MEDAKA — A MODEL ORGANISM FROM THE FAR EAST

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Genome sequencing has yielded a plethora of new genes the function of which can be unravelled 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. Already, the popularity of medaka among developmental biologists has led to important insights into vertebrate development.

ZOOGEOGRAPHY
The discipline of biology that deals with the geographical distribution of animals.

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Medaka, *Oryzias latipes*, is a small, egg-laying freshwater fish native to Asia that is found primarily in Japan, but also in Korea and eastern China. Many colour prints, known as Ukiyo-e, from the Edo period of the seventeenth to the nineteenth centuries, show that medaka, which in Japanese means a tiny fish with big eyes, once used to be an integral part of everyday life in Japan[1].

The physiology, embryology and genetics of medaka have been extensively studied for the past 100 years[2]. It was first described scientifically under the name of *Poecilia latipes* and appeared in the *Fauna Japonica* in 1850, which was written by Phillip Franz von Siebold — a physician, surgeon and naturalist[3] (FIG. 1). In 1906, Jordan and Snyder[4] renamed this species *Oryzias latipes*, a name that was chosen to reflect the preferred habitat of medaka — the rice (*Oryza sativa*) fields. This habitat also gave rise to the common English name of medaka — ricefish.

Medaka was first used to show Mendelian inheritance in vertebrates from 1913 onwards (REFS 5–7). However, the systematic genetic analysis of medaka dates back to 1921, when it was the first vertebrate in which the occurrence of crossing over between X and Y chromosomes was shown[5]. Subsequently, genetic studies in medaka have focused on pigmentation and sex determination, as discussed in more detail below.

The key reference for medaka as a genetic model system was published in 1975 — *Medaka* (*Killifish*): *Biology and Strains* by Tokio Yamamoto[2]. As well as reviewing medaka research at that time, it covered topics ranging from systematics and ZOOGEOGRAPHY, to early embryonic development and the genetic control of sex differentiation[2]. Subsequent important advances in medaka research have included the establishment of inbred strains (reviewed in REF. 8), the development of transgenesis protocols[9] and the establishment of MULTI-LOCUS TESTER STRAINS[10]. The development of mutagenesis protocols[10] in 1991 led to the first systematic mutagenesis screens for developmental phenotypes[11] and, in combination with detailed descriptions of medaka anatomy[12–14], they have led to the characterization of many mutant phenotypes that were recovered from these screens. Genomic resources, combined with a detailed linkage map, have also facilitated cloning of the genes that are responsible for these mutant phenotypes[15–17], which shows the power and the potential of medaka as a genetic model system.

Comparative studies of more distantly related vertebrate species are essential if we are to identify the conserved, as well as the species-specific, genetic and molecular mechanisms that underlie development and evolution. Medaka and zebrafish are ideal for this purpose, as both separated from their last common ancestor ~110 million years (Myr) ago (FIG. 2). This evolutionary distance is reflected in many aspects of their biology, including early development and sex determination. Both fish models offer several advantages, and both combine the power of genetics with

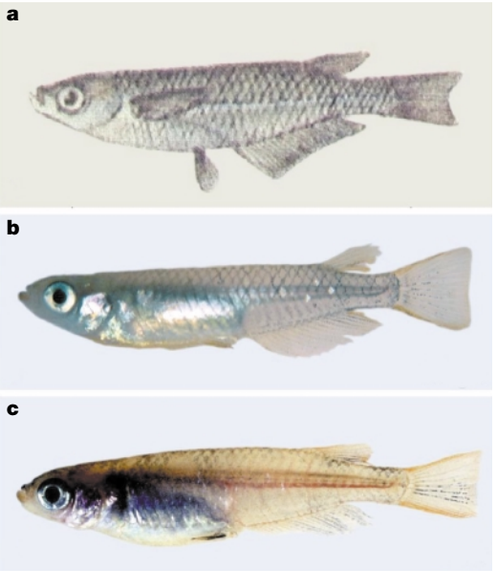

Figure 1 | **Views on medaka. a** | A hand-coloured lithograph of a male, probably derived from the northern population of medaka, from the first description of medaka that was published in 1850 (REF. 3). Males from the **b** | northern and **c** | southern inbred strains differ significantly in body shape and in pigmentation. Nevertheless, both strains can be interbred to produce fully fertile hybrids.

experimental embryology and molecular biology. Their short generation time of 2–3 months is comparable with the mouse, but is considerably shorter than that of *Xenopus laevis* (3 years) or *Xenopus tropicalis* (6 months). Furthermore, the synchronous extra-uterine development of many transparent eggs makes it easy to follow early developmental processes in both fish. Large-scale genetic screens for genes that are involved in vertebrate development and pathogenesis are in progress in medaka and have been completed in zebrafish[11,14,18]. Notably, the zebrafish screens have yielded some mutants with diseases that closely resemble human genetic diseases, and therefore can serve as models for them[19–21].

Whereas zebrafish and its features as an experimental system are well known among biologists, little is known about the applications or advantages of research on medaka outside Japan and the Far East[22]. Here, we review the potential of medaka as a genetic model system and emphasize its complementarity to other vertebrate model systems in studies of developmental biology and evolution. Like any model system, zebrafish has its limitations as to the types of question that it can be used to address, the most prominent example being sex determination. So far, nothing is known about sex determination in zebrafish and, remarkably, none of the many markers of the zebrafish genetic map have been found to be sex linked[23–25]. Conversely, medaka has clearly defined sex chromosomes, and sex determination is intensively studied, as discussed below. In comparison with zebrafish, medaka is easy to work with because it is very hardy and less susceptible to disease (BOX 1). Despite several large-scale mutagenesis screens in zebrafish, the number of mutations recovered that correspond to those that have been identified in mouse and human is still limited. This might be due, in part, to the additional gene duplications that have occurred in teleosts. Therefore, studies of medaka can complement those of zebrafish and will provide important additional phenotypes (H. Kondoh, personal communication) that will prove useful in modelling human diseases[16].

Medaka as a genetic resource

Spontaneous and induced mutants. Early work on medaka identified various spontaneous pigmentation mutants[5]. The first systematic screen for spontaneous mutants in medaka was carried out at Nagoya University in 1960. Thirteen spontaneous mutants were identified in an initial survey of natural populations and of breeding stocks from local commercial breeders. By 1975, 37 spontaneous mutations had been reported, and the mutant phenotypes ranged from abnormal pigmentation and body size to fin or scale morphology defects[26]. By 1990, the number of spontaneous mutations that had been identified had risen to 76. All of the original mutants are still maintained at the medaka stock centre at Nagoya University (BOX 2). Most mutants recovered from this screen were defective in pigmentation, but other mutants — such as *double anal fins* (*Da*), which has a mirror-image duplication of ventral body structures, and *pectoral fin-less* (*pl*) — despite their severe morphological phenotypes[27,28], were viable.

