REVIEW: DEVELOPMENT

Organogenesis—Heart and Blood Formation from the Zebrafish Point of View

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Organs are specialized tissues used for enhanced physiology and environmental adaptation. The cells of the embryo are genetically programmed to establish organ form and function through conserved developmental modules. The zebrafish is a powerful model system that is poised to contribute to our basic understanding of vertebrate organogenesis. This review develops the theme of modules and illustrates how zebrafish have been particularly useful for understanding heart and blood formation.

During the evolution of multicellular organisms, the homeostatic function of organs provided animals with a selective advantage. In vertebrates, most of organogenesis occurs during embryonic development and is often completed before birth or before hatching. At the onset of organ development, cells in the embryo are associated with one of three germ layers: the ectoderm, mesoderm, and endoderm (*1*) (Fig. 1). Each organ has its embryonic origins from one of these layers, although distinct cell populations from each layer will occasionally mix to form an organ. For instance, the gastrointestinal epithelium is derived from endoderm, but the intestine also contains connective tissue and muscle cells that are derived from mesoderm. This tripartite segregation of germ layers is associated with the migratory events of gastrulation and is linked to the spatial and temporal formation (or patterning) of the embryonic axes. In most vertebrates, the position of the organs along the dorsal-ventral axis is conserved. The notochord and muscle are located dorsally, whereas kidney and blood form in more ventral tissues. The examination of morphological similarities and differences between animal phyla has been used historically to develop a basic understanding of organ development. This comparative approach, taken together with new molecular and genetic tools, has led to the reevaluation of classical concepts in the developmental biology of organ formation.

Organs are formed from groups of cells within a developmental field. The concept of a field implies a homogenous multipotential stage before differentiation occurs (*2–5*). Cells within the field are specified and selected to become a particular type or lineage. Despite the apparent morphologic homogeneity, molecular techniques have revealed that cells within a field can express distinct genes before organ development, a condition called prepatterning. Cell lineage commitment within the field can occur early and quickly. The specified cells within the field undergo morphogenesis, the process of cell movement and coalescence as tissues change form. Morphogenesis is guided by both soluble and cell-associated ligand-receptor interactions. During or after morphogenesis, organs form into recognizable units through cell-specific differentiation and proliferation. As a general rule, the cells of the embryo remain plastic with respect to cell-specific commitment until late in the process of organ formation.

Stem cells and early progenitors are deposited in specific sites to ensure organ development throughout embryonic, fetal, and adult life. The differentiation process is controlled by combinatorial mechanisms regulating lineage-specific gene transcription, reinforced by environmental cues that induce, select, or promote the survival of certain cells. Although the general organ shape is determined early during embryogenesis, the organization and function of most organs are continuously refined during development. Many organs form in successive waves, executing a distinct gene program at each developmental stage. During embryogenesis, the muscle and blood express embryo-specific genes for myosin heavy chain and globin, respectively (*6*, *7*). Later in fetal development, as the embryonic genes are silenced, the fetal and adult-specific genes are expressed. The precise control of gene expression in conjunction with morphogenesis establishes the organ as a functional unit.

Similarities of organ development and function between organisms can lead to unifying views of gene programs (Fig. 2), whereas striking differences can be used to understand the process of evolution. For instance, *Drosophila* heart formation is regulated by the *tinman* gene, and vertebrate heart development depends on *tinman* homologs, the NKX genes (*8–10*). From a comparative standpoint, the similarity of this gene program strongly suggests a common mechanism of heart formation in animals. Yet, there are distinct differences. The *Drosophila* heart is mostly rudimentary; it is single chambered and does not beat with regularity.

