# Genetic control of hematopoietic development in *Xenopus* and zebrafish

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ABSTRACT **Blood development has been highly conserved during evolution. Hematopoietic cells in amphibian and fish embryos, as in mammalian embryos, emerge and progressively differentiate in several locations. Hematopoiesis, including of the immune system, is similar in the amphibian, *Xenopus*, to mammals and the embryos are ideal for tissue transplantation and lineage labelling experiments, which have enabled the elucidation of the distinct origins of embryonic and adult hematopoietic cells, as well as their migration pathways and organ colonisation behaviours. The zebrafish hematopoietic system is less well understood, but these embryos have recently emerged as a powerful system for both genetic analysis and imaging. In this review, we summarise our current knowledge of the cellular and genetic basis of ontogeny of the hematopoietic system in *Xenopus* and zebrafish embryos.**

KEY WORDS: *hematopoiesis, hemangioblast, transcriptional regulation, adult hematopoietic stem cell*

## Introduction

Evolutionary conservation of hematopoietic development means that amenable model organisms, such as amphibia and fish, can be exploited to elucidate mechanism. Due to oxygen diffusion from the surrounding environment, fish and frog embryos are less hypoxic and therefore less dependent on a fully functional cardio-vascular system, thus allowing the study of factors that cause embryonic arrest in more oxygen dependent embryos. *Xenopus* and zebrafish embryos are easy to culture and their large number and size allows extensive experimental manipulation. Genetic pathways can be readily studied using a variety of techniques to perturb them. Blood development in *Xenopus* is very similar to that in mammals, including a similarly developed immune system (reviewed by Du Pasquier *et al.*, 2000). In addition, *Xenopus* embryos can be used to generate fate maps and for transplantation between cytogenetically distinct individuals. Over the last decade or so, the zebrafish system has emerged as a powerful genetic model in which to study blood development. Zebrafish are well suited to saturation mutagenesis and also imaging of cell fates and movements, often using transgenic reporter lines. Studies in these two model organisms are contributing significantly to our knowledge of developmental hematopoiesis.

In order to understand the genetics of hematopoietic development, it is essential to know the cellular hierarchies. We need to identify all the intermediate cell types and determine their locations as development takes place. This knowledge can provide clues as to the signals that the cells might be exposed to, and it also enables us to determine which particular combinations of transcription factors are competing for dominance in a given nucleus at any given time.

## Hematopoietic development in *Xenopus*

### The ventral blood island (VBI)

In *Xenopus* embryos, the first hematopoietic cells (erythro-cytes) differentiate in the ventral blood island (VBI). The VBI is the ventralmost tissue in the *Xenopus* tadpole and therefore blood has been generally regarded as a ventral mesoderm derivative. In

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agreement, when incubated to the tail bud stage, only ventral marginal zone (VMZ, prospective ventral mesoderm) and not dorsal marginal zone (DMZ, prospective dorsal mesoderm) explants give rise to erythrocytes. This view has recently been challenged following the discovery of a dorsal mesoderm contribution to the VBI (Ciau-Uitz *et al.*, 2000; Lane and Smith, 1999; Tracey *et al.*, 1998; Walmsley *et al.*, 2002). The first evidence was provided by explants cultured for less time, to neurula instead of tailbud stages. At neurula stages, the DMZ rather than the VMZ expressed blood genes (Tracey *et al.*, 1998). Furthermore, injection of a lineage tracer into the marginal zone of 4-cell embryos conclusively demonstrated that the DMZ contributes to the most anterior portion of the VBI, the aVBI, whereas the VMZ contributes to the posterior portion, the pVBI (Tracey *et al.*, 1998). This finding was confirmed by fate mapping blastomeres of the 32-cell embryo which showed that the aVBI derives from the dorsal blastomeres, C1 and D1, and the pVBI derives from the ventral blastomere, D4, in regularly dividing embryos (Ciau-Uitz *et al.*, 2000; Figs. 1A, 2A).

Gene expression in the precursors of the aVBI is first detected just after gastrulation in a population of hemangioblasts located in the most anterior ventral mesoderm (Tracey *et al.*, 1998; Walmsley *et al.*, 2002; Fig. 1B). Before gastrulation, these cells are located in the BMP deficient dorsal blastopore lip. However, as gastrulation proceeds they involute to become part of the mesodermal leading edge which migrates under the roof of the blastocoel (bcl). Finally, at the end of gastrulation they meet up with the ventral mesodermal leading edge, along the way encountering BMP which activates expression of hematopoietic and endothelial genes (Kumano *et al.*, 1999; Walmsley *et al.*, 2002; Fig. 1B). The aVBI precursors maintain their hemangioblast character until the end of neurulation (Fig. 1C), after which endothelial, myeloid and erythroid populations segregate as they differentiate. This anterior, embryonic hemangioblast population gives rise to a minority of the VBI erythrocytes but to a large number of myeloid cells (Costa *et al.*, 2008; Tashiro *et al.*, 2006; Walmsley *et al.*, 2008; Fig. 1D).

The pVBI gives rise to early and late larval erythrocytes, leukocytes and a few, short term lymphoid cells (T cells) (Maeno *et al.*, 1985b; Rollins-Smith and Blair, 1990; Smith and Turpen, 1985; Turpen *et al.*, 1997). However, hematopoietic development in the pVBI differs from that in the aVBI. For example, while the aVBI derives from hemangioblast precursors, these have not yet been found for the pVBI (Walmsley *et al.*, 2002; Walmsley *et al.*, 2008). Furthermore, hematopoietic gene expression in the pVBI initiates long after that in the aVBI, a delay caused at least in part by FGF signalling (Walmsley *et al.*, 2008). Thus, genes associated with erythroid differentiation, such as globin, are expressed first in the aVBI and only later in the pVBI. Similarly, myeloid differentiation in the pVBI or explanted VMZ occurs only after myelopoiesis in the aVBI or explanted DMZ has taken place, with the aVBI generated myeloid cells patrolling the tadpole, while myeloid cells from the pVBI are not seen until the early larval stages, after initiation of heart beating (Tashiro *et al.*, 2006; Fig. 2 B-C).