For decades, medaka was an important test system for environmental research. It is widely used for carcinogenesis studies[29] and for testing ENDOCRINE DISRUPTORS in ecotoxicology[30–32]. Medaka was tested for its susceptibility to physical and chemical mutagens, and it was in the course of these experiments that conditions for systematic mutagenesis were established[10,33]. These conditions turned out to be useful for mutagenizing not only medaka but also zebrafish[34]. The key issue in these environmental studies was the establishment of the mutagenic potential of the substance under study; this was done by counting the number of mutations that were induced at specific loci or the number of dominant-lethal phenotypes that were recovered after exposure to various mutagenic agents. More than 300 viable alleles of the known pigmentation genes were isolated in screens of multi-locus tester strains that involved more than 3 million loci[35]. New screens for developmental mutants were carried out recently[11,14] in parallel with the environmental screens, and eye and brain phenotypes were recovered at a high rate, showing that a morphology-based screen was possible in medaka[11]. Although some of the mutant phenotypes were partly reminiscent of those recovered from zebrafish screens, some, such as *Da* or *koepke*, were strikingly different[11] (FIG. 3), which indicates a non-overlapping spectrum of embryonic mutant phenotypes.

MULTI-LOCUS TESTER STRAIN
Strain that is homozygous at several recessive loci and is used to detect induced recessive mutations in the germ line; the higher the number of marker loci, the higher the detection efficiency.

ENDOCRINE DISRUPTOR
An exogenous substance that changes endocrine function and causes adverse effects at the level of the organism, its progeny and/or the population.

INBRED STRAIN
A strain is considered inbred when it is homozygous at 99% of loci. This level of homozygosity can be achieved after at least 20 generations of successive brother–sister matings.

AFLP
A mapping method in which genomic DNA from different strains is PCR amplified using arbitrary primers. DNA fragments that are amplified in one strain, but not the other, are cloned, sequenced and used as polymorphic markers.

MORPHOLINO
Chemically modified oligonucleotide that behaves as an RNA analogue and has been used to interfere with gene function in fish. Because it interferes with translation, a morpholino is designed to complement the region around the translational start site of the target gene.

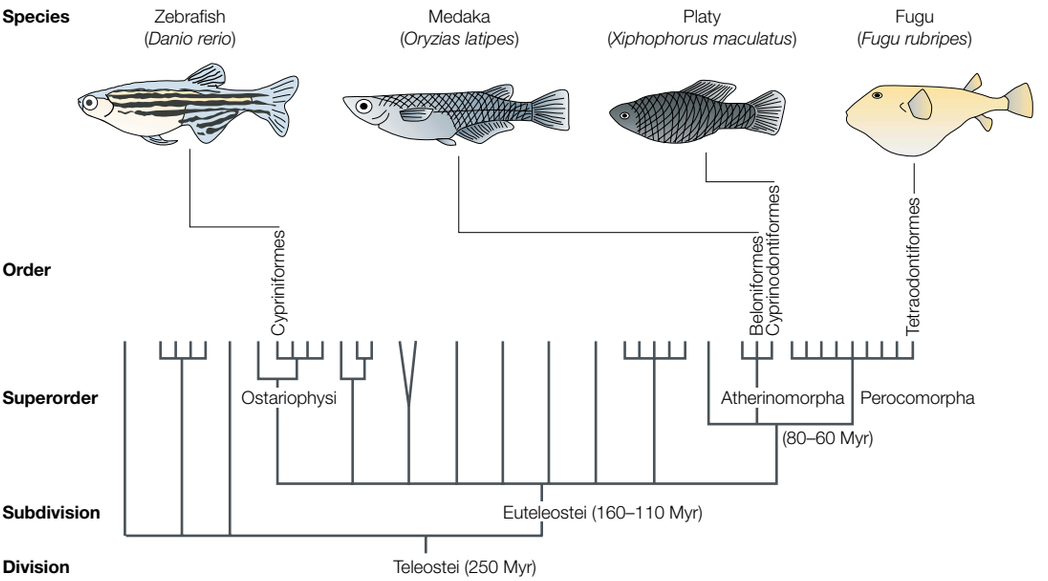

Figure 2 | **Evolutionary relationships between fish models.** This evolutionary tree (based on data from *Fishes of the World* by J. S. Nelson[115]) illustrates that the last common ancestor of medaka and zebrafish lived more than 110 million years (Myr) ago. Notably, medaka is a much closer relative to fugu than it is to zebrafish, or than zebrafish is to fugu.

Box 1 | **Medaka biology**

Medaka is a small (3–4 cm) egg-laying freshwater fish; its eggs are fertilized and develop externally. Both the eggs and the embryos are transparent. Medaka is very hardy and tolerates a wide range of salinities and temperatures (10–40 °C); it is easy to breed and highly resistant to common fish diseases.

Males and females are easily distinguished by a clearly dimorphic dorsal fin. Spawning tightly correlates with light cycles; between 30 and 50 eggs are spawned every day, and up to 3,000 eggs are produced during a mating season. Because the eggs are connected to the female body by attachment filaments, reproductively active females can be easily identified and propagated. Under laboratory conditions, generation time is between 6 and 8 weeks for medaka, compared with 8 and 10 weeks for zebrafish.

Early medaka development is rapid; whereas zebrafish larvae hatch after 2–3 days, medaka embryos are enclosed in a tough chorion that protects them in their natural habitat until they hatch as feeding young adults after 7 days (see REF. 108 for further details). In the laboratory, medaka embryos are raised in Petri dishes at a maximum density of 200 eggs per 9-cm (diameter) dish. Early development is synchronous and the fully transparent embryos are easily staged under the dissecting microscope, according to a set of morphological criteria[108]. All known zebrafish techniques[109], including single-cell injections, or transplantation and MORPHOLINO knockdown technology[104], also apply to medaka. For up to 3 months, early medaka embryos can be maintained at temperatures as low as 4 °C to slow down their development — a feature that is especially useful in transplantation and microinjection studies. For long-term storage and stock preservation, reliable and highly efficient protocols for sperm freezing and *in vitro* fertilization have been developed[110,111]. See the Medakafish homepage for an overview of techniques.

Successful mating and spawning of female medaka only occurs in response to male mating behaviour. To obtain unfertilized oocytes, sterile males can be used to induce spawning and, if kept under high salt conditions, these oocytes can be fertilized *in vitro* within 8 hours of spawning. Sterile males can be obtained by interbreeding different *Oryzias* species[112]; for example, when *Oryzias celebensis* males are mated with *Oryzias latipes* females, the male offspring are sterile, but still do well in the mating dance, and can therefore induce the spawning of unfertilized oocytes in *O. latipes* females.

Polymorphic and inbred medaka strains. The first step towards identifying the genes that underlie mutant phenotypes involves the establishment of linkage between the mutated locus and a polymorphic marker. Morphological polymorphisms were initially found in different wild populations of medaka[36], and the molecular analysis of these populations indicated the presence of two genetically distinct, highly polymorphic populations (northern and southern)[37]. Sequence comparisons of orthologous loci showed that single base-pair polymorphisms between these two strains occur at a frequency of 3% in introns and ~1% in exons[38,39]. These polymorphisms are invaluable for genetic mapping[40,41] — for any gene of interest, polymorphisms between the two populations can be detected by PCR, followed by a restriction-enzyme digest. To improve the ease and resolution of mapping, polymorphisms between strains should be maximized, whereas polymorphisms within strains should be minimized. So, much effort has been devoted to the establishment of INBRED STRAINS in medaka. The first such strains were established in 1985 by Hyodo-Taguchi and Egami (reviewed in REF. 8). Unlike in other lower vertebrate genetic systems, inbred and highly fertile strains of medaka are available (BOX 2).

