Annu. Rev. Genomics Hum. Genet. 2002. 3:311–40
doi: 10.1146/annurev.genom.3.031402.131506

First published online as a Review in Advance on June 12, 2002

# FROM ZEBRAFISH TO HUMAN: Modular Medical Models

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**Key Words** developmental biology, genetic screen, chemical screen, integrative physiology, pathophysiology

■ **Abstract** Genetic screens in *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Danio rerio* clarified the logic of metazoan development by revealing critical unitary steps and pathways to embryogenesis. Can genetic screens similarly organize medicine? We here examine human diseases that resemble mutations in *Danio rerio*, the zebrafish, the one vertebrate species for which large-scale genetic screens have been performed and extensively analyzed. Zebrafish mutations faithfully phenocopy many human disorders. Each mutation, once cloned, provides candidate genes and pathways for evaluation in the human. The collection of mutations in their entirety potentially provides a medical taxonomy, one based in developmental biology and genetics.

## INTRODUCTION

The genetic screen is a means to completely analyze the genome, but based principally on phenotype rather than sequence (52). By identifying unitary defects associated with mutations, and in a manner complete enough to reflect a large proportion of all genes involved in the target process, the screen can provide the logical elements of a complex biological process, as such screens did for early embryonic patterning in *Drosophila melanogaster* (118). Many attributes of vertebrate development similarly have been put into a rational edifice by analysis of mutations isolated in large-scale genetic screens in the zebrafish (*Danio rerio*). Many of these contributions to developmental biology have been reviewed elsewhere (47, 53, 69, 77, 123, 142). One fortunate and perhaps surprising attribute of many mutations is that, in a relatively "modular" manner, they perturb selectively one attribute of organ formation or function (54). For example, individual mutations might eliminate the ventricle of the heart without affecting the atrium (24) or might eliminate the glomerulus of the kidney without affecting the tubules (38). The selective elimination of particular elements in a Lego-like manner reveal the essential units of organotypic form and function. Once cloned, these genes provided entrance points to the responsible pathways.

Zebrafish organs are functionally and morphologically similar to the human, so it was hoped that the screens additionally might unveil mechanisms and pathways directly relevant to human disease and therapy (36). In some ways it is premature for this type of analysis because most genes cloned have been studied with regard to their roles in early embryonic development rather than to later organ formation. However, with the eye of the clinician and allowing some poetic license, the phenotypes of many mutations do resemble common human disorders.

We here explore the arenas where zebrafish genetics and medicine intersect. Descriptive and broadstroked as it is, this view does lead to the conclusion that model-organism genetics could provide organizing principles for medicine. The effects of some mutations bear remarkable resemblance to human illness, thereby suggesting that diseases might be categorized as perturbations of evolutionarily conserved ontogenetic mechanisms rather than, as is now standard, compendia of affected tissues. In addition to establishing a developmental "logic" to disease, the mutant genes, once cloned, provide entrance points to critical disease pathways in medicine as in developmental biology.

We organize the essay around the questions: What are some human disorders now lacking good therapies? Are there zebrafish mutations with phenotypes that can reasonably be viewed as resembling these diseases? Can the zebrafish be useful in discovery of new therapies? We recognize that, although this may be a practical approach, it is less systematic than one based on unifying principles and pathways of developmental genetics and that its extrapolations are subject to leaps of evolutionary faith with far less substantive underpinnings. Furthermore, this approach is woefully incomplete. However, a more fulsome treatment, crossing a textbook of medicine with a treatise on the zebrafish, would be lethal. Therefore, we limit ourselves to a few disorders across the spectrum of diseases.

The essential science of day-to-day medicine is integrative organ physiology. By integrative physiology we mean issues of how form and function interact both during organ assembly and in later organismal homeostasis. These organismal questions may seem unrefined to those now used to the lapidary logic underpinning more "classical" questions of developmental biology, those of patterning and cell fate, questions chiseled by genetic screens in *Drosophila* and *Caenorhabditis elegans*. Unfortunately, fruitflies and worms lack directly extrapolatable complex organs (although they do have their own organs that are of interest and clear relevance to cell-fate acquisition). The zebrafish, by contrast, has all of the tissues (except for lungs) afflicted by common human diseases.

The specific benefits and weaknesses of the zebrafish as a model system have been exhaustively reviewed elsewhere (31, 46, 152). In brief, the zebrafish attracts researchers because (*a*) it is a vertebrate with simple husbandry requirements, (*b*) it is relatively fecund and generates clutches of 100–200 embryos weekly, (*c*) its fertilization and development are external, and (*d*) its embryo is transparent. In particular cases it has been useful to generate haploid embryos (using inactivated sperm), although these embryos frequently manifest abnormalities, especially in later organ development. Homozygous diploids can be generated by interruption of the second meiotic division with hydrostatic pressure using a French press.

Large-scale genetic screens have been performed in zebrafish after use of ethyl-nitrosourea (ENU) to generate point mutations. More than 7000 mutations in 600 genes (37,68) (not all yet subjected to complementation) have been so generated. The original screens utilized simple dissecting microscopes to find mutations. Subsequent screens have used probes to highlight particular tissues (25,50,67,71,144). Injection with pseudotyped retrovirus can generate insertional mutations and has the considerable advantage of expediting mutation cloning. However, insertional strategies provide sparser coverage of the genome and, hence, less-complete coverage with regard to any particular attribute, such as the development of an organ's form and function.

For positional cloning, genetic maps have been constructed using both microsatellites (95,130,146) and genes (75,85,174), as have physical maps (61,74, 108), and the complete genome sequence of zebrafish is to be completed shortly. Already it is clear that zebrafish orthologs exists for most human genes, and in fact, there are large regions of conserved synteny between chromosomes of fish and mammal (9). There is believed to have been a genome duplication at the base of the teleost radiation 450 million years ago (mya), as well as a second duplication 100 mya, with as many as 20% of genes having been duplicated in the fish as compared to the human (129,130). While it is possible that these evolutionary "accidents" might obfuscate the effect of a single nucleotide change or insertional event, these duplications appear to have occurred at sufficient evolutionary distance, such that the duplicated genes can be functionally resolved. Rather than creating a tetraploid state where function is duplicated, the ancestral gene's functions can be split between the two zebrafish genes, and mutations can therefore have separable phenotypes as has been shown for Na(+), K(+) ATPase, *Hox*, and *Pax* genes [(58,129,145) and references therein] or one copy may become inactivated, leading to a pseudogene.

There are a few peculiarities of genetic screens that are eminently suited to clinical extrapolation. Because phenotype is the entrance filter for genetic screens, combinations of defects are not necessarily uninformative, but rather, by their complexity, may reflect syndromes, i.e., patterns of multiorgan dysfunction that coexist because of shared underlying pathways. In addition, many common human diseases, even if heritable, are not due to null mutations. Unlike targeting of genes for disruption in mice, which intentionally renders them null at least at first pass, ENU often causes partial loss-of-function or hypomorphic mutations, which reveal more subtle effects. ENU-induced mutations are thus potentially more relevant to human disease than null mutations in which early or widespread dysfunction may obscure the role of a gene in later organ formation. Furthermore, because functional analysis comes first in genetic screens, ENU-induced mutations make no presuppositions about protein domain structure, so the mutation may reveal previously undescribed important domains.

We here examine a few organ systems and question the clinical relevance of the zebrafish by using two criteria, the degree of resemblance between wild-type fish and human tissues and the faithfulness with which mutant phenotype mimics disease. We also briefly examine the possibility that zebrafish may be used more

directly for drug and other therapeutic discovery. We ask the reader to keep in mind some caveats throughout the review: The phenotypes we discuss are embryonic and inherited (usually) in a recessive lethal manner, whereas the human diseases are generally postnatal or adult in onset and rarely strictly Mendelian in inheritance. Hence, the hope is that the mutant genes point to pathways that explain pathophysiology of disease and, when such diseases are heritable, can serve as candidate genes that might otherwise be difficult to ascertain by positional cloning in humans. We focus here on the genetic screens, but it is additionally possible to interfere specifically with gene expression in zebrafish by injection of morpholino antisense oligomers. Although such defects are transient and not heritable, morpholinos can be used to assess the effects of diminishing function of orthologs of genes, including those known to be involved in disease, such as dystrophin, presenilin, and apolipoprotein E (6, 97, 111).