The absence of a pVBI hemangioblast may suggest an interesting parallel with the mouse yolk sac, which gives rise to two
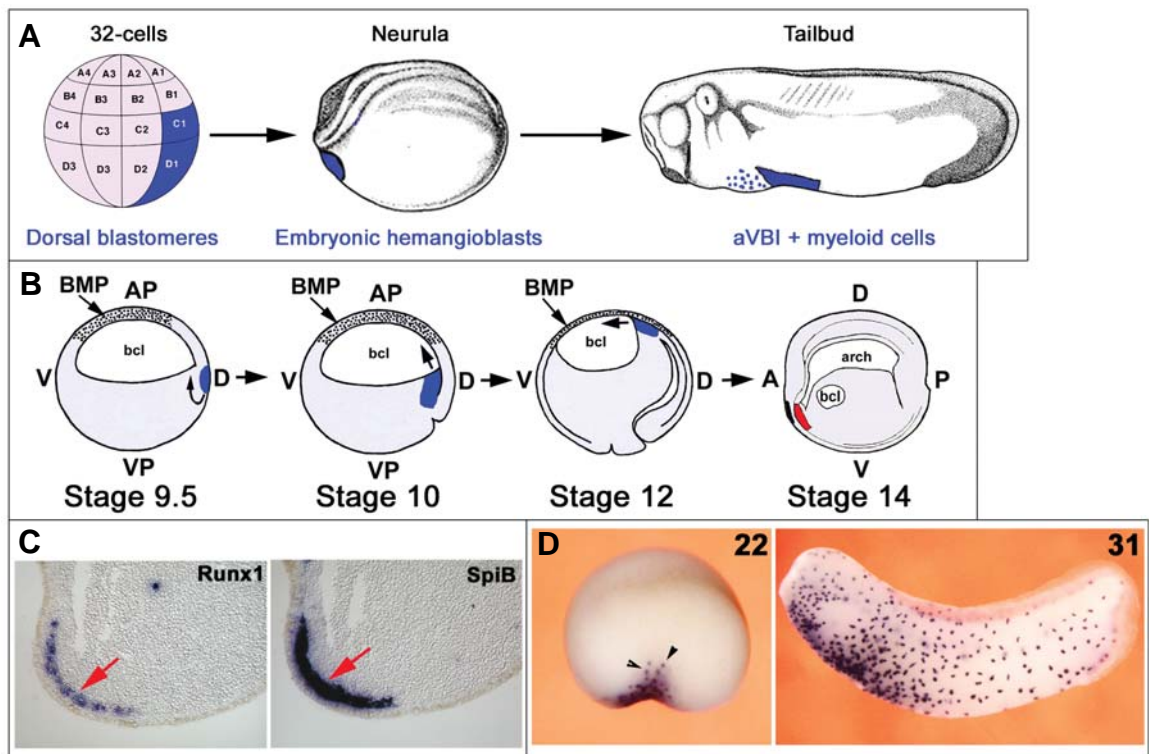


**Fig. 1**. **Ontogeny and hematopoietic potential of the anterior portion of the ventral blood island (aVBI). (A)** *The aVBI and its precursor, the embryonic hemangioblasts, derive from dorsal blastomeres of the 32-cell stage embryo.* **(B)** *Embryonic hemangioblast (red cells) are specified during gastrulation when their mesodermal precursors (blue cells) encounter BMP signalling during their migration from dorsal to ventral.* **(C)** *Erythroid (Runx1) and myeloid (SpiB) genes are co-expressed (red arrows) at the hemangioblast stage.* **(D)** *Numerous myeloid cells differentiate from the embryonic hemangioblast at tail bud stages. These myeloid cells are highly migratory and initiate migration at stage 22 (24 hpf arrowheads).*

waves of hematopoiesis, the first deriving from a hemangioblast precursor and the second from hemogenic endothelium (Goldie *et al.*, 2008; Lancrin *et al.*, 2009). The first yolk sac wave gives rise to embryonic erythrocytes and myeloid cells whereas the second wave gives rise to multipotent progenitors and embryonic erythrocytes expressing adult globin chains (Palis, 2008). By analogy, the aVBI gives rise to embryonic erythrocytes and myeloid cells whereas the pVBI gives rise to multipotent progenitors differentiating into leukocytes (myeloid and a few lymphoid) in addition to embryonic erythrocytes. Thus, the aVBI appears to be equivalent to the first wave of yolk sac hematopoiesis while the pVBI is equivalent to the second wave. Whether the pVBI multipotent progenitors derive from hemogenic endothelium is currently unknown.

### The dorsal lateral plate (DLP)

Studies in chimeric embryos resulted in the discovery of a second source of blood cells apart from the VBI in amphibian embryos, including *Xenopus* (Kau and Turpen, 1983; Maeno *et al.*, 1985a; Turpen *et al.*, 1981). This second source was narrowed down to the dorsal lateral plate mesoderm (DLP) (Maeno *et al.*, 1985b), which was found to contain adult hemangioblasts that give rise to HSCs and thereby all the hematopoietic lineages of the adult (Chen and Turpen, 1995; Kau and Turpen, 1983; Turpen and Knudson, 1982; Walmsley *et al.*, 2002). The ontogeny of adult and embryonic blood has been controversial, but a few years ago we obtained evidence in *Xenopus* that embryonic and adult blood cells have distinct origins (Ciau-Uitz *et al.*, 2000). We showed that in regularly dividing embryos, the progeny of blastomere C3 includes the DLP and the HSCs associated with the ventral wall of the dorsal aorta but, critically, not the VBI (Ciau-Uitz *et al.*, 2000; Walmsley *et al.*, 2002; Fig. 3A and Fig. 4). Similarly, blastomeres giving rise to the VBI do not contribute to the DLP.

An alternative fate map has concluded that all C and D blastomeres contribute to the VBI and that blastomeres contributing to the DLP also contribute to the VBI and vice versa (Lane and Sheets, 2002; Lane and Smith, 1999). Reasons for this discrepancy include the use of irregularly dividing embryos and non-specific biotin staining (discussed in (Walmsley *et al.*, 2005)). However, even under these less-than-ideal conditions the number of C3-derived cells found in the VBI was extremely low and likely to be within experimental error. Also, some of the analyses were carried out at late stages of development when the presence of highly migratory myeloid cells, and other migratory cells, could lead to spurious interpretation. As an example, Mills *et al.* (1999) found that at the 32-cell stage all blastomeres, including those which in other studies have been shown to exclusively give rise to ectoderm, contribute to the vasculature. This apparent disparate result could be explained by the detection of migrating neural crest cells which have been found in the vasculature and the pronephros (Collazo *et al.*, 1993). Interestingly, though, even Mills *et al.,* found contributions to the trunk dorsal aorta, where HSCs are found (see below), only from C3, in total agreement with our data.