Genes required for organ development are conserved throughout evolution, but minor differences in gene expression or function can modify organs in a major way. For instance, it has been hypothesized that such subtle alterations during evolution led to the development of arms and legs from the basic structure of lobed-fin fish. There are a large number of blood-specific genes shared among the vertebrates, indicating that the basic hematopoietic program is conserved (Fig. 2); however, the adult red blood cells of fish, amphibians and birds are nucleated,

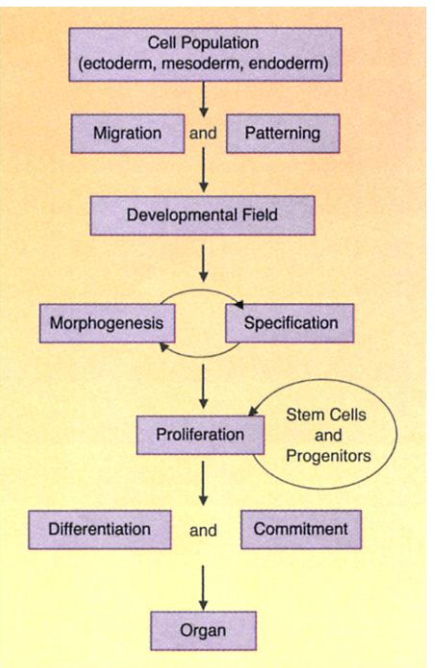

Fig. 1. A schematic view of developmental modules used in organogenesis.

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whereas mammalian red blood cells are enucleate (*11*). Despite these differences in red blood cell morphology, the oxygen-carrying function of most blood cells is very similar. Even among the mammals, differences can be informative. For instance, globin switching is the process by which stage-specific globins are expressed in red blood cells during distinct developmental periods. All genes identified to date involved in globin gene switching are conserved. Despite this, mice undergo one globin switch from embryonic to adult globins, whereas humans execute two switches—an embryonic to fetal switch, followed by adult globin expression at birth (*12*). It is interesting that the zebrafish has two globin switches, similar to humans. The aim of molecular genetic studies of organogenesis is to identify the conserved genes that regulate organ formation and also to characterize genes that establish the subtle differences between species.

The Zebrafish as a Model for Organogenesis

The zebrafish (*Danio rerio*) has several advantages as a model for studying vertebrate developmental processes (*13–15*). These include its small size, easy care, and rapid generation time. In addition, the embryo develops from eggs that are externally fertilized. The embryos are transparent, allowing for a continuous observation of developing organs under the light microscope. Mutagenesis screens have thus examined defects in early organogenesis and late organ function. Given the intense biomedical interest in human organ function and disease, a model system is often judged by how well it predicts human biology. The fish is a vertebrate, and thus the genetic program is more similar to that of mammals than invertebrate models. The evolutionary divergence of fish from the mammalian lineage occurred roughly 300 million years ago. This level of divergence is useful, because most organs among the vertebrates appear generally similar in form and thus, genes involved in zebrafish organogenesis will likely have human orthologs. In this regard, several zebrafish models of human disease have been characterized (*16*). A review of all organs is beyond the scope of this manuscript, and thus we have chosen to examine closely two representative organs: the heart and blood.

Heart formation and function. During evolution, the vertebrate heart developed specialized chambers, outflow tracts to an intricate vasculature, valves to ensure directionality, specialized endothelial cells called the endocardium, musculature to drive a high-pressure system, and an electrical system to regulate rhythm (*8*, *9*). The fish and mammalian hearts resemble each other in form. There is inflow of blood from a major vein to an atrium. The blood moves to a muscular ventricle that delivers it to the aorta. Valves are present to direct flow, and the heartbeat is associated with pacemaker activity. Advances continued throughout vertebrate evolution, and differences are evident between the fish and mammalian heart. There are only two chambers in a fish heart, compared with four chambers of the mammalian heart. The gills are utilized to exchange oxygen, a role that the mammalian lung performs. The air-filled swim bladder is thought to be derived from a primitive lung during evolution, indicating that the swim bladder is homologous to the mammalian lung (*17*). The pulmonary vasculature is derived from the branchial (gill) vasculature. The conservation of general form predicts that the induction of the heart tissues, specification of the chambers, and establishment of the heartbeat will be similar among the vertebrates.