## THE FISH AS PATIENT

### Heart: Form, Contractility, and Rhythmicity

HEART FAILURE Heart failure affects nearly five million Americans, with 400,000 new cases each year (15, 115). Approximately 20% of cases are familial. The vast majority are dilated cardiomyopathies (with diminished contractility, lower cardiac output, and generally thin ventricular walls) and of unknown cause (as opposed to the hypertrophic cardiomyopathies, which often exhibit increased contractility and thickened ventricular walls evidencing myofibrillar disarray, disorders that are generally due to mutations in myofibrillar genes). Drug therapy is limited for all causes of heart failure and is palliative at best, with definitive cure for the most severe cases limited to those fortunate enough to receive heart transplantation.

The embryonic zebrafish heart at 24 h post fertilization (hpf), as shown in Figure 1 and as can be viewed in video at http://cvrc.partners.org/AR/supplement.html/, is nearly identical to the two-chambered human heart at three weeks of gestation. It is spontaneously contractile, emptying atrium and then ventricle sequentially to generate unidirectional blood flow. Growth of a muscular septum late in human embryonic development, which divides the primitive atrium and ventricle into left and right parts, differentiates the adult human from the adult zebrafish heart, the latter retaining a single atrium and a single ventricle.

More than 35 mutations in the zebrafish prevent the normal acquisition of contractile function, without disturbing the generation of normal chamber cell fate or formation, and therefore functionally mimic dilated cardiomyopathies. *pickwick*, a mutation in *titin*, the myofibrillar element around which the actin-myosin arrays assemble and contributor of most of the elastic force to the muscle, has been cloned (176). It is interesting to note that a human dilated cardiomyopathy family has also recently been documented to have a *titin* mutation (62, 78). Other mutations cause increased amount of heart tissue as well as hyperplastic hearts (W. Rottbauer,

personal communication). These "heart failure" mutations in zebrafish will reveal not only candidate genes for disease propensity in humans, but also potential factors and pathways that could be pharmacologically manipulated to improve contractile function.

ARRHYTHMIAS Another common set of ailments with only rarely defined molecular pathogenesis is arrhythmia. Arrhythmia means that the heart beats at the wrong rate or rhythm, that it incorrectly propagates the electrical impulse to the chambers, or that it expresses a defect in automaticity. Although there certainly are channel disorders that predispose to certain arrhythmias (60, 73, 164), how such molecular disorders lead to abnormal cardiac rhythmicity is unclear. In many cases, an arrhythmia is associated with an anatomic abnormality, such as chamber enlargement or scar after injury. Sudden cardiac deaths (the majority of which are arrhythmia related) account for 300,000–400,000 deaths annually in the United States, half of all cardiac deaths. Taken on its own, sudden cardiac death would be the third leading cause of death in the United States (110).

The zebrafish heart manifests the same pattern of electrical excitation as does the human heart, with impulses generated in the sino-atrial node, propagated through the atrium, pausing in the atrio-ventricular (A-V) node, and thence to the ventricle (Figure 1*D*). Mutations that mimic the most common arrhythmias, including pacemaker problems *(slow mo* and *reggae*), A-V block (*hiphop* and *breakdance*), and atrial fibrillation (*island beat, isl*), have been described (7, 24, 138, 150). The atrial fibrillation of the *isl* mutation is due to a mutation in the gene for the cardiac-specific L-type calcium channel (138), an observation of relevance because chronic atrial fibrillation in humans is associated with diminution in expression of this channel gene (19, 147).

CONGENITAL HEART DISEASE Congenital anomalies of the heart affect 8 of 1000 live births (11). Three percent of these are due to inadequate chamber growth (45). Although there is a high sibling recurrence, suggesting a genetic component, these diseases in general are not transmitted in a Mendelian fashion.

Several mutations in zebrafish disrupt generation of early heart form. For example, *heart and soul* causes the ventricle to form inside of the atrium and is due to a mutation in PKCλ (72, 125). *handsoff* and *pandora* have diminutive ventricles, the former due to mutation in the bHLH transcription factor *dHAND* (177). *jekyll* is due to a defect in the enzyme UDP-glucose dehydrogenase and lacks an A-V valve (162). *casanova* is a *sox*-related gene that lacks endoderm and causes cardia bifida (32, 88). Most of these mutations are unlikely to speak directly to human disease because comparable defects would be lethal at or prior to three weeks of gestation, before a pregnancy would even have been noted. Later in development the structure of the heart is not directly comparable between the two species because in the human there is septation, both between the chambers and within the outflow tract, the former achieved by localized muscle growth and the latter by neural-crest migration (89, 161). However, it is likely that mutations interfering

with muscle growth or neural-crest migration may reveal genes related to these cardiac septation processes as well.

## Vascular Disease, Angiogenesis, and Oxygen Deprivation

VASCULOGENESIS AND ANGIOGENESIS Blockage of large bore arteries causes ischemia, with, for example, consequent myocardial infarction or stroke. If it occurs during development, poor vascularization can retard growth of distal tissues. This type of large-vessel assembly requires not only generation of endothelial tubes, but also their envelopment by connective tissue and smooth muscle. A similar process in the embryo is the establishment of the large vessels, a process termed vasculogenesis (21,56,59,179,180). The generation of smaller vessels, termed angiogenesis, is important clinically because it sustains the growth of tumors and underlies disorders such as diabetic retinopathy (57). More is known about the molecular mechanisms of angiogenesis than about vasculogenesis.

One of the curiosities of vascular disease is that it strikes regionally in vessels otherwise apparently homogeneous. Atherosclerosis, for example, affects arteries and not veins and does so primarily at junction points, ostensibly because these are the regions subjected to the highest sheer stress by hemodynamic force. Evidence that there are regional molecular differences among vessels is accumulating (5). Obviously such intervascular differences would be of importance for the design of targeted pharmaceuticals.

The zebrafish embryo provides an opportunity to visualize both vasculogenesis and angiogenesis in real time and to define relevant pathways by mutational analysis. Vasculogenesis involves medial migration of angioblasts from the lateral mesoderm, to form the single central artery and vein of the trunk in the zebrafish embryo. From these vessels sprout smaller intersomitic branches. Mutations have revealed clear regional differences along the course of, as well as between, vessels. The *gridlock* (*grl*) mutation selectively blocks blood flow at the anterior bifurcation of the trunk artery, the aorta (168). This is believed to be the region affected in coarctation and other aortic arch anomalies in humans. *grl*, and the mammalian homologue *hey2*, is a divergent member of a transcription factor family *hairy enhancer of split* (180) and is part of a pathway that is needed to generate the artery. The *grl* mutation is hypomorphic. Complete absence of g*rl* gene product prevents formation of the entire artery (179). That the anterior aortic bifurcation, a region subject to congenital anomalies, also is particularly sensitive to *grl* diminution is a good example of how hypomorphic mutations can be revealing in terms of disease. Whether this gene actually is involved in human aortic disease is now under investigation.

Even within small vessels two or three endothelial cells in length, there are clear regional disparities. The microenvironment regulates the fate of endothelial cell in particular locales, such that mutations selectively perturb regions of vessels. For example, the normal growth of intersomitic vessels appears constrained by signals from the somites and is diminished in the *out of bounds* mutation (26). This could be of clinical relevance to the search for inhibitors of angiogenesis.

ADAPTATION TO ISCHEMIA Chronic ischemia often is better tolerated by the affected end organ than is the acute cessation of blood flow. Additionally, several brief episodes of transient ischemia appear to precondition the end organ better to tolerate periods of ischemia. The molecular and genetic mechanisms of preconditioning are poorly understood.

The zebrafish embryo has a particularly dramatic response to ischemia. Embryos maintained in absolute anoxia enter a state of metabolic arrest, or "suspended animation," for periods of up to 24 h (120). This ability is lost between 24 and 48 hpf. During the period of arrest, there is a cessation of motion and heartbeat. At the cellular level, proliferation appears to be arrested predominantly in the S- and G2 phases of the cell cycle. Adult zebrafish can be primed to endure hypoxia, mimicking the process of ischemic preconditioning. There is an extension of survival times in severe hypoxia (5% $O_2$) when adult zebrafish are allowed to acclimate to nonlethal hypoxia (10% $O_2$) compared with nonpretreated controls (134). These observations suggest that it may be feasible to screen for genes relevant to preconditioning.