Independent evidence that the DLP and VBI have different origins, comes from the ontogeny of the pronephric system. DLP hemangioblasts localise immediately dorsal to the pronephric anlagen and they co-express some pronephric genes (Walmsley *et al.*, 2002; Fig. 3B). Hemangioblasts of the zebrafish posterior
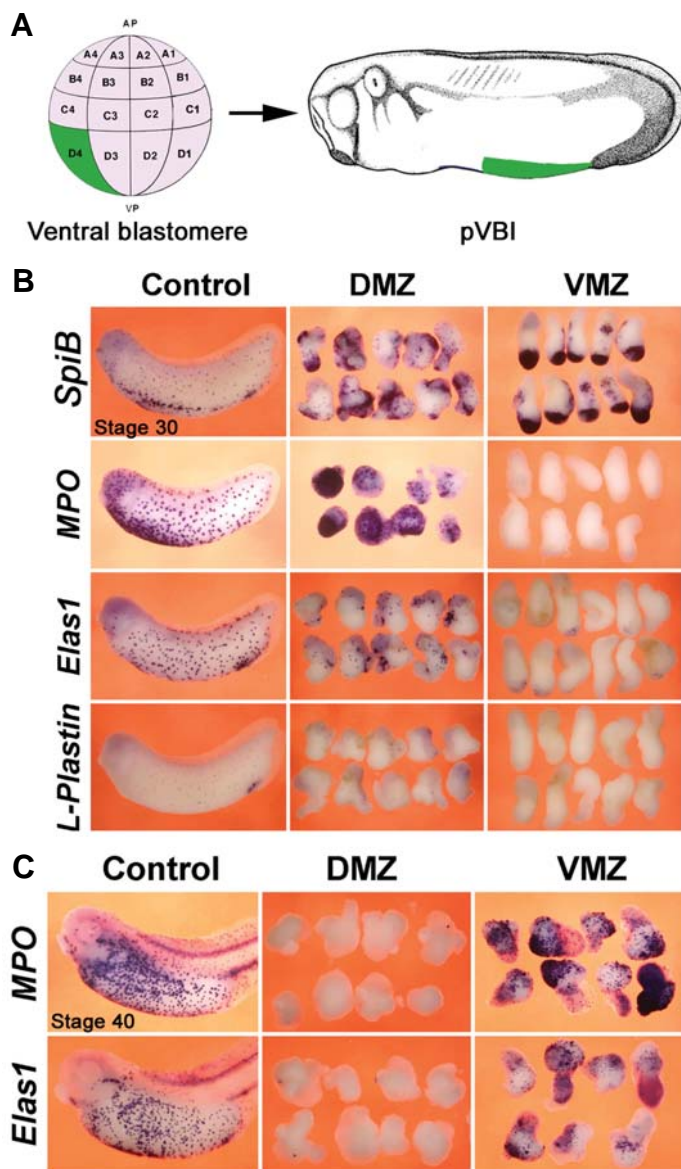
**Fig. 2**. **Ontogeny and hematopoietic potential of the posterior portion of the ventral blood island (pVBI)**. **(A)** *Ventral blastomere D4 gives rise to the pVBI.* **(B)** *At tail bud stages (stage 30, 35hpf) all differentiated myeloid cells (MPO, Elas1, L-plastin) derive from the dorsal marginal zone (DMZ) indicating their anterior hemangioblast origin. At this stage, the ventral marginal zone (VMZ) contains myeloid precursors (SpiB) but no migrating mature myeloid cells.* **(C)** *At early larval stages (stage 40, 66hpf), myeloid cells are no longer produced by the DMZ while a second wave differentiating from the VMZ becomes abundant.*

lateral plate mesoderm (PLM) are also closely associated with pronephric tissue, and a bi-potential progenitor giving rise to pronephric cells and hemangioblasts has been proposed (Gering *et al.*, 1998). Dale and Slack (1987) found that at the 32-cell stage the pronephric anlagen derive mainly from blastomeres C3, with smaller contributions from B3 and D3. Moody (1987) arrived at a similar conclusion and, in addition, pointed out that C1 and D1 do not contribute at all to pronephric tissue while D4's contribution is only small, presumably in the cloaca region. Orthotopic grafts just

before gastrulation also indicated that the pronephric precursors are distinct from VBI precursors, and placed the pronephric progenitors (DLP) about 90° from the dorsal and ventral midlines (Fig. 3C; Dale and Slack, 1987).
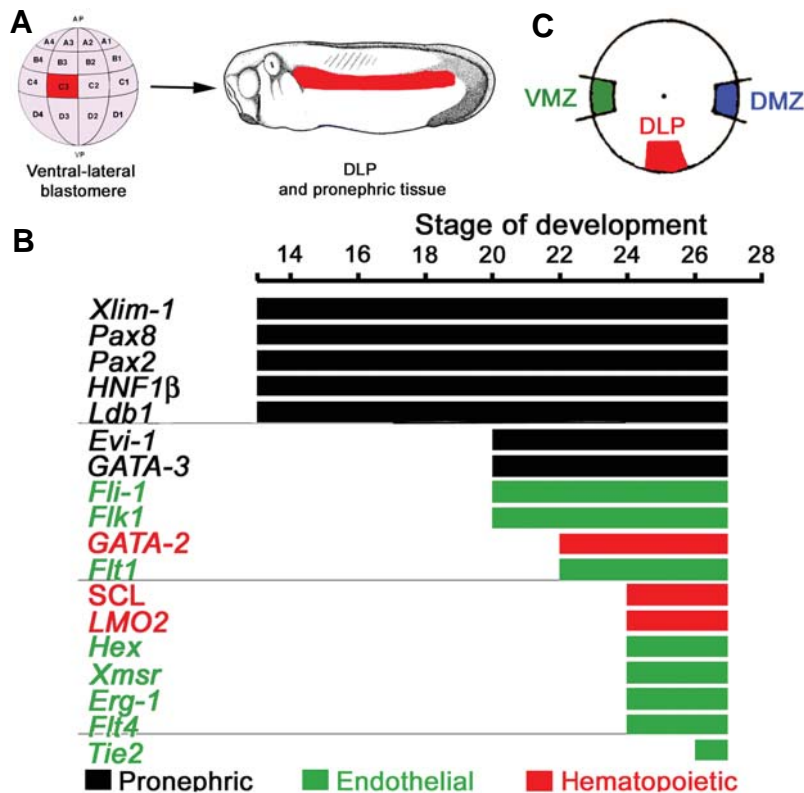
### The dorsal aorta (DA)

Hematopoietic clusters containing HSCs are observed associated with the ventral wall of the DA at stages 42- 44 (80-92hpf) (Ciau-Uitz *et al.*, 2000). Early transplantation experiments between cytogenetically distinct embryos demonstrated that hematopoietic cells adhering to the surface of both the DA and pronephric duct derived from DLP mesoderm (Turpen and Knudson, 1982). The DLP origin of the DA has recently been confirmed by Shibata *et al.* (2008), who transplanted DLPs labelled either with GFP or LacZ and found that their progeny populated the DA. Significantly, surgical elimination of the DLP results in larvae without a DA (Cleaver and Krieg, 1998). Also, the blastomere of the 32-cell stage embryo that gives rise to the DLP, namely C3, also gives rise to the DA of the trunk and associated intra-aortic hematopoietic clusters containing HSCs (Ciau-Uitz *et*

al., 2000; Walmsley *et al.*, 2002). The hypochord produces and releases diffusible isoforms of vascular endothelial growth factor (VEGF) A, which is thought to guide the migration of DA precursors from the DLP to the midline (Cleaver and Krieg, 1998). In agreement, just before the DA precursors are found coalescing ventral to the hypochord, a stream of endothelial cells are seen between the DLP and the midline (Ciau-Uitz *et al.*, 2000).

