[Frontiers in Bioscience 8, d1227-1253, September 1, 2003]

ZEBRAFISH: A GENETIC MODEL FOR VERTEBRATE ORGANOGENESIS AND HUMAN DISORDERS

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1. ABSTRACT

Mutations may be tolerated without noticeable effect or may present with a specific phenotype that reveals information about the function of the mutated gene. This information is an inexhaustible source for understanding biology and let us ask particular questions about the molecular mechanisms of development, degeneration and disease. The zebrafish (*Danio rerio*) has been proven to be instrumental in the genetic analysis of spontaneous and induced mutations and has provided invaluable clues to the elucidation of complex molecular processes in vertebrate biology. Since completion of the two large-scale mutagenesis screens carried out at the Max-Planck Institute in Tuebingen and at the Massachusetts General Hospital in Boston, many of the recovered mutations have been cloned and the function of the mutated genes studied. Special interest laid in the analysis of mutations affecting structures and organ systems characteristic for vertebrates such as the notochord, neural crest, heart, vasculature, blood and kidney. This review updates our knowledge of heart, vessel, blood and kidney organogenesis in zebrafish and extrapolates our insights to human disorders by assessing common genetic pathways.

2. INTRODUCTION

Zebrafish (*Danio rerio*) has been proven to be a powerful experimental system in elucidating complex biological processes. Many features of this tropical freshwater fish were recognized as advantageous for studying vertebrate development and eventually contributed to the success of zebrafish as a model organism. The suitability of zebrafish for embryological studies and systematic genetic analysis has provided insight into the genetic program of the vertebrate body plan as well as into disease mechanisms. The formation of the germ layers, the shaping of the body, the generation of organ systems and specific cell types, and the organization of blood vessels and neural circuits can be followed extensively in the optically transparent and externally developing embryos. Because respiration in zebrafish can occur via passive oxygen diffusion, zebrafish do not require a functional blood and cardiovascular system for survival during early embryogenesis. This allows the analysis of hematopoietic and cardiovascular defects without the confounding context of a dying embryo (1, 2). Finally, its high fecundity (200 eggs per clutch), brief generation time (four months) and small size (3-4 cm long as an adult) make the zebrafish feasible for large-scale genetic screening (3, 4). Based on the mutant phenotype of interest, novel genes or hitherto unknown gene function can be discovered in an unbiased way.

Mutations in zebrafish have been generated mainly by the chemical mutagen N-ethyl-N-nitrosourea (ENU), a DNA-alkylating agent, that induces base pair substitutions in premeiotic germ cells (5, 6). Although there is some effect of sequence context, the probability of inducing a mutation in a specific gene is closely related to the size of the gene. Chemical mutagens are therefore preferred over gamma- or X-rays (7), since the latter are known to induce breaks in DNA strands, thereby producing large deletions and translocations that may affect more than one gene. A third approach to induce mutations uses insertional mutagenesis of mouse retroviral vectors that, when injected into fertilized zebrafish eggs, integrate in the genome and disrupt gene structure (8, 9).

By means of mutagenesis screens for embryonic defective phenotypes, hundreds of zebrafish mutants have been recovered (10). Inventive screening techniques have exploited the versatility of zebrafish and have unveiled mutations that are normally not noticeable by visual inspection (reviewed in 11). The ongoing cloning and functional analysis of the mutated genes is steadily contributing to our understanding of vertebrate development and disease mechanisms. Many of the mutant

phenotypes resemble human disease states and can point to critical steps in pathogenesis or provide entry points into these processes. In fact, the significant homology between zebrafish and human genes causing a related disease phenotype established zebrafish as a model organism for human disease (reviewed in 12-14).

With the sequencing of the zebrafish genome, gene identification starting with DNA sequence will become increasingly important www.sanger.ac.uk/Projects/D_rerio/). A significant challenge for the future era of zebrafish genetics will be to perform functional analysis of genes "discovered" in the genome sequence. This will require methods of disrupting gene function when only the sequence and local position of a gene is known, as accomplished in the mouse by the embryonic stem cell-based gene knockout technique. Although short-term zebrafish embryo cell cultures maintained in presence of a rainbow trout stromal spleen cell line have been reported (15), cell mediated gene transfer and targeted inactivation of a gene in zebrafish is still in its infancy. However, the ability to perform gene knockdowns, target-selected mutagenesis and transgenesis in zebrafish provides three invaluable methods to study gene function and to create prospects for modeling human disease.

Injection of morpholino phosphorodiamidate oligomers (morpholinos) into zebrafish zygotes of the 1- to 4-cell stage produces a targeted, transient loss of gene function (gene knockdown). Morpholinos have a morpholine ring instead of the ribose moiety, are uncharged and can base pair with RNA, thereby acting as antisense reagents (reviewed in 16). They are designed to interfere with mRNA processing or to inhibit translation. Binding of morpholinos to a splice junction or to the 5'-end of a gene can affect proper splicing of the pre-mRNA or inhibit the ribosomal scanning process and translational initiation, respectively. The effectiveness of morpholinos in reproducing a permanent loss-of-function phenotype, caused by chemically-induced mutations, has been demonstrated and validates this gene targeting strategy (17-20). However, mistargeting has been observed and the inclusion of specificity controls are essential in studying morphant embryos (21).

Methods to obtain mutants of a gene of which only the sequence is known have been developed for plants, *Caenorhabditis elegans*, *Drosophila melanogaster*, mice and more recently also for zebrafish (22-28). The suitability of zebrafish for large-scale mutagenesis combined with the availability of a well-developed genomic infrastructure and a high-throughput sequencing facility has opened the opportunity to screen for zebrafish mutants of the gene of interest (target-selected mutagenesis). By using established DNA and sperm libraries from mutagenized individuals, zebrafish mutants of the *rag1* (*recombination activating gene 1*) gene were recovered (27). In this proof-of-principle study, male zebrafish were mutagenized with *N*-ethyl-*N*-nitrosourea (ENU) and crossed with wild-type females to rise a generation of heterozygous F_1 fish. Sperm from fertile F_1 males was cryopreserved and genomic DNA from the same individuals was isolated, thereby generating parallel sperm and DNA archives of ENU-mutagenized fish. By sequencing the *rag1* locus of all F_1-fish, 15 mutations were found of which one encoded a premature stop codon resulting in deficient V(D)J joining.

The engineering of transgenic zebrafish that express green fluorescent protein (GFP) under control of a cell- or tissue-specific promoter has created another useful tool to study developmental processes in the zebrafish. Transgenic lines allow visualization of gene expression patterns with single cell resolution from early developmental stages throughout adulthood, and can be crossed to existing mutant lines to investigate potential alterations in the expression of the transgene. Transgenic lines can also be used in mutagenesis screens to select for mutants with altered GFP expression, thereby facilitating the identification of new mutations in a pathway of interest. Furthermore, GFP-expressing cells can be isolated by fluorescence activated cell sorting (FACS) and subsequently used for the production of cDNA libraries and microarrays (29-31).

This review summarizes zebrafish mutant phenotypes that resemble human disease and puts them into the context of embryonic development. We report on the progress made in identifying zebrafish genes involved in organogenesis and discuss some of the ways in which mutagenesis, transgenesis and morpholino-induced knockdown strategies have stimulated each other to identify new genes, to comprehensively analyze gene function and to study the integration of genes into pathways. Zebrafish models of human disease with particular relevance to heart, vessel, blood and kidney disorders will be presented.

3. MODELS OF HUMAN DISEASE CATEGORIZED INTO IMPAIRED ORGAN FUNCTION

Organ development originates from the three germ layers ectoderm, endoderm and mesoderm that are defined during gastrulation, or can even be traced to late blastula stages where endodermal and mesodermal progenitors are still intermingled (endomesodermal field). Cells at this stage ingress and migrate and thereby segregate into developmental fields that later give rise to the brain, heart, vasculature, blood, kidney, gastrointestinal tract and all other organ systems of an intact organism. Beside this, formation of an embryonic organ involves the commitment/specification, differentiation and proliferation of multiple cell lineages that assemble into a specific form (morphogenesis). These processes are guided by both soluble and cell-associated ligand-receptor interactions, including morphogens, which build up a gradient of signaling activity. Many organs form in successive waves and their organization and function are continuously refined during development. This implies the processing of a distinct gene program at each developmental stage (reviewed in 32).

3.1. Cardiac development and disorders

The vertebrate heart functions in a manner analogous to the contractile dorsal vessel of *Drosophila* that pumps hemolymph through the interstices of the body. In fact, many factors that determine the heart formation

process in vertebrates are homologous in *Drosophila* (reviewed in 33). However, there are evolutionary innovations in the vertebrate heart that make its function highly efficient. The fish heart is regarded as the prototype of the vertebrate heart since it displays all components essential for the function of the "modern" heart. For instance, the ventricle, the thick-walled, muscular heart chamber, delivers blood at sufficiently high pressures to perfuse all tissues for gas exchange and circulation. The valves at the boundary between the two heart chambers, the atrium and ventricle, prevent blood from backflow. The pacemaking system regulates heart contractility, rhythm and rate of the heartbeat. Finally, the endothelium of the heart (endocardium) permits continuation with the endothelium of the vasculature and presumes the co-evolution of the cardio-vascular system. Divergent to fish, air-breathing animals with lungs as gas exchanging organs have achieved intrachamber septa to separate deoxygenated and oxygenated blood. For a more comprehensive review of comparative developmental biology of cardiogenesis in different organisms such as *Drospohila*, zebrafish, *Xenopus laevis* and mouse, we refer to reference (33).

During gastrulation, cardiogenic precursors (precardiac cells) from both sides of the vertebrate embryo migrate medially and reside on either side of the midline as part of the lateral mesoderm. A distinct group of cells, the endocardial precursor cells, sit medially between them. The bilateral heart primordia (myocardiocytes), which emerged from the precardiac cells, then fuse at the midline enclosing the endocardial cells, and form the primitive heart tube, consisting of two concentric layers. The outer layer, the myocardium, will form the heart muscle, and the inner layer, the endocardium, will become the inner lining of the heart. The heart starts beating regularly soon after tubular fusion, orientates the venous end to the left of the midline, and loops to the right side of the embryo. The heart tube is patterned along the anterior-posterior axis to form two major chambers, the ventricle and the atrium. Cardiac cushions form at the atrio-ventricular boundary and develop into valves to prevent retrograde blood flow (Figure 1) (reviewed in 33-35).

Despite continued improvement in the ability to diagnose and treat human congenital heart diseases, the understanding of the genetic causes and methods of prevention has been limited. While surgical repair of complex congenital heart defects in the neonatal period is routinely conducted, deficiencies in very early cardiac development will result in a nonfunctional cardiovascular system and embryonic lethality. Through a better understanding of the molecular and cellular mechanisms responsible for normal heart development, congenital heart disease may be successfully treated in the future. Several key regulators in cardiac development have been identified in zebrafish and shown to play a conserved role in humans.