## Kidney: Cysts and Renal Failure

Chronic renal failure affects 400,000 Americans, most of whom will come to require long-term dialysis (158). During embryogenesis, the human kidney is generated as sequential kidney types (pronephros, mesonephros, and metanephros), each replacing the prior one during development. The teleost embryo retains the pronephric kidney, which is necessary for osmotic balance in the embryo. Although the global structure of the pronephric kidney differs from the metanephric, it has the three major modular elements of all kidneys: the glomerulus [the region where vessels appose renal podocytes and filtration occurs (see Figure 2)], tubules, and ducts, the latter two being where urine is modified by absorption and secretion.

Polycystic kidney disease (PKD), the most common form of heritable end-stage renal disease, accounts for 3% of patients who need chronic dialysis (158). The mechanism of the cyst formation is not clear. In 85%–95% of cases of autosomal dominant PKD, there is a mutation in PKD1 (90). Found in areas of cell-cell junctions in epithelial and endothelial cells, polycystin 1, the gene product of PKD1, is a glycoprotein that spans the membrane 11 times and is thought to serve as an important mediator of protein-protein interactions. At least 15 mutations in the zebrafish cause cysts to form (38). Using an insertional mutagenesis strategy, Sun & Hopkins recently cloned a cyst mutant that encodes *vHnf1*, which is the orthologous to the gene mutated in human glomerulocystic kidney disease (154).

Renal failure also occurs from vascular disease, although the route to parenchymal pathology is not clear. It is interesting that, at least during development, assembly of the glomerulus depends on maintenance of normal flow in the vessels to the kidney. Perturbations that block flow, be they genetic, physical, or pharmacological,

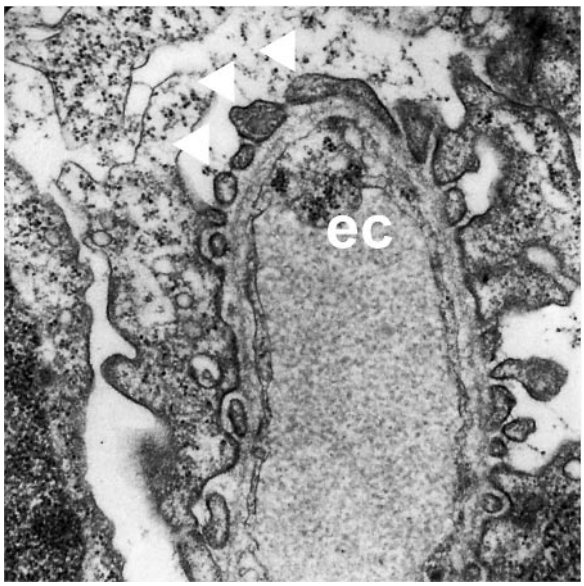


**Figure 2** The zebrafish glomerulus. This electron micrograph of a zebrafish glomerulus shows an endothelial cell (ec) and interdigitation with surrounding podocytes and foot processes (*white arrowheads*) (courtesy of M. Elger & H. Hentschel).

block normal assembly of the glomerulus. The mechanism seems to involve stretch receptors in the vessel wall and is mediated by matrix metalloproteinase-2 (MMP2) (143). In this case, it appears to be a sensible linkage, in that generation of the specialized fenestrated filtering endothelium of the glomerulus does not form without assembly of the urine-modifying organ. It is of interest that in patients with diabetic renal disease, which has an important vascular component, there is downregulation of MMP2 (30).

## Skeleton: Osteoporosis and Congenital Malformations

OSTEOPOROSIS Osteoporosis is the most common form of metabolic bone disease in human adults, affecting approximately 20 million Americans. Osteoporosis is a disorder of bone remodeling with an imbalance between bone production by osteoblasts and bone removal by osteoclasts. It is manifest clinically by loss of 50% trabecular bone and 30% cortical bone mass. Derived from hematopoietic precursors of the macrophage lineage, the human osteoclast is traditionally described as a multinucleated cell that is responsible for resorbing bone. Increased osteoclast number and heightened activity are associated with the increase in bone turnover and the decrease of bone mineral mass that is associated with osteoporosis. Although complex in etiology, osteoporosis clearly has a genetic component.

**Figure 3** The zebrafish skeleton. A radiograph of an adult zebrafish skeleton shows ossification of cranial and axial skeleton as well as fins and rays (courtesy of S. Fisher).

In teleosts, as in other vertebrates, the skeleton arises via condensation of mesenchymal cells in areas that first give rise to chondrocytes, followed by osteocytes and osteoblasts. The early genetic screens done to date in zebrafish were not suited to discovery of primary defects in mineralization because this process occurs relatively late in development, and screening was done principally during the first few days of embryogenesis. The earliest calcification occurs in the head skeleton 5 days post fertilization (dpf) and then proceeds caudally, such that all skeletal elements demonstrate evident calcification by 23 dpf (41) (see Figure 3). The first mononuclear osteclastic cells are noted in zebrafish only after 20 dpf, with functional multinuclear osteoclasts appearing after 40 dpf, and bone remodeling not evident until 60 dpf (172). Caudal skeletal defects are seen in *chordin* mutations [identification of these defects is possible because 4% of *chordino* homozygotes survive into adulthood (51)]. Zebrafish bone differs functionally from mammalian bone in that it does not serve a homeostatic role in calcium metabolism; rather, the gill is the primary organ of calcium regulation. However, at least most of the specific elements genes important to mammalian osteogenesis have zebrafish orthologs: These include CBFA1/RUNX2, which regulates mammalian osteoblast differentiation; FGFR3, which has been linked to achondroplasia, Crouzon's syndrome, and Thanatophoric dysplasia; PTH receptor, which is involved in Blomstrand's chondrodysplasia and Jansen's disease; and BMP and IHH, which have been implicated in the human disease brachydactyly [reviewed in (160)].

OSTEOGENESIS IMPERFECTA Osteogenesis imperfecta is a rare, autosomally dominant disorder characterized by extreme bone fragility. The underlying defect is characterized by a disorder in connective-tissue formation with specific structural defects identified in collagen type I, resulting in decreased amounts of normal collagen. The zebrafish mutation *chihuahua*, appears to affect collagen formation (S. Fisher, personal communication) and is manifest by a rounded forehead and

small jaw. There is normal cartilage formation during development, but adult bone is fragile and easily broken.

Current analyses of the skeleton have relied principally on postmortem analysis by the histological stains alcian blue for cartilage and alizarin red for bone. The fluorescent calcium chromophore, calcein, makes it possible to label bone in living embryos and so may facilitate screens in older embryos for defects in bone metabolism (41).



CRANIOFACIAL SYNDROMES Holoprosencephaly (HPE) is estimated to affect 1/250 conceptions and occurs in 1/15,000 live births (170). Manifestations range from a single central incisor to severe facial abnormalities with cyclopia, overlying proboscis, and cleft palate accompanied by fusion of the cerebral hemispheres. The genetics of HPE are complex, with chromosomal abnormalities in one quarter to one half of affected patients (170). The first of these to be cloned on chromosome 7 affected the *sonic hedgehog* (*shh*) gene (12, 137). The zebrafish mutant *sonic you* (*syu*) carries point mutations in the zebrafish ortholog of *shh*. *syu* embryos have small eyes with reduced spacing between them, a small head, and pectoral fin defects (140). Other mutations affecting downstream elements of the *shh* signaling pathway also cause cranial abnormalities. For example, *you too*, a mutation in the zebrafish *gli-2* ortholog, causes posteriorly turned-in eyes without eye fusion (83). Mutations in other pathways also lead to a single, central eye, including *cyclops*, which affects a *nodal*-related gene in the squint pathway (133), and *one-eyed pinhead*, which encodes an epidermal growth factor–related molecule of the nodal pathway. These mutants may help to identify specific pathways underlying the nonheritable forms of HPE.

