Int. J. Dev. Biol. 46: 493-502 (2002)

Pathways in blood and vessel development revealed through zebrafish genetics

PHILIP S. CROSIER*, MAGGIE L. KALEV-ZYLINSKA, CHRISTOPHER J. HALL, MARIA VEGA C. FLORES, JULIA A. HORSFIELD and KATHRYN E. CROSIER

Division of Molecular Medicine, Faculty of Medical and Health Sciences, The University of Auckland, New Zealand

ABSTRACT Studies in zebrafish have potential to contribute to understanding of the vertebrate hematopoietic and vasculogenic systems. Our research has examined the roles of several molecules in pathways that lead to the development of blood and vessels in zebrafish, and has provided insights into the regulation of these processes. Gdf6a/radar, a member of the bone morphogenetic protein (BMP) family, is expressed in the zebrafish hypochord and primitive gut endoderm; structures that flank the developing dorsal aorta and posterior cardinal vein. This pattern of expression positions Gdf6a/radar as a candidate regulator of vasculogenesis. Support for such a role has come from experiments where Gdf6a/radar function was depleted with antisense morpholino oligonucleotides. This resulted in vascular leakiness, suggesting that Gdf6a/radar is involved in maintenance of vascular integrity. The transcription factor Runx1 is known to play a critical role in mammalian definitive hematopoiesis. When Runx1 expression domains and function were analyzed in zebrafish, the importance of this gene in definitive hematopoiesis was confirmed. However there was also evidence for a wider role, including involvement in vascular development and neuropoiesis. This work has laid the foundation for an ethylnitrosourea (ENU) mutagenesis screen based on *runx1* whole-mount *in situ* hybridzation, that aims to identify genes operative in the *runx1* pathway. An additional member of the Runx family, Runx3, is also involved in developmental hematopoiesis, with a function distinct from that of Runx1. We hypothesize that Runx1 and Runx3 form a continuum of transcriptional control within the hematopoietic system. An added attraction of zebrafish is that models of human disease can be generated, and we have shown that this system has potential for the study of Runx1-mediated leukemogenesis.

KEY WORDS: *angioblast, BMP, Gdf6, hemangioblast, hematopoietic stem cells, runx, vasculogenesis, zebrafish*

Introduction

An understanding of stem cell biology requires definition of the molecular mechanisms that permit a single multipotent cell to differentiate into a complex population of functionally and morphologically distinguishable cells. The hematopoietic system provides a powerful model for addressing questions in stem cell development. However, within this system, there remain significant challenges in identifying the molecular switches that regulate the earliest events in hematopoietic stem cell formation. Work in this area has highlighted the tight interrelationship between the development of blood and the vasculature from mesodermal precursors. Insights into the genetic programs that regulate developmental hematopoiesis and vasculogenesis are being made through use of a variety of model systems. The zebrafish is one of the more recently adopted vertebrate models that is amenable to genetic manipulation and provides a bridge between less complex systems such as *Drosophila* and higher vertebrates for understanding early events in blood and vascular development.

Developmental Hematopoiesis: the Pathway to Blood

The blood system arises during development from ventral mesoderm. The pathway of events leading to the early establishment of the hematopoietic system can be viewed as a multi-step process beginning with mesoderm induction. Next, ventral meso-

Abbreviations used in this paper: AGM, aorta gonad mesonephros; BL-CFC, blast colony-forming cell; BMP, bone morphogenetic protein; ENU ethylnitrosourea; ES cell, embryonic stem cell; Gdf, growth and differentiation factor; hpf, hours post-fertilisation; ICM, intermediate cell mass; TGFβ, transforming growth factor β; RD, runt domain; SST, signal sequence trap.

***Address correspondence to:** Dr. Phil. S. Crosier. Division of Molecular Medicine, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag 92019, Auckland, New Zealand. Fax: +64-9-373-7010. e-mail: ps.crosier@auckland.ac.nz

0214-6282/2002/$25.00

Printed in Spain
www.ijdb.ehu.es

derm is patterned across the dorso-ventral axis during gastrulation, followed by specification of ventral mesoderm to a hematopoietic fate (reviewed in Davidson and Zon, 2000). During gastrulation, the marginal zone gives rise to cells or tissues as varied as notochord, somites, pronephros, and blood. The mesoderm is patterned as a result of complex antagonistic interactions between regulators that promote ventral fates and molecules secreted by the Spemann organizer, or dorsal mesoderm, that induce dorsal fates. A substantial amount of data points toward members of the TGF-β family playing a role in this process with, for example, a gradient of bone morphogenetic proteins (BMPs) being established by the differential action of BMP antagonists such as cerebrus, chordin, follistatin and noggin (De Robertis *et al.*, 2000). In zebrafish, it is thought that signaling molecules from the yolk cell syncytial layer induce adjacent cells in the marginal zone to adopt a mesodermal fate (Solnica-Krezel, 1999).

Studies of the blood island yolk sac led to recognition of the close spatial and temporal association between the developing hematopoietic and endothelial systems. The concept then arose of a common precursor cell, called the hemangioblast, which derives from ventral mesoderm and has two fates; namely hematopoietic stem cells and angioblasts (Haar and Ackerman, 1971; Sabin, 1920). While the isolation and characterization of the hemangioblast has been difficult, there is an increasing amount of evidence for the existence of such a cell. There are a number of molecules, including cell surface receptors and transcription factors, that are common to the hematopoietic and endothelial lineages. These include CD34 (Young *et al.*, 1995), Flk1 (Millauer *et al.*, 1993), Flt-1 (Fong *et al.*, 1999), GATA-2 (Orkin, 1995), PECAM-1 (Watt *et al.*, 1995), Tie2 (Takakura *et al.*, 2000) and SCL (Kallianpur *et al.*, 1994). It has been demonstrated that cells of both the hematopoietic and endothelial lineages can be cultured from differentiated mouse embryonic stem (ES) cells via a common, transient progenitor (Choi *et al.*, 1998). This work demonstrated that blast cell colonies (BL-CFC) contained primitive and definitive hematopoietic precursors as well as endothelial cells.

The targeted disruption of genes in mice has demonstrated that several genes are essential for both hematopoietic and endothelial development. These include Flk-1 (Shalaby *et al.*, 1995), TGFβ1 (Dickson *et al.*, 1995) and SCL (Robb *et al.*, 1995; Shivdasani *et al.*, 1995; Visvader *et al.*, 1998). In zebrafish, the spontaneous mutant *cloche* has defective blood and endothelial development, with abnormalities in endocardial structure (Stainier *et al.*, 1995). The expression of several blood markers including *scl*, *lmo2*, and *gata-1*, as well as the vascular markers *flk1*, *fli1* and *tie1*, are all severely reduced or absent in *cloche* embryos (Gering *et al.*, 1998; Liao *et al.*, 1998). Injection of *scl* is able to partially rescue the *cloche* phenotype, providing evidence that Scl acts downstream of *cloche* (Liao *et al.*, 1998). It is likely that the product of the *cloche* gene plays a role in hemangioblast development.

During vertebrate development, there are overlapping waves of hematopoiesis that originate from distinct anatomical sites. In many vertebrates, regions of the ventral mesoderm migrate onto the embryonic yolk sac and form blood islands that give rise to both primitive erythroid and primitive myeloid hematopoietic programs (Cumano and Godin, 2001; Orkin, 2000). In mammals, a transition occurs from primitive yolk sac hematopoiesis to a multilineage hematopoietic programme in the fetal liver that gives rise to definitive erythroid, myeloid and lymphoid cells (Cumano and Godin, 2001; Orkin, 2000).

Despite recognition that the yolk sac represents the first site of both hematopoietic and endothelial development in the mammalian embryo, the precise nature of the genetic pathways that regulate the commitment of mesoderm to these fates remains poorly understood. Studies in mice and zebrafish have pointed to a role for Scl in the development of a putative hemangioblast. Targeted disruption of *Scl* has shown that it is required for the development of both primitive and definitive hematopoiesis, and has a role in remodelling the yolk sac vascular plexus (Porcher *et al.*, 1996; Robb *et al.*, 1995; Shivdasani *et al.*, 1995; Visvader *et al.*, 1998). Replating studies with *Scl* $^{-/-}$ ES cells have shown that embryoid bodies derived from these cultures do not develop bilineage blast colonies. Instead they form a transitional colony that contains mesodermal cells together with hematopoietic and vascular precursors. They appear to represent a developmental step, intermediate between ventral mesoderm and the hematopoietic/endothelial lineages (Choi *et al.*, 1998; Robertson *et al.*, 2000). An interpretation of this work is that the transitional cell represents a stage of hemangioblast development that requires SCL for further maturation. In zebrafish, overexpression of *scl* results in expansion of cells that express hematopoietic and vascular markers (Gering *et al.*, 1998). These data together with that which shows partial rescue of the *cloche* hematopoietic and vascular defects by *scl* (Liao *et al.*, 1998) supports a role for Scl in hemangioblast function.