If fusion of the bilateral heart primordia (myocardiocytes) during gastrulation fails, two "hearts" on either side of the midline will form. This phenotype is known as cardia bifida. The zebrafish mutants *one-eyed pinhead* (*oep*), *bonnie and clyde* (*bon*), *faust* (*fau*), *casanova* (*cas*), *hands off* (*han*) and *miles apart* (*mil*) all display cardia bifida and therefore may be deficient in the same developmental pathway. In fact, the genes mutated in *oep, bon, fau* and *cas* were shown to be part of the Nodal signaling network, which ensures segregation of endoderm from mesoderm out of an endomesodermal field (36). Endoderm precursors move medially, together with myocardiocytes, to form pharyngeal structures and the gut tube. The exact requirement of the endoderm for myocardiocyte migration, however, needs yet to be determined. The *oep* locus encodes a member of the EGF-CFC (epidermal growth factor-Cripto/FRL1/Cryptic) family that is required for endoderm and anterior axial (prechordal) mesoderm formation as well as for the specification of myocardial precursors (37, 38). *bon* encodes a Mix family homeodomain transcription factor that is expressed in both endodermal and mesodermal progenitors. *bon* is required for the formation of gut tissue from endodermal progenitors and is involved in the specification of cardiac mesoderm (39, 40). *fau* encodes the zinc-finger transcription factor Gata5 that is expressed in mesodermal and endodermal progenitors, similar to *bon* (41). *gata5* is required for the specification of myocardial precursors, the development of the ventricle, and the formation of gut and pharyngeal endoderm giving rise to gut, liver, pancreas, thyroid and thymus (42). *cas* encodes a Sox-related protein that is necessary and sufficient for endoderm specification (43, 44). In the absence of *cas* activity, endodermal progenitors differentiate into mesodermal precursors. Though the mesodermal program seems not to be affected in *cas* mutants, mesodermal organs such as the heart, vasculature, blood and kidney display morphogenetic defects. These deficiencies may stem from the absence of endoderm that provides signals, or serves as a substrate for mesodermal morphogenesis (45). *han* encodes a Hand2-related bHLH (basic helix-loop-helix) transcription factor that is expressed broadly in the lateral plate mesoderm, which is a discrete group of cells that give rise to a number of mesodermal derivatives such as the heart, vasculature, blood, connective tissue, smooth muscle and chondrogenic portion of the limbs (46). Although *hand2* is expressed in an extensive portion of the lateral plate mesoderm, it is required specifically for myocardial and ventricular differentiation as well as for pectoral fin patterning. The human congenital Holt-Oram syndrome displays developmental defects in both heart and upper limbs and is caused by mutations in *TBX5*, a T-box transcription factor (47, 48). Related to Holt-Oram syndrome, *han* mutants fail to maintain *tbx5* expression in the heart field and pectoral fin-forming region. This suggests that Hand2, its cofactors and targets are involved in a pathway for both heart and forelimb development, and may represent candidate genes that are affected in humans with a combination of upper limb and cardiac malformations (Figure 2) (46). The recent cloning of the zebrafish *heartstrings* (*hst*) mutation revealed the ortholog of the human *TBX5* gene and thereby provided the fish model for the human Holt-Oram syndrome (49). Relative to humans, the *hst* mutation affects the whole heart, whereas Holt-Oram syndrome patients have predominantly atrial defects. However, the syndromic deficiencies of *tbx5*

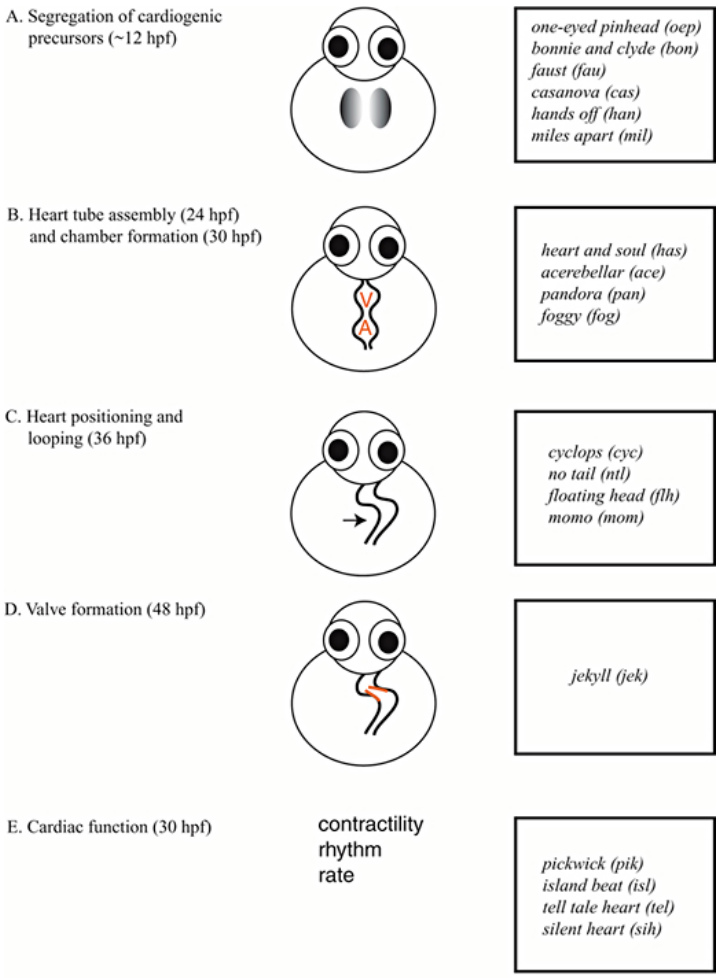

Figure 1. Zebrafish heart development. Mutants with cardiac defects affecting various aspects of heart specification are indicated in the boxes. A. Heart progenitor cells align along the embryonic axis by the 5-somite stage (~12 hpf). Endocardial precursors lie most medially, myocardial precursors most laterally. B. Starting at 19 hpf, myocardial precursors migrate medially and form a tube concomitantly enclosing endocardial cells. By 30 hpf, visibly distinct ventricular (V) and atrial (A) chambers form. C. By 36 hpf, the venous end of the heart orients to the left of the midline and the heart undergoes looping morphogenesis. D. By 48 hpf, specialized endocardial cells form cushions at the arterioventricular boundary, which give rise to functional valves. E. By 22 hpf the heart tube starts beating and around 24 hpf circulation begins.

mutations, including absence of forelimbs, are remarkably well retained between fish and humans. The *mil* locus encodes a G-protein-coupled receptor that binds sphingosine-1-phosphate and that critically promotes myocardial migration towards the midline. The function of *mil*, however, is required in cells other than the migrating myocardiocytes (cell non-autonomous function of *mil*). The cells that require *mil* function to permit myocardial cell migration have yet to be identified. (50).

Zebrafish mutants that affect heart tube assembly include *heart and soul* (*has*), *acerebellar* (*ace*), *pandora* (*pan*) and *foggy* (*fog*). *has* mutants have an abnormal heart chamber orientation with ventricular muscle within the atrium, and display epithelial defects within the organs of the digestive tract, as well as the eye and neural tube. The *has* locus encodes the ortholog of protein kinase C-lambda (PKC-lambda), which is required for epithelia polarization and organ morphogenesis (51, 52). *ace* was originally classified as a brain mutant (53), but *fibroblast growth factor 8* (*fgf8*), the mutated gene in *ace*, has also been shown to be responsible for proper ventricle formation (54). *pan* and *fog* encode zebrafish orthologs of the transcription elongation factors Spt6 and Spt5, respectively, which are both crucial in generating myocardial cells (52, 55, 56). The identification of the *pan* and *fog* mutations exemplifies the unbiased nature of genetic screens in revealing novel functions of previously known genes.

The organ positioning establishes a conserved left-right (LR) asymmetry in the body plan of all vertebrates. The first anatomic indication of LR asymmetry occurs with the leftward positioning of the venous end of the heart tube and the subsequent rightward looping. Aberrations in the process of cardiac positioning and looping are associated with many congenital heart diseases

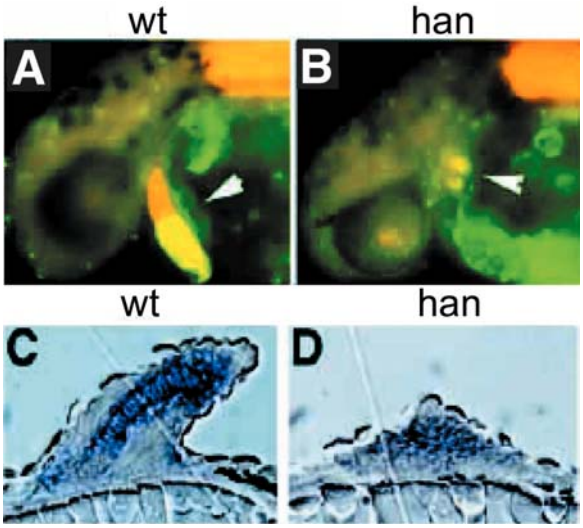

Figure 2. Myocardial and pectoral fin defects in *hands off (han)* mutants. An example for an animal model of the human Olt-Horam syndrome, which is characterized by heart and limb defects. Lateral views, anterior to the left. A, B. Immunofluorescent images of embryos at 36 hpf showing ventricle in red and atrium in yellow staining. A. Wild-type embryos have a heart tube (arrowhead) with two distinct chambers, an anterior ventricle (red) and a posterior atrium (yellow). B. *han* mutants have two small clusters of myocardial tissue (arrowhead) that appear to be primarily atrial (yellow). C, D. Longitudinal sections through pectoral fin buds of embryos at 48 hpf. C. In wild-type embryos, the pectoral fin is elongating and a chondrogenic condensation is forming. D. In *han* mutants, the fin bud is small and undifferentiated. Reprinted with permission from D. Yelon (46) and The Company of Biologists Ltd.

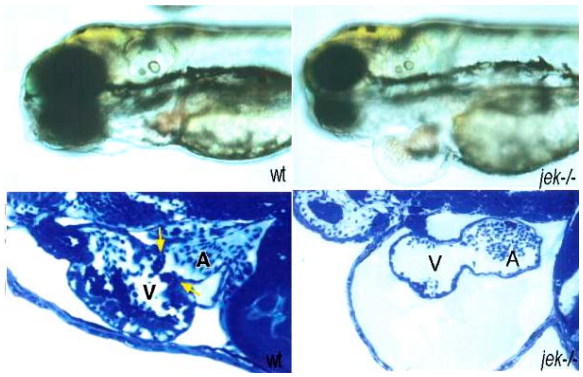

Figure 3. The *jekyll* (*jek*) mutation perturbs the development of the cardiac valves at the arterio-ventricular boundary. Wild type (wt) sibling embryos form valves (arrows) at the boundary between the atrium (A) and the ventricle (V). Valves are absent in siblings homozygous for the *jek -/-* mutation. As a secondary effect, they have an enlarged pericardium. Reprinted with permission from D.Y.R. Stainier (70) and The American Association for the Advancement of Science.

and laterality defects (reviewed in 57). Zebrafish mutants with randomized heart looping such as *cyclops* (*cyc*) (58, 59), *no tail* (*ntl*) (60), *floating head* (*flh*) (61) and *momo* (*mom*) (62) are equally defective in establishing midline structures. *ntl*, *flh* and *mom* mutants lack a differentiated notochord and *cyc* mutants are deficient in floor plate (ventral neuroectoderm) and prechordal plate (anterior axial mesoderm) development (63-65). Asymmetric expression of *cyc*, *lefty2*, *pitx2* and *bmp4* in the heart field seems to be crucial for cardiac laterality as *ntl*, *flh*, *mom* and *cyc* show bilateral or absent expression of those genes (62, 66). A recent study has shown that protein disulfide isomerase P5 (PDI-P5), expressed in embryonic midline structures, is required for left/right asymmetries (67). Depletion of PDI-P5 by morpholino knockdown strategy resulted in bilateral expression of normally asymmetrically expressed genes, and in loss of asymmetric development of the heart, liver, pancreas and gut without disrupting midline development. Rescuing laterality defects in the midline mutants *ntl*, *flh*, *mom* and *cyc* by providing exogenous PDI-P5 would reveal the necessity and sufficiency of PDI-P5 in LR organ positioning. EGF-CFC, the gene product of human *CRYPTIC* and zebrafish *oep*, was also shown to be essential for the establishment of the LR axis (68). *CRYPTIC* loss-of function alleles from patients with randomized organ positioning failed to rescue the *oep* phenotype whereas wild-type *CRYPTIC* was able to rescue several aspects of the *oep* phenotype including the development of two separate eyes (69). Such as yeast cell-cycle mutants have been rescued with human ortholog genes, this study demonstrates the conservation of fundamental processes in vertebrate evolution and appreciates the relevance of zebrafish as a model for vertebrate development and human disease mechanisms.