Genetic mapping. The high degree of polymorphism between the inbred strains derived from the southern and northern populations was the basis for the establishment of a genetic map for medaka. A preliminary genome-wide linkage map of medaka was made by linking phenotypic markers to randomly amplified polymorphic DNA fragments[41,42]. Further systematic mapping efforts, which used cDNA and AFLP (amplified fragment length polymorphism) sequences as a source

Box 2 | **Medaka strains and mutants**

Strains

Southern population

AA2 (T5): The AA2 strain is a multiple recessive tester strain with three marker loci (*b*/*b*; *lf*/*lf*; *gu*/*gu*), whereas the T5 strain is a derivative tester strain with two additional specific marker loci (i^b/i^b; *wl*/*wl*), each of which lies on a different chromosome[29]. (For information on Tokyo Stocks contact A.S.)

Cab: This strain was originally obtained from Carolina Biological Supply (North Carolina) and established as an inbred strain in Germany[14]. It has the variegated pigmentation phenotype because it carries the *b*′ allele at the *B* locus. (For more information contact J.W.)

Northern population

HNI: This is an inbred strain that was derived from a wild northern population[11], and has become one of the standard mapping strains[33,35].

Kaga: Another strain, derived from the northern population, which was established at EMBL, Heidelberg, from the isolated Kaga stock at the University of Tokyo. (For more information contact J.W.)

Most of the inbred strains produced by Hyodo Taguchi, including HNI, have been maintained at the National Institute of Radiological Sciences and are available on request. (For information on Chiba stocks contact Y. Ishikawa at ishikawa@nirs.go.jp.)

Induced mutants

So far, more than 300 induced alleles of major pigmentation genes have been recovered from mutagenesis screens carried out by a group led by A.S. at the University of Tokyo. More than 70 embryonic-lethal mutants that were identified in a morphology-based pilot screen[14] are kept as live stock or sperm stocks[100] at EMBL, Heidelberg. (For more information contact J.W.) Furthermore, ~90 induced embryonic-lethal mutants are kept as live stock or sperm stocks at the National Institute of Radiological Sciences, Chiba. (For information on Chiba stocks contact Y. Ishikawa as above.)

***Spontaneous mutants* (*Nagoya stocks*)**

The medaka stock collection at Nagoya University was founded by H. Tomita in 1960. The stock centre was later expanded to accommodate an increasing number of induced and spontaneous medaka mutants. At present, more than 80 mutants that mostly affect pigmentation, but also gross morphology, eye, fin and skeletal development, are maintained at Nagoya University. (For information on Nagoya stocks contact Y. Wakamatsu at wakamatsu@bio.nagoya-u.ac.jp.)

***Isolated stocks of wild populations* (*Tokyo and Niigata stocks*)**

Wild populations can provide valuable insights into the intraspecific variation and microevolution of fishes. More than 80 wild populations of medaka have been maintained by random mating at the University of Tokyo since 1985. Current stocks at the University of Tokyo include 14 northern Japanese populations, 54 southern Japanese populations, 5 northern–southern Japanese hybrid populations, 6 Chinese and Korean populations, 2 tester strains, 22 induced specific-locus mutant strains and 2 congenic strains. Although they are primarily a genetic resource, their preservation has also become important from a conservation point of view since 1999, when medaka was listed as an endangered species. (For more information on Tokyo stocks contact A.S., or A. Shimada at kirita@biol.s.u-tokyo.ac.jp; for information on some stock resources that are preserved at Niigata University contact M. Sakaizumi at sakaizumi@scux.sc.niigata-u.ac.jp.)

of markers, consolidated the map to 24 linkage groups that represent the 24 haploid medaka chromosomes[39]. So far, more than 1,300 markers have been mapped, resulting in an average marker distance of less than 0.85 cM, which is roughly equivalent to 600 kb (H. Mitani *et al.*, personal communication).

These genetic resources were a crucial prerequisite for the recent, successful positional cloning of the first medaka mutants in 2001 (REFS 15–17). One of them was the *b* gene, first described by Aida in 1921 (REF. 5). B turned out to be related to a sugar-transport protein that has a key function in controlling the melanin content of pigment cells[15]. The second gene was *eyeless*[43]; the temperature-sensitive, lethal *eyeless* phenotype was found to be caused by an intronic insertion in the *eyeless* locus, which encodes the homeodomain protein Rx3, that brought about transcriptional inactivation[16]. Conserved synteny between medaka linkage group 21, zebrafish linkage group 9 and human chromosome 2q was the basis for the cloning of *rs3*, which is mutated in the *reduced scales* mutant[17] and encodes the ectodysplasin-A receptor (EDAR). Notably, none of the above mutant phenotypes has been found in recent zebrafish mutagenesis screens, illustrating the complementarity of the two systems and again hinting at the lack of overlap between phenotypic classes in these fish.

By mapping medaka genes that are orthologous with genes that had already been mapped in other vertebrates, the medaka map can be anchored to the genetic maps of other species. On the basis of criteria for synteny conservation — that three or more orthologous genes map to the same linkage group — so far, ~40 regions of conserved synteny have been detected between the human, zebrafish and medaka genomes. Conservation of synteny provides important clues for predicting gene function, but it can also provide important insights into genome evolution. For example, gene order in regions of conserved synteny between medaka, zebrafish and humans frequently changes, which indicates that inversions have occurred more frequently than translocations in vertebrate genome evolution.

Medaka and transgenesis

Transient approaches and transgenic lines. As in amphibians and in other fish, transgenesis in medaka involves injecting foreign DNA into the cytoplasm of the 1–2-cell-stage embryo[44]. Whereas injected DNA is distributed in a mosaic fashion, mRNA or morpholino oligonucleotides that are injected into two-cell-stage embryos are uniformly distributed; their activity begins from the time of injection up to somitogenesis and early organogenesis stages[45–47] (see also M. Carl, F. Loosli and J.W., unpublished data).

Transgenic tools for analysing maternal genes have so far only been available in medaka. This is because immature medaka oocytes can be matured and fertilized *in vitro* under controlled conditions[48], and synthetic maternal mRNAs or molecules that block maternal mRNA translation can be injected into the oocyte at the GERMINAL VESICLE STAGE.

Transgenic lines. The first stable transgenic fish lines were generated in medaka by injecting DNA into germinal-vesicle-stage oocytes[9] and later routinely by cytoplasmic injection into one-cell-stage embryos.

Of the medaka embryos that are injected with plasmid DNA, 1–5% will stably integrate the foreign DNA and become transgenic founders. Various useful transgenic lines have been generated in this way; for example, one stable line has been produced that

GERMINAL VESICLE STAGE
A stage during oocyte maturation in which the oocyte nucleus is located close to the surface of the egg cell and is clearly visible.