It is important to note that hematopoiesis does not take place all along the DA but is restricted to the single medial DA encompassing the trunk, starting anteriorly just posterior to the glomus (pronephros) and ending posteriorly at the level of the cloaca (Fig. 4). A 32-cell stage fate map of the *Xenopus* vasculature shows that vessels which run along the anterior-posterior axis, such as the DA, have a strong anterior-posterior map (Mills *et al.*, 1999). In other words, dorsal blastomeres give rise to the anterior portion of these vessels whereas their most posterior portion derives from ventral blastomeres. Specifically for the DA, dorsal blastomeres (A1, B1 and less significantly D1) contribute to the paired DA, which are anterior to the liver primordium and the pronephros, whereas ventral blastomeres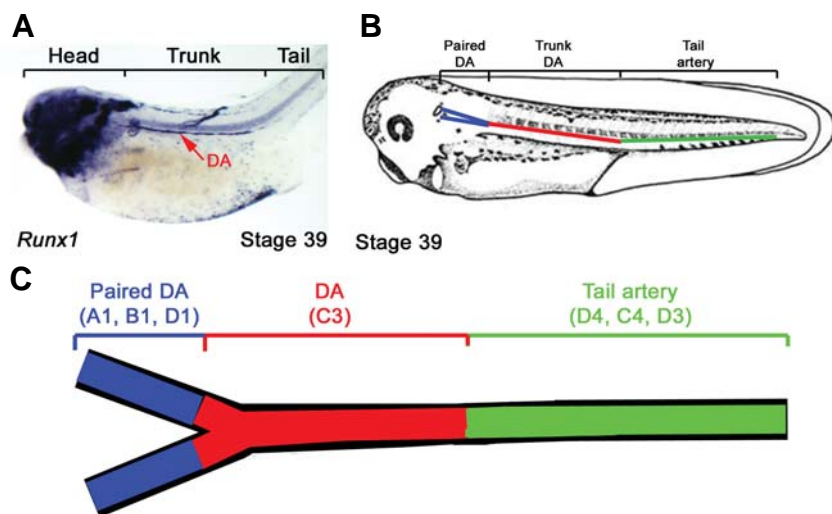 (D4, C4 and D3) contribute to the tail artery. The trunk DA, where HSCs are found, was mapped to blastomere C3 (Mills *et al.*, 1999; Fig. 4C). Significantly, none of the blastomeres giving rise to the VBI (C1, D1 and D4) (Ciau-Uitz *et al.*, 2000) contribute to the trunk DA, and C3 which gives rise to the DLP and HSCs, does not contribute to the VBI. Thus, regarding the origins of the VBI and the DA, these two fate maps are in complete agreement and conclusively show that embryonic and adult blood have distinct origins in the *Xenopus* embryo.

### Hematopoietic development in zebrafish

In recent years, the zebrafish has emerged as a powerful genetic model for vertebrate developmental biology. The isolation of numerous mutant lines, together with a range of other genetic perturbation approaches, has revealed that the molecular basis of hematopoiesis is highly conserved between teleosts and higher vertebrates, including humans (de Jong and Zon, 2005). Also, the generation of a handful of transgenic lines has facilitated the *in vivo* analysis, in real time, of developing hematopoietic cells (de Jong and Zon, 2005). As in other vertebrates, there are two major waves of hematopoiesis in zebrafish. The first wave, primitive hematopoiesis, takes place in two distinct areas of the embryo: the anterior lateral plate mesoderm (ALM) and the posterior lateral plate mesoderm (PLM, Fig. 5 A-E). At early somitogenesis stages, the ALM gives rise to a transient hemangioblast population that differentiates into myeloid and endothelial cells. In its turn, the PLM gives rise to a second population of hemangioblasts that differentiate into erythroid and endothelial cells. Red blood cells do not differentiate in the PLM, instead the erythroid derivatives of the PLM migrate to the midline to differentiate in the intermediate cell mass (Fig. 5E). Their differentiation potentials indicate that the ALM and PLM are genetically distinct, and it has been demonstrated, for example, that ALK8 signalling is essential for the ALM but dispensable for PLM specification (Hogan *et al.*, 2006). Also, specifi-



**Fig. 3**. **Ontogeny of dorsal lateral plate (DLP) hemangioblasts and their molecular make-up. (A)** *The C3 blastomere of the 32-cell stage embryo gives rise to the DLP which later generates HSCs in the ventral wall of the dorsal aorta.* **(B)** *Expression hierarchy in the DLP mesoderm. DLP hemangioblasts are located ventral to the somites and immediately dorsal to the pronephric duct and they express pronephric genes (Walmsley* et al., *2002). Pronephric genes are expressed from mid gastrula but it is not until early tail bud (Stage 20, 20hpf) that endothelial expression is seen in a subset of pronephric cells. A few hours later, endothelial expressing cells initiate hematopoietic expression to become hemangioblasts.* **(C)** *Orthotopic grafts at the beginning of gastrulation (stage 10, 11hpf) indicate that the region giving rise to the pronephros and the DLP is located 90° from both the DMZ and VMZ, illustrating the distinct origins of the VBI and DLP.*

**Fig. 4**. **Ontogeny of the dorsal aorta (DA) and hematopoietic stem cells (HSCs) in *Xenopus*. (A)** *Runx1 expression and HSC emergence is restricted to the DA encompassing the trunk.* **(B)** *The DA is anatomically subdivided into paired DA, trunk DA and tail artery. HSCs only emerge in the trunk DA.* **(C)** *Diagram summarizing the blastomere origins of the anatomical subdivision of the DA.*

cation of the ALM appears to be intimately interconnected with the development of cardiac tissue: the elimination of the ALM programme causing an expansion of the heart (Schoenebeck *et al.*, 2007).

The second wave of hematopoiesis, definitive hematopoiesis, gives rise to HSCs, which generate all blood cell types in the later embryo and throughout adult life. In the adult, the head kidney serves as the main hematopoietic organ (Willett *et al.*, 1999), and its function is equivalent to that of the bone marrow in mammals. The derivatives of the HSCs include erythroid (erythrocyte), myeloid (macrophage, neutrophil, monocyte) and lymphoid (B

and T cell). Apart from megakaryocytes and natural killer (NK) cells, all other differentiated blood lineages have been found and characterized in zebrafish embryos and/or adults, and they exhibit a high degree of conservation in both molecular and cellular features when compared to their counterparts in mammals.