Primitive hematopoiesis in the zebrafish occurs within the embryo, in a region located between the notochord and endoderm of the trunk called the intermediate cell mass (ICM). It also develops anteriorly in the paraxial mesoderm over the yolk cell and posteriorly in a small ventral cluster of cells in the developing tail referred to as the posterior blood island (Al-Adhami and Kunz, 1977; Detrich *et al.*, 1995; Thompson *et al.*, 1998). The earliest expression of hematopoietic genes, including *gata-2* and *scl*, occurs in bilateral stripes within the lateral plate mesoderm at approximately the 3 somite stage or 11 hours post-fertilisation (hpf). *Gata-1* expression appears slightly later at the 5 somite stage and by 12 somites βE_3-*globin* expression commences. At the 18 somite stage (around 18 hpf), the bilateral stripes have fused anteriorly and by 23 hpf, they have converged to form the ICM (reviewed in Amatruda and Zon, 1999). The ICM is intraembryonic and is functionally equivalent to the extraembryonic yolk sac blood islands of higher vertebrates. The embryonic hematopoietic system undergoes further development when cells from the ICM migrate anteriorly prior to the onset of circulation. It has been proposed that these cells populate the dorsal mesentery and the ventral wall of the dorsal aorta (Detrich *et al.*, 1995). It has been further suggested that the ICM forms two developmentally distinct compartments, where cells from the anterior ICM exit onto the yolk and establish the primitive circulation, and cells from the posterior ICM enter the circulation later (Amatruda and Zon, 1999; Thompson *et al.*, 1998). A number of gene expression domains potentially demarcate these regions where, for example, *gata-2* and *scl* expression, but not *gata-1*, extend ventrally at 24 hpf (Amatruda and Zon, 1999; Detrich *et al.*, 1995; Thompson *et al.*, 1998). By using techniques such as lineage tracing, the sites and timing of the establishment of zebrafish hematopoiesis and the nature of ICM sub-compartmentalization will be elucidated. In 48 hpf embryos, cells that express c-*myb* are found scattered along the ventral wall of the dorsal aorta; an expression pattern analogous to that observed in the progenitors of definitive hematopoiesis within the aorta-gonad-mesonephros (AGM) region of mammals (Thompson

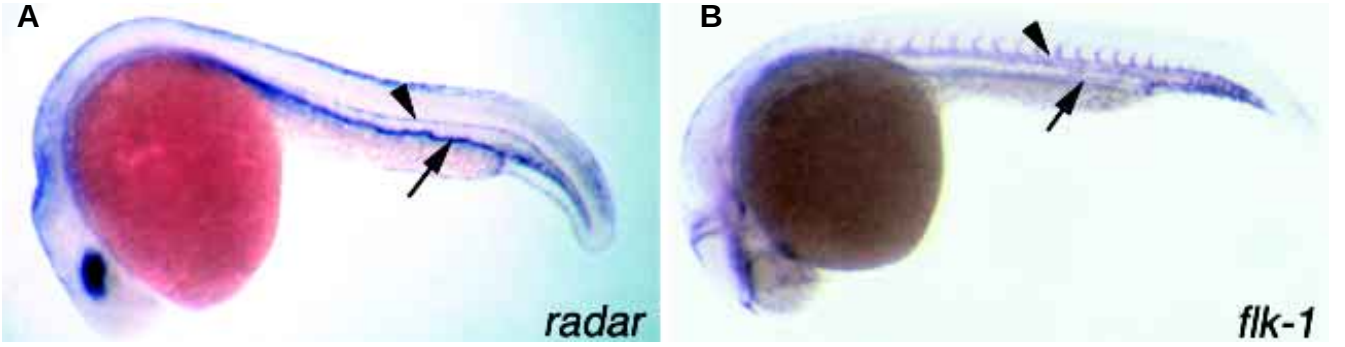

Fig. 1. *Gdf6a/radar* is expressed in domains that flank the developing axial vasculature. *Whole mount* in situ *hybridizations were performed with* gdf6a/radar *and* flk-1 *RNA probes. Lateral views, anterior to left.* **(A)** *Expression of* gdf6a/radar *in wild type, 24 hpf embryo. Arrow, hypochord; arrowhead, primitive gut endoderm.* **(B)** *Expression of the endothelial cell receptor tyrosine kinase* flk-1 *in wild type, 24 hpf embryo. Arrow, posterior cardinal vein; arrowhead, dorsal aorta.*

et al., 1998). These cells may represent the first definitive hematopoietic precursors in zebrafish. Later on, the site of definitive hematopoiesis shifts to the kidney, although some hematopoietic activity may occur in the spleen (Amatruda and Zon, 1999).

This review describes our application of both forward and reverse genetics in zebrafish to obtain further understanding of molecular events involved in the development of the hematopoietic and vascular systems. We focus on a member of the BMP family of genes, *Gdf6a/radar*, that appears to be involved in vascular development and on two members of the Runx family of transcription factors that function in early hematopoiesis. We also outline our strategy for establishing the zebrafish as a valid model in which to study human leukemia.

Beyond the Horizon: Gdf6/radar and its Role in Vasculogenesis

The BMPs and growth and differentiation factors (GDFs) form the largest gene group within the TGF-β family (see review by Hogan, 1996). The BMPs are instructive molecules that function during embryonic development and in adult tissue homeostasis (Massague, 2000). There are two broad areas of interest concerning the biological function of BMPs. One centers on the mechanisms by which cell fates and axes are determined by the antagonistic interactions between BMPs and other secreted proteins, such as noggin, chordin and follistatin. The second relates to how cells interpret BMP signals. The interaction of BMPs and antagonistic molecules has often focussed on mechanisms by which a morphogen gradient is interpreted by cells. This process leads to the formation of different cell types arranged in a defined spatial distribution (Gurdon and Bourillot, 2001). With regard to BMP signaling, there have been numerous cross-talk and feedback loops described, all of which result in an activated Smad translocating from the cytoplasm to the nucleus and binding to the promoter of a specific target gene (Massague, 2000).

We have an interest in the function of members of the Gdf5, 6, 7 subgroup of BMPs (Davidson *et al.*, 1999). The mouse *Gdf5, 6*, and *7* genes form a closely related subgroup of the TGF-β family and were originally isolated from genomic DNA by a degenerate PCR approach. Homologous genes have been identified in mammals, *Xenopus* and zebrafish (Bruneau and Rosa, 1997; Chang and Hemmati-Brivanlou, 1999; Chang *et al.*, 1994; Davidson *et al.*, 1999; Rissi *et al.*, 1995; Storm and Kingsley, 1996; Wolfman *et al.*, 1997). In mammals, members of this subgroup have been implicated in cartilage and tendon formation during embryonic development. *Gdf5* was mapped to the region of mouse chromosome 2 that contains the *brachypodism* mutation (Storm *et al.*, 1994). Mutations in *Gdf5* were found to be responsible for the brachypodism phenotype, whereas mutations in the human ortholog (known as *CDMP1*), cause the phenotypically similar human disorder Hunter-Thompson type chondrodysplasia (Thomas *et al.*, 1996). Targeted disruption of the mouse *Gdf7* locus results in hydrocephalus and a defect in the development of dorsal commissural neurons (Lee *et al.*, 1998).

The zebrafish *gdf5, 6*, and *7* genes have been isolated and genetically mapped (Bruneau and Rosa, 1997; Davidson *et al.*, 1999; Rissi *et al.*, 1995). We have described the phylogenetic relationships of this gene family in zebrafish, and the expression of *gdf7* (Davidson *et al.*, 1999). There has been an ancestral duplication of the *Gdf6* gene resulting in two genes, *Gdf6a* (*radar*) and *Gdf6b* (*dynamo*). There is a complex embryonic pattern of expression of *gdf6a/radar* in the eye, neural tube, dorsal fin and hypochord (Rissi *et al.*, 1995). We have re-examined the expression pattern of this gene, focussing our attention on its expression in the primitive gut endoderm, the ventral trunk and the hypochord (Fig. 1A). These expression domains lie immediately adjacent to, and flank, the developing axial vasculature that includes the dorsal aorta and posterior cardinal vein (Fig. 1B). The pattern of expression has led us to consider whether Gdf6a/radar has a role in early hematopoiesis at the level of the hemangioblast, or whether it may function later in hematopoiesis and/or vasculogenesis.

Several studies have demonstrated that in zebrafish, the trunk, notochord, hypochord and endoderm are required for formation of the dorsal aorta and posterior cardinal vein (Fouquet *et al.*, 1997; Sumoy *et al.*, 1997). The hypochord is found in fish, lampreys and amphibians and is derived from endoderm. It is a transient, rod-like structure consisting of a row of single cells positioned ventral to the notochord, and it has been shown that signals from the notochord directly influence hypochord development (reviewed in Cleaver and Krieg, 2001). The position of the hypochord and its close association with the dorsal aorta has led to the idea that it is involved in patterning development of this vessel. A number of genes with defined vasculogenic roles, such as *VEGF* and *Ang-1*, are expressed in the hypochord (Cleaver *et al.*, 1997; Eriksson and Lofberg, 2000; Pham *et al.*, 2001).