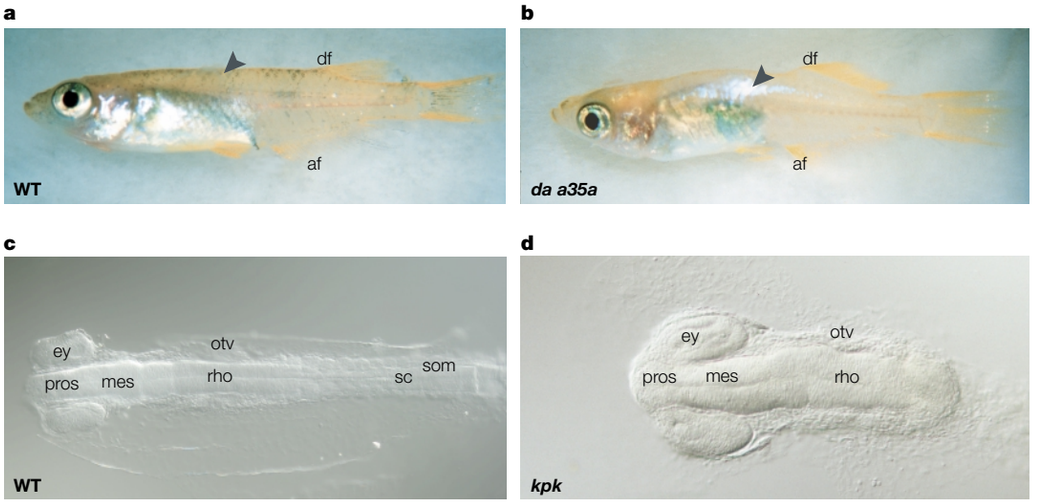

Figure 3 | **Mutant phenotypes that are unique to medaka. a** | A wild-type (WT) adult medaka. af, anal fin; df, dorsal fin. **b** | A *double anal* (*da*) mutant adult that has a mirror image duplication of the ventral body structures. The arrowhead shows the shiny pigmentation on the back of the mutant and the large dorsal fin, which in *da* shows anal fin morphology. **c** | A wild-type medaka embryo at 24 hours post-fertilization (hpf). **d** | A *koepke* mutant embryo at 24 hpf, which does not form trunk and tail tissue posterior to the hindbrain, but its head develops normally. ey, eye; mes, mesencephalon; otv, otic vesicle; pros, prosencephalon; rho, rhombencephalon; sc, spinal cord; som, somites. Reproduced with permission from REF. 11 © (2000) Elsevier Science.

expresses GFP (green fluorescent protein) from the *vasa* promoter[49] (FIG. 4). The endogenous maternal *vasa* mRNA is present in all cells of the early embryo until the MIDBLASTULA TRANSITION. Thereafter, zygotic *vasa* expression begins, but is exclusively confined to the germ line. In the *vasa*–GFP transgenic medaka (*olvas*–GFP; FIG. 4b), the green fluorescent germ cells can be clearly seen from their first appearance in the embryo, and can be followed as they migrate to their final location in the female and male gonads. Crossing the transgene into a transparent strain of medaka makes it possible to monitor the processes of oocyte maturation and spawning *in vivo*[50]. These transgenic strains can be used to study germ-cell development and, when used in mutagenesis screens, they could help to identify genes that are involved in germ-cell-fate determination.

Viral vectors have been used in zebrafish to increase the frequency of transgenesis and as a tool for insertional mutagenesis. Infecting early zebrafish embryos with PSEUDOTYPED RETROVIRUSES in particular has been used to obtain sufficient integration events to allow insertional mutagenesis and efficient cloning of the mutants that have arisen from the integration of the retroviral genome[51]. In medaka, two alternative techniques that involve co-injecting the transgene with an endonuclease are being developed, as discussed below.

One strategy that has recently been developed substantially increases the efficiency at which transgenic medaka lines are created. It involves *in ovo* linearization of circular plasmid DNA in which the transgene of interest is flanked by two rare endonuclease (I-SceI)-recognition sites (V. Thermes *et al.*, unpublished data). When this construct is co-injected with the endonuclease at the early one-cell stage of embryonic development, the transgene integrates into the medaka genome. The promoter-dependent expression pattern can then be studied in more than 80% of the injected embryos and the TRANSGENESIS FREQUENCY increases to more than 30% (a marked improvement on the 1–5% frequency for the standard random integration protocols (P. Ristoratore *et al.*, unpublished data).

Transgenic approaches have so far been limited by germ-line mosaicism in the founder fish. In embryos in which a transgene is co-injected with I-SceI, the GERM-LINE TRANSMISSION RATE is boosted to almost 50%, which indicates that integration happens in the founder fish at the one-cell stage. Transgenic lines that are generated in this way are stable and, so far, continue to show stable expression over five generations. At a low efficiency, this method leads to ENHANCER-TRAPPING effects — new patterns of expression that are detected are most probably due to enhancer elements that lie close to the integration site. The potential of this procedure is now being tested in other species, including zebrafish (F. Loosli and J.W., unpublished data), but so far this approach to transgenesis and enhancer trapping is unique to medaka. Because this method markedly increases the efficiency of transgenesis and speeds up the analysis of transgenic embryos, transgenesis in medaka can be done in a reasonable time frame, with a relatively small number of animals.

Gene and enhancer trapping. The systematic use of P-element-mediated enhancer trapping has been a key technique in the analysis of development in *Drosophila*[52–54]. In other insects, in zebrafish and in mammalian cells, P elements are not naturally mobile[55–57] because the host factors that are required for P-element transposition are absent in these species.

MIDBLASTULA TRANSITION
A stage during embryogenesis when zygotic transcription starts and cell divisions become asynchronous.

PSEUDOTYPED RETROVIRUS
Retrovirus, the host range of which has been altered; they were initially developed for human gene therapy.

TRANSGENESIS FREQUENCY
The frequency of injected embryos that are stably transgenic and transmit the transgene to the next generation.

GERM-LINE TRANSMISSION RATE
The rate of transgenic F_1 offspring that arise from a cross between a transgenic founder fish and a wild-type fish. It reflects germ-line mosaicism, depends on the time point of integration and cannot exceed 50%.

ENHANCER TRAPPING
A strategy that uses targeting vectors to trap or isolate enhancers of nearby genes. The inserted vector sequence acts as a tag that facilitates cloning of the neighbouring gene.