Cell-fate mapping in the zebrafish established the origin of the heart field (*18–21*). The two heart primordia are found anteriorly, along the edge of the embryo. The heart develops from lateral mesoderm in two bilaterally symmetric fields (Fig. 3A). Cells within a field have already become specified to an atrial or ventricular cell fate before the fusing at midline. After the fields fuse, the general shape of the heart is formed. Subsequently, the heart loops to the left (*22*).

Several zebrafish mutants have defects with endoderm formation, and as a consequence have alterations in the development of the heart (Fig. 3A). The *bonnie and clyde* (*bon*) mutant is defective in the *Mix* gene (*23*), and the *faust* mutant is defective in the gene that expresses GATA-binding protein 5, *GATA5* (*24*, *25*). In each of these mutants, the cardiac progenitors fail to migrate to midline. Another mutant, *casanova* (*cas*), has defective heart and gut tissue. The mutant gene encodes a novel member of the Sox family of transcription factors (*26*, *27*). Thus, endoderm must supply signals critical for normal heart development.

The migration of the heart progenitors is influenced by bioactive glycolipids. The zebrafish mutant *miles apart* (*mil*) has two beating hearts because of a failure of fusion of the heart primordia (Fig. 3B) (*28*). The defective gene encodes a lysosphingolipid G protein–coupled receptor, and one ligand for this receptor is sphingosine-1-phosphate (S1P). The defect in *mil* is not autonomous with respect to the cardiac progenitors, and S1P receptor activation is likely to activate additional signals in the control of cell migration. A mouse deficient in a different sphingosine-1-phosphate receptor has a defect in the migration of

