Lmo2 and Scl/Tal1 convert non-axial mesoderm into haemangioblasts which differentiate into endothelial cells in the absence of Gata1

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Accepted 16 September 2003

Development 130, 6187-6199
Published by The Company of Biologists Ltd 2003
doi:10.1242/dev.00875

Summary

The LIM domain protein Lmo2 and the basic helix-loop-helix transcription factor Scl/Tal1 are expressed in early haematopoietic and endothelial progenitors and interact with each other in haematopoietic cells. While loss-of-function studies have shown that Lmo2 and Scl/Tal1 are essential for haematopoiesis and angiogenic remodelling of the vasculature, gain-of-function studies have suggested an earlier role for Scl/Tal1 in the specification of haemangioblasts, putative bipotential precursors of blood and endothelium. In zebrafish embryos, Scl/Tal1 can induce these progenitors from early mesoderm mainly at the expense of the somitic paraxial mesoderm. We show that this restriction to the somitic paraxial mesoderm correlates well with the ability of Scl/Tal1 to induce ectopic expression of its interaction partner Lmo2. Co-injection of *lmo2* mRNA with *scl/tal1* dramatically extends its effect to head, heart, pronephros and pronephric duct mesoderm inducing early blood and endothelial genes all along the anteroposterior axis. Erythroid development, however, is expanded only into pronephric mesoderm, remaining excluded from head, heart and somitic paraxial mesoderm territories. This restriction correlates well with activation of *gata1* transcription and co-injection of *gata1* mRNA along with *scl/tal1* and *lmo2* induces erythropoiesis more broadly without ventralising or posteriorising the embryo. While no ectopic myeloid development from the Scl/Tal1-Lmo2-induced haemangioblasts was observed, a dramatic increase in the number of endothelial cells was found. These results suggest that, in the absence of inducers of erythroid or myeloid haematopoiesis, Scl/Tal1-Lmo2-induced haemangioblasts differentiate into endothelial cells.

Key words: Haematopoiesis, Blood, Erythropoiesis, Myelopoiesis, Vasculogenesis, Pronephric duct, Heart, Lateral mesoderm, Somitic mesoderm

Introduction

In mammalian and avian embryos, primitive erythrocytes form in the extraembryonic yolk sac where clusters of mesodermal cells give rise to both haematopoietic cells (HCs) and angioblasts, early progenitors for endothelial cells (ECs). Inside the embryo in the aorta-gonad-mesonephros (AGM) region, definitive HCs develop in association with the floor of the dorsal aorta. This close relationship of HCs and ECs suggests the existence of a common progenitor, the haemangioblast (Keller, 2001).

The concept of the haemangioblast has gained support from several findings. Firstly, angioblasts and haematopoietic progenitors share the expression of several genes (Keller, 2001). Secondly, targeted disruption of the gene for the vascular endothelial growth factor (VEGF) receptor-2, *flk1*, in the mouse (Shalaby et al., 1995) and mutation of the zebrafish *cloche* locus affect both the blood and endothelial lineages (Stainier et al., 1995). Thirdly, single Flk1-positive ($Flk1^+$) cells (so called blast colony forming cells; BL-CFCs) derived from embryonic stem (ES) cells can give rise to blast colonies (BL-C) that contain both blood and endothelial progenitors in vitro (Choi et al., 1998; Faloon et al., 2000; Nishikawa et al., 1998). However, haemangioblasts have not yet been isolated from a vertebrate embryo. The best in vivo evidence for the existence of the haemangioblast comes from lineage labelling studies in the chick (Jaffredo et al., 1998; Jaffredo et al., 2000). These studies also suggest that definitive HC clusters directly differentiate from the ventral endothelial lining of the dorsal aorta. Consistent with this idea, ECs isolated from the AGM region and the vitelline and umbilical arteries of murine embryos possess haematopoietic stem cell activity (North et al., 2002), and ECs selected from the AGM region, the foetal liver and the foetal bone marrow of human embryos develop into myelo-lymphoid cells in culture (Oberlin et al., 2002).