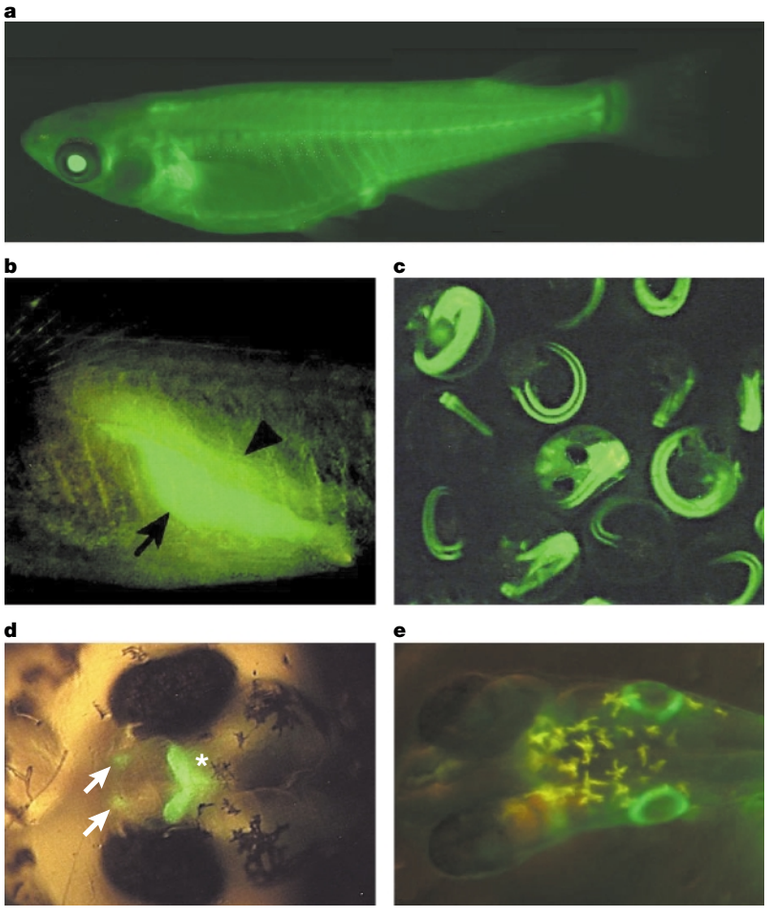

Figure 4 | **Medaka fish that are transgenic for green fluorescent protein.** Different transgenesis approaches have been used to generate several stable transgenic medaka lines that express green fluorescent protein (GFP) uniformly or in specific tissues or regions of the developing embryo. **a** | Adult medaka in which constitutive GFP expression is driven from the cytoskeletal actin promoter. **b** | GFP expression from the *olvas–GFP* transgene in the adult testis. The testes (arrows) and fat tissues (arrowhead) are viewed through the abdominal body wall, anterior is to the left. **c** | Strong GFP expression in the developing muscle driven by an actin promoter. Different GFP intensities reflect differences between individual fish in the copy number of the transgene in this clutch of embryos from heterozygous parents. (Photo courtesy of Jean-Stephane Joly, Institute de Neurosciences, Gif-sur-Yvette, Paris. Reproduced with permission from REF. 50 © 2001 National Academy of Sciences, USA.) **d**,**e** | Enhancer-trap lines that express GFP specifically **d** | in the dorsal diencephalon (habenulae, shown by asterisk) and in nasal epithelium (arrows), or **e** | in the otic vesicle, which will give rise to the ear.

By contrast, the transposition of *Tc1*-like transposable elements (TcEs) does not require host factors and is sufficient for TcE transposition[58]. An artificially reconstructed member of this transposon superfamily — *Sleeping Beauty* (*SB*), which has been shown to mediate integration of reporter constructs in vertebrate tissue culture[59] — was used as a basis for developing a transposon-mediated germ-line integration system in medaka[60]. The *SB* system does not work in zebrafish, probably because of an endogenous defence mechanism against *SB* infection. The transgenes delivered in this way integrate into 35% of embryos at the 1–16-cell stage and are stably transmitted to, and expressed in, the subsequent generations. One-third of the transgenic lines generated in this way show new spatial and temporal expression patterns that are indicative of transgene integration near regulatory elements. The efficiency of this approach, combined with a high rate of enhancer trapping, has so far led to the generation of more than 50 transgenic lines with specific GFP expression patterns[60] (C. Grabber *et al.*, unpublished data; FIG. 4). These lines provide unique tools for the functional analysis of mutants *in vivo*, as well as in gain- and loss-of-function experiments that involve RNA or morpholino injection.

Another active transposon, *Tol2*, of the *hobo* class has been found in medaka[61]. Following their observation that *Tol2* can actively jump out of the medaka *albino* locus[62], Koga and colleagues decided to use this element as a tool for transgenesis, in an analogous way to the use of the *SB* system. Unfortunately, *Tol2* turned out not to be suitable as a transgenesis tool because laboratory strains of medaka contain *Tol2* elements in their genome, which means that *Tol2* -mediated integrations are unstable owing to transgene transposition between *Tol2* sites. But medaka species, such as *Oryzias luzonensis*, that do not carry endogenous Tol-like elements have now been identified (H. Hori, personal communication), which makes *Tol2* -mediated transgenesis possible in these species. In zebrafish, *Tol2* has so far been used to show transposase activity in transient assays, in which transposase mRNA was co-injected with a targeting plasmid[63].

Stable transgenic lines that express GFP from different promoters are particularly useful in lineage and fate-tracing experiments. Additionally, labelled cells from dissociated embryos can be enriched by FACS SORTING for either subsequent propagation in cell culture or for specific mRNA extraction; enriched mRNA can then be used in subtractive cloning or expression profiling experiments[64]. Medaka and zebrafish lines that ubiquitously express GFP have also been successfully used as donor lines for cell transplantation experiments[43]. In future, GFP lines with organ-specific expression will be important tools for genetic screens that are aimed at improving our understanding of organogenesis. It will be possible to study the activity of specific genes, such as insulin, in transgenic lines *in vivo*, in which GFP expression is controlled by the enhancer of the gene. The analysis of GFP expression in response to drug treatment, in those lines, will allow drug screening in high-throughput approaches.

Embryonic stem cells and chimaeras. Targeted gene disruption in transgenic animals is so far possible only in the mouse. The application of this technology to other laboratory animals has been prevented by a lack of embryonic stem (ES) cell lines for these species. ES cells retain their full developmental potential and can be re-transplanted into host embryos, in which they can differentiate into all cell types except the trophoblast in mouse, but most importantly they can contribute to the germ line and can therefore be passed to the next generation. ES-like cell lines have been established from zebrafish

FACS SORTING
A method whereby dissociated and individual living cells are sorted, in a liquid stream, according to the intensity of fluorescence that they emit as they pass through a laser beam.

MIDBLASTULA
Early stage of fish embryonic development corresponding to ~1,000 totipotent cells that form a cap on the yolk.

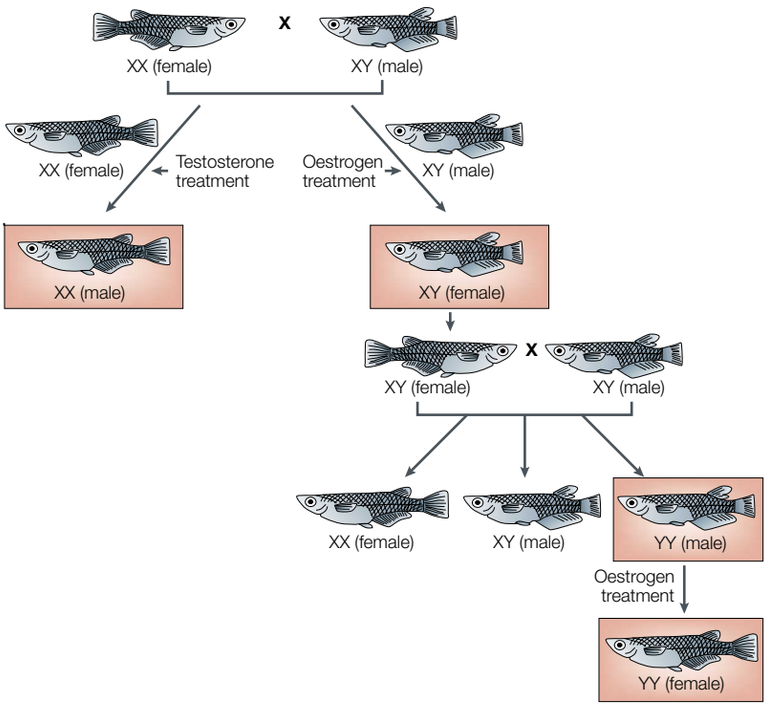

Figure 5 | **Producing sex-reversed medaka.** Medaka breeding normally results in XX females and XY males in the expected 1:1 ratio. If, during the sensitive period around hatching, medaka embryos are treated with either androgens or oestrogens, fully fertile XX males and XY females, respectively, can be obtained. Crosses between such sex-reversed XY females and normal XY males give rise to YY males among other offspring. When such YY male embryos are treated with oestrogen, fertile YY females can be produced.