Recent lineage tracing has revealed the migratory pathways of HSCs in zebrafish embryos. As in mammals, the first HSCs are found associated with the ventral wall of the DA. At least partly via the circulation, these HSCs migrate and colonize a recently discovered hematopoietic organ, the caudal hematopoietic tissue (CHT), which is believed to be functionally equivalent to the mammalian fetal liver (Murayama *et al.*, 2006). HSCs expand in the CHT and some of them differentiate into erythrocytes and myeloid cells while others further migrate to the thymus and the kidney, the main hematopoietic organ in adult fish (Jin *et al.*, 2007; Murayama *et al.*, 2006). In addition, recent work provides evidence for a novel migration route in which DA-derived CD41+cmyb+ cells colonize the zebrafish kidney by migrating along the pronephric tubules (Bertrand *et al.*, 2008). The pancreas is the site for B cell differentiation in zebrafish (Danilova and Steiner, 2002), however the origins of the precursors and their migratory routes are not known.

## Cellular intermediates and signalling

### *The hemangioblast*

During development, blood and endothelial cells develop in close proximity to each other suggesting that they derive from a shared common progenitor cell, termed the hemangioblast (Sabin, 1920). Consistent with such a notion, many hematopoietic and endothelial genes are co-expressed during development, and deletion of some of them causes both blood and endothelial defects. The first cellular evidence for hemangioblasts was provided by studies of embryonic stem (ES) cells, where individual cultured cells give rise to colonies containing both hematopoietic and endothelial cells (Choi *et al.*, 1998). Although this
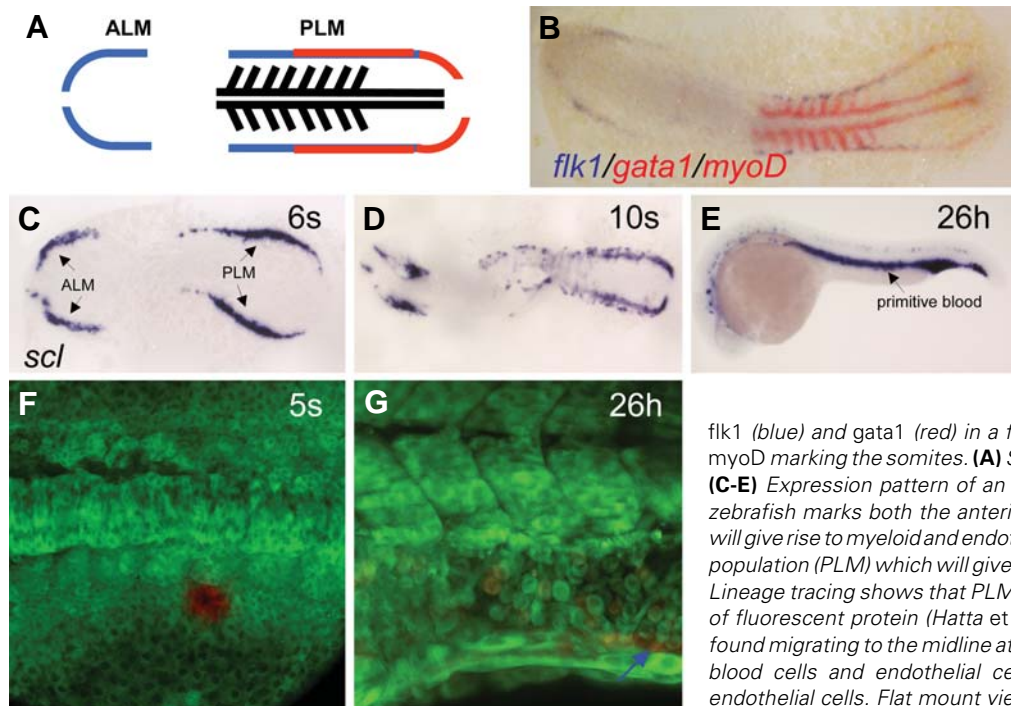


**Fig. 5**. **Origins of hemangioblast and blood/endothelial cells in zebrafish embryos. (A,B)** *Expression patterns of flk1 (blue) and gata1 (red) in a flat mounted 8-somite stage embryo, with myoD marking the somites.* **(A)** *Schematic diagram;* **(B)** *in situ hybridization.* **(C-E)** *Expression pattern of an established hemangioblast marker, scl, in zebrafish marks both the anterior hemangioblast population (ALM) which will give rise to myeloid and endothelial cells, and the posterior hemangioblast population (PLM) which will give rise to erythroid and endothelial cells.* **(F,G)** *Lineage tracing shows that PLM cells labeled at 5s* **(F)** *by photo conversion of fluorescent protein (Hatta et al., 2006) in the confocal microscope, are found migrating to the midline at 18s (not shown) and differentiated into red blood cells and endothelial cells at 26 hpf* **(G)**, *with arrow indicating endothelial cells. Flat mount view (A-D); lateral view (E-G).*

shows that blood and endothelium can derive from a common progenitor, the number of such bipotential progenitor cells *in vivo* appears to be too low to account for all the blood and endothelium.

In the mouse, single cells isolated from the posterior primitive streak give rise to blood and endothelial cells, indicating that hemangioblasts are generated during gastrulation (Huber *et al.*, 2004). Similarly, single cell labelling and lineage tracing experiments in zebrafish identified cells with hematopoietic and endothelial potential in the gastrulating embryo (Vogeli *et al.*, 2006). These experiments indicate that bipotential progenitors are located in the ventral blastomeres at shield stage, however the low numbers of such cells suggest that only a subset of hematopoietic and endothelial cells arise from hemangioblasts. Labelling cells during gastrulation could target mesodermal cells with a broader potential and give rise to many mesoderm-derived tissues. To explore the potential of more committed cells, we labelled cells at the early somitogenesis stage when hemangioblast populations are defined by the co-expression of genes such as *scl*, *fli1* and *gata2* (Fig. 5 F-G). Using a photo convertible fluorescent protein (green to red), a group of posterior lateral plate (PLM) cells were labeled under the confocal microscope at 5s (Fig. 5F). Time lapse imaging shows that these cells migrated to the midline and eventually differentiated into blood and endothelial cells at 26 hpf (Fig. 5G). Thus, clearly the PLM contains precursors of both blood and endothelium at early somitogenesis stages (as already shown by Vogeli *et al.*, 2006; Zhang and Rodaway, 2007). However, the existence of 'hemangioblasts' in the PLM awaits the labeling of single cells within this population.