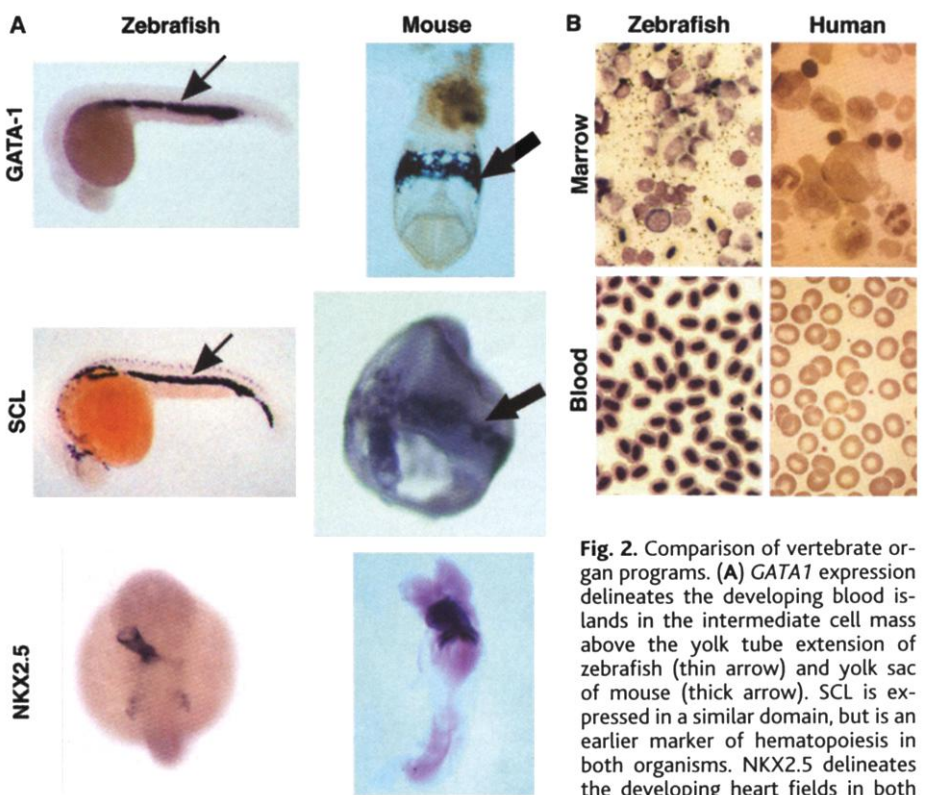

Fig. 2. Comparison of vertebrate organ programs. (**A**) *GATA1* expression delineates the developing blood islands in the intermediate cell mass above the yolk tube extension of zebrafish (thin arrow) and yolk sac of mouse (thick arrow). SCL is expressed in a similar domain, but is an earlier marker of hematopoiesis in both organisms. NKX2.5 delineates the developing heart fields in both zebrafish and mouse (anterior is to the top of the figure). (**B**) The marrow of zebrafish and humans is very similar. Note the prominent erythroblasts and myeloblasts in both organisms. In contrast, the peripheral blood of fish contains nucleated red blood cells, whereas erythroid cells enucleate in mammals. [Photograph of mouse NKX2.5 courtesy of R. Harvey; photograph of mouse GATA1 and SCL courtesy of S. Orkin and Y. Fujiwara]

smooth muscle cells that participate in blood vessel organization (*29*). This finding suggests a broad use of glycolipids in the development of organs, and it highlights environmental cues, such as the extracellular matrix, that can be critical to morphogenesis of many tissues.

Because the fusion of cardiac primordia is required for normal atrioventricular septum formation, abnormalities in the heart chambers may occur in the mutants with cardia bifida. Studies in mouse have pinpointed some genes specifically involved in chamber identity. For instance, the *hand2* and *Mef2c* mouse knockouts have defective right ventricles (*8*, *9*). The *hands off* zebrafish mutant has a defect in the *hand2* gene (*30*), and, as seen in the mouse knockout, a ventricle is defective. A null allele leads to cardia bifida, which, unlike the mouse, suggests an earlier role in heart formation. Large-scale screens have been used to identify more genes involved in atrial and ventricular identity (*31*–*33*). The *pandora* and *lonely atrium* mutants almost completely lack the ventricle, and *santa*, *valentine*, and *heart of glass* have altered ventricular wall growth. The specialized screens should provide an in-depth view of the genes required for chamber formation.

Blood and blood vessels. Distinct types of blood cells are derived from the hematopoietic stem cell, a pluripotent cell that can renew itself. Stem cells become blood as a result of a highly conserved gene program that regulates cell proliferation and differentiation (*11*). Unlike the genetic program, the site of hematopoiesis is not conserved throughout vertebrate evolution. In mammals, the yolk sac is the first site of embryonic hematopoiesis. The zebrafish forms blood in the intermediate cell mass (ICM), a relatively dorsal location above the yolk tube extension (*34*, *35*). The timing of blood differentiation depends on the relative rate of development in each species. Fish form blood in 24 hours, whereas mice and humans form blood in 7.5 and 19 days, respectively (*11*). Definitive hematopoiesis initiates in the ventral wall of the dorsal aorta in all vertebrates that have been studied. These definitive stem cells colonize larval or fetal hematopoietic sites. The fish forms blood in its kidney in larval and adult life, whereas the mammal forms blood in the fetal liver and the bone marrow in fetal and adult life, respectively. Most vertebrates form T lymphocytes in the thymus, whereas B cells are often found in the marrow. Thus, the genes involved in the differentiation of hematopoietic cells are conserved, whereas the factors that regulate cell migration and homing may not be as conserved.

Blood is formed from ventral mesoderm, and this process involves the bone morphogenetic protein (BMP) signal transduction pathway (Fig. 3B). Disruption of BMP signaling leads to a reduction or a lack of ventral mesoderm resulting in an absence of blood cells. This is well demonstrated by the studies of the zebrafish mutants *swirl* (BMP2b) (*35*, *36*), *snailhouse* (BMP7) (*38*, *39*), *somitabun* (SMAD5) (*40*), and *minifin* (tolloid) (*41*). Similar mesodermal defects are found in murine knockouts of some of these orthologs (*42*–*46*). Thus, early patterning of the vertebrate embryo is similar throughout evolution.