The bones of the lower face and jaw are derived embryologically from the branchial arch apparatus and depend on the migration of neural crest for their specification and morphogenesis. The *sucker* (*suc*) mutation in zebrafish disrupts lower-jaw morphogenesis. *suc* encodes *endothelin-1*, normally expressed in a core of arch mesoderm and epithelium. Mutation of the mouse ortholog of *endothelin-1* causes a similar phenotype (96). The zebrafish adds an additional ability to track in real time the migration and maturation of these skeletal precursors from their origins in the neural crest, thereby establishing the timing of the affected developmental step and the cell autonomy of the mutation. Such tracking also may help to define the targets for teratogenic effects of certain pharmaceuticals or toxicants affecting facial bone development and possibly the agents to revert such problems.

The human cranial vault and other rostral elements of the head skeleton are affected by mutations in the fibroblast growth factor receptors (FGFR). Mutation of the FGFR3 gene is associated with Muenke's syndrome (113) and with a subset of individuals suffering from Crouzon's syndrome (109). Unlike most bones in the body, the mammalian cranium is composed of bone that forms not from cartilage but from direct ossification of the dermis in a process termed intramembranous ossification. Although the specific mechanism by which the zebrafish cranium

forms has yet to be described, the cloning of the zebrafish FGFR3 and its localization in early head structures suggests that this molecule may be important in the development of the zebrafish cranium as well (148).

REGENERATION Fish grow throughout their lives, and therefore their organs must continue to grow proportionally. In addition, adult teleosts can regenerate many tissues, including the fin. The zebrafish fin is composed of several tissue types. The rays are bone and grow by addition of new segments to the end of the distal tip. Each ray is composed of two hemirays surrounding a core of fibroblasts, pigments cells, nerves, and blood vessels (82). Epidermis surrounds the outer layer of the fin. All of these tissues are reconstituted after injury. Mutations have been identified that affect the development of the fin or the size and regeneration of the adult fin (4, 81, 159). The latter group includes mutations that are dominant and temperature sensitive. Furthermore, the ability to pharmacologically modulate this process by addition of small molecule inhibitors to the environmental water has been used to demonstrate the essential role of FGF signaling in blastema formation (128).

## Blood: Aplasia, Hematopoiesis, and Clotting

BONE MARROW APLASIA In humans, failure to generate blood cells of all lineages may occur as a heritable disorder or, secondarily, as a consequence of infection or complication of chemotherapy or radiation toxicity to the bone marrow. The incidence of primary aplastic anemia ranges between two to seven per million, depending on geographic area (with the highest occurrence in Southeast Asia) (178). The affected genes are known for a subset of these [e.g., Fanconi's anemia type I (49), Dyskeratosis congenita (*dyskerin*) (70), and paroxysmal nocturnal hemoglobinura (*pig*-A) (106)], but the specific pathogenesis of the majority of cases has yet to be elucidated. Additionally, despite an excellent understanding of the pathways that drive hematopoietic lineages, the stem cell remains elusive. Currently, it is believed that there is a shared embryonic precursor for both the original hematopoietic and angioblast lineages, a cell termed the hemangioblast. Where this cell resides is speculative.

It is hoped that the zebrafish mutation *cloche* (*clo*) will provide molecular access to the hemangioblast. *clo* mutant embryos lack nearly all blood cells and vessels (151). Injection of *scl* and *hhex* genes, but not *vegf*, rescues blood formation, suggesting that *clo* is essential to the formation of the hemangioblast (100, 101). SCL, identified in a chromosomal translocation associated with acute lymphocytic leukemia, acts at the stem cell level to specify all blood lineages in humans and mice and appears to be acting downstream from *clo*.

ERYTHROCYTES: IRON METABOLISM, HEME SYNTHESIS, AND PORPHYRIAS Iron is an essential component of hemoglobin, as well as key metabolic enzymes, and its levels are tightly regulated during development and adult life. Hereditary

hemochromatosis, a human autosomal recessive disorder of iron overload caused by mutations in the HFE gene, is manifest by diabetes, bronze skin, hepatic failure, and cardiomyopathy. Over 10% of certain northern European populations carry at least one mutant allele of HFE (22). HFE is expressed in the placenta and the crypt enterocyte and is linked to iron transport through its association with the transferrin receptor. Adult mammals absorb iron from the duodenum in two steps. The divalent metal transporter, DMT1, whose expression is upregulated in hereditary hemochromatosis (55), mediates apical absorption of iron into the enterocyte. The second transporter, ferroportin 1 (FP1), was discovered as the gene mutated in the *weissherbst* mutant zebrafish and is manifest by hypochromic erythrocytes, anemia, and reduced levels of iron in the red blood cells (RBC) (35). Human FP1 is expressed on the basolateral surface of the duodenal enterocyte, where it is the transporter exporting cellular iron into the vessels. Both DMT1 and FP1 are upregulated in models of hereditary hemochromatosis (181).

Porphyrias are disorders caused by defects in the heme synthesis pathways, with buildup of toxic byproducts, porphyrins, that are specific to defects in particular enzymes in that pathway. The porphyrias may be manifest in childhood or adulthood, with hepatic hemosiderosis followed by cirrhosis, RBC fragility, and hemolytic anemia and in some cases with intermittent psychosis. There is speculation that "the madness" of King George III resulted from this inherited disease (105). To date, three zebrafish mutations have been identified that involve the heme biosynthetic pathways. The zebrafish *yquem* mutation has autofluorescent RBCs and anemia (163). This mutation affects uroporphyrinogen decarboxylase and is the first animal model of the hepatoerythropoietic variant of porphyria. *dracula* has a more pronounced anemia when raised in ambient light conditions, with rapid lysis of intact erythrocytes under epifluorescent illumination (510–560 nm wavelength), and results from a mutation in *ferrochelatase*, mimicking the human condition erythropoietic protoporphyria (27). The third mutation, *sauternes* (*sau*), encodes a mutation in the first enzyme of the heme biosynthetic pathway, $\delta$-aminolevulinate synthase (18). Pharmaceutical reagents have been difficult to design for the porphyrias, so these piscine models may be of particular use in the design of assays for novel pharmacotherapeutic agents (see below).

Hereditary spherocytosis is an autosomal dominant defect in red cell morphology, which affects 2 in 10,000 individuals. A defect in the RBC membrane-associated protein, $\beta$-spectrin, causes abnormal erythrocyte shape, increased fragility, and anemia. Erythropoiesis is normal, but there is reduced red cell survival owing to hemolysis with consequent anemia. The zebrafish mutation *riesling* is in the zebrafish ortholog of $\beta$-spectrin. By 4 dpf, it exhibits profound anemia and abnormal erythroid morphology.

LEUKOCYTES AND PLATELETS The original genetic screens did not focus on white blood cells, which are fewer in number and harder to visualize than are RBCs. However, it is clear that the zebrafish has circulating granulocytes, including a heterophil (similar to a neutrophil), which expresses myeloperoxidase, an eosinophil,

as well as a set of tissue macrophages that serve as a primitive reticuloendothelial system (102). The heterophils appear to be recruited to sites of injury and to be involved in the inflammatory response. A zebrafish platelet has also been identified, which aggregates in vitro to traditional platelet agonists such as ristocetin, collagen, and arachadonic acid. Aspirin inhibits this response, suggesting that cyclooxygenase is involved in the aggregation response much as it is in the mammalian platelet (79). It is interesting to note that immunochemical evidence suggests that the zebrafish platelet analog also has the surface glycoprotein receptors (Ib and IIb/IIIa) known to be important in vascular disease and as the targets of the IIb/IIIa pharmaceutical antagonists. A subset of lymphocytes has also been described in the zebrafish. These cells appear relatively late in development and for their maturation require both thymic education and extrinsic activation of recombination activating genes (*rag*) (171).

## Brain: Neuronal Survival and Degeneration

PARKINSON'S DISEASE PD is a neurodegenerative disorder caused by the death of a particular population of dopaminergic cells and affects at least 500,000 adults in the United States. Pathological findings include loss of dopamine-producing cells and deposits, called Lewy bodies, in the cells of the substantia nigra (SN) and locus coeruleus (LC). Hence the loss of specific populations of catecholaminergic neurons appears to be critical to the pathophysiology of PD. Parkinsonism is characterized primarily by signs and symptoms related to muscle rigidity and tremor, presumably owing to neuronal loss and neurotransmitter depletion in the nigrostriatal pathway and putamen. Although the vast majority of cases appear to be sporadic, there are some families with an apparently heritable component to PD, and three mutations have been associated with an early-onset form of PD (94, 103, 127). However, the cause of most cases of PD remains unknown, and why these particular catecholaminergic neuronal populations should be susceptible to it remains an active area of investigation.