To determine the function of Gdf6a/radar, we have adopted

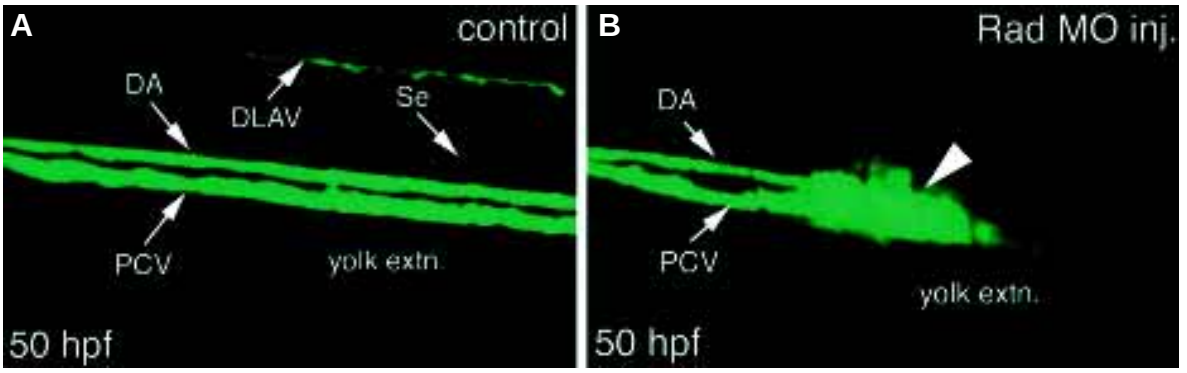

Fig. 2. Loss of vessel integrity in Gdf6a/radar-depleted embryos. *Microangiography was undertaken with fluoresceinated latex beads in 50 hpf wild type* **(A)** *and 50 hpf* gdf6a/radar *morpholino-injected embryos* **(B)**. *Lateral views, anterior to left. Leaks occurred in the trunk vasculature as indicated by the large arrowhead in B. DA, dorsal aorta; PCV, posterior cardinal vein; Se, intersegmental vessel; DLAV, dorsal longitudinal anastomotic vessel.*

several approaches. When Gdf6a/radar is overexpressed, embryos exhibit a ventralized phenotype typified by expansion of the ICM (Hall *et al.*, unpublished observations). Furthermore, a delay in the normal midline convergence of cells that express *scl* is observed, together with absence of the notochord. These features are reminiscent of those seen in the mutants *floating head* and *no tail* (Sumoy *et al.*, 1997), and raise the possibility of a chemotactic/migratory role for Gdf6a/radar during establishment of the ICM.

A further insight into Gdf6a/radar function has been obtained using antisense morpholino-modified oligonucleotides (morpholinos) to inhibit gene translation. Embryos depleted of Gdf6a/radar were phenocopies of the rdr^{D1} deletion mutant (Delot *et al.*, 1999), where areas of neuroectodermal cell degeneration occurred suggesting a role for Gdf6a/radar in maintaining the identity of the dorsal-most neural tube and possibly some neural crest cells (Hall *et al.*, unpublished observations). A striking result has been the identification of circulatory defects in the morpholino-injected embryos. Visualization of the defects was possible using microangiography, and these ranged from an absence of trunk circulation, to subtle changes within the intersegmental vessels. Fig. 2 shows an example of the vascular leakiness that was observed. Interestingly, embryos generated by morpholino-knockdown of VEGF-A (Nasevicius *et al.*, 2000), show many features in common with the GDF6a/radar-depleted embryos, raising the possibility of an intersecting role for these molecules in axial vessel formation. We hypothesize that GDF6a/radar functions in the maintenance of vessel integrity, rather than in the initial formation of vessels. This work raises questions as to the interplay between the GDF6a/radar and VEGF/Flk-1 signaling pathways.

A model for GDF6a/radar function in vasculogenesis is shown in Fig. 3, where signals from the hypochord and primitive gut endoderm establish vascular integrity. Interest lies in defining the signaling pathways that intersect with GDF6a/radar to augment its vascular function. It is very apparent that growth factor/cytokine signaling pathways are not insulated biological systems but are components of a dense, highly interconnected network. Candidate molecules for interaction with GDF6a/radar are the Tie2 ligand Ang-1, the Flk-1 ligand VEGF and possibly the ETS-domain transcription factor Fli-1. Studies in Ang-1 and Fli-1 knockout mice have suggested roles for these molecules in establishing vascular integrity (Sato *et al.*, 1995; Suri *et al.*, 1996). A further function for Ang-1 is its role as a survival factor for endothelial cells (Kwak *et al.*, 1999). Fli-1 is expressed in sites of zebrafish vasculogenesis, and genetic analyses of several mutants have confirmed its likely role within the vascular compartment (Brown *et al.*, 2000). Ang-1 is expressed in the hypochord, VEGF is expressed in both the hypochord and ventral somites, and Fli-1 is endodermally expressed. A recent intriguing observation, indicative of convergence within this field, is the connection between VEGF expression and Cbfa1/Runx2. It has been shown that Cbfa1/Runx2 is a necessary component of a genetic program that regulates VEGF during bone formation (Zelzer *et al.*, 2001). The expression domains of all of these genes fall within the range of GDF6a/radar signaling.

Runx1 as a Critical Regulator of Developmental Hematopoiesis/Vasculogenesis

The discovery of genes such as AML1 (RUNX1) and SCL, that are frequently rearranged in human leukemia, led to predictions that these molecules may have important functions in normal hematopoiesis (reviewed in Downing *et al.*, 2000). These predictions have spawned many investigations, using different model systems, that have placed such genes at particular sites within the hematopoietic transcriptional hierarchy. In this section we review our studies of two members of the Runx family of transcription factors, using the zebrafish as a system where early events in hematopoiesis/vasculogenesis are particularly accessible.

Runt domain (RD) proteins are a family of conserved transcription factors found in *Drosophila* (*Runt* and *Lozenge*), spiders, sea urchins, *Xenopus* (Xaml) and other vertebrates (Downing, 1999; Speck, 2001). A preferred nomenclature to describe the vertebrate genes has been adopted where they are called Runx genes. All previous names are collated as follows: RUNX1 = AML1, PEP2aB, CBFa2; RUNX2 = AML3, PEP2aA, CBFa1 and RUNX3 = AML2, PEP2aC, CBFa3. The defining feature of this family is the RD, a conserved 128 amino acid motif responsible for both sequence-specific DNA binding and for dimerization with an unrelated partner protein. This partner protein, termed core binding factor β (CBFβ), is encoded by a single gene in mammals, while the *Drosophila* genome contains at least two homologous genes, *Brother* (*Bro*) and *Big-brother* (*Bgb*) (Canon and Banerjee, 2000). The CBFβ proteins do not directly bind DNA, but modulate

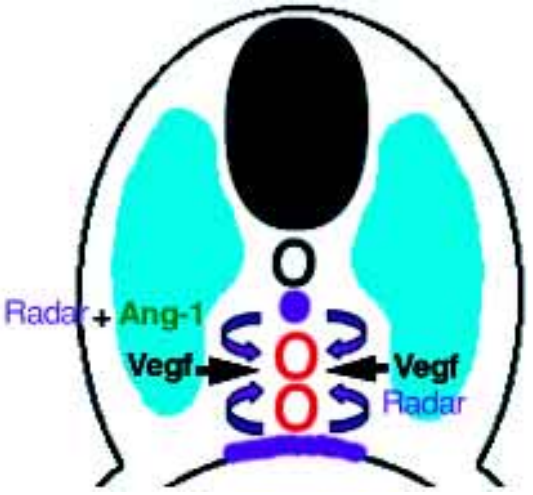

Fig. 3. A model for the role of Gdf6a/ radar signaling, *from the hypochord and primitive gut endoderm, in establishing vascular integrity. Gdf6a/radar is expressed in the hypochord and primitive gut endoderm (purple). Gdf6a/radar (purple arrows), possibly together with Vegf and Ang-1, provide signals for the maintenance of integrity of the dorsal aorta and posterior cardinal vein (red circles).*

DNA binding by RD proteins through an undescribed mechanism (Wheeler *et al.*, 2000). The mechanism of transcriptional regulation by RD proteins centers around their role as activators or repressors, with an emerging view that these functions are dependent upon the organization of a particular promoter/enhancer in a specific cell type at a certain time (Wheeler *et al.*, 2000). Evidence for a RD transcriptional activation role arose through investigation of the sex-lethal (*Sxl*) early promoter, the pair-rule gene *fushi tarazu* and, in vertebrates, the regulation of T cell receptor genes, the M-CSF receptor, myeloperoxidase, osteocalcin and murine leukemia virus enhancer cores (Wheeler *et al.*, 2000). There is evidence that Runx3 cooperatively activates BMP signaling, with Smad binding leading to transcriptional activation (Massague, 2000). The repressive effects of RD proteins are most widely seen in the regulation of a number of genes during embryogenesis, such as *engrailed*, *orthodenticle*, *hairy* and *even-skipped*, with the repressive mechanism operating in the last two genes via a co-repressor *groucho* (Canon and Banerjee, 2000; Wheeler *et al.*, 2000).