The formation of cardiac valves is an innovation in vertebrate evolution, which allows the ventricle to pump blood into the outflow tract without retrograde flow into the atrium. Several human congenital and sporadic heart diseases, such as tricuspid atresia (no connection between the right atrium and the right ventricle), and pulmonary and aortic stenosis (outflow obstruction) display valve malformations. For valve formation, endocardial cells undergo an epithelial to mesenchymal transition and migrate into the cardiac jelly (the extracellular matrix between the myocardium and endocardium) where cushions, the primordia of the valves, form. The *jekyll* (*jek*) mutation has provided an entry point to the molecular mechanism of valve morphogenesis. *jek* mutants do not exhibit distinct gene expression in myocardial and endocardial cells at the atrioventricular boundary and fail to form cushions in the valve-forming region (Figure 3). The *jek* locus encodes *uridine-diphosphate (UDP)-glucose dehydrogenase*, an enzyme required for the synthesis of several proteoglycans such as heparan sulfate, chondroitin sulfate and hyaluronic acid (70). This implies that jek is part of a pathway that involves glycosaminoglycan production and is required for proper specification of cells at the atrioventricular border to form cushions.

Congestive heart failure can result from various disease states with inadequate cardiac output. In *pickwick*

(*pik*) mutants arterial pressure is reduced because little if any blood is ejected from the heart. By candidate gene cloning, the causative mutation was revealed to affect *titin* (*ttn*), the largest-known protein spanning the half-sarcomere in heart and skeletal muscle (71). In fact, *pik* embryos fail to generate higher-order sarcomeres and form thin cardiac myocytes. Concurrently, it has been shown that mutations of *TTN* in humans cause familial dilated cardiomyopathy (increase in ventricular chamber volume), a phenotype that is also displayed by *pik* mutants (72).

Another gene that impacts both form and function of the heart is represented by the zebrafish *island beat* (*isl*) locus. The mutated gene encodes an alpha-1C L-type calcium channel subunit (C-LTCC) that is responsible for calcium entry into cardiac myocytes (73). In *isl* mutants the ventricle fails to grow and does not contract, while the atrium exhibits rapid, isolated and discoordinated contractions in a pattern resembling atrial fibrillation. *isl* mutants have a reduced number of myocardial cells in their ventricle. This seems to be a consequence of loss of calcium signaling, since *tell tale heart* (*tel*) mutants similarly display a silent ventricle and arrhythmia, whereas the number of myocardial cells is comparable to wild-type siblings. *tel* mutants were reported to be defective in a myofibrillar protein that is part of the sarcomeres (73). The zebrafish mutant *isl* may provide further insight into the role of calcium in ventricular growth and contraction as well as into alternative calcium sources accessible to atrial cells (74) (reviewed in 75-78).

Mutations in the *TNNT2* gene encoding the thin-filament contractile protein cardiac troponin T can be the cause of familial dilated (increase in cardiac chamber volumes) and hypertrophic (increase in muscle mass) cardiomyopathy (79, 80). Both malformations can initiate heart failure and sudden death (81). Tnnt2, together with alpha-tropomyosin (Tpma) and cardiac troponins C and I (Tnni3), form a calcium-sensitive regulatory complex within sarcomeres (81). The zebrafish mutant *silent heart* (*sih*) was shown to be deficient in tnnt2 (82). In the absence of tnnt2, cardiac sarcomeres fail to assemble and the heart muscle is nonfunctional. Concomitantly, *tpma* and *tnni3* were found to be significantly reduced in *sih* mutants, suggesting an interdependence of these proteins in expression and/or stability. The resulting sarcomere loss and myocyte disarray are the life-threatening hallmarks of *TNNT2* mutations in humans (81).

3.2. Vascular development and disorders

Vascular malformations have been observed in hereditary and sporadic human diseases. Many of the inherited defects occur as part of a syndrome, indicating that several deleterious genes may be involved, or that one defective gene may affect several organ systems. The occurrence of sporadic defects may be due to metabolic stress (i.e. low pO_2, low pH and hypoglycaemia), mechanical stress (i.e. pressure generated by proliferating cells), inflammatory responses or mutated genes involved in the control of blood vessel formation (reviewed in 83, 84). The study of how embryonic vasculature arises and how it is guided has provided insight into many hallmarks of vascular diseases and tumor growth, which is dependent on *de novo* vascularization.

Vasculature as well as blood are derived from mesoderm. Both mesodermal lineages differentiate synchronously in close association and mature into endothelial and hematopoietic cells, respectively (85). Analyses on expression of genes and surface markers, as well as grafting and cell lineage tracing in zebrafish, chick and mouse, led to the hypothesis of the existence of a hemangioblast, the common progenitor of endothelial and hematopoietic cells (86-89) (reviewed in 90). Further support for the hemangioblast hypothesis comes from studies of zebrafish *cloche* mutants in which blood vessels, including endocardium, fail to develop and markers specific for endothelial and hematopoietic cells are all absent (91-95). The requirements for hemangioblast specification, however, are largely unknown. Migration pattern analysis (fate-mapping) revealed at least three distinct populations of mesoderm-derived cells in the marginal zone of the blastula that give rise to the endothelial lineage, and only one of these populations may have the potential to specify endothelial and blood progenitors (85). In the following, the factors involved in early events of endothelial and blood lineage specification are introduced and their relational properties discussed.

Expression of *flk-1*, a receptor tyrosine kinase of the vascular endothelial growth factor (VEGF) receptor family, is the earliest marker for endothelial cells and their progenitors (angioblasts). In zebrafish, *flk-1* transcripts are first detected during early somitogenesis (6-somite stage) (92). At this stage, *flk-1* expression can be detected in two physically separate populations of endothelial progenitors (angioblasts) in the lateral plate mesoderm. One population appears in the head, the other in the trunk region. In agreement with this pattern, lineage analysis revealed that future endothelial cells are first located throughout the marginal zone of the late blastula but then, during gastrulation, separate into two populations. Some of these cells migrate dorso-anteriorly towards the head region, whereas blood as well as trunk and tail angioblasts take a more posterior route (85). At the 20-somite stage, a continuous band of *flk-1*$^+$ cells stretches from the anterior head region to the tailbud, and by the 24-somite stage, *flk-1* expression marks the future dorsal aorta and the axial vein. *flk-1*$^+$ cells then emerge between somites by sprouting from the dorsal aorta, and at 24 hours post fertilization (hpf) *flk-1* is present in all endothelial cells lining the vasculature (92). Analysis of zebrafish *flk-1* mutants revealed that *flk-1* is not required for the differentiation of angioblasts nor the assembly of vessels that are formed before 36 hpf, such as the dorsal and caudal aorta, the caudal and posterior cardinal vein, and several main vessels of the head (96). However, disruption of *flk-1* impairs the formation or function of vessels that are probably generated by sprouting, such as the intersegmental vessels, the central arteries that penetrate the brain, the subintestinal vein that supplies the digestive system and the pectoral arteries and veins of the fins. Mice deficient in *Flk-1* die *in utero* because of a lack of both, endothelial and hematopoieitc cells (97). The analysis of chimeras generated by

aggregation of embryonic stem cells homozygous for the *Flk-1* mutation with wild-type embryos revealed that *Flk-1*$^{-/-}$ cells were incapable of contributing to endothelial or hematopoietic cells at any stage of development. (98). However, *Flk-1*$^{-/-}$ embryonic stem cells are able to differentiate into hematopoietic and endothelial cells *in vitro* (98, 99). The data from mouse genetics suggests that Flk-1 is critically involved in the migration/expansion of the progenitors of the extraembryonic blood islands (giving rise to both hematopoietic stem cells as well as angioblasts) from the posterior primitive streak to the yolk sac and, possibly to the intraembryonic sites of hematopoiesis. The ablation of the entire vasculature by loss of VEGF function in zebrafish and the more severe phenotype of *Flk-1* knockout mice suggest that there might be a second VEGF receptor in zebrafish partially compensating the loss of the other isoform, or having a unique role in early vasculogenesis (96).

Neuropilin-1 (NRP1) is a cell-surface receptor for both VEGF and class 3 semaphorins, which mediate neuronal cell guidance, and is expressed by endothelial cells and neurons (100-102). Neuropilins do not seem to be receptor kinases but may act as co-receptors for Flk-1 that enhance VEGF activity (102). Morpholino-mediated knockdown of NRP1 in zebrafish embryos resulted in impaired circulation in intersegmental vessels, whereas circulation via trunk axial vessels was not affected (103). Combined morpholino injection of NRP1 and VEGF at concentrations that individually do not inhibit blood vessel development affected both intersegmental and axial vessel formation, suggesting that NRP1 and VEGF are interdependent for the development of the vascular system (103).

The receptor tyrosine kinase *flt-4* shows the same spatial and temporal expression pattern as *flk-1* in zebrafish. In contrast to *flk-1*, *flt-4* is more strongly expressed in the caudal vein than in the dorsal aorta (94).

tie-1 and *tie-2* belong to another family of endothelium-specific receptor tyrosine kinases. They have nearly identical expression patterns within developing and mature blood vessels, with the exception that *tie-1* is additionally expressed in intersegmental vessels (104). *tie-1* and *tie-2* transcripts appear in succession to *flk-1* by the 10-somite stage in angioblasts within the lateral plate mesoderm (105).

The stem cell leukemia gene (*SCL)* was discovered by investigating chromosomal translocations in human T-cell leukemias. SCL is a basic helix-loop-helix transcription factor that was found to be ectopically activated in a T-cell environment due to an aberrant chromosome translocation involving the T-cell receptor enhancer (reviewed in 106). Loss-of-function studies in mice have shown that SCL is essential for the formation of hematopoietic stem cells (107-109), for subsequent erythroid development (110), and for yolk sac angiogenesis (111). In zebrafish, *scl* expression starts at 1-3 somites and appears in bilateral stripes of anterior and posterior cell populations that are part of the lateral plate mesoderm (86, 93). At the 6-somite stage, *scl* is co-expressed with *flk-1* in single cells of the head mesenchyme and the posterior lateral plate mesoderm. Later during somitogenesis, *flk-1*-expressing cells assemble on the midline below the notochord where the dorsal aorta will develop. More ventrally, a parallel stripe of *scl*+ cells comprises the intermediate cell mass (ICM), the location of embryonic erythropoiesis. Cells of the ICM differentiate into red blood cells and enter circulation by 24-26 hpf. The population of *scl*+/*flk-1*+ cells in the posterior lateral plate mesoderm of 6- to 10-somite stage embryos are considered to consist of progenitors for both, blood and endothelium, and may represent hemangioblasts (112). Overexpression of *scl* resulted in programming early mesodermal cells into hematopoietic and endothelial progenitors at the expense of somitic and pronephric duct cell fates (86). A sub-population of *scl*-overexpressing progenitors differentiated into hemoglobinized blood cells, whereas it remained unclear whether terminal endothelial differentiation also occurred.