In zebrafish, genes involved in the systhesis of catechol precursors are expressed at approximately 22 hpf and are restricted to one or two cells of the ventral diencephalon. Six hours later, the number of cells expressing catechol biosynthetic enzymes has increased to five or six in the tract of the postoptic commissure. By 36 hpf, the cells of the LC are evident in the ventrolateral region of the hindbrain rhombomere-1, sending axonal branches to the medulla. Other neurons that produce dopamine or its deriviative epinephrine and norepinephrine are found in the adult zebrafish, including in the pituitary, the olfactory system, the amacrine cell layer of retina, the branchial arch-associated tissues, and the gut-associated ganglia. There appears to be no counterpart in zebrafish to the SN.

Genetic screens have revealed genes essential to development of the catecholaminergic system, such as the LC, some of which had not previously been known to be involved in development of these cells. Mutations that interrupt the genes for the *paired*-like homeodomain protein, *phox2a* [*soulless* (66)], *fgf8*

[*acerebellar* (135)], *bmp2b* [*swirl* (93)], and *bmp7* [*snailhouse* (66)] all disrupt the formation of LC progenitors. A mutation in *smad5* [*somitabun* (66)], which also affects BMP signaling, leads to the development of supernumerary noradrenergic neurons. Other mutations that selectively affect subpopulations of catecholaminergic neurons have been isolated. For example, the zebrafish mutant *soulless* lacks LC and arch-associated neurons but has normal hypothalamic dopamine-producing cells (66).

ALZHEIMER'S DISEASE AD is the eighth leading cause of death in the United States (110), and its prevalence is increasing because of longer lifespans in developed countries. In less than 10% of cases, AD is familial and due to mutations in amyloid precursor protein (APP), presenilin (PS)-1, or PS-2. In most cases, especially in those of late onset, it does not evidence clear heritability, although there is an association between AD and the ApoE4 allele of apolipoprotein E. Zebrafish express APP, PS-1, and apoE. Zebrafish PS-1 is maternally inherited and ubiquitously expressed embryonically (97). ApoE is expressed in the brain and retina of the day-3 embryo as well as in the epidermis and fin bud (111).

## Behavior: Circadian Rhythms and Addiction

CIRCADIAN RHYTHMICITY Molecular clocks mediate entrainment of organismal behavior to light-dark cycles. Many disorders manifest circadian exacerbations [e.g., asthma (10) and angina (114)]. In mammals, lights exposure triggers release of melatonin from the superchiasmatic nucleus (SCN), which then acts to synchronize peripheral clocks. However, most cells have an intrinsic circadian rhythmicity of gene expression that can be changed by light even in cell culture. In mammals, the peripheral oscillators can be uncoupled from the central SCN-driven one by starvation (8, 43).

Adult zebrafish exhibit circadian rhythmicity of gross locomotor activity (76) and are active during light. Embryos inherit some clock components from maternal message that may help to set the phase of the cycle (29), and they manifest nocturnal increases in melatonin at 2 dpf (84). CLOCK, a presumptive master regulator of circadian rhythms, and its binding partners zfBMAL1 and zfBMAL2 cycle in circadian rhythm but, of interest, manifest varying temporal patterns in different tissues (23). CLOCK oscillations persist in culture of zebrafish tissues such as heart, kidney, and spleen, where they can be reset by changes in the light-dark cycles (169), perhaps reflecting the fact that the tissues normally are accessible to light through the transparent embryo.

ADDICTION Substance abuse and addiction has long been thought to have a heritable component (165), although the responsible genes are unknown. Genes encoding enzymes of the dopaminergic pathway have been suggested as candidates based on their involvement in reward schema and genetic segregation analyses (13). Because many substances, including alcohol and cocaine, can permeate

embryos when added to the water, it is feasible to analyze their effects upon behavior. Genetic screens in zebrafish recently have been explored as a means to begin genetic dissection of addiction susceptibility. Using a conditioned-place paradigm in which cocaine is coupled with a distinct visual cue, three mutations have been identified (*dumbfish*, *jumpy*, and *goody-two-shoes*), with diminished responsiveness to cocaine (28).

## Ear: Deafness and Vertigo

The human ear is the chief organ both of balance and auditory perception. It is estimated that 1 in every 150 people has some form of hearing loss (141). Ménière's disease, a disorder of episodic vertigo and progressive hearing loss, affects 2 in 1000 in the United States (173). The majority of disorders leading to vertigo as well as hearing loss are associated with aging, although some do have a clear genetic basis. Type I Usher syndrome is the most common cause of combined deafness and blindness in the industrialized world, with a frequency estimated at 4.4/100,000 in the United States (14). It is an autosomal recessive disorder in which afflicted patients suffer from profound congenital deafness, vestibular areflexia, and progressive retinitis pigmentosa. Type I Usher syndrome has been mapped to three loci, with 75% of patients having type Ib, which results from a defect in the structural protein myosin VIIa (167). This gene also is affected in the *shaker-1* mouse (63). Mutations in *cochlin* account for some familial Ménière's disease/DFNA9 (136), suggesting a close association between disorders of hearing and balance.

The ear of the zebrafish is grossly different from the human but develops in a similar manner and shares certain important features. In both species during embryonic development, a thickening of the surface ectoderm lateral to the caudal hindbrain, termed the otic placode, invaginates and migrates medially to give rise to the otic vesicle. In humans, this occurs around the fourth week of development and in the zebrafish at the 14-somite stage. In the zebrafish, two otoliths are visible by 19 hpf, and full development of the semicircular canals, the organ of position sense, begins at 44 hpf and is complete at 64 hpf. In humans, the otic vesicle gives rise not only to the semicircular canal, but also to a spiral organ, the cochlea, which transduces acoustic impulses based on frequency. Although the zebrafish ear does not have a cochlea per se, the hair-cell epithelium within the semicircular canals appears to play a similar function. The stereocilia line the apical surface of the cochlea and semicircular canals and transduce mechanical movement into neural signals. Each hair-cell unit comprises a single, tall kinocilia and multiple smaller stereocilia tethered together by fine filaments. It is believed that the channels are in the ends of the stereocilia, with channels gated by tension at the link between the stereocilia and channel.

Genetic screens in the zebrafish have identified mutants in mechanosensation. In the mutants *sputnik* and *mariner*, the stereocilia are detached from the kinocilia and transduction is abnormal (116). It is interesting to note that the *mariner*

mutation has been identified as a defect in the zebrafish ortholog of myosin VIIa (48) and, hence, demonstrates conservation both structurally and functionally of the mechanosensory apparatus across species. In other zebrafish mutants, the hair-cell apparatus appears ultrastructurally intact. *orbiter*, *mercury*, and *gemini*, like *sputnik* and *mariner*, lack hair-cell microphonics, although they appear otherwise morphologically and physiologically normal (116). Hence, their cloning may reveal elements of sensorineural hearing loss. *astronaut* and *cosmonaut* have normal microphonics and thus may reflect genetic susceptibility to central hearing loss (116).



## Eye: Retinitis Pigmentosa and Macular Degeneration

Retinitis pigmentosa (RP), a heritable disorder of photoreceptor degeneration, affects 100,000 individuals in the United States and 1 in 4,000 individuals worldwide. Patients with RP develop progressive night blindness during adolescence culminating in blindness later in adulthood. In those with a genetic basis, RP can be caused by a variety of mutations, including mutations in genes involved in light transduction [*rhodopsin* (40), *arrestin* (3), subunits of rod cGMP phosphodiesterase (39)] and of chromophore [retinal pigmented epithelium (112)] and retinaldehyde binding proteins (107).

