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SCL-GFP transgenic zebrafish: *In vivo* imaging of blood and endothelial development and identification of the initial site of definitive hematopoiesis

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Abstract

The bHLH transcription factor *SCL* plays a central role in the generation of hematopoietic cells in vertebrates. We modified a PAC containing the whole zebrafish *scl* locus, inserting GFP into the first coding exon of *scl*. In germline-transgenic zebrafish generated using this construct, GFP expression completely recapitulates the endogenous expression of *scl* in blood, endothelium and CNS. We performed *in vivo* timelapse imaging of blood and endothelial precursor migration at the single-cell level and show that these cells migrate from the posterior lateral plate mesoderm to their site of differentiation in the intermediate cell mass. The anterior lateral plate domain of GFP expression gives rise to primitive macrophages and the blood vessels of the head. In later embryos, GFP expression identifies clusters of hematopoietic cells that develop between the dorsal aorta and posterior cardinal veins after primitive erythrocytes have entered circulation. Two treatments that block definitive hematopoiesis (treatment with dioxin (TCDD), and injection of an antisense morpholino oligonucleotide targeted to *runx1*) ablate these hematopoietic clusters. This indicates that these clusters represent the first site of definitive hematopoiesis in zebrafish. This site is anatomically homologous to the proposed source of hematopoietic stem cells in amniotes, the aorta–gonad–mesonephros (AGM) region. A second transgenic line, containing the promoter of *scl* driving GFP, lacks expression in the definitive clusters.

Keywords: TAL1; AML1; Globin; Fugu; Pufferfish; Recombinase; Recombineering; Transgenesis; mRFP; Vasculature; Haematopoiesis

Introduction

Hematopoiesis in vertebrate embryos occurs in two waves: primitive and definitive (reviewed in Godin and Cumano, 2002). Primitive hematopoiesis gives rise to erythrocytes and phagocytes in the early embryo, and does not appear to involve self-renewing hematopoietic stem cells (HSC). Subsequently, definitive hematopoiesis gives rise to HSC, which generate the full range of blood cell types in the later embryo and throughout adulthood. Primitive and definitive cells appear to have separate embryonic origins (Ciau-Uitz et al., 2000; Dieterlen-Lievre, 1975).

In amniotes, the site of primitive hematopoiesis is the yolk sac. The primitive erythrocytes and yolk sac vasculature form from 'hematic cords' of extraembryonic mesoderm (Sabin, 1920), which led Murray (1932) to coin the term 'hemangioblast', for a proposed bipotential progenitor cell for endothelial cell (EC) and blood cell formation.

The zebrafish is becoming an increasingly important model for the study of blood development (reviewed in Davidson and Zon, 2004). Its transparency, and development outside the mother, allow *in vivo* studies that are impractical in amniote embryos. In zebrafish, primitive hematopoiesis is also closely associated with endothelial development. Erythroblasts are formed in the intermediate cell mass (ICM) of the trunk (between the paired somites, ventral to the notochord, and dorsal to the endoderm) (Al-Adhami and Kunz, 1977). This tissue also forms the major vessels of the trunk: the dorsal aorta (DA) and posterior cardinal vein (PCV). Primitive macrophages emerge from the anterior lateral mesoderm (ALM) adjacent to the midbrain (Herbomel et al., 1999), a region that is also thought to contribute to the head vasculature.

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Expression of blood and endothelial-specific genes that are later found in the ICM is initiated in the posterior lateral mesoderm (PLM) (Brown et al., 2000; Detrich et al., 1995; Fouquet et al., 1997; Gering et al., 1998). The movement of these expression domains to the midline is generally thought to result from cell migration. However, gene expression is dynamically regulated during this process. Genes that are later confined to either the blood or the endothelium are initially co-expressed in lateral stripes in the PLM (Brown et al., 2000; Gering et al., 1998), and as the expression domains move from the lateral mesoderm to the ICM, a progressive separation of blood and endothelial gene expression occurs. Therefore, while strongly suggestive, movement of gene expression does not prove cell movement. There is only limited direct evidence for such migration: Childs et al. (2002) showed that patches of cells in the PLM, in which a caged fluorescein marker had been activated using a laser, could give rise to cells in the axial vasculature. Therefore, in this study, we have imaged migration of PLM cells at single-cell resolution in living zebrafish embryos.