and medaka MIDBLASTULA embryos[65–67]. After a short period *in vitro*, medaka and zebrafish ES-like cells can still contribute to the germ line[68]. However, they cannot be cultured for long enough to carry out gene targeting by homologous recombination, and therefore they cannot be used for targeted gene inactivation. Long-term culture conditions that give enough time for the genetic manipulation of cells and for the *in vitro* selection of those that carry the constructs have been established only in medaka so far[67]. These cell lines show all the *in vitro* characteristics of mouse ES cells: a EUPLOID KARYOTYPE, unlimited growth *in vitro*, expression of ES-cell markers and clonal growth from single cells. Importantly, they can also be cryo-preserved and genetically manipulated without losing ES-like characteristics. After appropriate stimuli, such as high- or low-density culture, EMBRYOID BODY aggregation or growth-factor treatment, they can differentiate *in vitro* into many cell types that include melanocytes, neurons and muscle cells[67]. As *in vitro* differentiation systems, they are useful tools in many cell-culture experiments that require standardized conditions and controllable manipulations. Medaka ES cells also remain pluripotent *in vivo* — after transplantation into host embryos, they contribute to the development of all principal tissues and organs, and differentiate into highly specialized cell types, such as the epithelial cells of undulating fins, contracting muscle cells of the heart, neurons and retinal cells[69]. The frequency at which chimeric embryos are created using these cells is very high — up to 90%; however, the DEGREE OF CHIMERISM is lower than that which is routinely achieved in the mouse. Because of this low degree of chimerism, the contribution of the ES-like cells to the germ line has not been achieved. Therefore, it is not yet possible to use these ES-like cells to generate targeted gene disruptions in medaka.

A more immediate solution to the germ-line-transmission problem might come from nuclear transfer, in which nuclei from cells of embryos or fish larvae are transplanted into enucleated eggs. Experiments along these lines were already being carried out as early as the 1960s (reviewed in REF. 70) and this methodology, originally developed for CYPRINID FISHES, has recently been established for medaka[71]. Although nuclear transplants are experimentally difficult to carry out, they can be used to obtain fertile fish that transmit the donor genome. Experiments that use totipotent nuclei from cultured medaka ES cells are now under way. A combination of nuclear transplantation and ES-cell technology, in which the tools for homologous recombination are being developed[72], will hopefully soon lead to knockout technology in medaka.

Insights into development and evolution

With so many techniques being developed for it, the future of medaka as a model system looks bright, but medaka has also proved to be a useful developmental biology model in the past. Most of this early research focused on sex determination and eye development, but brain development, especially its morphology, has also been studied[13,73,74].

Sex determination. From the evolutionary point of view, fish are a particularly interesting group of organisms for studying sex determination, because the reproductive strategies of members of this CLASS range from hermaphrodism to GONOCHORISM and show different types of sex-determining mechanism — from environmental to genetic (for a review, see REF. 75). By comparing the structure and expression of sex-determination genes in different fish species, the relative importance of these genes, their position in the sex-determination cascade and their conservation in evolution can be studied. So far, nothing is known about sex determination in zebrafish and, remarkably, none of the many markers of the zebrafish genetic map seems to be sex linked[23–25]. The situation is much more convenient in medaka; males and females differ by several secondary sex characters[2], some of which — such as the shape and size of the dorsal and anal fins — can be easily scored. Additionally, strains with sex-linked pigmentation patterns have been established. For example, in one strain, a wild-type *lf* (*leucophore free*)[26,76] allele is expressed from the Y chromosome and a mutant recessive *lf* allele from the X chromosome. This expression allows individual fish to be sexed on the basis of their pigmentation, as early as the second day after fertilization. Like mammals, medaka has an XX, XY sex-determination system, with the male-determining locus on the Y chromosome.

EUPLOID KARYOTYPE
Complete chromosome set, without any deletions or additions of whole chromosomes or chromosome fragments.

EMBRYOID BODY
Ball-like structure that forms from embryonic stem cells when they are kept in suspension culture.

DEGREE OF CHIMERISM
The proportion of differentiated embryonic cells that are derived from the embryonic stem cells.

CYPRINID FISH
A large group of freshwater fish species that includes the common carp, goldfish and zebrafish.

CLASS
A taxonomic group.

GONOCHORISM
The presence of separate male and female sexes in a species (opposite to hermaphrodism).

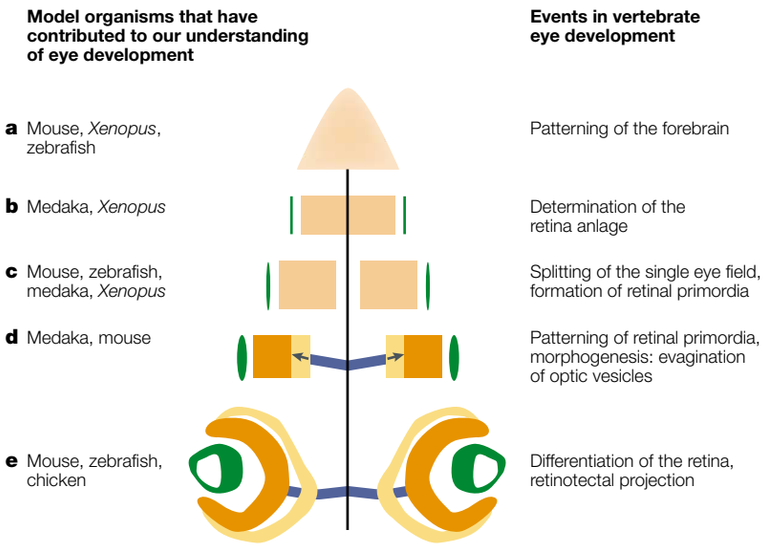

Figure 6 | **Schematic representation of vertebrate eye development.** The central line represents the temporal axis, as well as the midline of the developing embryo. **a** | Eye development initiates at the late gastrula stage, simultaneously with the patterning of the prospective forebrain. The tissue from which the neuroectodermal part of the eye will form (retina, optic stalk) is shown in orange. **b** | Eye development starts at the end of gastrulation with the determination of the eye field; tissue that will give rise to the lens is shown in green. **c** | As a result of the signals that emanate from the midline, the initially single retinal anlage is split into two retinal primordia. These signals also establish a proximo-distal pattern in the primordia. **d** | Proliferation and active morphogenesis lead to the evagination of the optic vesicle, which then invaginates to form the optic cup. Subsequently, the neuroretina (dark orange), pigmented epithelium (pale orange) and the optic stalk (blue) form. Arrows indicate secreted signals that initiate retinal differentiation. **e** | The neuroretina (dark orange) differentiates and sends axons along the optic stalk (blue) to the target areas in the optic tectum. At this stage of eye development, the differentiated lens (green) is fully embedded into the eye cup.

The X and Y chromosomes of medaka — the second largest pair of medaka chromosomes — are considered to be at a very early stage of evolution. They are morphologically indistinguishable[77] and crossing-over occurs throughout the entire length of the sex chromosomes. Therefore, like the autosomes, the linkage map of sex chromosomes mostly contains evenly distributed markers. The sex-determining locus is the only exception to this, at which a pronounced clustering of markers indicates a region of reduced recombination[39,78]. Unlike in higher vertebrates, full sex reversals can be obtained; the first sex reversals were reported in 1958 by Yamamoto[79]. Treatment with steroid sex hormones during the larval period has generated YY males, XY females, XX males and even YY females[2] (FIG. 5). Experiments such as these uncovered two important phenomena. First, sex can be artificially reverted as long as the gonad is morphologically indifferent; in genetic females, the gonad remains morphologically undifferentiated until hatching, whereas in genetic males the gonad remains in this state until 2 weeks after hatching. Second, the uncompromised fertility of XX males points to the fact that there are no genes that are essential for the function of the testes or sperm production within the Y-specific region of the Y chromosome.