The ETS-domain transcription factor Fli-1 is involved in the induction of erythroleukemia by Friend virus in mice and is target of a chromosomal translocation in humans resulting in Ewings sarcoma (113). Mice with a targeted null mutation in the *Fli-1* locus die during early embryogenesis due to loss of vascular integrity leading to internal bleeding (114). *Fli-1-/-* embryonic stem cells do not contribute to the megakaryocytic lineage in chimeric mice, and *Fli-1* mutant embryonic stem cells fail to produce megakaryocytes *in vitro*, suggesting the requirement of Fli-1 for megakaryocyte commitment (114, 115). In zebrafish, *fli-1* parallels the spatial expression pattern of *scl* during early stages of segmentation (1-10 somites, 10-14 hpf). Later during somitogenesis (19-24 hpf), *fli-1* is expressed in the same locations as *flk-1*, namely in the tail, trunk and head, where *fli-1* expressing cells align and form the walls of major vessels including the dorsal aorta, axial vein and intersegmental vessels (116).

The homeobox gene *hhex* is a regulator of early endothelial and blood differentiation and overlaps with the temporal and spatial expression pattern of *flk-1*, *flt-4*, *scl* and *fli-1*. *hhex* first is expressed at the 3-somite stage in an anterior and a posterior population of cells. *hhex* later is expressed in the nascent ICM, which contains both endothelial and blood precursors. After the onset of circulation (24 hpf), *hhex* expression diminishes in endothelial and blood progenitors and disappears completely by 30 hpf. *hhex* and *scl* can induce each other as well as the endothelial gene *flk-1* and the erythropoietic transcription factor *gata-1* (117). Overexpression of *hhex* as well as *scl* in *cloche* mutants, which fail to express any blood or endothelium specific genes, results in the expression of *flk-1*, *tie-1* and the erythroid marker *gata-1*, implying that *hhex* and *scl* act downstream of the hemangioblast level defined by the *cloche* locus (93, 117). Analysis of the zebrafish *hhex* deletion mutant *b16* showed that *hhex* is not essential for early endothelial and blood differentiation and suggests that *scl* might compensate for the absence of *hhex* function (117).

Chromosomal translocations affecting the human *RUNX1* (also known as *AML1* for acute myeloid leukemia-1, *CBFA2* for core binding factor A2, or *PEBP2alphaB* for

polyomavirus enhancer binding protein 2alphaB) locus, encoding a transcriptional regulator of the Runt family, are frequently involved in human acute myeloid leukemias (reviewed in 118). In addition, point mutations in *RUNX1* have been described in myelodysplasias (119) and haploinsufficiency of *RUNX1* can cause familial thrombocytopenia (120). Runx1 was also shown to play a critical role in angiogenesis and definitive hematopoiesis (reviewed in 121, 122). Mice lacking Runx1 protein die during embryogenesis due to hemorrhaging in the nervous system, and lack definitive erythropoiesis and myelopoiesis (123, 124). In zebrafish, *runx1* was demonstrated to function downstream of the hemangioblast level, to be involved in both primitive and definitive hematopoiesis as well as in neuropoiesis (125). *runx1* and *scl* are coexpressed in the lateral plate mesoderm at 12 hpf (5-somite stage) and later (18-24 hpf) in the forming ICM. Embryos injected with *runx1*-morpholinos showed disrupted vasculature, lacked normal circulation and accumulated immature hematopoietic progenitors in the ICM, which were *scl*-positive and failed to differentiate, and maintained *scl* expression for an extended period of time. *flk-1* expression was also perturbed and resulted in missing segments of vasculature, abnormal axial vessels and deficient formation of intersomitic vessels (125). The specific role of *runx1* and its partner CBF-beta in primitive and definitive hematopoiesis and leukemogenesis will further be outlined in the following section on blood development and disorders.

Once angioblasts are specified bilaterally to the anterio-posterior axis in the lateral plate mesoderm, they migrate medially to form the primordia of the large axial vessels, the dorsal aorta and the axial vein. Subsequently, the cord-shaped primordia lumenize and turn into a tubular structure. This process is known as vasculogenesis. Vessels that perfuse organs with endoderm-derived epithelial layers also form by vasculogenesis. In contrast, head internal carotid arteries, trunk intersomitic vessels and vessels that perfuse organs with ectorderm-derived epithelial cells form primarily by angiogenesis, in which existing vessels give rise to sprouts that invade avascular territories. Angiogenesis also allows remodeling of primary vessels including splitting of vessels at branch points.

The dorsal aorta forms ventral to the notochord, while the posterior cardinal vein forms in between the aorta and the trunk endoderm. The notochord was demonstrated to be a key organ in the process of axial vessel formation. If the notochord fails to form, such as in the zebrafish mutants *floating head* (*flh*) and *no tail* (*ntl*), a dorsal aorta fails to develop, while some characteristics of the axial vein can be observed (126, 127). *one-eyed pinhead* (*oep*) mutant embryos have a notochord, but lack the prechordal plate (anterior axial mesendoderm), ventral neuroectoderm, gut and other endodermal derivatives (128). Whereas in *oep* mutants the dorsal aorta is visible ventral to the notochord, the axial vein, which would normally run immediately above the gut, is not formed. This implies that signals form the endoderm might be responsible for inducing the morphogenesis of the axial vein (116). Mutants of the *you*-class have neural defects, U-shaped somites and display disrupted circulation due to a lack and disorganization of vessel formation. Of this class, *you-too* (*yot*) mutants are deficient in *gli2*, a zinc finger transcription factor downstream of the Hedgehog family of signaling molecules (129). In addition to the neural and somitic defects, *yot* mutants are disrupted in the formation of the dorsal aorta (130). *sonic-you* (*syu*), harbor a mutation in *sonic hedgehog* (*shh*), which is expressed in the notochord, the ventral CNS and endoderm, and signals to adjacent tissues such as somites and hypochord (131). Since neither the dorsal aorta nor the axial vein are formed in *syu* mutants, the notochord and the gut likely signal to induce axial vessel morphogensis either via *shh* or by factors whose expression are dependent on *shh* (116). A target of *shh* signaling was shown to be vascular endothelial growth factor (*vegf*) (130). *vegf* is expressed in the ventromedial portions of the somites adjacent to the notochord. *vegf* binds with high affinity to the receptor tyrosine kinase *flk-1*, thereby activating downstream signaling effectors. Overexpression of *vegf* resulted in increased production of *flk-1*, *tie-1*, *scl* and *gata-1* transcripts as well as terminally differentiated red blood cells, indicating a stimulation of both endothelial and hematopoietic lineages (132). In *vegf* loss-of-function embryos, generated by morpholino-based gene knockdown, neither a dorsal aorta nor an axial vein was formed, no circulating red blood cells were detectable and the whole vasculature was little or not functioning (133). However, *flk-1* and *fli-1* were expressed in locations where the axial vessels were normally situated, but not in intersegmental regions. This suggests that *vegf* is required for angiogenesis and proper axial vessel formation but not for angioblast specification (133). Similar results were obtained with a chemical genetic approach in which zebrafish embryos treated with the FLK-1 inhibitor PTK787/ZK222584 lacked blood vessels (134). Previous studies have shown the effectiveness of this drug in inhibiting neovascularization and tumor growth in mouse models (135, 136). The effectiveness of PTK787/ZK222584 in the zebrafish system underscores the utility of zebrafish for small molecule screens and preclinical drug testing.

The tie-2 ligands, known as angiopoietins, also play critical roles in vertebrate vascular embryogenesis. Zebrafish *angiopoietin-2* (*ang-2*) is expressed in head and anterior trunk ventral mesenchyme and in the pronephric glomeruli. *angiopoietin-1* (*ang-1*) is expressed in head ventral mesenchyme at the 5- to 10-somite stage and later, at 24 hpf, in the region of the hindbrain and aortic arch arteries, in the trunk such as in the ventromedial region of the somites, in the mesenchyme surrounding trunk axial vessels and in the hypochord (105). The hypochord of the zebrafish embryo is a transient structure that emerges at the 9-somite stage as a single row of cells immediately ventral to the notochord and vanishes continuously after the onset of circulation. In the gap between the hypochord and the endoderm underneath, angioblast cells aggregate and start to form the dorsal aorta (137). The hypochord secretes VEGF, which appears to be required for angioblast migration and subsequent dorsal aorta formation (130). The signaling activity of the hypochord coupled with the temporal and spatial correlation between the appearance of the hypochord and the formation of the dorsal aorta

suggests that the hypochord plays a role in the formation and stabilization of the dorsal aorta and in the sprouting of intersegmental vessels.

Cell lineage analysis revealed that the lateral plate mesoderm between the 7- and 12-somite stages contains precursors for the aorta, the posterior cardinal vein, the dorsal longitudinal anastomotic vessel (DLAV), the sub-intestinal venous vessels and the intersegmental vessels (ISVs). It was further demonstrated that no definable region of the lateral plate mesoderm preferentially provides progeny to any type of these vessels (138). This contrasts with results provided by Zhong *et al.* showing that individual angioblasts in the lateral plate mesoderm contribute to either a venous or an arterial cell fate (139). However, individual angioblasts destined for the ISVs migrate to the dorsal aorta, move between somites and assume one of three cell fates when connecting the dorsal aorta with the DLAV. These three cell fates include a dorsally situated T-shaped cell that is anchored in the DLAV and branches ventrally into the intersomitic space, an inverted T-shaped cell that is based in the aorta and branches into the intersomitic space, and a connecting cell that emerges in between the two T-shaped cells. In *out of bounds* (*obd*) mutants, the patterning of ISVs is not restricted to the intersegmental space but is largely random, and sprouts traverse somite domains that are normally forbidden to vessel formation. The dorsal portions of the ISVs appear to be less affected than the ventral-most regions where sprouts originate from abnormal positions along the aorta. The patterning defect in *obd* appears to be cell non-autonomous as shown by reciprocal transplantation between wild-type and *obd* homozygous mutant embryos (138).