The function of the *Runx1* gene in normal hematopoietic development was first established when targeted disruption of this gene in mice caused a complete block in the establishment of definitive hematopoiesis (Okuda *et al.*, 1996; Wang *et al.*, 1998b). The mice lack definitive erythroid, myeloid and megakaryocytic cells. Yolk sac hematopoiesis was initiated, however embryos died *in utero* by 11-12.5 days of gestation. Prior to death, the fetal liver rudiment contained primitive, nucleated erythroblasts. A second developmental defect described in these mice was hemorrhaging in the central nervous system (CNS), preceded by perivascular edema and apoptosis in the CNS capillaries, suggesting a vascular defect. Recent work by Takakura *et al.* (2000) has suggested that the hemorrhagic phenotype observed in *Runx1* knockout mice is a non-cell autonomous defect in angiogenesis, caused by a lack of hematopoietic stem cells capable of secreting vascular regulators such as Ang-1.

The phenotype of *Runx1* knockout mice positions Runx1 at the base of a transcriptional hierarchy, where it is expressed at the earliest stage of the definitive hematopoietic program. By using β-galactosidase-marked Runx1, thus enabling the tracking of Runx1 expression in mouse development, it has been shown that expression first occurs at day 7.5 in the extraembryonic mesodermal cells and then transiently in primitive erythrocytes of the developing blood islands at day 8.0 (North *et al.*, 1999). At day 8.5 of gestation, Runx1 expression was observed in a small population of endothelial and hematopoietic cells dispersed throughout the yolk sac, perhaps representing definitive hematopoietic precursors. Following a further two days of gestation (day 10.5), Runx1 is expressed in the AGM where definitive hematopoietic stem cells develop. Analysis of both AGM and fetal liver cells demonstrated Runx1 expression in a population with the c-kit$^+$/CD34$^+$ cell surface phenotype, which is that of long-term repopulating stem cells.

The expression of Runx1 in developing primitive erythroblasts is interesting, given the absence of reported defects in primitive hematopoietic development in *Runx1* knockout mouse embryos. These data suggest that either Runx1 is expressed, but not essential, in primitive hematopoiesis, or that an as yet undetected defect exists in this lineage. Studies in other systems have shed some light on this issue. Forced expression of the *Xenopus* Runx1 homolog Xaml has been shown to block both primitive and definitive hematopoiesis in *Xenopus* (Tracey *et al.*, 1998). Transgenic mice expressing a chimeric protein CBFβ-MYH11 have impaired primitive erythropoiesis, as well as a block in definitive hematopoiesis (Castilla *et al.*, 1996). CBFβ-MYH11 was originally identified from a leukemic translocation and is thought to function by sequestering Runx1 in the cytoplasm. In a recent study, embryoid bodies from Runx1 $^{-/-}$ ES cells were analyzed for primitive erythroid, definitive erythroid, myeloid and blast colony-forming cell potential. Besides demonstrating a complete block in

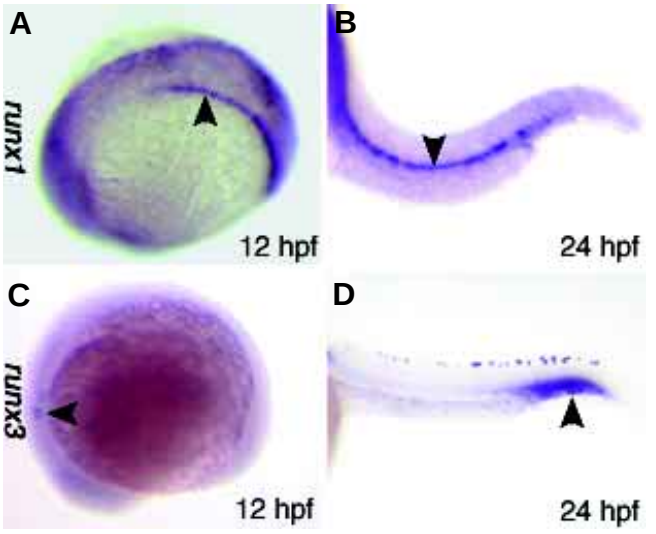

Fig. 4. Expression of *runx1* and *runx 3* in early hematopoiesis. (A) *Dorsal view of whole embryo, anterior to left, 12 hpf*. **(B,D)** *Posterior halves of embryos, lateral views, anterior to left, 24 hpf.* **(C)** *Whole embryo, anterior to left, 12 hpf. Arrowhead in A, lateral plate mesoderm; arrowhead in B, ventral wall of dorsal aorta; arrowhead in C, trigeminal ganglia; arrowhead in D, intermediate cell mass.*

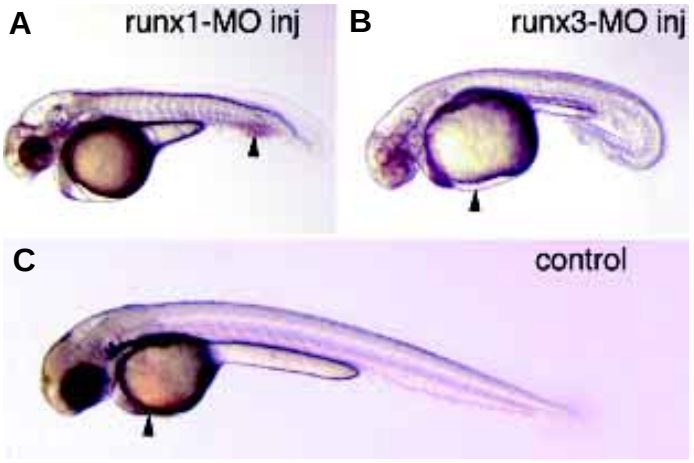

Fig. 5. *Runx1* and *runx3* morpholino-injected embryos demonstrate abnormalities in hematopoiesis. *Lateral views of* runx1 **(A)** *and* runx3 **(B)** *morpholino-injected embryos, 24 hpf, and control embryo, 24 hpf* **(C)**. *All anterior to left. Arrowhead in (A), accumulation of erythroid cells in ventral tail; arrowhead in (B) indicates marked reduction in circulating erythroid cells (compare with arrowhead in C).*

definitive hematopoiesis, the Runx1$^{-/-}$ embryoid bodies generated significantly fewer primitive erythroid precursors and BL-CFC than wild type controls (Lacaud *et al*., 2001). This work suggests that Runx1 may function at an earlier stage in the hematopoietic pathway than predicted from knockout embryo studies, perhaps at the level of the hemangioblast.

Research from our group provides further insight into the function of Runx1 within the developing hematopoietic and vascular systems. Following the isolation of zebrafish *runx1,* its spatial and temporal pattern of expression in wild type and mutant embryos has been investigated, along with definition of function using transgenic approaches and morpholino-mediated gene knockdowns (Kalev-Zylinska *et al*., 2002). Results from this work form the basis of an ethylnitrosourea (ENU) genetic screen, with mutants selected on the basis of loss of *runx1* expression as assessed by whole mount *in situ* hybridization. This is being undertaken to identify genes involved in the regulation of *runx1*.

Two zebrafish homologs of Runx genes (*runxa* and *runxb*) have been reported previously (Kataoka *et al*., 2000). The Runx1 ortholog we have isolated is identical at the amino acid level to *runxa*. Zygotic *runx1* expression commences at 12 hpf in two bilateral stripes within the lateral plate mesoderm (Fig. 4A). Runx1 is expressed robustly during development of the ICM. At 24 hpf, expression in the posterior ICM begins to diminish and a new expression domain occurs in the ventral wall of the aorta (Fig. 4B). *Runx1* expression overlaps with that of *scl* in the lateral plate mesoderm, however expression domains become increasingly distinct as development proceeds. In addition, we have shown that *runx1* partially rescues hematopoietic defects in *cloche* embryos, positioning *runx1* downstream of *cloche* in a pathway of hematopoietic development (Kalev-Zylinska *et al.,* 2002).

Runx1 also appears to be required for vascular development in zebrafish. Evidence for this comes from embryos injected with *runx1*-morpholinos. The most striking abnormality of *runx1*-depleted embryos was the lack of normal circulation at 48 hpf, with accumulation of erythroid cells in the aorta and ventral tail (Fig. 5 A,C). In parallel, *flk-1* expression was shown to be perturbed, with missing segments of intersomitic vessels and atypical vessel architecture (Kalev-Zylinska *et al.,* 2002).

A characteristic of the runx1-morpholino embryos was enlargement of the ICM region, where cells accumulating at 24 hpf were *scl*-positive with blast-like morphology. These cells remained the dominant population until 48 hpf, and no *scl*-positive cells were observed in the circulation. This suggests that loss of Runx1 results in an arrest or marked delay in blood maturation. In addition, embryos showed evidence of a block in the establishment of definitive hematopoiesis, with a marked reduction in c-*myb* expression within the dorsal aortic wall. These results provide confirmation that Runx1 function in the zebrafish largely recapitulates that observed in other vertebrates, however the gene also appears to play a role in vasculogenesis. Although not discussed here, our analysis of Runx1 function has provided insights into the role of this gene in neurological development.