In mouse embryos many endothelial cells of the yolk sac arise from unilineage angioblast precursors and not from hemangioblasts (Furuta *et al.*, 2006; Ueno and Weissman, 2006). Furthermore, even though all hematopoietic cells, and some endothelial cells, had been thought to arise from hemangioblast precursors (Palis, 2008), recent lineage tracing has questioned this (Ueno and Weissman, 2006). In zebrafish embryos, most of the blood and the endothelium appears to come from cells which earlier express *scl*, a criterion used in some mouse circles as a definition of the hemangioblast (Chung *et al.*, 2002; Gering *et al.*, 1998). In the frog, while many of the endothelial cells derived from the anterior ventral blood island mesoderm, the vitelline vessels, derive from *scl* positive cells, those in the posterior and in the head appear not to go through an *scl* expressing stage. We therefore conclude that the extent to which individual vertebrate species make blood and endothelium from a hemangioblast precursor may vary. Regardless of whether it goes through a hemangioblast intermediate or not, all blood and endothelial development appears to require *bmp* signaling (Walmsley *et al.*, 2002).

### Hemogenic endothelium

Emergence of the first HSCs is intimately associated with the ventral wall of the DA and, in mammals, the vitelline and umbilical arteries (Ciau-Uitz *et al.*, 2000; Gering and Patient, 2005; Jaffredo *et al.*, 1998; Wang *et al.*, 1998). A body of evidence indicates that HSCs derive from cells with an endothelial phenotype and suggests that HSCs differentiate from the endothelium, hemogenic endothelium, of mature blood vessels (Chen *et al.*, 2009; Taoudi *et al.*, 2008; Zovein *et al.*, 2008). Interestingly, HSC emergence has only been associated with arteries (de Bruijn *et al.*, 2000). Arteries and veins are functionally and structurally distinct and

recent reports indicate that their genetic programming is also different. Studies from zebrafish and other vertebrates demonstrate that a complex network of molecular pathways is involved in the specification of veins and arteries during embryogenesis (Gering and Patient, 2005; Lawson *et al.*, 2001; Lawson *et al.*, 2002; Wang *et al.*, 1998). Genetic mutants combined with other manipulations including chemical inhibitors have clearly demonstrated that a hedgehog (HH)-VEGF-Notch pathway is required for arterial specification of the DA. In this signalling cascade, HH expressed in the notochord, hypochord and floor plate, induces VEGF expression in nearby somites and, in its turn, VEGF activates Notch in the DA to induce arterial differentiation in part by suppressing venous fate (Lawson *et al.*, 2002). How VEGF regulates Notch is currently unclear. Nevertheless, recent work in the mouse shows that VEGF activates ERK signaling to induce expression of Dll4 and Hey via Foxc1/2, transcription factors (TFs) that directly bind to the Dll4 promoter (Hayashi and Kume, 2008; Seo *et al.*, 2006). Furthermore, the amount of exogenous VEGF added to cultured endothelial cells greatly influences their differentiation into veins or arteries, with lower levels of VEGF favouring vein differentiation and higher levels inducing arterial specification (Lanner *et al.*, 2007). This indicates that VEGF availability is a key factor during arterial specification. Strikingly, a HH-VEGF-Notch signalling cascade is also required for the emergence of HSCs in the ventral wall of the DA (Gering and Patient, 2005). However, how exactly this cascade operates to control HSC emergence is currently unknown and whether arterial specification is a prerequisite for HSC emergence or a parallel developmental process is not known. However, recent data from our lab shows that signalling by *bmp4* from underneath the DA is required to induce the HSC programme in the floor of the DA (Wilkinson *et al.*, 2009). In its absence, the arterial programme is intact, consistent with the increasing evidence that an arterial endothelial cell is the immediate precursor to the HSC, and with the conversion from one to the other being driven by BMP signaling. Distance of the DA floor from continued HH signaling also appears to be a factor (Wilkinson *et al.*, 2009).

Hemogenic endothelium has also been described in the mouse yolk sac (Goldie *et al.*, 2008) and in cultured ES cells (Lancrin *et al.*, 2009). Clonal analyses indicate that Flk1$^+$c-Kit$^+$CD45$^-$ side population cells isolated from the murine yolk sac represent hemogenic endothelium with multi lineage hematopoietic potential. This rare cell population constitutes approximately 0.05-0.1% of yolk sac cells and localizes to the yolk sac capillaries, particularly on the arterial side of the yolk sac vasculature, but also to the vitelline vein, with the greatest concentration in the latter (Goldie et al., 2008). Since hemogenic endothelium is thought to be associated to arteries rather than to veins, the presence of Flk1$^+$c-Kit$^+$CD45$^-$ cells in the vitelline vein is intriguing and further clarification of their hematopoietic potential is required. Interestingly, yolk sac hemogenic endothelium expresses retinoic acid receptors and, in contrast to the first wave of yolk sac hematopoiesis, their hematopoietic differentiation is dependent on retinoic acid signalling (Goldie *et al.*, 2008). More recently, Tie2$^{hi}$c-Kit$^+$CD41$^-$ cells isolated from ES cells and in gastrulating murine embryos have been shown to represent hemogenic endothelium (Lancrin *et al.*, 2009). These cells are located in developing yolk sac blood islands of early head-fold embryos and have the capacity to produce both primitive red cells and pro-definitive

hematopoietic colonies giving rise to red cells expressing adult hemoglobin isoforms and myeloid cells. Hierarchically, hemogenic endothelium may represent an intermediate between hemangioblasts, found in the primitive streak and from which they arise, and hematopoietic precursors found in the yolk sac (Lancrin *et al.*, 2009).