After patterning defines those cells that will have a ventral fate, a putative cell called the hemangioblast is derived. This cell is bipotential, able to differentiate into a hematopoietic stem cell or a vascular endothelial cell (*47*, *48*). The zebrafish expresses hematopoietic and vasculogenic genes in two stripes of ventral mesoderm after the 3-somite stage (*34*, *48*). This population is thought to represent hemangioblasts. Distinct hematopoietic and vasculogenic cells can first be seen between the 5- and 7-somite stages, when *Gata1* and *Flk1* are expressed, respectively. The development of the blood and blood vessel lineages in vivo can be visualized in real time by studying zebrafish transgenics for green fluorescent protein

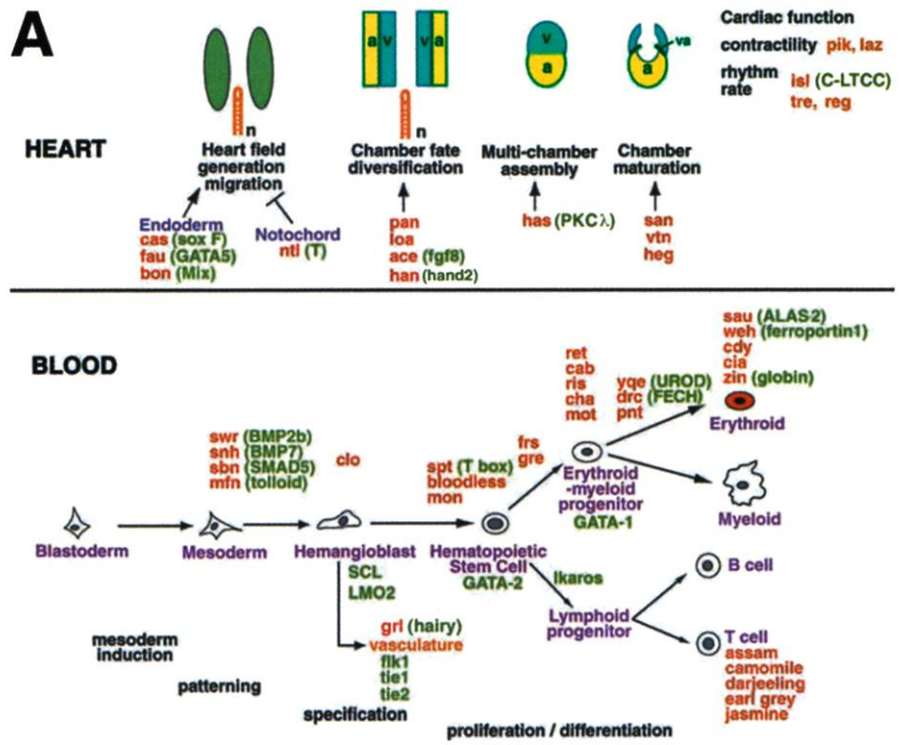

Fig. 3. Organ differentiation. (**A**) The differentiation scheme for heart and blood is highlighted by stages of development. In general, during organogenesis, there is tissue induction, specification, proliferation, and differentiation. Genes involved in the differentiation scheme are highlighted in green. Mutant zebrafish that affect this particular stage of development are highlighted in red. When the gene for the mutant has been isolated, it is written in parentheses. *spt* and *grl* are members of the T box and hairy-related families, respectively. For the heart: a, atrium; v, ventricule; va, valve; n, notochord (heart panel). Diagrams adapted from Chen *et al.* (*9*) and Amatruda *et al.* (*112*). (**B**) The mutant *miles apart* affects the developing heart (*mil*, arrow). The *cloche* mutant has no blood or endothelium, as delineated by SCL expression. [Photograph of *mil* embryo courtesy of D. Stainier]