The Neglected Triplet of the Family, Runx3, finds its Place

In contrast to Runx1 and Runx2, where a considerable amount of information has been obtained from a broad range of studies including gene knockouts, the function of Runx3 has been less well characterized. Evidence thus far suggests a role within the hematopoietic system and in leukemogenesis. Runx3 is expressed in hematopoietic cell lines and its expression is up-regulated in a leukemic cell line following retinoic acid-induced differentiation (Le *et al.*, 1999). Recently, evidence has been presented that supports a role for Runx3 during mouse embryogenesis (Levanon *et al*., 2001). Interaction between the TGFβ signaling molecule Smad3 and Runx3 has been demonstrated (Hanai *et al*., 1999; Zhang and Derynck, 2000). Expression of *RUNX3* in mononuclear blood cells may have prognostic value in patients with the M2 subtype AML; preservation of expression was shown to confer a more favourable outcome (Kornblau *et al*., 1997).

As part of our interest in understanding the role of Runx family genes in hematopoiesis, we isolated zebrafish *runx*3. At 12 hpf *runx3* is not expressed in hematopoietic tissues, but is observed in the developing nervous system (Fig. 4C). This contrasts with *runx1* expression that is present at this stage in the lateral plate mesoderm. At 24 hpf, *runx3* expression is positioned in the anterior and posterior ICM (Fig. 4D). As with *runx1*, expression of *runx3* is reduced in *cloche* embryos, placing the gene in a pathway downstream of *cloche* (Kalev-Zylinska *et al.,* unpublished). When the function of Runx3 is abrogated by morpholino oligonucleotides, embryos demonstrate a marked reduction in circulating blood cells (Fig. 5 B,C). This phenotype has been quantitated using video-assisted image capture to count circulating blood cells on the yolk. Analysis of c-*myb* expression at 48 hpf revealed a reduction in this marker of definitive hematopoiesis in the dorsal aorta. Expression of the *flk-1* vascular marker was normal in these embryos. Our interpretation of this work is that Runx3 is required for the maintenance of early blood cell numbers and for definitive hematopoiesis, but not for establishment of the vasculature (Kalev-Zylinska, unpublished). These studies have also provided insight into the role of Runx3 in neurological development, and raised interesting questions regarding the convergent regulation of hematopoiesis and neuropoiesis.

Zebrafish permit Further Dissection of Hematopoiesis and Leukemogenesis

With the completion of the human genome sequence, and the current efforts to sequence the zebrafish genome, one of many challenges is to develop approaches that systematically determine gene function. By adopting phenotype-driven mutagenesis screens, large numbers of mutant phenotypes can be screened to assess gene function without making any prior assumptions regarding the genes that constitute a given pathway. Mutagenesis screens represent a powerful approach to identify novel genes and pathways.

Previous large scale mutagenesis screens in zebrafish have produced a sizeable number of mutants, many of which are currently being genetically mapped. Approximately 26 zebrafish mutants have been described that affect hematopoiesis. These mutants were recognized on the basis of anemia, and as the genes underpinning these mutants have been isolated, their function has been predominantly in erythropoiesis (Amatruda and Zon, 1999). The mutant genes have been cloned by a combination of positional and candidate strategies. These include heme biosynthetic enzymes (Brownlie *et al.*, 1998; Wang *et al.*, 1998a; Childs *et al.*, 2000), a structural protein (Liao *et al.*, 2000) and a novel iron transporter (Donovan *et al.*, 2000). There are few mutants available that affect early events in hematopoietic stem cell development. The *runx1* screen that we are undertaking has potential to fill this gap.

Zebrafish mutants provide models for human diseases, and will be important in developing an understanding of the pathophysiology of disease. The examples above provide validation that forward genetic approaches result in mutants that resemble human hematopoietic disease. The same principles are being applied for diseases occurring in other organ or tissue systems including heart, CNS, kidney, pancreas and muscle. In addition, these approaches are being applied to gain insight into behavioural abnormalities.

An approach we have developed for the exploitation of zebrafish as a model of human disease is to express genes with known pathophysiological consequences in zebrafish embryos and monitor the resulting cellular and genetic outcome. The RUNX1 gene was first isolated from the chromosome 21 breakpoint in t(8,21)(q22;q22) acute myeloid leukemia (AML; Miyoshi *et al.*, 1991). Approximately 40% of patients with the M2 subtype of AML have this translocation, which results in the formation of a chimeric protein known as RUNX1-CBF2T1 (formerly AML1-ETO). Other translocations and point mutations have been described that involve RUNX1 (Downing, 1999). Studies in mice have shown that expression of a RUNX1-CBF2T1 fusion protein during embryogenesis causes embryonic lethality; probably by dominant interference with normal RUNX1 function (Okuda *et al.*, 1998; Yergeau *et al.*, 1997).

We have expressed a human RUNX1-CBF2T1 transgene in zebrafish embryos (Kalev-Zylinska *et al.*, 2002). This has resulted in two major abnormalities: the defective development of blood and circulation, and internal hemorrhages. RUNX1-CBF2T1-injected embryos lacked a normal circulation and accumulated blood cells in the aorta and ventral tail (Fig. 6 A,B). Numbers of blood cells were reduced in the injected embryos and overall the defects were remarkably similar to the *runx1* morpholino-injected embryos shown in Fig. 5A. Hemorrhages were found in the pericardium and CNS, where areas of intracerebral and intraventricular bleeding were observed. Cells that accumulated in the ventral tail of RUNX1-CBF2T1-injected embryos had a blast-like morphology, with dysplastic features (Fig. 6 C,D). This work has demonstrated that the zebrafish is likely to be a valuable alternative model for studies of t(8,21)-mediated leukemogenesis. We intend taking a genetic approach towards identifying molecules that interact with RUNX1-CBF2T1 to progress leukemia. To move in this direction, we are developing a zebrafish line with an inducible RUNX1-CBF2T1 transgene.

Lessons from Zebrafish

Since adopting the zebrafish system in the mid 1990s, we have undertaken both forward and reverse genetic approaches to achieve our goal of understanding the molecular events involved in the regulation of developmental hematopoiesis and vasculogenesis. In addition to the work described here, we have undertaken a search for cDNAs encoding secreted molecules using a signal sequence trap (SST) screen. This was based on analysis of patterns of expression of SST clones using whole mount *in situ* hybridization and has resulted in the isolation of a range of novel and known molecules, a number of which have been genetically mapped (Crosier *et al.*, 2001). Some of these genes have been investigated in more detail; for example, a member of the cadherin family and its role in zebrafish pronephric development has been described (Horsfield *et al.*, 2002). In other research, we have isolated zebrafish orthologues of a range of known mammalian BMPs including BMP 9, 10 and 11 and have investigated their function (Bland *et al.,* unpublished). A continued interest in protein tyrosine kinases in development has led to the isolation, genetic mapping and partial functional characterization of zebrafish

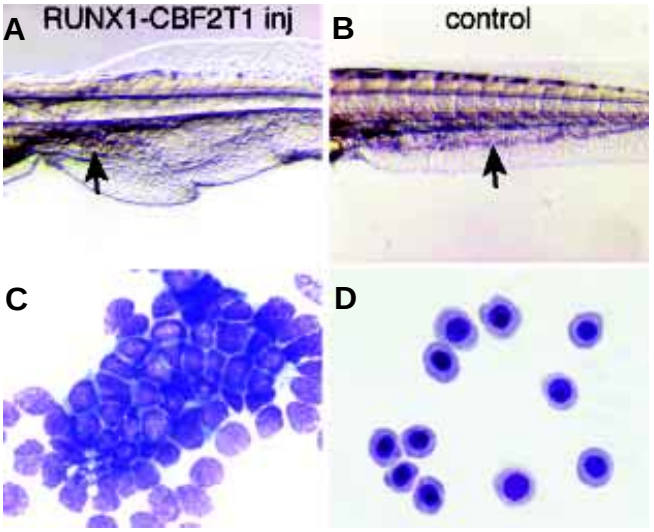

Fig. 6. Expression of a human *RUNX1-CBF2T1* transgene causes disordered hematopoiesis. *Lateral views of tail region, at 48 hpf of RUNX1-CBF2T1-injected* **(A)** *and control* **(B)** *embryos. Anterior to left. Arrow in A, entrapped cells; arrow in B, normal caudal circulation.* **(C)** *Cells aspirated from ICM of RUNX1-CBF2T1-injected embryo at 48 hpf show blast-like morphology.* **(D)** *Normal circulating cells at 48 hpf.*