Age-related macular degeneration, which affects two million Americans, results from loss of retinal cells in the region of the fovea centralis and surrounding macula lutea, where high-resolution vision takes place. It is predicted that, with the aging of the population, blindness resulting from macular degeneration will exceed the blindness due to diabetes and glaucoma combined. Macular degeneration in the elderly is associated with loss of retinal pigment epithelium (RPE) cells and function. Photoreceptors synthesize 10% of the outer segment each day, added at the base. The tip is removed and degraded by the RPE. The RPE also serves to support the photoreceptors in other ways, for example, by adjusting the ionic composition of extracellular fluid, processing vitamin A derivatives used for photoreception, providing nutrients, and inactivating free radicals generated by light absorption (153). Most cases of macular degeneration are of unknown cause and occur late in life. There are less-common heritable macular diseases that have an earlier age of onset (153), such as dominant familial drusen, a defect in the retinal specific protein VMD2 (of uncertain function), which causes vitelliform macular dystrophy (126); a mutation in TIMP-3, which causes accumulation of an abnormal membrane and interferes with vitamin A diffusion between the photoreceptor and the RPE and leads to Sorsby fundal dystrophy (166); and Stargardt Disease (2). A key issue for understanding macular degeneration is the nature of trophic signaling between the RPE and photoreceptors.

Retinal development both in zebrafish and in humans begins as a reflection of the neural tube called the optic vesicle. This appendage migrates eccentrically and forms the optic cup, the inner lining of which gives rise to the retina. In zebrafish, the photoreceptors are well differentiated by 72 hpf, when they have

been demonstrated to be functional through behavioral assays (44). The mature retina in zebrafish is similar to that in humans (Figure 4), in that it has three nuclear layers and two synaptic layers. The most medial layer contains photoreceptors: rods for transducing dim light and cones for color and bright light. Transduction occurs in the outer segment of the photoreceptors. The middle nuclear layer has bipolar cells, horizontal cells, and amacrine cells, the former transmitting from receptor to ganglion cells and the latter two responsible for lateral transmission. The axons from the ganglion cells compose the optic nerve. The zebrafish retina differs from that in humans in some aspects of development. For example, the central nervous system of teleosts is a rod of tissue, a neural keel, rather than a hollow tube. Zebrafish have five visual pigments, one rod and four cones: The cones transduce UV (362 nm), blue (415 nm), green (480 nm), and red (560 nm) wavelengths (16). In contrast, the human retina possesses one rod and three types of cone cells: blue (420 nm), green (530 nm), and red (560 nm). The layout of rods and cones throughout the retina also differs. In humans, the center of vision, the fovea, and its immediate surrounding region, the macula, are almost entirely cones, with rods distributed in the periphery. In zebrafish, there is a mosaic of regularly repeating cones distributed evenly across the retina.

The retina is affected by several mutations in zebrafish. There appear to be two prominent patterns of photoreceptor cell loss in mutants (34). One type (*niezerka*, *mikre oko*, and *not really finished*) begins peripherally, has severe glial-cell defects, and is cell nonautonomous. The others [*brudas* (*bru*), *elpsai* (*eli*), *fleer* (*flr*), *photoreceptors absent*, and *oval* (*ovl*)] begin centrally, exhibit fewer glial deficits, and are cell autonomous (33, 34). In teleosts, cells are added throughout life to the margin, so the central cells are the oldest, raising the speculation that the central-loss type affects the oldest photoreceptors and might represent an intrinsic defect in cell survival. It is presumed that mutations that affect principally the peripheral photoreceptors are owing to loss of limiting extracellular survival factors, possibly from glial cells. It is interesting to note that *eli*, *flr*, *and ovl* also have kidney defects—a combination noted in the human Senior-Loken and RHYNS (retinitis pigmentosa, hypopituitarism, nephronophthisis, skeletal dysplasia) syndromes. *bru* mutants also evidence progressive paralysis, which is a features of the human ceroid lipofuscinosis, Batten disease. The dominant mutation *night blindness a* (*nba*) (99) is of special interest vis-à-vis late-onset disease because the fish is normal until two to three months and then develops night blindness owing to late-onset photoreceptor degeneration. In homozygous mutant fish, retinal degeneration is much more pronounced and not restricted to photoreceptors, indicating it is not a photoreceptor-specific defect.

The zebrafish mutation *mosaic eyes* (80) may be relevant to macular degeneration. In *mosaic eyes* embryos, retinal-cell differentiation occurs, but the retina is disorganized. Cell-transplant studies show that the proper organization of the retina requires *mosaic eyes* function in the RPE.

Although initial screens used visual inspection of retinal architecture for identification of mutant embryos, retinal function screens can also be performed using

assays such as the optokinetic response or electroretinogram (17). The recent generation of a transgenic fish using the opsin promoter to direct green fluorescent protein expression to the rod photoreceptors provides an opportunity to extend these analyses to more subtle alterations of photoreceptor structure and function (87).

## Gastrointestinal Tract

COLORECTAL CANCER Although much has been learned about the genetics of colorectal cancer (92, 98), it still is the second leading cause of cancer death in the industrialized world. Several attributes suggest that the responsible cell (20, 156, 175) may have features of an epithelial stem cell, but to date the means to isolate and study such cells have been elusive.

The zebrafish gut develops from a cord of endodermal tissue, as opposed to the mammalian embryonic gut, which is a tube generated by invagination of the endoderm. There is no stomach in the zebrafish, but there are regions that predominantly participate in the digestion of proteins, fat, and ions, mimicking the anatomic divisions in humans (119). The zebrafish intestine microanatomically resembles the human intestine in many regards. An epithelial layer lines the lumen, with cell division in the intervillus spaces providing a renewing population for sloughed epithelium. A number of zebrafish mutants that prevent normal maturation of the intestine have been identified (1, 24, 119). The *pescadillo* mutation has been cloned in zebrafish, and the human homolog is overexpressed in malignant cells (91). *npo* (*nil per os*) encodes a novel gene expressed in the crypt region of adult fish in the presumptive stem cells (A.N. Mayer & M.C. Fishman, manuscript in preparation).

DYSMOTILITY The most common illnesses diagnosed by gastroenterologists are due to dysfunctional gut motility. These include irritable bowel syndrome, gastroesophageal reflux, and chronic constipation. Less common but immediately life threatening to a neonate is Hirschsprung's disease, a functional obstruction of the large intestine due to defective embryonic migration of enteric neurons. The enteric nervous system (ENS) governs gut motility through chemical mediators serotonin, acetylcholine, and nitric oxide, but pharmacological attempts to modulate gut motility have met with limited success.

Intestinal motility in the zebrafish results from contractions of an inner circular and outer longitudinal layer of smooth muscle (119). Peristalsis begins by 4 dpf and becomes coordinated one day later (119). To date, the genes isolated from zebrafish screens that speak most directly to motility are those that affect neural-crest development and thus lead to reduced or absent enteric neurons. For example, the *colourless* (*cls*) mutant lacks enteric neurons and glia and, predictably, appears to lack normal gut peristalsis (R. Kelsh, personal communication). In that it also has a pigmentation defect, *cls* resembles the human Waardenburg-Shah syndrome (86). *cls* encodes a *sox10* homolog (42) and performs a primary role in specification of neural-crest derivatives.

MALABSORPTION Nutrient absorption requires the enzyme-mediated breakdown of ingested macromolecules into absorbable units. Failure to accomplish this effectively leads to malabsorption and eventually malnutrition and can lead to severe fluid imbalances if ion and water transport is deranged as well. In fact, a leading cause of childhood mortality throughout the world is dehydration, secondary only to infectious diarrhea (65).

One approach to studying digestive physiology in vivo in the zebrafish involves the use of fluorescent substrates (50). Fluorescence resonance energy transfer analysis of phospholipase A2 activity in live zebrafish larvae was used to isolate a mutation, *fat free*, defective in this activity. Because the *fat free* mutant embryos lack any gross anatomic abnormalities, this work provides support for the use of zebrafish to genetically dissect a physiologic process.