In zebrafish, primitive erythrocytes and ECs of the major trunk vessels, the dorsal aorta and the axial vein, form in close association in the intermediate cell mass (ICM) in the posterior midline of the embryo 1 day after fertilisation. These cells originate from the posterior lateral mesoderm (PLM) of the post-gastrula embryo (Zhong et al., 2001) (Fig. 1D). Here, overlapping expression patterns of blood and endothelial genes suggest the existence of haemangioblast-like cells (Brown et

al., 2000; Gering et al., 1998; Thompson et al., 1998). Expression patterns of blood and endothelial genes also overlap in the anterior lateral mesoderm (ALM, Fig. 1D) which gives rise to ECs (Roman and Weinstein, 2000) and myeloid HCs (Hsu et al., 2001). In this paper, we will refer to such cells co-expressing blood and endothelial genes as haemangioblasts.

One of the genes expressed in both the ALM and the PLM is the stem cell leukaemia/T-cell acute lymphoblastic leukaemia gene *scl/tal1*, which encodes a basic helix-loop-helix transcription factor (Gering et al., 1998). It was first discovered through its involvement in chromosomal translocations in leukaemic T-cells. It has since been found to be expressed in early blood progenitors and in cells of the erythrocyte, megakaryocyte and mast cell lineages, as well as in endothelial and neuronal progenitors (Baer, 1993; Begley and Green, 1999). *scl/tal1*$^{-/-}$ mouse embryos die at day 9.5 from a complete lack of primitive haematopoiesis (Robb et al., 1995; Shivdasani et al., 1995) and *scl/tal1*$^{-/-}$ ES cells cannot take part in either primitive or definitive haematopoiesis in chimaeric animals (Porcher et al., 1996; Robb et al., 1996). Furthermore, a conditional knockout of *scl/tal1* in the adult revealed that *scl/tal1* is important for the specification of the haematopoietic stem cell rather than its maintenance (Hall et al., 2003; Mikkola et al., 2003). The loss-of-function studies did not reveal an essential role for *scl/tal* in vasculogenesis. However, although ECs appear to develop normally, they fail to form a primary vascular plexus and to undergo subsequent angiogenic remodelling (Elefanty et al., 1999; Visvader et al., 1998).

Gain-of-function studies suggest an earlier role for *scl/tal1* consistent with its expression in early lateral mesoderm prior to the separation of the blood and endothelial lineages. We reported previously that Scl/Tal1 can induce ectopic development of cells co-expressing both early blood and endothelial genes mainly from the paraxial mesoderm and that these cells appeared to give rise to blood and later some ECs (Gering et al., 1998). In a parallel study, forced expression of *scl/tal1* was shown to partially rescue expression of blood and endothelial genes in the zebrafish *cloche* mutant (Liao et al., 1998). Both studies suggest that Scl/Tal1 can specify blood and ECs possibly through the specification of the haemangioblast.

Scl/Tal1 forms complexes with other transcription factors. It heterodimerises with ubiquitously expressed bHLH transcription factors encoded by the E2a gene, which bind E-boxes in regulatory sequences and activate gene transcription (Hsu et al., 1991; Hsu et al., 1994). Scl/Tal1 also interacts with the LIM domain transcription factors Lmo1 and Lmo2, with whom it shares involvement in T-cell leukaemias (Rabbitts, 1998; Valge-Archer et al., 1994; Wadman et al., 1994). Like *scl/tal1*, *lmo2* is expressed in endothelial and haematopoietic progenitors and in cells of the erythroid and megakaryocytic lineage (Warren et al., 1994), and is essential for primitive and definitive haematopoiesis, as well as vascular remodelling (Warren et al., 1994; Yamada et al., 1998; Yamada et al., 2000). In erythroid cells, Lmo2, which itself does not bind DNA, acts as a bridging molecule in a complex that contains Ldb1, Scl/Tal1, E2a and Gata1 and binds composite E-box-GATA sequence motifs (Wadman et al., 1997). In early blood progenitors, Gata2 can replace Gata1, and Sp1 takes the complex to an essential Sp1 site within the c-kit promoter (Lecuyer et al., 2002). Later in erythroid maturation, the retinoblastoma protein has been reported to replace the GATA factor (Vitelli et al., 2000). Consistent with an important role for the Lmo2-Scl/Tal1-Gata1/2 complex in erythropoiesis, forced expression of *scl/tal1*, *lmo2* and *gata1* can turn activin or FGF-treated *Xenopus* animal caps into erythrocytes and can induce widespread erythropoiesis in *Xenopus* embryos (Mead et al., 2001). However, these studies were carried out at concentrations sufficient to induce Bmp4 and the embryos were ventralised. In addition, only differentiated erythroid cells were monitored and the effects on other blood cell types or endothelial cell differentiation were not addressed. Whether the red cells followed their normal differentiation pathway, developing from haemangioblasts, was also not determined.