Because the sex chromosomes have only recently diverged from their autosomal ancestors, the whole genetic sex-determination system might also be at an early stage of evolution. Although no homologue of *SRY* (sex-determining region Y) has been found in medaka, homologues of genes that lie downstream of *SRY* in the mammalian sex-determination cascade have been cloned and characterized[80,81]. None of them, however, maps to the sex chromosomes. It remains unclear which sequence on the Y chromosome directs the development of the primordial gonad towards male differentiation, or which inhibits ovary differentiation.

Eye development. The analysis of vertebrate eye development is one of the best examples of the complementary use of vertebrate model systems. Eye development starts at the end of GASTRULATION with the determination of the EYE FIELD (FIG. 6). Studies in zebrafish, *Xenopus* and mouse have shown that the establishment of the eye field during the patterning of the anterior NEURAL PLATE depends on the repression of Wnt (wingless related) and Bmp (bone morphogenetic protein) signals[82,83], and requires the activity of Otx (orthodenticle-related homeobox gene) transcription factors[84]. The subsequent patterning of the eye field leads to the defined expression of two conserved transcription factors — *Six3* (*sine oculis* homeobox homologue 3) and *Pax6* (paired box gene 6) — in the anterior neural plate. The analysis of *Pax6* loss-of-function mutations in mouse, and *Six3* gain-of-function experiments in medaka, indicates that *Pax6* and *Six3* have key roles in the specification of retinal fate. When *Six3* is ectopically expressed in medaka embryos, ectopic eyecups are formed in response to a regulatory feedback loop, in which *Six3* and *Pax6* have key roles[46]. Conversely, in mouse *small eye* mutants, in which *Pax6* is absent, eyes do not form at all. Experiments in medaka have also shown that *Six3* affects cell proliferation and thereby regulates the size of the retina ANLAGE[16,46]. Consistently, the closely related *Xenopus Optx2* (optix homeobox gene 2 or Six6) controls the size of the optic vesicles by regulating proliferation[85,86].

Experimental data from mouse, *Xenopus*, zebrafish and medaka collectively indicate that MIDLINE signalling is required for the split of the single retinal anlage into the two retinal primordia. Members of several of the known families of signalling molecules, such as Shh (sonic hedgehog)[45,87–89], Fgf (fibroblast growth factor)[47] and Nodal[90,91] are involved in this process. Whereas Nodal signalling is required for active cell movement leading to the physical division of the initially single eye field, Fgf signalling sets a competence domain in which Shh signalling is required to promote proximal eye fate (FIG. 6).

The identification and analysis of the medaka temperature-sensitive *eyeless* mutant provided important insights into the subsequent evagination of the optic primordia that leads to the formation of the optic vesicles. *Eyeless* mutants completely lack optic

GASTRULATION
A morphogenetic process in embryogenesis during which the mesoderm is formed.

EYE FIELD
An epithelial eye precursor in the anterior neuroectoderm.

NEURAL PLATE
Ectodermally derived precursor of the brain and spinal cord that forms at the beginning of neurulation.

ANLAGE
A precursor tissue before its determination and differentiation.

MIDLINE
The notochord and its anterior extension, the prechordal plate, which are located in the ventral midline of the developing embryo.

Box 3 | **Medaka genomic resources**

Gridded BAC, cosmid and cDNA libraries

Gridded BAC (bacterial artificial chromosome) libraries have been established from the southern and the northern strains of medaka. The Cab BAC library (BOX 1) is available at the RZPD (Resource Centre/Primary Database) (J.W. and C. Amemiya, unpublished data, and http://www.rzpd.de, RZPD library number 756). A second BAC library from the southern population has also been established[99]. The HNI BAC library has been constructed as part of the Medaka Genome Project funded by the MEXT, Japan (Priority Area number 813; contact S. Asakawa at asa@dmb.keio.ac.jp for more information).

Gridded cosmid libraries from both strains are also available (RZPD: Cab, library number 74; Kaga, library number 75). Gridded cDNA libraries were the basis for random *in situ* screens that have so far identified more than 500 unique expression patterns.

Expressed sequence tags

ESTs were established from four cDNA libraries that were prepared from the whole body, liver, ovary and a cultured cell line of the HNI strain. The 5′-terminal DNA sequences of 13,000 cDNA clones that map to 4,000 genes were determined, and ~50% of medaka ESTs greater than 300 bp showed significant homology with previously identified genes from other species. All the data, which are now accumulating at a rate of 10,000 ESTs per year, are available through an internet-based searchable EST database that also includes the mapping information (http://mbase.bioweb.ne.jp/).

Medaka Genome Initiative

As part of the Medaka Genome Initiative, several Japanese and European laboratories collaborate to establish genetic and physical mapping resources and to sequence the medaka genome. Genetic mapping is based in Tokyo (http://mbase.bioweb.ne.jp/), in the group of A.S. (contact shima@k.u-tokyo.ac.jp, or Hiroshi Mitani at mitani@u-tokyo.ac.jp).

Genes that are being mapped are identified by whole-mount *in situ* hybridization carried out in the laboratories of J.W. at EMBL in Heidelberg and Franck Bourrat in Paris (franck.bourrat@iaf.cnrs-gif.fr). Normalized cDNA libraries generated at the Max-Planck Institute for Molecular Genetics in Berlin, in the laboratory of Heinz Himmelbauer (himmelbauer@molgen.mpg.de), are the source of probes for these *in situ* hybridization experiments. Genes that are identified in this way are used to link the two complementary BAC contig maps: one from Berlin by Himmelbauer and the other from Keio University by N. Shimizu (shimizu@dmb.med.keio.ac.jp) and S. Asakawa (asa@dmb.keio.ac.jp).

Medaka mutants that have been identified in a collaborative, large-scale, hybridization-based screen in Kyoto (the Kondoh Differentiation Project; Hisato Kondoh and Makoto Furutani-Seiki) are then rapidly assigned to the BAC contig maps.

Sequencing of the medaka genome has already been initiated and N. Shimizu and colleagues at Keio University are now focusing on the BAC contig that covers chromosome 1. Sequencing data are deposited in a cross-searchable database that is developed and hosted by the Kondoh Differentiation Project.

NEURULA
A developmental embryonic stage at the end of gastrulation that corresponds to the formation of the neural plate.

PARALOGUE
Homologous gene that originates by gene duplication.

ORTHOLOGUE
Homologous gene in different species the lineage of which derives from a common ancestral gene without gene duplication or horizontal transmission.

vesicles, and the retinal progenitor cells that normally form in the lateral wall of the forebrain do not evaginate[43]. Temperature-shift experiments indicate that *eyeless* is required during the late gastrula and early NEURULA stages, before optic vesicle evagination[43]. *Eyeless* mutants carry a mutation in *Rx3* — a member of the Rx gene family of retina-specific homeobox transcription factors. Functional studies in mouse and *Xenopus* have consistently indicated that the Rx genes have a regulatory role in the proliferation of retinal progenitor cells within the optic vesicle[92,93]. In the absence of *Rx3*, there is no sign of morphogenesis, and the specified retinal precursors do not proliferate and eventually die. So, this transcription factor seems to control optic vesicle morphogenesis and its size, and these studies show that the early patterning of the eye anlage and eye morphogenesis are uncoupled in the *eyeless* mutation. Complementing the work in mouse, epistasis experiments in medaka have shown that *Rx3* acts downstream of *Six3* and *Pax6*, and is required for the control of morphogenesis and the size of the optic vesicles[16].