## Transcriptional control

Hematopoietic cell fate decisions are ultimately controlled by transcription factors (TFs) regulating gene expression. A substantial number of TFs have been implicated in hematopoietic development. Amongst them, *runx1* has been shown to be essential for HSC emergence in vertebrates, including *Xenopus* and zebrafish (Burns *et al.*, 2005; Gering and Patient, 2005; Kalev-Zylinska *et al.*, 2002; Our unpublished data). Other key hematopoietic TFs, such as *scl*, *lmo2* and *gata2*, affect both primitive and definitive hematopoiesis. These TFs play multiple roles in hematopoiesis making the elucidation of these roles in hemangioblast and HSC emergence, for example, dependent on the development of new tools with stage and lineage specificity. Here we
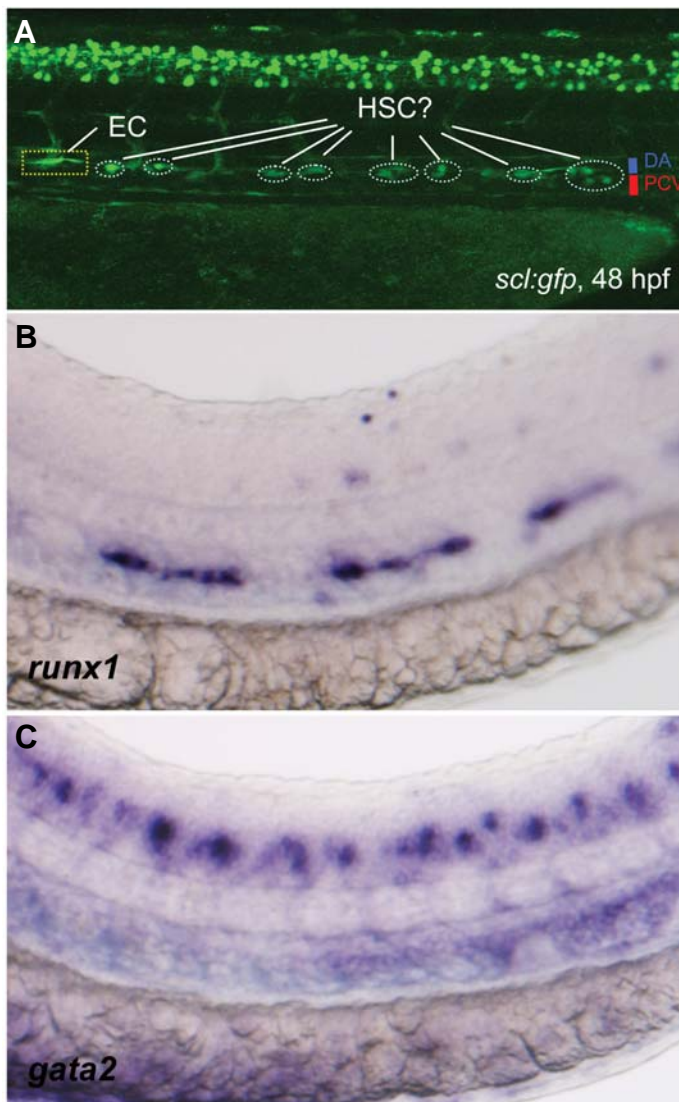
summarize the current knowledge of the functions of key TFs in hematopoietic development, although we start with a still-mysterious player, *cloche*.

### Cloche

In zebrafish, the *cloche* mutant is the earliest mutant identified so far with both hematopoietic and endothelial lineage defects (Stainier *et al.*, 1995). The recently cloned mouse homologue of a gene cloned from the *cloche* interval, lysocardiolipin acyltransferase (*lycat*), has been shown to be essential for the development of hematopoietic and endothelial lineages during *in vitro* ES cell differentiation (BL-CFC assay) (Wang *et al.*, 2007). Interestingly, protein modification by acyltransferase has emerged as an important mechanism for regulating cytoplasmic and secreted signalling molecules, such as Hedgehogs and Wnts (Linder and Deschenes, 2003; Linder and Deschenes, 2004; Wang *et al.*, 2007). This suggests that Lycat could modulate the secretion and extracellular availability of molecules required for hematopoiesis, for example, HHs, Wnts, Bmps, VEGF etc. Alternatively, Lycat could regulate the nuclear availability of TFs such as Scl, Runx1 and others to control the specification of the hematopoietic and endothelial lineages (Chamoun *et al.*, 2001; Lee and Treisman, 2001; Wang *et al.*, 2007).

### Scl/Lmo2

The T cell leukemia oncoprotein, Scl/Tal-1, is a basic-helix-loop-helix TF, required for production of embryonic red blood cells in the mouse yolk sac (Shivdasani *et al.*, 1995). In zebrafish, Scl plays early and pivotal roles during the specification of both blood and endothelial cells, possibly at the hemangioblast level (Dooley *et al.*, 2005; Gering *et al.*, 1998; Gering *et al.*, 2003; Patterson *et al.*, 2005). Ectopic expression of Scl mRNA in zebrafish embryos results in overproduction of hemangioblasts (Gering *et al.*, 1998). Morpholino injections showed that Scl is required for both primitive and definitive hematopoiesis and, in addition, for DA formation, which may explain the lack of HSCs in *scl* morphants (Patterson *et al.*, 2005). To better understand the development of *scl*+ cells in zebrafish, we generated an *scl:GFP* transgenic line. The location of *scl:GFP* cells in the DA (Fig. 6A) is similar to those expressing *runx1*, a well established marker for definitive HSCs in vertebrates (Fig. 6B), indicating that, in addition to hemangioblasts, HSC clusters in zebrafish express high levels of SCL.

*Lmo2* encodes a cysteine-rich, two LIM-domain protein that is required for yolk sac erythropoiesis (Warren *et al.*, 1994). The Lmo2 protein has a central and crucial role in hematopoietic development and is highly conserved. However, its role in vasculogenesis was only identified very recently in zebrafish. Lmo2-deficient embryos show a phenotype similar to *scl* morphants, that is, a lack of primitive and definitive blood and deficient DA development (Patterson *et al.*, 2007; Patterson *et al.*, 2005). Simultaneous overexpression of Scl and Lmo2 converts non-axial mesoderm into hemangioblasts which differentiate into

**Fig. 6**. **HSCs in association with the ventral wall of the dorsal aorta (DA). (A)** scl:gfp *transgenic embryos show GFP expression in the putative HSCs in the DA at 48 hpf.* **(B)** *In situ* hybridization at 26hpf showing runx1 *expressing cells (HSCs) associated with the ventral wall of the DA.* **(C)** gata2 *is expressed in primitive blood, vessels and spinal chord neurons at 26 hpf. Lateral views, anterior to the left.*

endothelial cells in the absence of Gata1 (Gering *et al.*, 2003), further supporting a role for an Scl-Lmo2 complex in the specification of hemangioblasts. Importantly, rescue of *scl* morphants only occurs if the rescuing Scl can interact with Lmo2 (Patterson *et al.*, 2007).

### Gata2

Gata-2 is an essential TF in the hematopoietic system, including hematopoietic stem and progenitor cells. Mice lacking Gata2 suffer from severely impaired primitive erythropoiesis and a complete lack of HSCs and other committed progenitors (Tsai *et al.*, 1994). In zebrafish, *gata2* is expressed predominantly in primitive blood and weakly in vessels (Fig. 6C). However, no convincing demonstration of an early and critical role for zebrafish Gata2 has been provided (Galloway *et al.*, 2008). In contrast, studies in *Xenopus* embryos clearly demonstrate that Gata2 is required for hemangioblast formation by controlling several important regulators (such as Scl and Lmo2) but not Fli1 (Liu *et al.*, 2008).