Investigations into the question of how angioblasts decide whether to contribute to arteries or veins has provided evidence that Notch signaling genes play a role in arterial-venous cell fate decision. Notch genes encode large transmembrane receptors for ligands of the *Drosophila* Delta and Serrate family, and are involved in cell fate decisions of a wide array of developmental processes throughout the animal kingdom (reviewed in 140). As a consequence of ligand binding, the intracellular domain of the Notch transmembrane receptor is released by proteolysis, translocates to the nucleus and associates with the *Drosophila* Suppressor-of-Hairless [Su(H)] family of transcription factors, thereby converting Su(H)-related proteins from repressors into activators (reviewed in 141). Primary targets of this signaling cascade are genes of the *Drosophila* hairy and Enhancer-of-split family of trancriptional repressors (HES genes) that elicit their action in neurogenesis, neural cell fate, vascular development, mesoderm segmentation and myogenesis (reviewed in 142). Aberrant Notch signaling has been linked to several human diseases including a number of cancers, Alagille's syndrome and the neural degenerative disease, CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy). CADASIL is characterized by an early onset of stroke and dementia in adults, and was reported to be caused by mutations in the *NOTCH3* gene (143). A further study ascribes the etiology of CADASIL to a vascular defect (144). In zebrafish, notch signaling plays an important role in the expression of artery-specific genes and repression of venous markers within developing arteries (145). *notch5* (the ortholog of human and mouse *NOTCH3*) and the transmembrane ligand *ephrinB2* are typically expressed in the dorsal aorta but not in the posterior cardinal vein. Embryos injected with mRNA encoding a dominant-negative Su(H) protein are inhibited in Notch activity and fail to induce the arterial-specific markers *ephrinB2* and *notch5* (145). The zebrafish mutant *mindbomb* (*mib)* exhibits many of the molecular and morphologic vascular defects that can be observed in embryos ectopically expressing dominant-negative Su(H) protein. For instance, *mib* mutants do not express *ephrinB2* and *notch5* in the dorsal aorta. In addition, *mib* mutants express the venous-specific markers *flt-4* and the putative receptor for ephrinB2, *ephrinB4*, ectopically in the dorsal aorta (145). Activation of the Notch pathway, either throughout the embryo or targeted to the endothelium using the *fli-1* promoter, results in ectopic expression of the artery markers *ephrinB2* and *notch5* in the posterior cardinal vein, as well as in loss of the venous-specific marker *flt-4* (130, 145). A recent study has shown that *shh* and *vegf* act upstream of the notch pathway to induce arterial differentiation (130). Zebrafish embryos lacking *shh* activity fail to express *vegf* in their somites and show absence of *ephrinB2* expression in their arteries. In turn, exogenous *shh* upregulates somitic *vegf* expression and induces ectopic formation of arteries within the trunk of zebrafish embryos. Loss of *vegf* function was shown to perturb arterial-venous differentiation, whereas exogenous *vegf* can rescue arterial differentiation in the absence of shh signaling. *vegf* overexpression does not reconstitute arterial markers in *mib* mutants, which are inhibited in notch activity, whereas activation of the notch pathway is sufficient to rescue arterial differentiation in *vegf*-morpholino knockdown embryos. Taken together, these results suggest that shh signaling regulates arterial differentiation by inducing *vegf* expression, whereas notch signaling acts downstream of vegf to induce arterial differentiation.

Mutants homozygous for the *gridlock (grl)* locus have no circulation to the posterior trunk and tail because there is a small interruption of the vessel at the base of the aorta where the two anterior lateral dorsal aortae merge to form the single midline dorsal aorta (146). *grl* encodes a protein that belongs to the HES family of basic helix-loop-helix proteins (147). The *grl* mutation was shown to change the stop codon to glycine extending the affected protein by 44 amino acids. *grl* transcripts are detected as bilateral stripes of the lateral plate mesoderm that converge toward the midline to form the primodia of the dorsal aorta at the 24-somite stage. When blood flow begins, *grl* is expressed strongly throughout the dorsal aorta (147). Graded reduction of *grl* expression, by injecting increasing doses of antisense morpholino oligomers, progressively ablates regions of the aorta, as demonstrated by decreased expression of *flk-1* and the arterial marker *ephrinB2*. Concomitantly, an increased expression of the venous-specific receptor *ephrinB4* was observed, indicating a contiguous expansion of venous regions. Overexpression of

grl caused a marked diminution in the vein, as revealed by *flk-1* and *flt-4* labelling, but did not affect the size of the aorta. Whereas *grl* was shown to be unaffected by notch signaling after 24 hpf (145), *grl* expression was reduced at the 3-somite stage by inhibiting the notch pathway, and increased at the 5-somite stage by activating the notch pathway (139). These data suggest that the arterial-venous decision is mediated by the notch pathway and *grl* plays an early role in determining whether angioblasts adopt an arterial or a venous fate.

The zebrafish mutant *violet beauregarde* (*vbg*) is characterized by an abnormal circulation pattern in which blood flows through a limited number of dilated cranial vessels and fails to perfuse the trunk and tail. These cranial vessels were shown to contain twice as many endothelial cells as their wild-type counterparts. *vbg* encodes the zebrafish ortholog of activin receptor-like kinase 1 (Acvrl1; also known as Alk1) that is predominantly, if not exclusively, expressed in endothelial cells (148). In humans, mutations in ACVRL1 are responsible for autosomal dominant hereditary hemorrhagic telangiectasia (HHT) type 2 (149). Symptoms of this disease are recurrent nosebleeds, superficial vascular dilations that present as small red spots and arteriovenous malformations that can lead to stroke (150). The cause of the increased number of endothelial cells in *vbg* is unknown since ligands and downstream targets of Acvrl1 are presently ill-defined. However, it has been suggested that Acvrl1 signaling inhibits endothelial cell proliferation (148).

3.3. Blood development and disorders

Blood of adult vertebrates consists of erythroid, megakaryocytic, myeloid and lymphoid cells. The myeloid lineage includes monocytes (macrophages) and several types of granulocytes (neutrophils, eosinophils). The lymphoid lineage consists of T and B lymphocytes. It has been suggested that hematopoietic stem cells transform into two kinds of progenitors, one capable of generating erythroid/megakaryocytic and myeloid lineages, the other able to give rise to the lymphoid lineage (151). Human acute leukemias arise from blood cell progenitors developing along the lymphoid, myeloid or erythroid pathway, or derive from primitive stem cells with multilineage potential (reviewed in 152). Anemia, thrombocytopenia and immunodeficiency can be caused by the lack of functional erythrocytes, megakaryocytes, and lymphocytes or myeloid cells, respectively. In recent years, genetic approaches using the zebrafish have greatly contributed to the understanding of normal and disease-related hematopoiesis (reviewed in 153-156).

During development of the zebrafish embryo, hematopoiesis occurs in mainly two phases as in all other vertebrates. In the phase of primitive hematopoiesis, predominantly erythrocytes expressing embryonic globin, but also macrophage-like phagocytes (157) as well as myeloid cells (158, 159) are generated. In the phase of definitive hematopoiesis, at a later stage of development, the mature erythroid, the thrombocytic, all myeloid and the lymphoid lineages arise.

The site of embryonic (primitive) erythropoiesis in zebrafish is the intermediate cell mass (ICM), a cord-shaped structure that stems from converging cells of the ventral and lateral mesoderm (160). This hematopoietic tissue generates all circulating erythrocytes for the first 4 days post fertilization but also contains cells that give rise to the pronephros and the trunk vessels. The forming ICM can be identified morphologically at the 5-somite stage (10-11.5 hpf) as two bilateral symmetric bands of cells in an intraembryonic region between the somites and the yolk sac. These cells begin migrating towards the midline around the 10-somite stage, become situated more medially in between the notochord and endoderm by the 18-24 somite stage (about 18-21 hpf), and finally fuse to form a single cord predominantly above the yolk sac extension. As shown by electron microscopy, the 20 hpf ICM contains proerythroblasts, which become erythroblasts by 24 hpf. Between 24 and 30 hpf, the ICM disappears as erythroblasts begin circulating. Final maturation of erythroblasts to erythrocytes occurs in circulation (161). Macrophages appear in the zebrafish embryo as early as erythroid cells, but are located on the yolk anterior to the cardiac field, in a region directly below the hatching gland (157-159). Lineage analysis revealed that yolk sac macrophages originate from ventro-lateral mesoderm, like precardiac cells. This implies that their migratory route during gastrulation resembles that of cardiogenic precursors. Several hours before erythroblasts leave the ICM for circulation, young macrophages first accumulate in the anterior yolk sac blood sinus, then wander randomly to lateral sites and finally invade the mesenchyme of the head or become spread throughout the embryo by blood circulation. Similar to cells that co-express the endothelial marker *flk-1* and the hematopoietic marker *scl* in the lateral plate mesoderm at 6-10 somites (see section 3.2.), *flk-1* and *draculin* (*dra*) expression can be observed anterior to the heart field at 7-10 somites, where early macrophages arise. *dra* function has been associated with the ontogeny of blood because progenitors of the two hematopoietic domains, ICM and anterior heart field, map to the location of *dra* expression. In addition, *dra* is upregulated in proerythroblast and single scattered cells on the yolk sac, which are presumed to be mature macrophages (157). In this regard, the zone of *flk-1* and *dra* expression in the anterior cardiac field may contain a second kind of hemangioblast giving rise to head vessels and macrophages. In support and refinement of these findings, fate mapping revealed that cells of the rostral (most anterior) lateral plate mesoderm give rise to myeloid cells and sit immediately adjacent to the heart field, whereas erythroid cells are located more posterior, at somite 6 and beyond (158). The zebrafish ortholog of the transcription factor PU.1, which plays a critical role in mammalian myeloid development, has been proven to be a marker to examine early myeloid commitment in zebrafish. *pu.1* is first expressed around the 5-somite stage (12 hpf) in a rostral population of cells of the lateral plate mesoderm that is anatomically isolated from *gata-1* expressing erythrogenic cells (158, 159). Later, *pu.1* is also expressed in the caudal lateral plate mesoderm and may interact with *gata-1*, as observed in mammalian systems (158, 162, 163).

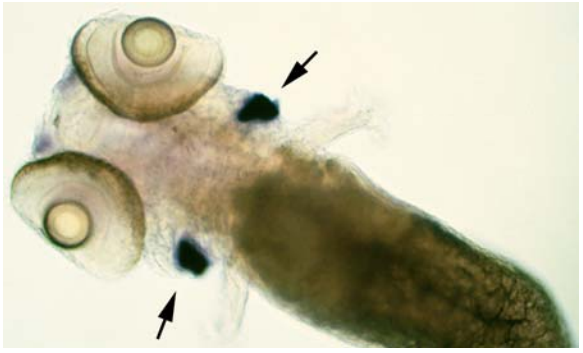

Figure 4. Dorsal view of a 5-day-old zebrafish. Arrows indicate prominent expression of *recombinase-activating gene 1* (*rag1*) in the bilaterally symmetric thymus. Reproduced with permission from N.S. Trede, unpublished data.

The location of hematopoietic tissue from the time the ICM disappears (24-30 hpf) until the kidney of the adult becomes the site of blood formation has not been conclusively identified. Based on the expression pattern of orthologous genes known to be involved in the initiation of definitive hematopoiesis in mice, it has been suggested that the ventral wall of the dorsal aorta is the location of definitive hematopoiesis in zebrafish. In mice, definitive hematopoiesis emerges first in the para-aortic, splanchnopleural and aorta-gonad-mesonephros (AGM) regions, then in the fetal liver and finally in the bone marrow (90). Pluripotent hematopoietic stem cells express *Scl*, *Lmo2*, *Gata-2*, *c-Myb*, *Runx1*, and *Cbf-beta* (Runx1 and Cbf-beta heterodimerize to form core binding factor [CBF]), and commit to a common lymphoid and myeloid progenitor. Gene knockout studies have shown that *c-Myb* $^{-/-}$ (164), *Runx1* $^{-/-}$ (123, 124), and *Cbf-beta* $^{-/-}$ (165-167) mice lack all definitive hematopoietic lineages, whereas targeted disruption of *Scl*, *Lmo2* and *Gata-2* affects both, primitive and definitive hematopoiesis (107-109, 168-170). Analysis of mice homozygous for a germ-line deletion in the *Ikaros* gene revealed an essential role for *Ikaros* in directing pluripotent hematopoietic stem cells to common lymphoid progenitors (171, 172). Cells at all stages of the B- and T-cell lineage were absent in these mice, indicating a requirement of *Ikaros* in providing lymphoid precursors. The specification of common myeloid precursors from hematopoietic stem cells remains obscure, and may be a default pathway in the absence of Ikaros. In zebrafish, *c-myb* is first expressed in primitive erythroid cells of the ICM and later, at 36-48 hpf, in cells scattered along the ventral wall of the dorsal aorta (94). *Ikaros* expression appears in lateral plate mesoderm at the 14-somite stage, in the ICM at 24 hpf, and between the dorsal aorta and the tail vein at 48 hpf (173). Cells that are located directly ventral to the aorta and express *c-myb* and *ikaros* may derive from the ICM and keep a continuous hematopoietic potential, or are a newly differentiated population of hematopoietic cells on the verge to launch the first wave of definitive hematopoiesis. A thorough resolution of the identity and potential of these presumptive stem cells, however, awaits lineage tracing and transplantation experiments.