In this report, we describe a role for a synergistic action of Lmo2 and Scl/Tal1 in the early mesoderm. We show that *scl/tal1* specifies haemangioblast-like cells in the very tissue in which it can ectopically induce the expression of its interaction partner Lmo2. Co-injection of *scl/tal1* and *lmo2* mRNAs extends the effect of Scl/Tal1 to other non-axial mesodermal tissues inducing the early blood and endothelial transcription programmes throughout the anteroposterior axis. Blood differentiation, however, is restricted to the pronephric mesoderm where Lmo2 and Scl/Tal1 are able to induce *gata1* expression. In the absence of Gata1, Lmo2-Scl/Tal1-induced haemangioblasts differentiate into ECs.

Materials and methods

Maintenance of fish

Breeding zebrafish were maintained and embryos were raised and staged according to Westerfield (Westerfield, 1993).

Preparation of mRNA for injection and antisense RNA probes

cDNAs for murine Lmo1, 2 and 4, and zebrafish Gata1, 2 and 5 were cloned into the expression vector pβUT2-MT (Gering et al., 1998). Oligos were used in PCR reactions on plasmid DNA to introduce suitable restriction sites at the ends of open reading frames:

murine *Lmo1*: 5′GATTCTAGACGCCTGGGGACTATGGTTTTG-GAC3′ and 5′GATCTCGAGCCTGAACCTGGGATTCAAAGGTG-CC3′

murine *Lmo2*: 5′GATTCTAGACCAATGTCCTCGGCCATCGAA-AGG3′ and 5′GATCTCGAGCGATGATCCCATTGATCTTGGTCC-A3′

murine *Lmo4*: 5′GATTCTAGAACCATGGTGAATCCGGGCAG-CAGC3′ and 5′GATCTCGAGCGCAGACCTTTTGGTCTGGCAG-TAG3′

zebrafish *gata1*: 5′CTCCTCTAGAGCCACCATGGAGAACTCC-TCTGAGCCTTC and 5′CTCCCTCGAGACACTAGTGTGGGCA-TCATGCC3′

zebrafish *gata2*: 5′GCCGATATCCACTTGAGATGGAGGTTG3′ and 5′CGGCTCGAGAGTGTGGTTCGGCCAG3′ (construct made by A. R. F. Rodaway)

zebrafish *gata5*: 5′CTCCTCTAGATATATCATGTATTCGAGCC-TG3′ and 5′CTCCCTCGAGACGCTTGAGACAGAGCACAC3′ (construct made by A. Gibson).