Specification of the eye field

Lack of prechordal plate mesoderm
cyc (Znr1), boz (dharma), sqt (Znr2)
oep (EGF-related), unf, sur (fast1)

Convergence extension movements alterations
kny, tri, slb (wnt11)

Neural degeneration of the retina
ome, nok, glo

Alterations in the retinal projections

Axons do not turn towards the tectal lobe
bal, gup, sly

Axons fail to cross the midline
bel, con, dtr, igu, uml, yot (Gli), blw

Affect only the retinal axons
ast

Affect axons sorting in the optic tract
box, dac, pic

Differentiation of retinal cell types

Photoreceptor cell loss
mok, nie, nrf (NRF)

Patchy loss of photoreceptors
krt, dis, sid

Progressive photoreceptors loss
eli, flr, pca, nba

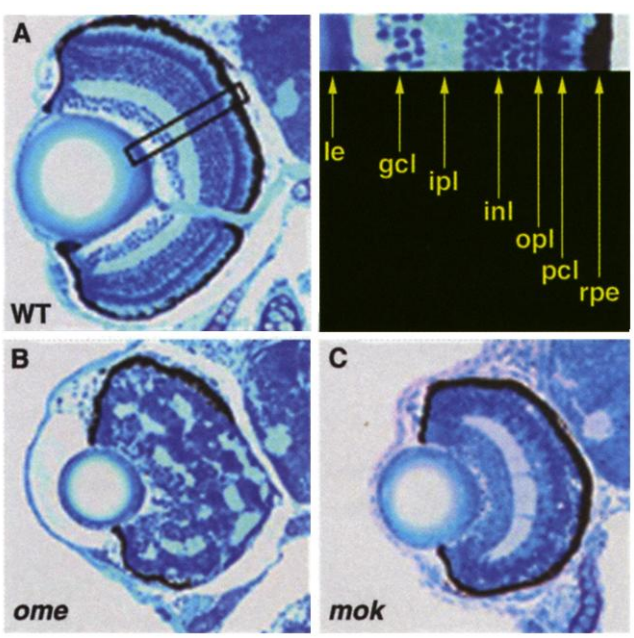

Fig. 4. Different categories of zebrafish eye mutants are listed at the top (*85, 86, 113–115*). Mutation abbreviation is followed by the name of the gene when cloned. (**A**) Cross section through a 5-day-old retina showing the different layers characteristic for this age depicted on the high magnification panel on the right; le, lens; gcl, ganglion cell layer; ipl, inner plexiform layer; inl, inner nuclear layer; opl, outer plexiform layer; pcl, photoreceptor cell layer; rpe, retinal pigmented epithelium. (**B**) In a 4.5-day-old *okomedusy* (*ome*) embryo, the retina shows a dramatic disorganization. Neuronal projections congregate, the photoreceptor cell layer and retinal pigmented epithelium are absent. (**C**) In a 5-day-old *mikre oko* (*mok*) embryo, the retinal pigmented epithelium is poorly differentiated and does not display the normal elongated shape. [Photographs courtesy of J. Malicki]

(GFP) driven by blood- or blood vessel–specific promoters (*50, 51*). Two proteins expressed in hemangioblasts are the basic-helix-loop-helix transcription factor SCL (Fig. 2) and the LIM domain protein LMO2 (*49, 52*). SCL and LMO2 are each required for the development of both blood and blood vessel lineages. Gene targeting experiments in mice reveal that both genes are required for hematopoietic progenitor formation and for angiogenesis. SCL overexpression in zebrafish leads to an expansion of hemangioblasts (*53*). SCL also rescues defective hematopoiesis and vasculogenesis in *cloche*, a mutant with no blood or blood vessels (Fig. 3B) (*52*). It is surprising that the DNA binding domain of SCL is not required for this rescue or for the rescue of hematopoiesis in SCL-deficient embryonic stem cells (*54*). The availability of a quick assay for SCL structure and function in *cloche*, taken together with the concordance of results between zebrafish and mouse assays, illustrates how the two systems (zebrafish and mice) are complementary for experimentation.