DIABETES The human pancreas is composed of two functionally and anatomically distinct tissues. The islet cells produce the endocrine hormones insulin and glucagon, and their destruction due to autoimmune disease is the etiology of Type I (juvenile onset) diabetes. Type II (adult onset) diabetes is characterized by peripheral insulin resistance and tends to cluster in families. The exocrine pancreas is the source of digestive enzymes. The zebrafish pancreas has exocrine and endocrine component cell types, with the insulin-producing cells sequestered in a solitary islet [Figures 5*D*,*E*]. The *syu* mutation abrogates endocrine pancreas formation via its action upstream of the *pancreatic and duodenal homeobox gene-1* (*pdx-1*) gene (139). Several other mutations (*slim jim*, *piebald*, *straight shot*, and *no relief*) that selectively remove the exocrine component have been isolated (119).

LIVER Many different types of hepatic injury—alcohol, infection, and toxins—cause a similar pattern of histological degeneration and ultimately lead to cirrhosis. The pathways leading to massive liver failure are poorly understood. The only remedy at such late stages is transplantation of the liver. Over 5000 liver transplants were performed in 1999, and nearly 15,000 patients in the United States currently sit on waiting lists (157).

At approximately 32 hpf, the zebrafish liver derives from the primitive gut tube as a morphologically distinct left ventrolateral diverticulum. Like its mammalian counterpart, the zebrafish liver produces bile, which is stored in a gall bladder, although there are no true portal lobules (119). The zebrafish liver is evident by 3 dpf under the dissecting microscope. Several zebrafish mutations with early liver degeneration have been isolated. For example, the *lumpazi*, *gammler*, and *tramp* mutations encode defects at three loci that lead to liver necrosis (24). The *beefeater* mutation shows liver necrosis and impaired glycogen utilization, as seen in the human glycogen storage diseases (119).

## Pigmentation

Physiological processes culminating in human hyperpigmentation occur ritually during the late spring and summer and are clearly related to the increasing

incidence of melanoma, which currently affects 44,200 individuals per year in the United States (149). Vitiligo, a disorder of localized melanocyte loss, occurs at a frequency of 5/1000 (117). Mammalian melanocyte precursors are derived from the dorsolateral pathway of neural crest and give rise to all dermal pigment-producing cells.

During zebrafish embryogenesis, pigment cells arise from the neural crest and give rise to xanthophores (yellow pigment), reflective iridophores, and melanophores (black pigment). Melanocyte specification in the neural crest occurs early, prior to migration, with pigment appearing within the melanoblasts often before the definitive location is attained [reviewed in (132)]. Mutations have been identified that disrupt the ontogeny of zebrafish melanocytes and identify specific elements of zebrafish melanogenesis. For example, embryos with *nacre*, a mutation in the microphthalmia transcription factor (*mitf*) gene, the human ortholog of which has been implicated in a subset of patients with Waardenburg syndrome type II (155), lacks all neural crest-derived pigmentation (104). In embryos with the mutation *sparse*, a mutation in the *kit* receptor tyrosine kinase, there is melanocyte deficiency until 21 dpf, at which time a *kit*-independent population of melanocytes appears and partially reconstitutes the stripes (122). This is the same gene affected in the human *piebald* trait (64). *rose*, a mutation in the *endothelin receptor type B1* gene, demonstrates normal melanocyte development early (until embryonic day 21) but fails to achieve the proper pattern and number until late in development (121). During the process of fin regeneration, it appears that the cells that replenish the melanocytes come from unpigmented stem cells (131). Presumably, this is a locally resident population originally derived from postmigratory neural crest.

## DRUG DISCOVERY

The zebrafish embryo is permeable to many small molecules (124). Some of these molecules are quite potent and exert discrete and specific effects during zebrafish embryogenesis. They can be added robotically to plates of embryos, and such chemical screens, therefore, can readily assay effects in the living embryo of tens of thousands of compounds (leading to the misnomer "chemical genetics").

There are several ways in which chemical screens interface with zebrafish genetics. First, some agents may phenocopy mutations and thereby reveal elements of a pathway. This may be particularly helpful when the effect of a mutation is pleiotropic, in that the different effects may be separately phenocopied by a chemical. The *heart and soul* (*has*) mutation in PKCλ (72, 125), for example, affects both cardiovascular and nervous systems. The heart of *has* embryos is mispatterned such that the ventricle is inside the atrium (72). Because cardiac and neural progenitors interact during development, it is difficult to know if the cardiac effect is primary. The small molecule, concentramide, causes an identical cardiac phenotype without any evident neural effect, so it is helpful in this regard (125). Second, the timing of critical events in development can be resolved using chemicals because such chemicals may be added at specific times (124). Third,

there are components of a pathway not accessible by mutation perhaps because the gene is used in many locales or because critical elements, such as posttranslational modifications, may more accurately be targeted by chemicals. Fourth, the chemical gives a biological means to isolate the target molecules. At present, isolation of the target may be arduous, but chemical libraries now are being synthesized with moieties amenable to conjugation for isolation of target ligands.

Chemical screens therefore complement genetic screens in elucidation of developmental pathways and provision of models of disease. In addition, chemical screens on zebrafish embryos conceivably could point to lead compounds as drugs. Toxicity, of course, is screened simultaneously. Agents might be recognized as potential pharmaceuticals by effects on normal growth and development or because they ameliorate the effects of a mutation.



## SUMMARY

Many phenotypes of zebrafish mutations resemble human diseases. These mutations provide an opportunity to parse illness by unitary pathways, and they have enhanced utility as models because the in vivo effects are exerted in the functioning environment.

## ACKNOWLEDGMENTS

The authors thank Iain Drummond, Marlies Elger, Shannon Fisher, Hartmut Hentschel, John Mably, Alan Mayer, Randy Peterson, and Fabrizio Serluca for their invaluable insights and generous use of figures. This work has been supported by grants 5T32HL07208 (J.T.S.), 5R01HL63206 (M.C.F.), and 2R01HL49579 (M.C.F.).

**The *Annual Review of Genomics and Human Genetics* is online at http://genom.annualreviews.org**

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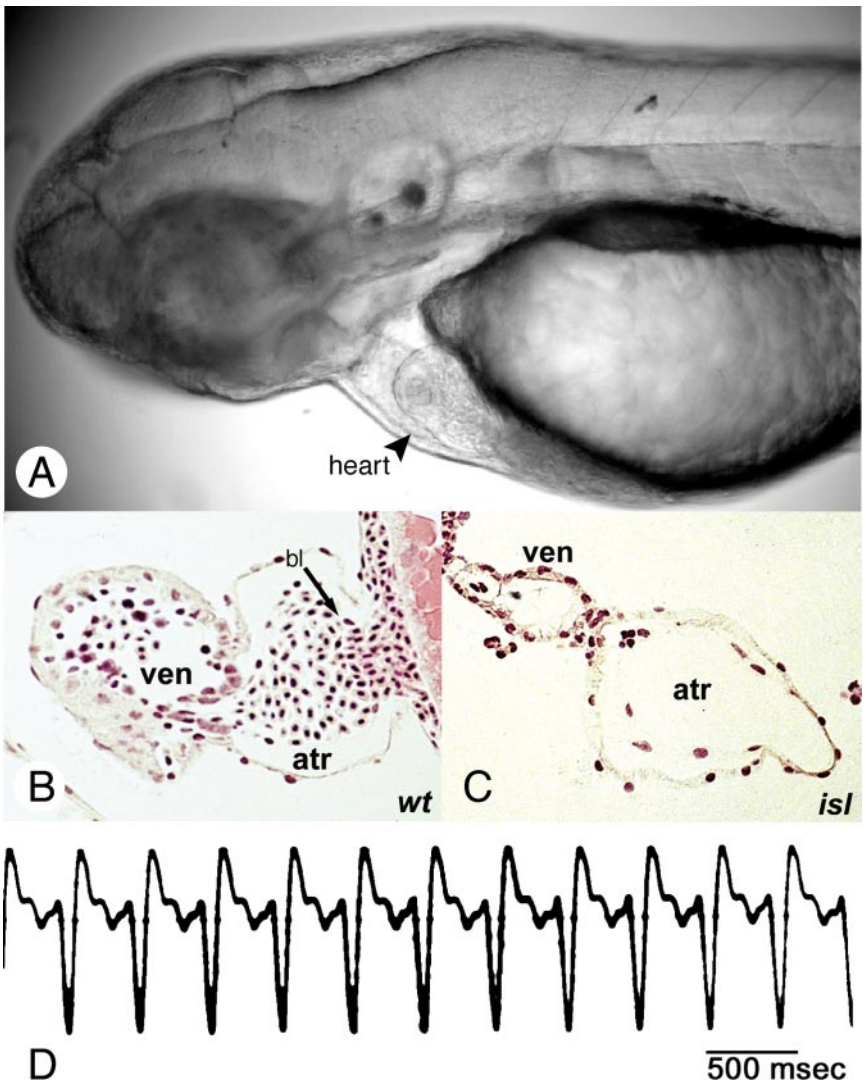


