleost genetic models to study vertebrate evolution. Finally, a panel of inbred lines is in the making that will serve as a medaka reference panel to study multigenic traits by GWAS.

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