The site of formation of the first definitive HSCs is still the subject of controversy. In amniotes, most evidence points to an intraembryonic site, the aorta–gonad–mesonephros (AGM) region (Godin et al., 1993, 1995; Medvinsky and Dzierzak, 1996; Medvinsky et al., 1993; Muller et al., 1994), from which later sites of blood formation, such as the fetal liver are colonized. However, extraembryonic sites for HSC formation, such as the yolk sac (Kumaravelu et al., 2002) and allantois, cannot definitively be ruled out. Within the AGM, clusters of presumptive hematopoietic cells are found in association with the ventral wall of the aorta (Emmel, 1916; Garcia-Porrero et al., 1995; Tavian et al., 1996). Tissue transplants (Pardanaud et al., 1996), *in vivo* labeling (Jaffredo et al., 1998) and marker-based sorting (de Bruijn et al., 2002; Nishikawa et al., 1998a,b) imply that hematopoietic cells are derived from the endothelium of the aorta: the 'hemogenic endothelium' hypothesis. Other experiments, however, suggest that the definitive HSCs might arise in mesenchyme ventral to the DA, and pass through the ventral wall of the DA to form the hematopoietic clusters (Bertrand et al., 2005; North et al., 2002). These two possibilities are not, however, mutually exclusive (Jaffredo et al., 2000).

In zebrafish, definitive hematopoiesis is poorly understood compared to primitive hematopoiesis. Primitive erythrocytes (which enter circulation at around 26 hpf) are gradually lost from circulation from 5 days post fertilization (dpf) (Weinstein et al., 1996), and are replaced by a population morphologically more similar to adult erythrocytes (Belair et al., 2001). Lymphocytes (which are regarded as a definitive cell type) are first detected in the thymus around 3 dpf (Willett et al., 1997, 1999). Cells with the morphology of hematopoietic blasts are found in the pronephric kidney (the site of adult hematopoiesis) from 4 dpf (Al-Adhami and Kunz, 1977; Willett et al., 1999). These data imply that definitive hematopoiesis initiates in the second or third day of development. Transcription factors implicated in definitive hematopoiesis in amniotes, including *c-myb*, *ikaros* and *runx1* (AML1), are expressed in the ICM region after the primitive erythroblasts have entered circulation (Burns et al., 2002; Gering and Patient, 2005; Kalev-Zylinska et al., 2002; Thompson et al., 1998; Willett et al., 2001). Interestingly, the expression of these genes initiates in the floor of the DA, implying that this may be hemogenic endothelium. Further evidence that these cells may be involved in definitive hematopoiesis in zebrafish is provided by morpholino oligonucleotide (MO)-mediated knockdown of *runx1* expression. This results in the loss of *c-myb* and *ikaros* expression in the floor of the DA and reduction or loss of lymphocytes in the thymus (Gering and Patient, 2005). An alternative site suggested is the ventral part of the post anal ICM, also called the ventral vein region (VVR), based on the prolonged expression of *scl* and *c-myb* in this area (Liao et al., 1998).

We have previously shown that bHLH transcription factor *scl* is one of the earliest markers of the cells in PLM that are thought to give rise to the ICM (Gering et al., 1998). In mice, SCL (also known as TAL1) plays a key role in the formation of both primitive blood (Kallianpur et al., 1994; Robb et al., 1995; Shivdasani et al., 1995) and definitive HSC (Porcher et al., 1996; Robb et al., 1996). In zebrafish embryos, forced expression of *scl* expands blood and endothelial tissues at the expense of other mesodermal derivatives (Gering et al., 1998, 2003), and MO-mediated knockdown of *scl* expression ablates primitive hematopoiesis (Dooley et al., 2005; Patterson et al., 2005). Loss of *runx1* and *c-myb*-expressing cells in the post-circulation ICM, and *rag1*-expressing lymphocytes in the thymus, implies that the knockdown also ablates definitive hematopoiesis (Dooley et al., 2005), but this could be secondary to the failure to form the dorsal aorta, from which these cells might be derived (Patterson et al., 2005).

To use the *in vivo* imaging and embryonic manipulations that are possible in zebrafish, but not in mammals, to study blood and endothelial development, we determined to generate a line of transgenic zebrafish-expressing GFP in blood and endothelial precursors. We wished to use *in vivo* imaging to examine the behavior of the cells that gives rise to the primitive blood, and, in particular, to prove that the ICM is generated by medial migration of PLM cells. Furthermore, we hoped that the line would enable us to identify the site of initiation of definitive hematopoiesis. Since *scl* is clearly a key regulator of primitive and definitive hematopoiesis, and of vasculogenesis, and is expressed from the early stages of these processes, we chose to use the control elements of the *scl* locus to drive GFP expression in transgenic zebrafish.

In this paper, we show that embryos transgenic for GFP under the control of the *scl* locus accurately recapitulate *scl* expression. We use these embryos to prove that the ICM (erythroblasts and trunk vessel angioblasts) is, indeed, formed by cells migrating from the PLM. The *scl* expression domain in the anterior lateral mesoderm (ALM) similarly gives rise to both blood cells (in this case macrophages), and angioblasts of the head vasculature. GFP expression (supported by co-expression of multiple hematopoietic markers) identifies clusters of cells between the DA and PCV as being a likely site of the initiation of definitive hematopoiesis. This identification is supported by the effects of drug treatment, and MO-mediated gene knockdown experiments that ablate definitive hematopoiesis.