Although well-developed renal tubules and glomeruli can be observed at 72 hpf, the first hematopoietic cells do not appear in the pronephros until 96 hpf (161). At this stage and during the next several days erythroblasts and myeloblasts are the most frequent blood cell lineages found. During week 2 and 3 post fertilization the amount of hematopoietic tissue of the pronephros greatly increases and the first lymphoid cells and all developmental stages of the neutrophilic lineage can be identified. In the adult fish, the hematopoietic tissue of both pronephros and mesonephros gives rise to all blood lineages (161).

Lymphoblasts can first be distinguished in the thymic primordium of the zebrafish at 65 hpf. *Ikaros* and *recombination activation gene 1* (*rag1*) expression is detected in thymic lymphocytes at 72 and 84 hpf, respectively. This suggests that lymphoid progenitors found in the thymic primordium do not come from the pronephros, which initiates its hematopoietic activity only around 96 hpf, but possibly derive from early hematopoietic centers such as the ICM or the ventral wall of the aorta (analogous to the mouse AGM region) (Figure 4) (161) (reviewed in 174).

Taken together, definitive hematopoiesis that generates erythrocytes, thrombocytes, myelocytes and lymphocytes is thought to begin in a location other than the kidney and is proposed to originate from cells of the ventral wall of the aorta or from the former ICM. The hematopoietic potential of the thymic primordium and the pronephros may derive from colonizing progenitors or stem cells that find appropriate conditions for self-renewal, further commitment, differentiation and proliferation (reviewed in 174).

The hematopoietic program of vertebrates has been meaningfully defined by zebrafish genetics using mutagenesis screens. Mutants with defects in ventral versus dorsal cell fate patterning, such as *chordino* and *swirl*, have disrupted genes in the bone morphogenic protein (BMP) pathway and show either an expansion or a lack of hematopoietic cells, respectively (reviewed in 32). This is consistent with cell lineage analyses demonstrating that blood derives from ventral mesoderm, which is specified by a gradient of BMPs (85).

Another early patterning zebrafish mutant called *spadetail* (*spt*) shows an increased number of axial at the expense of paraxial progenitor cells with the consequence that these mutants have almost no trunk somites (175). In addition, *spt* mutants fail to form ICM-derived blood, despite expression of early hematopoietic lineage commitment genes such as *lmo2* and *gata-2* (94). *spt* mutants are deficient in the T-box family transcription factor Tbx16, which is responsible for cell sorting rather than cell fate commitment (176). It prevents dorsal and ventral/lateral cells from becoming mixed to successfully take an axial versus paraxial mesodermal cell fate (177). Consistent with normal rostral development in *spt* mutants, the rostral population of *pu.1* expressing cells is preserved, whereas caudal *pu.1* as well as *gata-1* and *c-myb*

expression in the lateral plate mesoderm is absent in these mutants (94, 158).

The zebrafish *cloche* (*clo*) mutation affects the specification of both the endothelial and hematopoietic cell lineage (see section 3.2). *clo* mutants lack endocardium, head and trunk endothelium and nearly all blood cells (91). The gene responsible for the *clo* phenotype has yet to be identified, but some endothelial and hematopoietic target genes that are affected by the *clo* mutation have been characterized. The expression of *flk-1*, *fli-1*, *flt-4*, *tie*, *scl*, *lmo2*, *hhex*, *gata-2*, *gata-1*, *c-myb* is mostly absent in *clo* mutants and suggests that both primitive and definitive hematopoiesis, as well as endothelial development are disrupted (92-94, 117). Genes expressed in both endothelial and blood lineages from an early stage, such as *scl*, *hhex* and *runx1*, can partially restore the expression of endothelial and blood genes in *clo* mutants, and are therefore thought to function downstream of the hemangioblast level, whereas *clo* itself may specify the hemangioblast (93, 117, 125). Cell transplantation experiments revealed that *clo* is required cell autonomously in the formation of the endocardium, cell non-autonomously in the differentiation of *gata-1*-expressing cells and cell autonomously in the survival/proliferation of primitive erythrocytes at a later time point (95). The character of the non-permissive environment for *gata-1* expression in *clo* mutants is not known but may reflect the lack of defined endothelium. *vegf*, a critical factor for vascular development, is generally not affected in *clo* embryos (except that its expression in the pronephric glomeruli is missing) and *vegf* overexpression cannot rescue the *clo* phenotype. This suggests that *vegf* expression is independent of *clo* function (132). The transcription factors *lmo2* and *gata-2* are both involved at early stages of hematopoiesis (169, 170). *lmo2* is expressed in a similar pattern as *scl* and is absent in *clo* mutants (94). The expression of *gata-2* starts before the onset of *clo* function and is detectable in early lateral mesoderm and ventral ectoderm. Cells derived from these regions give rise to hematopoietic progenitors or neurons, and continue expressing *gata-2* through the first 24 h or 8 days after fertilization, respectively (178, 179). *Clo* mutants lack hematopoietic *gata-2* but maintain neural *gata-2* expression (94).

As mentioned in the previous section of this review, chromosomal translocations of the transcription factors *SCL* and *RUNX1* (*CBFA2*, *AML1*, *PEBP2alphaB*) are frequently involved in human leukemias. Chromosomal translocations that involve *AML1* result in chimeric proteins known as TEL-AML1, AML1-ETO (RUNX1-CBF2T1), AML1-MTG16 and AML1-EVI1 (reviewed in 118 and 152). AML1 can both repress and activate transcription. However, the mechanisms that convert AML-1 from an activator to a repressor and vice versa remain to be elucidated. The ETS family member TEL (translocation-ets-leukemia) exhibits repression domains that bind transcription factors such as Fli-1, thereby inhibiting its transcriptional activity (180, 181). The designation ETO (eight twenty one) refers to the t(8;21) translocation, which fuses the *ETO* gene on human chromosome 8 with the *AML1* gene on chromosome 21. ETO acts in concert with a nuclear complex that mediates transcriptional repression (182). MTG16 (myeloid transforming gene 16) is a ETO family member located on chromosome 16 with presumably the same function as ETO. EVI1 has been reported to be a transcription factor (183). Core binding factor beta (CBF-beta) is another gene involved in leukemia, due to a chromosomal inversion (184, 185). AML1 normally heterodimerizes with CBF-beta to form core binding factor (CBF) having a prominent role in definitive hematopoiesis. Mice homozygous for *AML1* or *CBF-beta* loss-of-function mutations are not viable and have identical phenotypes, namely nervous system hemorrhages and failure in definitive hematopoiesis (123, 124, 165-167). *CBF-beta* is frequently mutated through a chromosome 16 inversion, which generates a fusion gene between *CBF-beta* and *MYH11*, encoding smooth muscle myosin heavy chain (SMMHC). The resulting CBFB-SMMHC (this nomenclature refers to the protein, whereas *MYH11* refers to the gene) fusion protein was shown to dominantly inhibit AML1 function via its ability to complex with endogenous AML1 (184, 186, 187). Although both AML1 and CBF-beta fusion genes lead to alterations in the CBF transcription complex, they may function along different pathways because they present with different leukemic transformation properties. The *TEL-AML1* fusion gene is found in patients with acute lymphoblastic leukemia, whereas *AML1-ETO*, *AML1-EVI1* and *CBFbeta-MYH11* is associated with acute myeloid leukemias (reviewed in 152). TEL-AML1 blocks pro-B cells differentiation, whereas AML1-ETO, AML1-EVI1 and CBFbeta-SMMHC affect myeloblastic, myelodysplastic and myelomonocytic cells, respectively (reviewed in 152). In principle, any translocation event could lead to gain or loss of function of either fusion partner, to the formation of dominant interfering alleles, or to entirely new activities. The amino acid motifs contributed by the fusion partners AML1, TEL, ETO, MTG16, EVI1, CBF-beta and SMMHC may aberrantly recruit transcriptional (co-)activators, (co-)repressors, or histone deacetylases to certain promoters, or may bind to distinct DNA motifs, thereby altering gene expression profiles and contributing to a transformed state (188-192). Overexpression of the human RUNX1-CBF2T1 (AML1-ETO) fusion protein during zebrafish embryogenesis causes abnormal vascular development and defective hematopoiesis, similar to the abnormalities observed in *RUNX1*-morpholino injected embryos and *RUNX1-CBF2T1* knock-in mice (193, 194). These results are consistent with the idea that the RUNX1-CBF2T1 fusion protein dominantly inhibits endogenous RUNX1 function in zebrafish (125). However, results from the mouse and zebrafish model show that RUNX1-CBF2T1 alone is not capable to induce leukemia and suggest that additional mutations are necessary in the etiology of human t(8;21) acute myeloid leukemia (195, 196). Zebrafish express *runx1* first in cells of the lateral plate mesoderm at the 5-somite stage (12 hpf) in co-localization with *scl*. At later developmental stages, *runx1* appears in Rohon-Beard neurons, in the ICM and in the ventral wall of the dorsal aorta (125). *cbfb* (the zebrafish ortholog to *CBF-beta*) expression starts at the 3-somite stage and shows the same

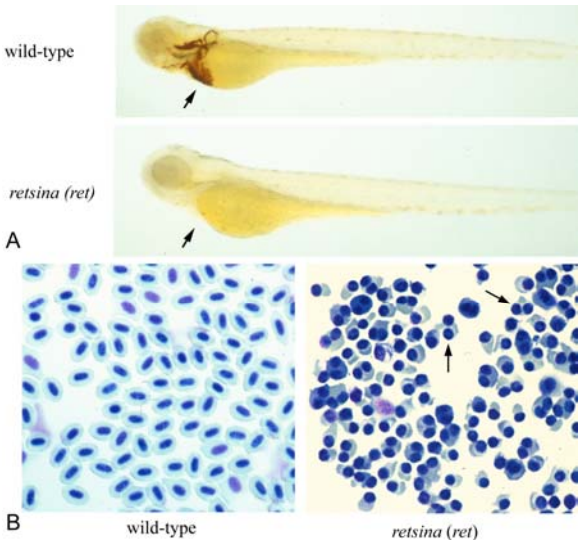

Figure 5. Erythrocyte development in *retsina* (*ret*) mutants. A) Wild type and *ret* embryos at 72 hpf were stained with o-dianisidine for hemoglobin visualization. *ret* embryos lack circulating erythrocytes, which is most significantly noticeable in the cardiac sinus (arrows). B) Peripheral erythrocytes from wild type zebrafish in comparison to concentrated peripheral blood from *ret* mutant embryos. Examples of bi-nucleated erythroblasts (arrows) are indicative of a defect in cytokinesis. Reprinted with permission from B.H. Paw (206).

expression pattern as *runx1* (197). Both factors are thought to function downstream of the hemangioblast level and contribute to both primitive and definitive hematopoiesis. Impaired primitive hematopoiesis in *runx1*-morpholino treated zebrafish and unaffected primitive hematopoiesis in *Runx1*-null mice could be reconciled with functional redundancy amongst runt family members in the mouse system.