Medaka genomics

Comparisons between species are particularly informative with respect to investigating gene function and regulation, as well as genome evolution. One interesting question that can be answered by a comparative genome analysis of both fish models concerns genome evolution. It is generally accepted that gene duplications are a principal driving force of evolution. Freed from selection, a gene that has been duplicated can diverge and eventually acquire a new expression pattern, and its encoded protein might adopt a new three-dimensional confirmation. In teleost fish, many examples of duplicate genes have been reported; whereas, surprisingly, in mammals only one gene of that kind is present[94]. This has led to the "more genes in fish" hypothesis[94] that can be partly explained by a whole-genome duplication event in teleost fish that occurred since their separation from the common tetrapod lineage[23,95]. Although convincing evidence for this can be found by looking at conserved synteny in certain gene pairs in fish and comparing them with their mammalian counterparts, a molecular phylogenetic analysis of these genes supports an alternative theory — that tandem gene duplications are the source of the additional genes[96]. Depending on the time when a single gene was duplicated in evolution, only one or both zebrafish and medaka genomes will contain both genes and enjoy the evolutionary advantage that a genetic duplication provides. Because many genes in fish are present in single copy, proponents of the whole-genome duplication hypothesis postulate that many of the duplicated versions of genes have degenerated since the initial duplication event[94,97]. If this theory were true, one would expect medaka and zebrafish to have considerably different sets of genes because this degeneration process would have affected different genes in the two fish lineages. Data provided by the detailed study of the genomes of both fish should resolve some of these evolutionary issues.

Whole-genome duplications, and/or individual gene duplications, that occurred in the teleost lineage[23,94,95] have resulted in altered gene-expression patterns, so that the complement of the expression of the fish PARALOGUES is equivalent to the expression of their mouse ORTHOLOGUES. For example, a mouse gene that is expressed in the neural tissue and muscles corresponds to two separate fish genes, one of which is expressed only in the neural tissue and the other only in muscle. Although gene sequences have diverged to some extent since the separation of both copies from the last common ancestor, the genes have retained the same function and, therefore, in most cases they can

still be considered homologous (for exceptions to this see REF. 94). This process has been described as gene subfunctionalization[98] and has had far-reaching implications for the difference in function that might be uncovered when these genes are mutated. For example, in mouse, inactivating a gene might lead to an early embryonic-lethal phenotype, which makes it difficult to interpret the function of the gene in later development. But if the same gene has more than one counterpart in fish, mutations in these fish orthologues might correspond to more than one distinct mutant phenotype, each of which can be studied individually. Conversely, functional redundancy might lead to non-scorable or subtle phenotypes in fish.

Because of the evolutionary distance between zebrafish and medaka (FIG. 2), it is likely that the repertoire of duplicated genes is different, and therefore the two fish model systems are unlikely to be redundant. To explore systematically these differences between medaka and zebrafish, a medaka genome project has been initiated. A genome project is much more than whole-genome sequencing — it is a coordinated generation of resources that contribute to our understanding of how a genome works. This philosophy has been taken up by some laboratories of the medaka community. The Medaka Genome Initiative is based on genetic and physical mapping resources that have already been established[38,39,99] (BOX 3). Laboratories that are part of this initiative collaborate to physically and computationally interconnect the resources (BOX 3). For example, normalized cDNA libraries have been generated as a source of clones that are individually analysed by whole-mount *in situ* hybridization[100–102]. These clones are also sequenced to generate expressed sequence tags (ESTs). ESTs that have unique expression patterns are mapped onto the genetic and physical maps to integrate these two maps.

The growing pool of medaka genomic resources should facilitate the rapid identification of candidate genes for mutants that have already been mapped, and the identification of a mutant for a gene with an interesting expression pattern, the molecular nature of which remains unknown. Conserved synteny between medaka and zebrafish will be informative for studies in both systems; although, at the moment, medaka profits most from the high-resolution genome maps of zebrafish[25,95,103]. As more medaka mutants are being identified in screens, such as the collaborative large-scale mutagenesis screen in Kyoto (Kondoh Differentiation Project H. Kondoh and M. Furutani-Seiki, personal communication), hybridization-based approaches are being used to assign them to individual medaka chromosomes[104]. Physical map information from RADIATION HYBRID PANELS and BAC (bacterial artificial chromosome) contig maps allows candidate regions or BACs to be identified, where the genes responsible for the mutant phenotype might lie. The expression patterns of genes or ESTs that map to these regions can then be used to identify candidate genes, which can eventually be analysed in rescue or morpholino knock-down experiments[105].

Although the complete genome sequence is not an immediate prerequisite for successful work with a model system, it will be very important for future comparative analysis. In zebrafish, whole-genome sequencing has been initiated after the successful shotgun approach[106]. At present, genomic sequencing in medaka is focusing on a BAC contig that covers chromosome 1 — the sex chromosome — and will be extended in the near future (A.S., unpublished data). The medaka genome consists of 24 sets of chromosomes, and the genome size is estimated at 650–1,000 Mb (REFS 107,108). So, the medaka genome is the smallest of all vertebrate genetic model systems — it is only one-third of the human genome and less than half the size of the zebrafish genome[108]. Given the significantly smaller genome size and the already well-established infrastructure of the Medaka Genome Project, completion of the medaka whole-genome sequence in the near future is a realistic expectation.

Conclusion

When the existing medaka and zebrafish mutants are compared, it becomes clear that some biological problems can be addressed more effectively in one or the other model. In many cases, the mutant phenotypes are unique, which indicates underlying differences in the genome and the degree of functional redundancy between these two fish. These model systems are therefore complementary rather than redundant, because when we study what makes a species unique, we need comparative approaches that involve different, and distantly related, vertebrate model systems.

The advantages of medaka biology, together with the sophisticated tools described above, make medaka an attractive complementary model system. Increasingly, transgenic strains of medaka that stably express variants of GFP in a tissue-specific manner will be used in genetic screens to isolate mutations that specifically disrupt individual aspects of many biological processes. Another field of medaka research that has great potential and is already under way is that of stem-cell culture. In addition to ES cells, the possibilities of culturing neural stem cells from the CILIARY MARGIN of the retina and from the cortical region of the optic tectum are also being looked into. Already there are medaka cell-culture systems that combine mutant analysis with cell biological and biochemical analysis.

Fish comparative genomic studies will greatly benefit from the complete sequence of the *Fugu* and *Tetraodon* genomes that will soon be available. With an evolutionary distance of ~60 Myr (compared with 110–160 Myr in zebrafish), medaka and *Fugu* are as close as the fast-evolving *Drosophila* species *D. melanogaster* and *D. hydei* that have been successfully used to establish conserved genomic features in Diptera[109]. Rapid completion of the genome sequencing of medaka will, therefore, be a crucial step and a valuable addition to future evolutionary and developmental biology studies.

RADIATION HYBRID PANEL
A set of DNA samples prepared from a collection of radiation hybrids, each of which is a clonal population of cells that are derived by the fusion of lethally X-irradiated donor cells with mammalian cells. Such cell lines can be used to create a physical map of the donor genome.

CILIARY MARGIN
An area at the margin of neuroretina and pigmented epithelium that contains cells with stem-cell characteristics.

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