Some differences exist among the vertebrates with respect to the gene program for hemangioblasts. In mammals, the vascular-endothelial growth factor receptor, Flk1, is expressed by the hemangioblast and has been used to enrich the population by cell sorting (*55, 56*). Gene targeting of *Flk1* in mouse provides evidence that *Flk1* is required for the migration of hemangioblasts to embryonic sites, but not for actual hematopoietic differentiation (*57–59*). In zebrafish, *Flk1* is not expressed in hemangioblasts at 3 to 5 somites, but only in differentiated angioblasts after 5 somites (*48, 60, 61*). It is possible that *Flk1* is required in these cells for migration of angioblasts in zebrafish. Thus, although the genes are conserved, subtly different expression patterns among vertebrate species may have implications for gene function during organogenesis or for organ function. Regardless of these species-specific differences in gene expression and function, a similarly structured vasculature forms.

Only a few mutants with defects in vasculogenesis have been studied thus far. In contrast to the lack of all blood vessels in *cloche*, the *gridlock* mutant has defective artery formation, whereas veins are normal (*62, 63*). The similarity of *gridlock* fish to human patients with coarctation of the aorta is striking. The *gridlock* gene encodes a novel basic-helix-loop-helix gene that is a member of the hairy family. This gene is expressed in the ventral-lateral mesoderm before the arteries and veins develop, and later the gene is expressed in an artery-specific pattern. This suggests that the artery fate is already determined in the mesoderm. Additionally, the similarity to the hairy family suggests that Notch signaling is important to establish vessel identity.

There are currently 26 complementation groups of zebrafish mutants with defective erythropoiesis (Fig. 3B) (*64, 65*). The analysis of mutants with defective hemoglobin formation has illustrated the conservation of this process, and the ability to find novel genes. The *weissherbst* gene was found to encode a novel iron exporter called ferroportin1 (*65*). This protein transports maternally derived yolk iron into the embryo for the erythroblasts to hemoglobinize. The human ortholog is expressed in the placenta and probably functions there to transport maternal iron to the fetus. In the adult vertebrate, ferroportin1 is expressed in the basolateral location in the intestinal cell where it exports iron into the circulation. Mutations in human ferroportin1 were recently found to cause hemochromatosis, a disease of chronic iron uptake (*67*). The *sauternes* (ALAS2 deficiency) (*68*) and *reisling* (β-spectrin deficiency) (*69*) blood mutants are excellent models of congenital sideroblastic anemia and hereditary spherocytosis, respectively. The *yquem* (UROD) (*70*) and *dracula* (ferrochelatase) (*71*) mutants have fluorescent blood cells and resemble the human disease, porphyria. Thus, studies of zebrafish mutants have novel and known genes involved in organogenesis and organ function. These genes are likely to provide useful insight into vertebrate physiology and disease.

Use of Forward and Reverse Genetics

The pathway of modules presented in Fig. 1 represents a schema for organ formation. The tissues in mutants defective in a module arrest at a common stage and fail to differentiate into a normal organ. For instance, let us analyze the development of blood and blood vessels. Ventral mesoderm makes hemangioblasts, and these become hematopoietic and endothelial cells (Fig. 3). *cloche* mutants have ventral mesoderm that cannot form hemangioblasts, whereas other mutants such as *moonshine* have hemangioblasts that cannot form blood. Identifying and analyzing such mutants establishes that the modules represent distinct key points during organogenesis.