**Figure 1** The zebrafish heart. (*A*) The location of the zebrafish heart on the ventral surface of the embryo (3 days post fertilization) is marked by an arrow. (*B*) An hematoxylin- and eosin- (H&E) stained section through a wild-type heart showing the positions of the atrium (atr) and ventricle (ven). Flow would proceed right to left in this figure, and blood (bl), predominantly nucleated erythocytes, is seen within the chambers. (*C*) An H&E-stained section of an *island beat* mutant shows the diminutive ventricle. (*D*) An electrocardiogram of the zebrafish heart showing regular electrical rhythmicity (*A*, courtesy of J.D. Mably; *B*, *C*, and *D*, courtesy of W. Rottbauer).

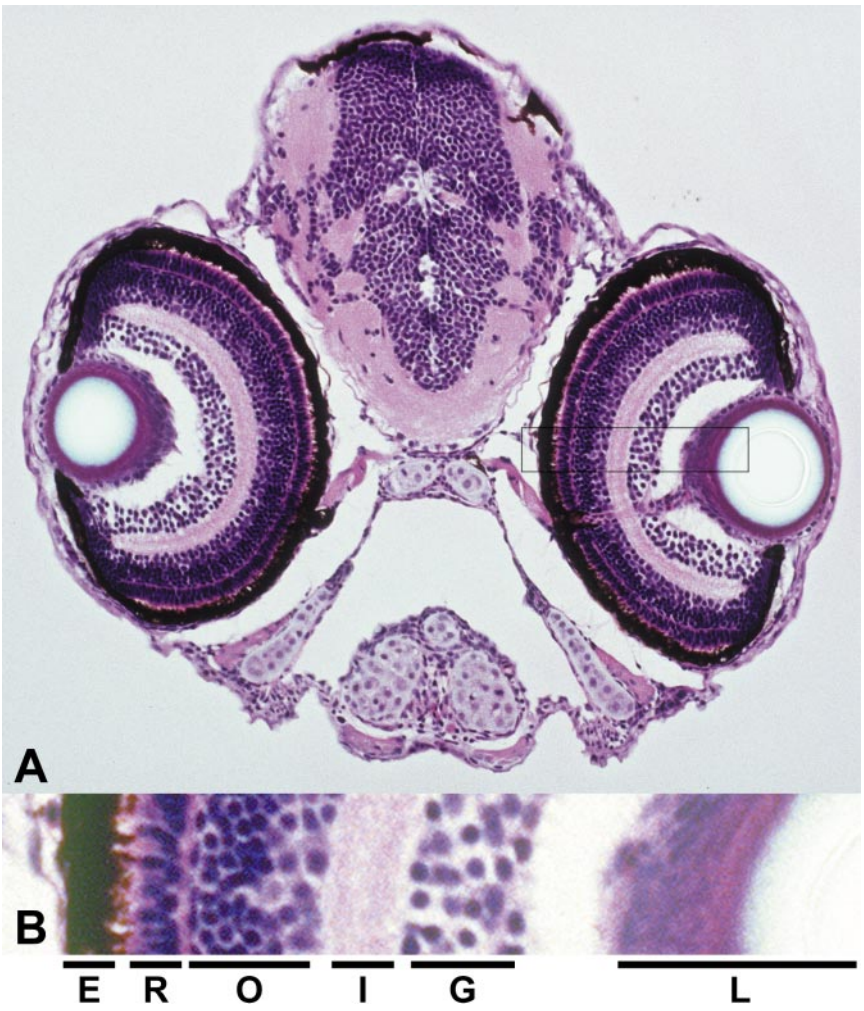


**Figure 4** Zebrafish retinal structure. (*A*) H&E staining of a transverse section through the head of a zebrafish embryo 72 h post fertilization (hpf), showing the eyes on the lateral aspect of the head. (*B*) Enlargement of section demarcated by box in panel *A* delineating the structure of the zebrafish retina. E, retinal pigment epithelial layer; R, rods and cones; O, outer plexiform layer; I, inner plexiform layer; G, ganglion cell layer; L, lens (courtesy of R. Peterson).

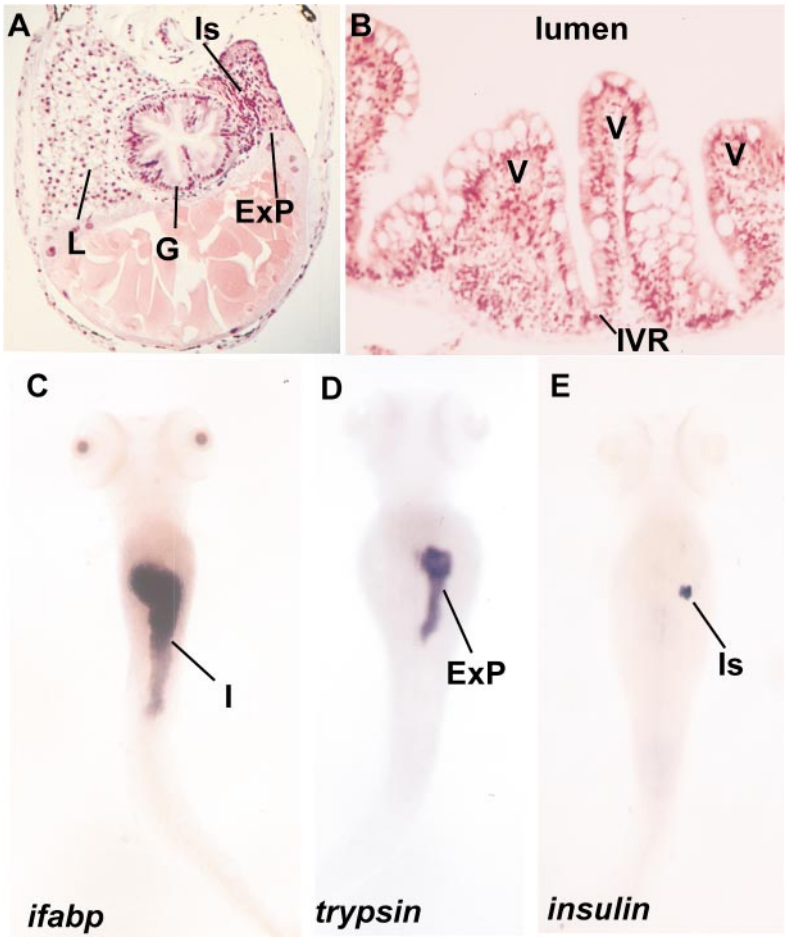


**Figure 5** The zebrafish digestive system. (*A*) H&E stain of a transverse section through foregut region of zebrafish larva 4 days post fertilization (dpf). The liver (L), intestine (G), and pancreas [exocrine pancreas (ExP) and island (Is)] have adopted their respective organotypic cytoarchitecture. As seen in mammals, nascent villi begin as protrusions of the intestinal mucosa into the lumen. The individual cell fates are mostly specified and differentiated. (*B*) H&E stain of the adult zebrafish intestine. Villus (V) architecture is similar to that seen in mammals, except that the progenitor cells are located in the intervillus (IVR) region rather than in crypts. (*C–E*) Whole mount in situ hybridization permits simultaneous visualization of organ morphology and gene expression. Probes are to intestinal fatty acid binding protein (*ifabp*), which stains intestinal enterocytes (I), (*panel C*); to trypsin, which stains exocrine pancreatic acinar cells, (*panel D*); and to insulin, which stains pancreatic $\beta$-islet cells (*panel E*) (figure courtesy of A.N. Mayer).

*Annual Review of Genomics and Human Genetics*
*Volume 3, 2002*

# CONTENTS

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ERRATA
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