### ETS transcription factors: Fli1 and Etsrp

Genes necessary for blood and endothelial development contain ETS binding sites in their promoters/enhancers, suggesting that ETS factors act at the top of the transcriptional network driving blood and endothelial development via direct regulation of several master regulators. Their precise roles in this network were evaluated in manipulated zebrafish and *Xenopus* embryos, where the populations of blood and endothelial precursors are well-defined (Liu *et al.*, 2008). By gain- and loss-of-function analyses we showed that Fli1 is both necessary and sufficient for hemangioblast formation *in vivo*. It works by inducing expression of key regulators of hematopoiesis and vasculogenesis, namely Scl, Lmo2, Gata2, Etsrp and Flk1, and it also positively feeds back on its own expression. The misprogramming of the hemangioblast population which takes place in the absence of Fli1 is followed by apoptosis. Epistatic experiments show that, although induction of Fli1 expression is not dependent on the activities of Scl, Gata2, Etsrp or Cloche, its activation of downstream target genes does depend on the activities of these important regulators, indicating a position either upstream of or parallel to them in the genetic hierarchy leading to blood and endothelial development. Taken together, these results identify Fli1, as a master regulator of the hemangioblast programme in vertebrate embryos.

Etsrp, a novel zebrafish ETS domain protein, has been shown to be necessary and sufficient for the initiation of vasculogenesis in zebrafish (Sumanas and Lin, 2006). However, recent observations by us and others have suggested that Etsrp may play an earlier role during hemangioblast formation in the ALM, since loss of Etsrp causes an absence of both myeloid and endothelial makers in the ALM (Liu and Patient, 2008; Sumanas *et al.*, 2008). This view is supported by functional analysis of the mouse homolog of Etsrp, ER71/ETV2 (Lee *et al.*, 2008). ER71 enforced expression in ES cells appears to be sufficient to induce the generation of Flk1^{+} cells and enhance hematopoietic and endothelial cell generation even when BMP, Notch and Wnt are inhibited. Furthermore, in ER71^{-/-} embryos a complete absence of Flk1^{+} hematopoietic and endothelial cells is observed (Lee *et al.*, 2008), indicating that ER71 is essential for the generation of Flk1^{+} mesoderm and its derivatives.

### A genetic regulatory network for hematopoietic development

The specification of hemangioblasts from mesoderm and their differentiation into blood and endothelial cells requires precise coordination of TF activity. A fuller understanding of development will require moving beyond the study of individual genes to determine how regulatory genes interact to form functional genetic regulatory networks (GRNs) (Levine and Davidson, 2005). Conservation of the vertebrate body plan has been attributed to the evolutionary stability of such GRNs, which consist of assemblies of subcircuits made up of TFs and their target cis-regulatory modules. Subcircuits that perform essential functions in building up body parts have been termed kernels of the GRNs (Davidson and Erwin, 2006). Disruption of TFs that make up the kernel often results in loss of the body part, and hence the basic architecture of the kernel is highly conserved through evolution (Davidson and Erwin, 2006).

*In vitro* data suggests that the *scl* gene may be regulated by a TF complex that consists of the ETS transcription factors, Fli1 and Elf1, and Gata2 (Gottgens *et al.*, 2002). Indeed, recent data suggests that *fli1*, *scl* and *gata2* may reciprocally regulate each other in hematopoietic progenitor and endothelial cell lines, possibly also in hematopoietic tissues (i.e. fetal liver and the AGM) and in BL-CFC, the *in vitro* equivalent of hemangioblasts (Pimanda *et al.*, 2007). It has been suggested that *fli1*, *scl* and *gata2* form an auto-regulatory feedback loop (a GRN kernel; Fig. 7) that regulates hemangioblast development and that this putative GRN kernel could well be conserved from invertebrates, like *Drosophila*, to vertebrates. Whether this GRN kernel is operational *in vivo* during hemangioblast specification in fish and frog embryos has recently been investigated (Liu *et al.*, 2008). These data, suggest that Fli1 acts at the top of the genetic regulatory hierarchy controlling hemangioblast specification, since it controls the expression of several master regulators, such as *scl*, *gata2*, *lmo2* and *fli1* itself (Fig. 7) (Liu *et al.*, 2008). It is likely that upstream signals, such as Bmp initiate *fli1* and *gata2* expression (Walmsley
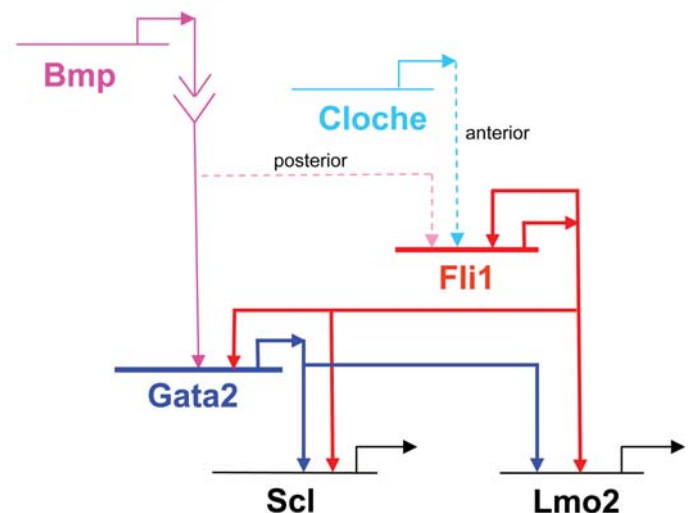


**Fig. 7. The hemangioblast kernel in vertebrate embryos.** *Solid lines, direct regulation; dashed lines, indirect or no evidence for direct regulation; chevron connection, signalling pathway. Adapted from Liu* et al., *2008.*

*et al.*, 2002), which then autoregulate and combine to initiate *scl* and *lmo2* expression, and then induce downstream targets. Autoregulation such as that seen for *fli1* can sustain the network after inducing signals have ceased. Steady-state kinetics of individual TFs, threshold concentrations at which they trigger expression and the requirement for combinatorial binding could all be critical factors in this circuit to either maintain the hemangioblast state or drive differentiation (Pimanda *et al.*, 2007).

## Perspectives

Over the past few years, we have gained a better understanding of hematopoietic development in both *Xenopus* and zebrafish, from the ontogeny of hematopoietic sites to the details of regulatory signalling pathways and transcription factor networks. Most of the information is applicable to mammals including humans, so it should benefit attempts to make these cells *in vitro* and manipulate them *in vivo*.

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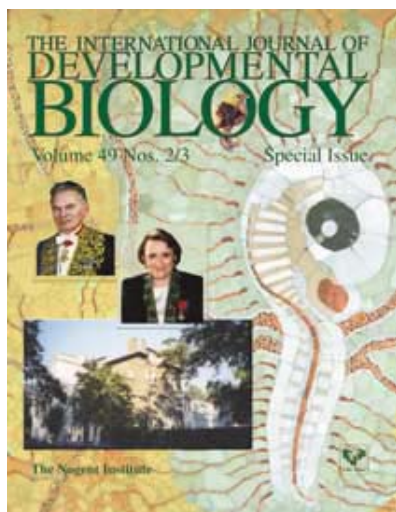**Pathways in blood and vessel development revealed through zebrafish genetics**
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