The cartilage (*72–75*), bone (*76, 77*), gastrointestinal system (*78*), muscle (*79*), skin (*80–82*), olfactory (*83*), and reproductive organs (*84*) are also currently being studied in the zebrafish. As an example, large-scale ENU mutagenesis screens [induced by *N*-ethyl-*N*-nitrosourea (ENU)] have produced over 100 mutations (*85*) affecting zebrafish eye development. Investigators first grouped fish mutants on the basis of morphological characteristics (*85, 86*), including cyclopia, growth retardation, small eyes with retinal degeneration, pigment defects with retinal degeneration, loss of laminar pattern in the retina, and loss of the outer retinal layers. Most of the mutants affect the development and function of the retina, whereas a few mutants have defects in the cornea and lens (Fig. 4). Another challenge is to correlate human disease with the basic biology of zebrafish. Human eye malformations have been associated with defects in genes that determine cell fate, such as the oculorenal syndrome and *pax2*, *aniridia*, and *pax6*, and Rieger syndrome (glaucoma and craniofacial dysmorphism) and *pitx2*, *cyclopia*, and *shh* [reviewed (*87*)], respectively. Several genetic defects of the zebrafish retina are reminiscent of human disorders. Mutations affecting the photoreceptors (*85, 88*) resemble retinitis pigmentosa and cone-rod dystrophies (*89, 90*). These are not rare maladies. Retinitis pigmentosa affects 3.5 to 4.5 per 1000 individuals in the industrialized world (*91*), and age-related macular degeneration affects over 15 million people in the United States (*92*). The zebrafish may help identify and characterize new diseases. For example, a novel form of color-blindness has been identified in the zebrafish that does not correspond to a mutation in an *opsin* gene and thus represents a novel gene involved in visualization of color (*93*).

New methods for the zebrafish system allow further testing of gene function. An efficient method of insertional mutagenesis in the zebrafish (*94–98*) has resulted in identification of more than 150 mutants affecting organ development (*99–103*). As a parallel approach, Sleeping Beauty, a synthetic transposon equivalent to an ancient element that disperses in the fish genome, is being successfully used as a gene-trap (*104, 105*). Epistatic relationships can be established in the zebrafish, either with the creation of double-mutants or with transgenic rescue phenotypes, such as the SCL rescue of *cloche* (*52*). Suppressor and enhancer screens are also possible in the zebrafish. Finally, antisense morpholinos (*106*) can be used to specifically inhibit gene function in the zebrafish and will be increasingly used to study organ development and function.

Large-scale in situ hybridization screens can be used to evaluate the cell-specific program within an organ. For example, within a collection of 5400 clones from embryonic cDNA libraries, 64 genes showed specific patterns within the vasculature or hematopoietic blood islands (*107*). One of these genes is defective in a zebrafish hematopoietic mutant (*108*). This technique has also helped define specific molecular markers for organs. Before this work was done, a *vasa* homolog was the only isolated gene expressed in the zebrafish germ cell lineage (*109, 110*); 10 new genes expressed in germ cells were isolated within the course of the in situ screen (*111*).

Conclusion

Organogenesis involves the specification, morphogenesis, and differentiation of tissue during embryogenesis. The developmental modules regulating organogenesis are not defined at a molecular level, and questions remain. For instance, what genes regulate the process of stem cell self-renewal? What genes regulate the size and shape of organs? The isolation of new genes and the detailed characterization of known genes, combined with development of infrastructure on genomics and genetics, will provide ample information on the distinct regulatory steps required for organs to form and function. With the full sequence of the zebrafish genome, the zebrafish will become a key model system for studying organogenesis and organ function. The system should also provide clues to understanding human pathophysiology.

Note added in proof: Additions have been made to Fig. 3 based on new findings since manuscript acceptance. The suggestion that Notch signaling is important to establish vessel identity has been supported by recent functional studies. Work on *gridlock (grl)* has established its function in artery-vein decisions, acting downstream of Notch (*116, 117*). The *heart and soul (has)* gene encodes protein kinase C-λ (PKCλ), which is involved in epithelial polarity in a number of tissues (*118, 119*). The *island beat (isl)* gene encodes the α1 C L-type calcium channel subunit (C-LTCC) (*120*). In Fig. 4, *kny* is a member of the glypican family (*121*).

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