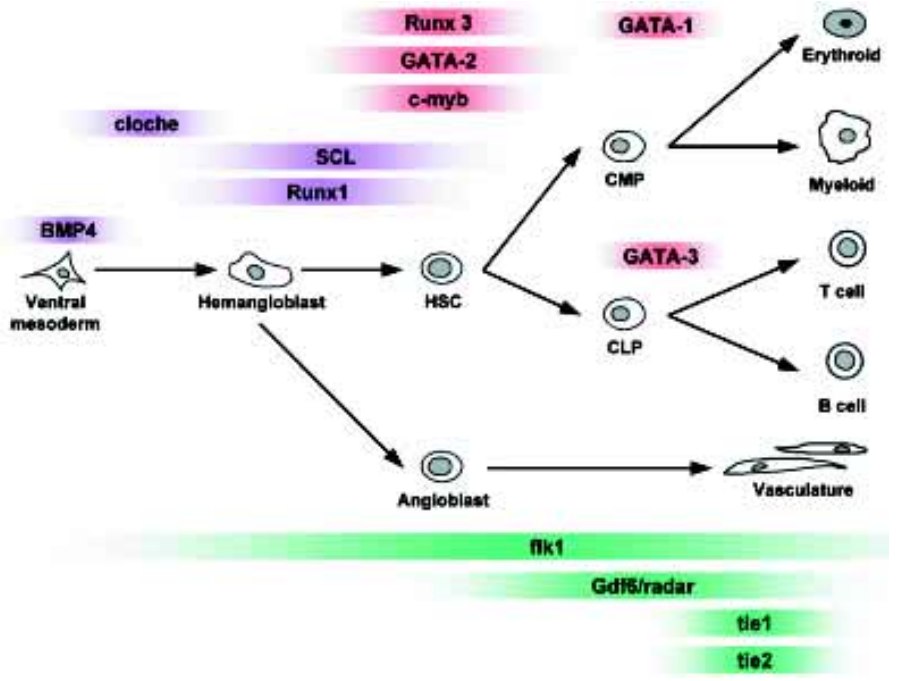

Fig. 7. Development of the hematopoietic and vascular hierachy. *Blood and vessel formation is initiated from ventral mesoderm and the hemangioblast. The hemangioblast gives rise to both angioblasts and hematopoietic stem cells (HSC). Angioblasts, along with vessel and smooth muscle progenitors (not shown), give rise to vessels. Blood cells (erythroid and myeloid) and immune cells (T cells and B cells) originate from the common myeloid (CMP) and lymphoid (CLP) progenitors respectively. The sites of action of transcription factors and growth factors/receptors that act within the zebrafish hematopoietic and vasculogenic program are depicted relative to the* cloche *mutant; hematopoietic (red), vasculogenic (green), or both programs (purple).*

orthologs for 7 of the 9 known members of the src-like protein kinase gene family (Mead *et al.,* unpublished).

We present a model to indicate where the genes discussed in this review might be positioned in a developmental hematopoietic hierarchy (Fig. 7). This forms the basis of several hypotheses being tested in our research. Following the formation of ventral mesoderm and its commitment to a blood and vascular fate, the product of the *cloche* gene most likely operates at the level of the early hemangioblast. Evidence suggests that Runx1 is expressed in the hemangioblast, and will provide a useful marker for this cell or cell cluster. Runx1 and Scl expression domains overlap early in the zebrafish lateral plate mesoderm, and although cells persist in the ICM that express both genes, the fields of expression diverge. Runx1 may be involved as part of a switch that distinguishes the primitive and definitive programs that emerge from the hemangioblast. The function of Runx 3 appears to be required later than that of Runx1 and may be part of this switch, operating to maintain levels of primitive hematopoietic cells. Further analysis of the interplay between these two RD proteins will be interesting. Gdf6a/radar is expressed in regions of early hematopoietic/vascular development, however our studies suggest that in this environment, the requirement for molecular function occurs after vessels have been established.

Working in the zebrafish system has led us to the following observations. First, the zebrafish does provide valuable additional information regarding the function of genes that have been investigated in other systems. The work with Runx1 and Runx3 convince us of this. Working in complementary model systems is a powerful approach to unlocking the secrets of stem cell biology. Second, the expression domains of Runx genes and the phenotypes resultant from abrogation of Runx gene function indicate that there are remarkable levels of similarity between the development of the hematopoietic and nervous systems. Third, models of human disease can be developed in zebrafish using, not only the analysis of mutants resulting from ENU screens, but by the expression of heterologous disease-associated genes. Finally, despite several hundred million years of evolutionary separation, the genes responsible for the teleost and mammalian hematopoietic programs are highly conserved. Humans are, after all, simply fish with limbs and lungs, …and bone marrow.

Acknowledgements

Our laboratory has been, or is currently, supported by grants from the Health Research Council of New Zealand, Cancer Society of New Zealand, Leukaemia and Blood Foundation of New Zealand, Auckland Medical Research Foundation, Lottery Health, and by Genetics Institute/Wyeth-Ayerst Research.

Work in the group has been enhanced by input from current staff including Peter Cattin, Latifa Khan, Scott Mead, Jackie Chau, Vicky Tsang and Latiful Khan. Former PhD students Jennifer Walshe, Paula Lewis (Biological Laboratories, Harvard University), Alan Davidson (The Children's Hospital, Boston), Heather Rooke (The Children's Hospital, Boston), Ross Bland (Thomas Jefferson University, Philadelphia) and former research fellows Anne Bardsley-Elliot, Marta Jansa Perez and Jeff Greenwood also made important contributions. The work has involved valued collaborations with Tony Celeste, Ashley Dunn, Ed Lavallie, John McCoy, John Postlethwait, Lisa Racie-Collins and Len Zon. Many people in the zebrafish community who have provided reagents and advice. Kristina Maconaghie assisted greatly in preparing this manuscript.

References

AL-ADHAMI, M.A. and KUNZ, Y.W. (1977). Ontogenesis of haematopoietic sites in Brachydanio rerio (Hamilton-Buchanan) (Teleostei). *Dev Growth Differ* 19: 171-179.

AMATRUDA, J.F. and ZON, L.I. (1999). Dissecting hematopoiesis and disease using the zebrafish. *Dev Biol* 216: 1-15.

BROWN, L.A., RODAWAY, A.R., SCHILLING, T.F., JOWETT, T., INGHAM, P.W., PATIENT, R.K. and SHARROCKS, A.D. (2000). Insights into early vasculogenesis revealed by expression of the ETS-domain transcription factor Fli-1 in wild-type and mutant zebrafish embryos. *Mech Dev* 90: 237-252.

BROWNLIE, A., DONOVAN, A., PRATT, S.J., PAW, B.H., OATES, A.C., BRUGNARA, C., WITKOWSKA, H.E., SASSA, S. and ZON, L.I. (1998). Positional cloning of the zebrafish sauternes gene: a model for congenital sideroblastic anaemia. *Nat Genet* 20: 244-250.

BRUNEAU, S. and ROSA, F.M. (1997). Dynamo, a new zebrafish DVR member of the TGF-β superfamily is expressed in the posterior neural tube and is up-regulated by Sonic hedgehog. *Mech Dev* 61: 199-212.

CANON, J. and BANERJEE, U. (2000). Runt and Lozenge function in Drosophila development. *Semin Cell Dev Biol* 11: 327-336.

CASTILLA, L.H., WIJMENGA, C., WANG, Q., STACY, T., SPECK, N.A., ECKHAUS, M., MARIN-PADILLA, M., COLLINS, F.S., WYNSHAW-BORIS, A. and LIU, P.P. (1996). Failure of embryonic hematopoiesis and lethal hemorrhages in mouse embryos heterozygous for a knocked-in leukemia gene CBFB-MYH11. *Cell* 87: 687-696.

CHANG, C. and HEMMATI-BRIVANLOU, A. (1999). Xenopus GDF6, a new antagonist of noggin and a partner of BMPs. *Development* 126: 3347-3357.

CHANG, S.C., HOANG, B., THOMAS, J.T., VUKICEVIC, S., LUYTEN, F.P., RYBA, N.J., KOZAK, C.A., REDDI, A.H. and MOOS, M., JR. (1994). Cartilage-derived morphogenetic proteins. New members of the transforming growth factor-β superfamily predominantly expressed in long bones during human embryonic development. *J Biol Chem* 269: 28227-28234.

CHILDS, S., WEINSTEIN, B.M., MOHIDEEN, M.A., DONOHUE, S., BONKOVSKY, H. and FISHMAN, M.C. (2000). Zebrafish dracula encodes ferrochelatase and its mutation provides a model for erythropoietic protoporphyria. *Curr Biol* 10: 1001-1004.

CHOI, K., KENNEDY, M., KAZAROV, A., PAPADIMITRIOU, J.C. and KELLER, G. (1998). A common precursor for hematopoietic and endothelial cells. *Development* 125: 725-732.

CLEAVER, O. and KRIEG, P.A. (2001). Notochord patterning of the endoderm. *Dev Biol* 234: 1-12.

CLEAVER, O., TONISSEN, K.F., SAHA, M.S. and KRIEG, P.A. (1997). Neovascularization of the Xenopus embryo. *Dev Dyn* 210: 66-77.