The ease of visual inspection of mutagenized zebrafish embryos for red blood deficiency lead to the isolation of 26 different mutants with defects in hematopoiesis (198, 199). Although the mutations affect several stages of hematopoietic development, mutant fish have mainly defects in erythropoiesis (154).

The *bloodless* (*bls*) mutation causes an absence of embryonic erythrocytes and lymphoid precursors, whereas primitive macrophages appear to develop normally. This is consistent with the existence of two segregated hematopoietic domains, the ICM and the anterior heart field, that define erythroblasts and primitive macrophages, respectively (157, 158). *bls* mutants, however, are able to generate red blood cells and lymphocytes later in development (5–7.5 dpf) when definitive hematopoiesis is thought to replace the primitive wave. Overexpression of *scl*, but not *bmp4* or *gata1*, partially rescues the ability to express blood specific markers. Cell transplantation experiments revealed that *bls* function is cell non-autonomously required for primitive hematopoiesis. The unique role of *bls* as a cell non-autonomous regulator of primitive hematopoiesis underscores the existence of different regulatory cues for primitive and definitive hematopoiesis (200).

Similar to *bls*, the *moonshine* (*mon*) mutation affects the formation of erythroid progenitors. However, mutant fish do not recover but suffer from severe anemia and die after 5 to 7 dpf. The expression of markers for myeloid and lymphoid lineages appears to be unaffected (Trede and Zon, unpublished observations). In addition to the hematopoietic deficiency, *mon* mutants exhibit alterations in fin morphology and pigment cell proliferation and migration (198).

In *vlad tepes* (*vlt*) mutants the generation of the erythroid lineage is disrupted due to a mutation in the *gata-1* gene that results in a truncated protein unable to bind DNA and mediate GATA-specific transactivation (201). Gata-1 is an indispensable transcription factor that is present in various vertebrates and regulates erythroid and megakaryocytic gene expression (reviewed in 202). Hematopoietic stem cell markers, such as *gata-2*, *lmo2*, *scl* and *cbfb*, myeloid markers, such as *pu.1*, *l-plastin* and *c/ebp1* (CCAAT/enhancer binding protein 1), and lymphoid markers, such as *ikaros* and *rag1*, are expressed normally in *vlt* mutants (201). The erythroid-specific gene *globin* is also normally expressed, whereas other erythroid markers such as *band3*, *sptb* and *alas2* (see below) are absent in *vlt* mutants. This may be explained by different requirements of *gata-1* for erythroid-specific gene expression.

Mutations in the *riesling* (*ris*), *chablis/merlot* (*cha/mot*) and *retsina* (*ret*) locus affect the generation of differentiated erythrocytes, which leads to a marked decrease of circulating red blood cells after 3 to 4 dpf. The gene defective in *ris* encodes zebrafish erythroid *beta-spectrin* (*sptb*), a protein involved in cytoskeleton assembly (203). The *sptb* mutation in zebrafish causes a red cell membrane disorder analogous to human hereditary spherocytosis, in which mutated erythroid spectrins lead to congenital hemolytic anemia (reviewed in 204). Human hereditary spherocytosis can also be caused by defects in alpha-spectrin, ankyrin, protein 4.1, band 3, glycohphorin C, actin and adducin, since these proteins are either part of the erythrocyte membrane or of the underlying cytoskeleton and bind, anchor, attach or cross-link each other to make erythrocytes withstand the mechanical deformation stresses of circulation. In fact, the zebrafish mutants *chablis* (*cha*)/*merlot* (*mot*) were shown to harbor allelic nonsense mutations in the erythroid-specific protein 4.1R, resulting in loss of membrane integrity, organization and stability (205). As a consequence, *cha/mot* mutants exhibit severe hemolytic anemia accompanied by cardiomegaly, splenomegaly, elevated bilirubin levels and erythroid hyperplasia in the kidney. *scl*, *gata-1* and *globin* expression is comparable to wild-type siblings indicating that erythropoiesis is not interrupted. *retsina* (*ret*) mutants are deficient in the cytoskeletal protein band 3, also known as anion exchanger 1 (AE1). The analysis of *ret* mutants revealed a novel evolutionary-conserved function of band 3 in erythroid-specific cytokinesis that is independent of its ion exchange function (Figure 5) (206).

Mutants with hypochromic blood include *weissherbst* (*weh*), *chardonnay* (*cdy*) and *zinfandel* (*zin*). They have reduced levels of hemoglobin with nearly normal numbers of blood cells. The mutated genes cause either defects in iron homeostasis or globin expression (reviewed in 207). The gene affected in *weh* mutants encodes an iron exporter known as *ferroportin1*, which is located in the basolateral membrane of polarized cells at the boundary between yolk and developing embryo, and between intestine and blood circulation in the adult (208-210). The divalent metal transporter *dmt1* (also called Nramp2 and DCT1) is located in the apical membrane of the same cells (211, 212). *dmt1* shuffles iron into boundary cells through the apical membrane, whereas *ferroportin1* exports iron to the developing embryo or to blood circulation through the basolateral membrane. *dmt1* is also expressed in specialized endosomes within erythroid precursors, where it releases transferrin-transported iron from the interior of the endosome to the cytoplasm (reviewed in 213, 214). Microcytic anemia (mk) mice, anemic Belgrade (b) rats as well as zebrafish *cdy* mutants harbor mutations in *dmt1* and may serve as animal models for human iron-importer-related disease (212, 215, 216). With increasing clinical vigilance for iron-deficiency phenotypes, it is likely to find humans with mutations in DMT1. Patients with Type 4 hemochromatosis carry mutations in the *ferroportin1* gene (217, 218) and have a characteristic pattern of iron loading in liver macrophages, which import iron from senescent red blood cells for recycling (208). The *zin* mutation is linked to one of the *globin* loci, representing a model for human thalassemia (219).

The zebrafish *sauternes* (*sau*) mutation affects the function of delta-aminolevulinate synthase (alas2), the enzyme required for the first step in heme biosynthesis (220). In humans, mutations in the *ALAS2* gene cause X-linked congenital sideroblastic anemia, a disease that resembles the *sau* phenotype (221, 222). The *yquem* (*yqe*), *freixenet* (*frx*), *dracula* (*drc*) and *desmodius* (*dsm*) mutants are also deficient in the heme biosynthetic pathway. The affected enzymes, however, are involved in later steps of heme biosynthesis leading to autofluorescent, light-sensitive blood that is reminiscent of human inherited or aquired porphyria syndromes (223). *yqe* mutants are deficient in *uroporphyrogen decarboxylase* (*UROD*), the enzyme accomplishing the fifth step of heme biosynthesis (224). *drc* are mutated in the *ferrochelatase* gene, which encodes the enzyme that catalyzes the transfer of iron to the heme moiety (225).

Zebrafish mutagenesis screens are similarly under way to determine the molecular determinants involved in the generation of myeloid, lymphoid and thrombocytic blood cells (thrombocytes in zebrafish are equivalent to platelets in mammals (226)). The cloned zebrafish homologs of mammalian myeloid genes such as *CCAAT/enhancer-binding protein (C/ebp)*, *pu.1*, *myeloperoxidase (mpo)* and *l-plastin* are useful molecular probes for identifying mutants deficient in myelopoiesis. *pu.1* was reported to be involved in early lineage commitment, *mpo* in granulopoiesis and *l-plastin* in monocyte/macrophage differentiation (158, 159, 227, 228). Using *rag1* as a probe for developing T-cells in the thymus, ENU-mutagenized zebrafish have been screened for absence of *rag1* expression and submitted to phenotypic and genetic characterization (reviewed in 174).

3.4. Kidney development and disorders

In vertebrates, the pronephros is the first kidney to form during development. In fish, it accomplishes blood filtration and osmoregulation at embryonic and larval stages. The pronephros consists of two glomeruli fused at the midline, two pronephric tubules connecting directly to the glomeruli via a neck segement, and paired bilateral pronephric ducts which convey the altered blood filtrate outside the animal. The glomerulus receives blood flow through a capillary network sprouting from the dorsal aorta. Selective filtration of the blood is achieved by capillary endothelial cells, glomerular podocytes and the properties of the glomerular basement membrane (GBM), which forms when the basement membrane of the endothelial cells and podocytes fuse to form the common trilaminar GBM. Podocytes display regularly spaced foot processes contacting the outer aspect of the GBM, whereas endothelial cells lining the inner aspect of the GBM have fenestrated membranes. Glomerular filtration begins between 36 and 48 hpf and coincides with the elaboration of podocyte foot processes and the ingrowth of glomerular capillaries from the dorsal aorta (229, reviewed in 230). As juvenile fish mature into adults, the mesonephric kidney develops and, beside blood filtration and osmoregulation, becomes the primary organ of hematopoiesis. Together with heart and blood, the pronephros was demonstrated to derive from ventral mesoderm (85).

The forming zebrafish pronephros expresses the paired-box transcription factors *pax2.1* and *pax8*, the homeobox gene *lim1*, the Wilms' Tumor suppressor gene *wt1,* and the homolog of the *Drosophila* single-minded gene *sim1* (231, 232). The first appearance of these transcription factors in bilateral stripes adjacent to the somites at the 1- to 8-somite stage and lineage analysis of labeled cells revealed that the early expression domain of these genes marks the origin and fate of cells that fashion the pronephros in the zebrafish. Pre-glomerular cells were shown to be $wt1^{+}/pax2.1^{-}/sim1^{-}$, pre-tubular cells $wt1^{+}/pax2.1^{+}/sim1^{-}$ and pre-nephric duct cells $wt1^{-}/pax2.1^{+}/sim1^{+}$. Concomitantly, pre-glomerular, pre-tubular and pre-nephric duct cells orientate along the anterior-posterior axis, occupying progressively more posterior domains of the pronephric field (231). Characteristic for an embryonic field, the pronephric progenitor region was shown to possess regulatory ability. After ablation of *wt1*-expressing tissue during the 8- and 10-somite stage, a complete pronephric kidney was able to form (231). This however contrasts with observations in *Wt1*-null mice in which the kidney fails to develop (233).

The close association of capillary endothelial cells and podocytes in the glomerulus suggests a mutual requirement for their own recruitment and differentiation. Analysis of *clo* mutants, which lack endothelium, revealed the presence of differentiated podocytes and the formation

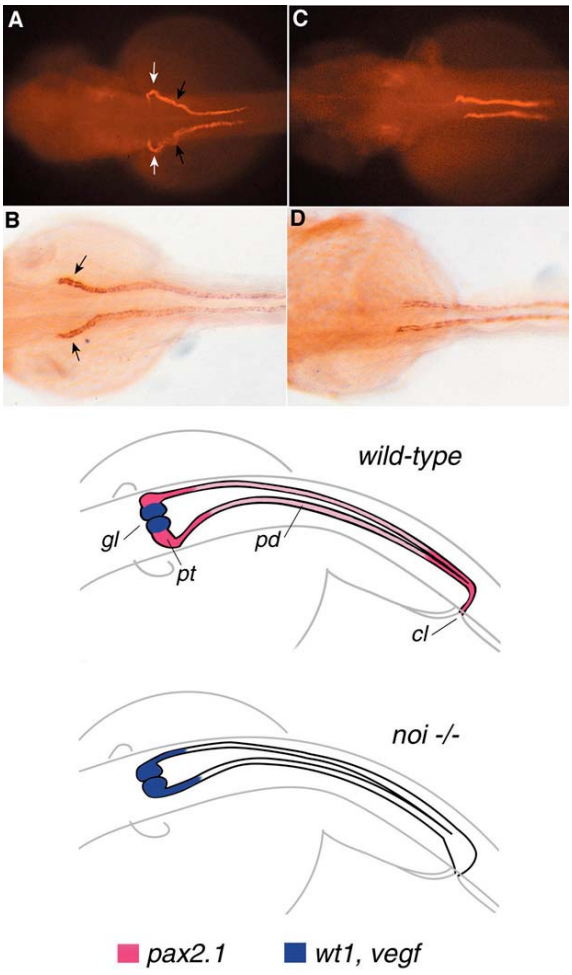

Figure 6. Upper panel. Tubules and anterior duct differentiation is affected in *no isthmus* (*noi*) mutants. Antibodies against pronephric markers stain the tubules (white arrows in A; black arrows in B) and anterior ducts in wild type siblings (A, B) but not in *noi* mutants (C, D). Reprinted with permission from I.A. Drummond (238) and The Company of Biologists Ltd. Lower panel. *pax2.1*/*wt1* regulatory interactions are important during pronephric patterning. During development, *pax2.1* and *wt1* are expressed in mutually exclusive domains. Strongest *pax2.1* expression (red) is found in the forming pronephric tubule (pt), the anterior pronephric duct and the cloaca (cl). *wt1* (blue) is strongly expressed in the glomerulus (gl). *pax2.1* is required to restrict the domain of wt1 expression. In absence of *pax2.1*, as in *no isthmus* (*noi*) mutants, *wt1* is expressed in domains where tubule and anterior duct cells occur, resulting in a failure of tubule formation. Reprinted with permission from I.A. Drummond (238) and The Company of Biologists Ltd.

of a trilaminar GBM (234). In addition, the expression of the podocyte differentiation marker *wt1* in the midline of 36-hpf *clo* embryos indicates that the dorsal aorta itself plays no role in maintenance of *wt1* expression or podocyte commitment. At 72 hpf, however, the differentiated glomerulus remains as a flattened sac of cells reminiscent of avascular nephron primordia, and the fragmented appearance of the GBM likely reflects the lack of endothelial contribution during GBM synthesis (234).

In the midline mutant *floating head* (*flh*) and the hedgehog signaling impaired mutants *sonic-you* (*syu*) and *you-too* (*yot*) the dorsal aorta is absent (as discussed in section 3.2.). Within these embryos, glomeruli remain at bilateral positions without fusing at the midline. The glomeruli contain morphologically identifiable podocytes and endothelial cells, meaning that podocytes recruit endothelial cells from an alternate source than the dorsal aorta in order to vascularize the glomeruli (235). However, endothelial cells in *flh* glomeruli lack fenestrations and are therefore partially compromised in blood filtration. Taken together, this suggests that the hedgehog signaling pathway is required for proper glomerular morphogenesis but not for the differentiation of podocytes.

In mutants with cardiac dysfunction such as *island beat (isl*), lacking a functional L-type calcium channel subunit, and *silent heart* (*sih*), being deficient in cardiac troponin T (see section 3.1), *wt1* expressing cells form, but remain bilateral even as late as 72 hpf and glomeruli fail to assemble at a midline position. The application of 2,3-butanedione monoxime (BDM), an inhibitor of myofibrilar ATPases, to wild type embryos phenocopies the glomeruli assembly defect of *isl* and *sih*. In addition, the expression of *matrix metalloproteinase-2* (*mmp-2*) is dramatically reduced in *isl* mutants and BDM-treated wild type embryos. Inhibition of *mmp-2* by tissue inhibitor of metalloproteinase-2 (TIMP-2) similarly blocks glomeruli assembly. These results demonstrate that medial migration and midline coalescence of podocytes is dependent on cellular interactions and signaling that occurrs outside the pronephric field (236).

The zebrafish mutant *no isthmus* (*noi*) is deficient in the *pax2.1* gene (53). Morphologically, these embryos fail to properly form the midbrain-hindbrain boundary, lack pronephric tubules and show defects in pronephric duct epithelial differentiation and cloaca morphogenesis (Figure 6a) (53, 237, 238). It was shown that the pronephric defects in *noi* arises from altered patterning events during the formation of the pronephric primordium (around the 8-somites stage and later on). In the absence of *pax2.1*, expression of the podocyte specific genes *wt1* and *vegf* expands to the region of the bilateral pronephric tubules and anterior ducts where *pax2.1* is normally present (Figure 6b**)**. This suggests that *pax2.1* is instructive for the development of pronephric tubules and the proper differentiation of the pronephric ducts and cloaca. In addition, *pax2.1* seems to repress podocyte-specific gene expression in ectopic regions where future tubule and anterior duct cells are located (238). The Denys-Drash syndrome (DDS) is a rare human urogenital disorder caused by dominant negative mutations in the Wilms' Tumor Suppressor gene, *WT1* (239). Podocytes of Denys-Drash patients show loss of WT1 nuclear localization and display upregulation of *PAX2*. Together with the *in vivo* role of *wt1* and *pax2.1* in zebrafish, these findings suggest that during nephron development *pax2.1* (the zebrafish

ortholog to human *PAX2*) and *wt1* act in a mutually antagonistic fashion to limit their respective expression domains.

Autosomal dominant polycystic kidney disease (PKD) in humans is primarily caused by mutations in polycystin and leads to renal failure (240). Polycystin is an integral transmembrane protein with an extracellular N-terminus that contains motifs characteristic of cell-cell and cell-matrix interactions. The precise physiologic function of polycystin and its role in PKD is elusive. Autosomal dominant PKD has also been associated with apical mislocalization of the Na^+/K^+ ATPase (241). Cystic maldevelopment was observed in zebrafish pronephric mutants and may be related to an inability to withstand filtration pressure followed by progressive distension of the pronephric tubule and duct epithelia (229). At presence, there is no evidence that any of the pronephric mutants harbor defects in homologs of human PKD genes. Instead, the zebrafish pronephric mutants *double bubble* (*dbb*) and *fleer* (*flr*) develop cysts and show predominant apical and reduced basolateral localization of Na^+/K^+ ATPase, whereas in wild-type Na^+/K^+ ATPase is restricted to the basolateral surfaces of pronephric duct epithelial cells (229). The murine juvenile cystic kidney (*jck*) mutation results in autosomal recessive PKD and was shown to affect a Nek-family kinase (Nek8) (242). Injection of zebrafish embryos with *nek8*-morpholinos resulted in the formation of pronephric cysts, copying the phenotype of *jck* mice. This exemplifies the utility of zebrafish in identifying molecular mechanisms leading to PKD and represents a straightforward strategy to reveal gene function in different model systems.

4. CONCLUSIONS AND OUTLOOK

Zebrafish as a model system for studying development is uniquely positioned in the gap between higher vertebrate and invertebrate model systems. Over the last 90 years, *Drosophila* genetics has immensely contributed to our understanding of molecular processes during development. Similarly, the more recent achievements in mouse genetics and the insights that have been gained from chicken and *Xenopus laevis* systems are invaluable. In zebrafish, typical chordate and vertebrate innovations such as the notochord, the neural tube, cranial structures, a continuous blood circulation system with a ventral heart, the kidney, gut, liver and pancreas can be prototypically analyzed. In *Drosophila* only elements of these structures have evolved, but still provide many clues to the understanding of the complexity of these structures. As exemplified by studying the function of the zebrafish *hand2* gene (46) and its ortholog in mouse (*Hand2*/*dHand*) (243), the comparative analysis of genes in different vertebrate model systems can reveal novel gene function. Since mouse *Hand2* is supposed to be redundant with *Hand1*, the complete function of *Hand2* was observed to be masked. By compilation of loss-of-function phenotypes from phylogenetically different vertebrates, redundancy can be overcome and a close approximation to complete gene function may be achieved.

In zebrafish, the achievements of molecular biology have been successfully combined with systematic genetic analyses. This lead to the discovery of novel genes, such as *ferroportin1* (208), novel gene functions, as analyzed in *pandora* (*pan*) (56) and *foggy* (*fog*) (55), and to the integration of pathways, such as Nodal and Notch, to which several genes contribute. An amazingly original contribution of zebrafish genetics on hand is the finding that housekeeping genes such as transcription elongation factors (*pan*/Spt6 and *fog*/Spt5) or protein disulfide isomerases (PDIs) (67), best known for catalyzing reducing as well as oxidizing reactions in disulfide bond formation, can have precise regulatory roles during development. The demonstration that these broadly expressed genes have prominent roles during emryogenesis reveals the strength of random mutagenesis and points out the importance of unbiased approaches for genetic analyses. Other invaluable contributions of zebrafish genetics to our understanding of gene function are the isolation of the *miles apart* (*mil*) (50) and *jekyll* (*jek*) (70) genes, encoding a sphingosine-1-phosphate-binding G-protein-coupled receptor and UDP-glucose dehydrogenase, respectively. The demonstration that both genes are involved in cell migration opens up new hypotheses about the role of lysosphingolipids and glycosaminoglycans in cell signaling and attraction.

With the expansion of zebrafish genomic infrastructure, gene-to-phenotype strategies are becoming complementary to phenotype-to-gene screens for functional analysis. The Targeting Induced Local Lesions IN Genomes (TILLING) aims at large-scale screening of genomic DNA for chemically induced mutations in the gene of interest. It was first described for plants and recently, it has been successfully adapted for zebrafish (23, 27). The advantage of TILLING over knockout strategies is the recovery of a traditional allelic series of point mutations including hypomorphic mutations, which have a special role in modeling human disease states.

Allelic mutants are especially valuable for the study of essential genes, where sub-lethal alleles are required for phenotypic analysis. Allelic variety is generally very desirable in functional genetics since hypomorphic alleles, gain-of-function alleles and conditional null alleles contribute to fully understand gene function. Hypomorphic alleles, showing a less severe phenotype, can uncover subtle aspects of gene function (tissue and stage specificity) that may be obscured by loss-of-function (or null) alleles.

Recalling the knowledge gained from studying zebrafish heart, vasculature, blood and kidney development, the relevance of the zebrafish system to medicine appears to be great. Zebrafish genetics provided many clues, as to how single genes regulate the assembly of these organs and how genes might fail, causing congenital disorders. Many inherited human diseases are not due to null mutations and present with a range of clinical severity. Chemically induced mutations giving a range of allelic strength are thus potentially more relevant to human disease than null mutations in which early or widespread dysfunction may hide the role of a gene in later organ formation. This again underscores the importance of allelic series that are produced by random mutagenesis.

5. ACKNOWLEDGMENT

This work has been supported by grants from the Swiss National Science Foundation (G.A.), U.S. National Institutes of Health and William Randolph Hearst Foundation (B.H.P). We thank Alan Davidson and Rebecca Wingert for critically reviewing the manuscript. We also thank Didier Stainier, Iain Drummond, Deborah Yelon, and Nikolaus Trede for permission to reproduce their figures in this review.

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Key Words: Zebrafish, Organogenesis, Heart, Vasculature, Blood, Kidney, Mutagenesis, Disease, Review

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