CROSIER, P.S., BARDSLEY, A., HORSFIELD, J.A., KRASSOWSKA, A.K., LAVALLIE, E.R., COLLINS-RACIE, L.A., POSTLETHWAIT, J.H., YAN, Y.L., MCCOY, J.M. and CROSIER, K.E. (2001). An in situ hybridization screen in zebrafish for the selection of genes encoding secreted proteins. *Dev. Dyn.* 222: 637-644.

CUMANO, A. and GODIN, I. (2001). Pluripotent hematopoietic stem cell development during embryogenesis. *Curr Opin Immunol* 13: 166-171.

DAVIDSON, A.J., POSTLETHWAIT, J.H., YAN, Y.L., BEIER, D.R., VAN DOREN, C., FOERNZLER, D., CELESTE, A.J., CROSIER, K.E. and CROSIER, P.S. (1999). Isolation of zebrafish gdf7 and comparative genetic mapping of genes belonging to the growth/differentiation factor 5, 6, 7 subgroup of the TGF-β superfamily. *Genome Res* 9: 121-129.

DAVIDSON, A.J. and ZON, L.I. (2000). Turning mesoderm into blood: the formation of hematopoietic stem cells during embryogenesis. *Curr Top Dev Biol* 50: 45-60.

DE ROBERTIS, E.M., LARRAIN, J., OELGESCHLAGER, M. and WESSELY, O. (2000). The establishment of Spemann's organizer and patterning of the vertebrate embryo. *Nat Rev Genet* 1: 171-181.

DELOT, E., KATAOKA, H., GOUTEL, C., YAN, Y.L., POSTLETHWAIT, J., WITTBRODT, J. and ROSA, F.M. (1999). The BMP-related protein radar: a maintenance factor for dorsal neuroectoderm cells? *Mech Dev* 85: 15-25.

DETRICH, H.W., 3RD, KIERAN, M.W., CHAN, F.Y., BARONE, L.M., YEE, K., RUNDSTADLER, J.A., PRATT, S., RANSOM, D. and ZON, L.I. (1995). Intraembryonic hematopoietic cell migration during vertebrate development. *Proc Natl Acad Sci USA* 92: 10713-10717.

DICKSON, M.C., MARTIN, J.S., COUSINS, F.M., KULKARNI, A.B., KARLSSON, S. and AKHURST, R.J. (1995). Defective haematopoiesis and vasculogenesis in transforming growth factor-β 1 knock out mice. *Development* 121: 1845-1854.

DONOVAN, A., BROWNLIE, A., ZHOU, Y., SHEPARD, J., PRATT, S.J., MOYNIHAN, J., PAW, B.H., DREJER, A., BARUT, B., ZAPATA, A., LAW, T.C., BRUGNARA, C., LUX, S.E., PINKUS, G.S., PINKUS, J.L., KINGSLEY, P.D., PALIS, J., FLEMING, M.D., ANDREWS, N.C. and ZON, L.I. (2000). Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. *Nature* 403: 776-781.

DOWNING, J.R. (1999). The AML1-ETO chimaeric transcription factor in acute myeloid leukaemia: biology and clinical significance. *Br J Haematol* 106: 296-308.

DOWNING, J.R., HIGUCHI, M., LENNY, N. and YEOH, A.E. (2000). Alterations of the AML1 transcription factor in human leukemia. *Semin Cell Dev Biol* 11: 347-360.

ERIKSSON, J. and LOFBERG, J. (2000). Development of the hypochord and dorsal aorta in the zebrafish embryo (Danio rerio). *J Morphol* 244: 167-176.

FONG, G.H., ZHANG, L., BRYCE, D.M. and PENG, J. (1999). Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. *Development* 126: 3015-3025.

FOUQUET, B., WEINSTEIN, B.M., SERLUCA, F.C. and FISHMAN, M.C. (1997). Vessel patterning in the embryo of the zebrafish: guidance by notochord. *Dev Biol* 183: 37-48.

GERING, M., RODAWAY, A.R., GOTTGENS, B., PATIENT, R.K. and GREEN, A.R. (1998). The SCL gene specifies haemangioblast development from early mesoderm. *Embo J* 17: 4029-4045.

GURDON, J.B. and BOURILLOT, P.Y. (2001). Morphogen gradient interpretation. *Nature* 413: 797-803.

HAAR, J.L. and ACKERMAN, G.A. (1971). A phase and electron microscopic study of vasculogenesis and erythropoiesis in the yolk sac of the mouse. *Anat Rec* 170: 199-223.

HANAI, J., CHEN, L.F., KANNO, T., OHTANI-FUJITA, N., KIM, W.Y., GUO, W.H., IMAMURA, T., ISHIDOU, Y., FUKUCHI, M., SHI, M.J., STAVNEZER, J., KAWABATA, M., MIYAZONO, K. and ITO, Y. (1999). Interaction and functional cooperation of PEBP2/CBF with Smads. Synergistic induction of the immunoglobulin germline Calpha promoter. *J Biol Chem* 274: 31577-31582.

HOGAN, B.L. (1996). Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev* 10: 1580-1594.

HORSFIELD, J.A., RAMACHANDRAN, A., REUTER, K., LAVALLIE, E.R., COLLINS-RACIE, L.A., CROSIER, K.E. and CROSIER, P.S. Cadherin-17 is required to maintain pronephric duct integrity during zebrafish development. *Mech. Dev.* In press.

KALEV-ZYLINSKA, M.L., HORSFIELD, J.A., FLORES, M.V., POSTLETHWAIT, J.H., VITAS, M.R., BAAS, A.M., CROSIER, P.S. and CROSIER, K.E. (2002). Runx1 is required for zebrafish blood and vessel development, and expression of a human RUNX1-CBF2T1 transgene advances a model for studies of leukemogenesis. *Development* 129: 2015-2030.

KALLIANPUR, A.R., JORDAN, J.E. and BRANDT, S.J. (1994). The SCL/TAL-1 gene is expressed in progenitors of both the hematopoietic and vascular systems during embryogenesis. *Blood* 83: 1200-1208.

KATAOKA, H., OCHI, M., ENOMOTO, K. and YAMAGUCHI, A. (2000). Cloning and embryonic expression patterns of the zebrafish Runt domain genes, runxa and runxb. *Mech Dev* 98: 139-143.

KORNBLAU, S.M., LE, X.F., ESTEY, E., ANDREEFF, M., GRONER, Y. and CHANG, K.S. (1997). Expression of AML-2 protein is highly prognostic in acute myelogenous leukemia (AML) FAB-M2. *Blood* 90: 309.

KWAK, H.J., SO, J.N., LEE, S.J., KIM, I. and KOH, G.Y. (1999). Angiopoietin-1 is an apoptosis survival factor for endothelial cells. *FEBS Lett* 448: 249-253.

LACAUD, G., ROBERTSON, S., PALIS, J., KENNEDY, M. and KELLER, G. (2001). Regulation of hemangioblast development. *Ann N Y Acad Sci* 938: 96-107; discussion 108.

LE, X.F., GRONER, Y., KORNBLAU, S.M., GU, Y., HITTELMAN, W.N., LEVANON, D., MEHTA, K., ARLINGHAUS, R.B. and CHANG, K.S. (1999). Regulation of AML2/CBFA3 in hematopoietic cells through the retinoic acid receptor alpha-dependent signaling pathway. *J Biol Chem* 274: 21651-21658.

LEE, K.J., MENDELSOHN, M. and JESSELL, T.M. (1998). Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. *Genes Dev* 12: 3394-3407.

LEVANON, D., BRENNER, O., NEGREANU, V., BETTOUN, D., WOOLF, E., EILAM, R., LOTEM, J., GAT, U., OTTO, F., SPECK, N.A. and GRONER, Y. (2001). Spacial and temporal expression pattern of Runx3 (AML2) and Runx1 (AML1) indicates non-redundant functions during mouse embryogenesis. *Mechanisms of Development* in press.

LIAO, E.C., PAW, B.H., OATES, A.C., PRATT, S.J., POSTLETHWAIT, J.H. and ZON, L.I. (1998). SCL/Tal-1 transcription factor acts downstream of cloche to specify hematopoietic and vascular progenitors in zebrafish. *Genes Dev* 12: 621-626.

LIAO, E.C., PAW, B.H., PETERS, L.L., ZAPATA, A., PRATT, S.J., DO, C.P., LIESCHKE, G. and ZON, L.I. (2000). Hereditary spherocytosis in zebrafish riesling illustrates evolution of erythroid β-spectrin structure, and function in red cell morphogenesis and membrane stability. *Development* 127: 5123-5132.

MASSAGUE, J. (2000). How cells read TGF-β signals. *Nat Rev Mol Cell Biol* 1: 169-178.

MILLAUER, B., WIZIGMANN-VOOS, S., SCHNURCH, H., MARTINEZ, R., MOLLER, N.P., RISAU, W. and ULLRICH, A. (1993). High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 72: 835-846.

MIYOSHI, H., SHIMIZU, K., KOZU, T., MASEKI, N., KANEKO, Y. and OHKI, M. (1991). t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. *Proc Natl Acad Sci USA* 88: 10431-10434.

NASEVICIUS, A., LARSON, J. and EKKER, S.C. (2000). Distinct requirements for zebrafish angiogenesis revealed by a VEGF-A morphant. *Yeast* 17: 294-301.

NORTH, T., GU, T.L., STACY, T., WANG, Q., HOWARD, L., BINDER, M., MARIN-PADILLA, M. and SPECK, N.A. (1999). Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. *Development* 126: 2563-2575.

OKUDA, T., CAI, Z., YANG, S., LENNY, N., LYU, C.J., VAN DEURSEN, J.M., HARADA, H. and DOWNING, J.R. (1998). Expression of a knocked-in AML1-ETO leukemia gene inhibits the establishment of normal definitive hematopoiesis and directly generates dysplastic hematopoietic progenitors. *Blood* 91: 3134-3143.

OKUDA, T., VAN DEURSEN, J., HIEBERT, S.W., GROSVELD, G. and DOWNING, J.R. (1996). AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* 84: 321-330.

ORKIN, S.H. (1995). Hematopoiesis: how does it happen? *Curr Opin Cell Biol* 7: 870-877.

ORKIN, S.H. (2000). Diversification of haematopoietic stem cells to specific lineages. *Nat Rev Genet* 1: 57-64.

PHAM, V.N., ROMAN, B.L. and WEINSTEIN, B.M. (2001). Isolation and expression analysis of three zebrafish angiopoietin genes. *Dev Dyn* 221: 470-474.

PORCHER, C., SWAT, W., ROCKWELL, K., FUJIWARA, Y., ALT, F.W. and ORKIN, S.H. (1996). The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. *Cell* 86: 47-57.

RISSI, M., WITTBRODT, J., DELOT, E., NAEGELI, M. and ROSA, F.M. (1995). Zebrafish Radar: a new member of the TGF-β superfamily defines dorsal regions of the neural plate and the embryonic retina. *Mech Dev* 49: 223-234.

ROBB, L., LYONS, I., LI, R., HARTLEY, L., KONTGEN, F., HARVEY, R.P., METCALF, D. and BEGLEY, C.G. (1995). Absence of yolk sac hematopoiesis from mice with a targeted disruption of the scl gene. *Proc Natl Acad Sci U S A* 92: 7075-7079.

ROBERTSON, S.M., KENNEDY, M., SHANNON, J.M. and KELLER, G. (2000). A transitional stage in the commitment of mesoderm to hematopoiesis requiring the transcription factor SCL/tal-1. *Development* 127: 2447-2459.

SABIN, F.R. (1920). Studies on the origin of blood vessels and of red corpuscles as see in the living blastoderm of the chick during the second day of incubation. *Contributions to Embryology* 9: 213-262.

SATO, T.N., TOZAWA, Y., DEUTSCH, U., WOLBURG-BUCHHOLZ, K., FUJIWARA, Y., GENDRON-MAGUIRE, M., GRIDLEY, T., WOLBURG, H., RISAU, W. and QIN, Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 376: 70-74.

SHALABY, F., ROSSANT, J., YAMAGUCHI, T.P., GERTSENSTEIN, M., WU, X.F., BREITMAN, M.L. and SCHUH, A.C. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376: 62-66.

SHIVDASANI, R.A., MAYER, E.L. and ORKIN, S.H. (1995). Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature* 373: 432-434.

SOLNICA-KREZEL, L. (1999). Pattern formation in zebrafish—fruitful liaisons between embryology and genetics. *Curr Top Dev Biol* 41: 1-35.

SPECK, N.A. (2001). Core binding factor and its role in normal hematopoietic development. *Curr Opin Hematol* 8: 192-196.

STAINIER, D.Y., WEINSTEIN, B.M., DETRICH, H.W., ZON, L.I. and FISHMAN, M.C. (1995). Cloche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. *Development* 121: 3141-3150.

STORM, E.E., HUYNH, T.V., COPELAND, N.G., JENKINS, N.A., KINGSLEY, D.M. and LEE, S.J. (1994). Limb alterations in brachypodism mice due to mutations in a new member of the TGF β-superfamily. *Nature* 368: 639-643.

STORM, E.E. and KINGSLEY, D.M. (1996). Joint patterning defects caused by single and double mutations in members of the bone morphogenetic protein (BMP) family. *Development* 122: 3969-3979.

SUMOY, L., KEASEY, J.B., DITTMAN, T.D. and KIMELMAN, D. (1997). A role for notochord in axial vascular development revealed by analysis of phenotype and the expression of VEGR-2 in zebrafish flh and ntl mutant embryos. *Mech Dev* 63: 15-27.

SURI, C., JONES, P.F., PATAN, S., BARTUNKOVA, S., MAISONPIERRE, P.C., DAVIS, S., SATO, T.N. and YANCOPOULOS, G.D. (1996). Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* 87: 1171-1180.

TAKAKURA, N., WATANABE, T., SUENOBU, S., YAMADA, Y., NODA, T., ITO, Y., SATAKE, M. and SUDA, T. (2000). A role for hematopoietic stem cells in promoting angiogenesis. *Cell* 102: 199-209.

THOMAS, J.T., LIN, K., NANDEDKAR, M., CAMARGO, M., CERVENKA, J. and LUYTEN, F.P. (1996). A human chondrodysplasia due to a mutation in a TGF-β superfamily member. *Nat Genet* 12: 315-317.

THOMPSON, M.A., RANSOM, D.G., PRATT, S.J., MACLENNAN, H., KIERAN, M.W., DETRICH, H.W., VAIL, B., HUBER, T.L., PAW, B., BROWNLIE, A.J., OATES, A.C., FRITZ, A., GATES, M.A., AMORES, A., BAHARY, N., TALBOT, W.S., HER, H., BEIER, D.R., POSTLETHWAIT, J.H. and ZON, L.I. (1998). The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. *Dev Biol* 197: 248-269.

TRACEY, W.D., JR., PEPLING, M.E., HORB, M.E., THOMSEN, G.H. and GERGEN, J.P. (1998). A Xenopus homologue of aml-1 reveals unexpected patterning mechanisms leading to the formation of embryonic blood. *Development* 125: 1371-1380.

VISVADER, J.E., FUJIWARA, Y. and ORKIN, S.H. (1998). Unsuspected role for the T-cell leukemia protein SCL/tal-1 in vascular development. *Genes Dev* 12: 473-479.

WANG, H., LONG, Q., MARTY, S.D., SASSA, S. and LIN, S. (1998a). A zebrafish model for hepatoerythropoietic porphyria. *Nat Genet* 20: 239-243.

WANG, L.C., SWAT, W., FUJIWARA, Y., DAVIDSON, L., VISVADER, J., KUO, F., ALT, F.W., GILLILAND, D.G., GOLUB, T.R. and ORKIN, S.H. (1998b). The TEL/ETV6 gene is required specifically for hematopoiesis in the bone marrow. *Genes Dev* 12: 2392-2402.

WATT, S.M., GSCHMEISSNER, S.E. and BATES, P.A. (1995). PECAM-1: its expression and function as a cell adhesion molecule on hemopoietic and endothelial cells. *Leuk Lymphoma* 17: 229-244.

WHEELER, J.C., SHIGESADA, K., GERGEN, J.P. and ITO, Y. (2000). Mechanisms of transcriptional regulation by Runt domain proteins. *Semin Cell Dev Biol* 11: 369-375.

WOLFMAN, N.M., HATTERSLEY, G., COX, K., CELESTE, A.J., NELSON, R., YAMAJI, N., DUBE, J.L., DIBLASIO-SMITH, E., NOVE, J., SONG, J.J., WOZNEY, J.M. and ROSEN, V. (1997). Ectopic induction of tendon and ligament in rats by growth and differentiation factors 5, 6, and 7, members of the TGF-β gene family. *J Clin Invest* 100: 321-330.

YERGEAU, D.A., HETHERINGTON, C.J., WANG, Q., ZHANG, P., SHARPE, A.H., BINDER, M., MARIN-PADILLA, M., TENEN, D.G., SPECK, N.A. and ZHANG, D.E. (1997). Embryonic lethality and impairment of haematopoiesis in mice heterozygous for an AML1-ETO fusion gene. *Nat Genet* 15: 303-306.

YOUNG, P.E., BAUMHUETER, S. and LASKY, L.A. (1995). The sialomucin CD34 is expressed on hematopoietic cells and blood vessels during murine development. *Blood* 85: 96-105.

ZELZER, E., GLOTZER, D.J., HARTMANN, C., THOMAS, D., FUKAI, N., SOKER, S. and OLSEN, B.R. (2001). Tissue specific regulation of VEGF expression during bone development requires Cbfa1/Runx2. *Mechanisms of Development* 106: 97-106.

ZHANG, Y. and DERYNCK, R. (2000). Transcriptional regulation of the transforming growth factor-β - inducible mouse germ line Ig alpha constant region gene by functional cooperation of Smad, CREB, and AML family members. *J Biol Chem* 275: 16979-16985.