PCR fragments were digested with *Xba*I and *Xho*I, and cloned in frame with the Myc-tag of pβUT2. To produce mRNA encoding zebrafish Scl/Tal1, β-galactosidase, nuclear localised GFP and murine MyoD, pFC6 (Gering et al., 1998), pCS2lacZ, pCSnlsGFP2 (a linker encoding a nuclear localisation signal with the sequence 5′GAAT-TCCCCAAAAAAGAAGAGAAAGGTAGAATTC3′ was introduced

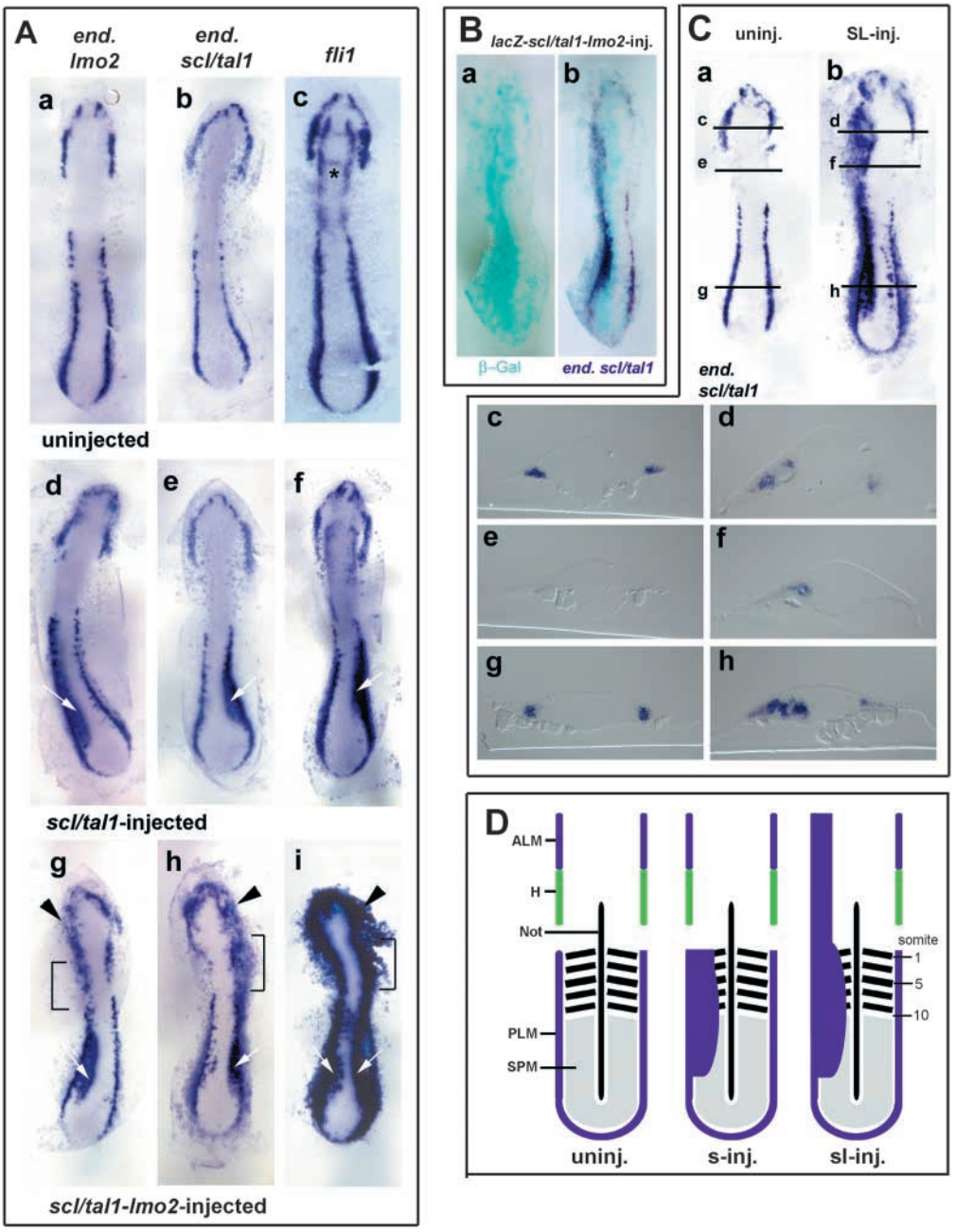

Fig. 1. Lmo2 and Scl/Tal1 induce early blood and endothelial cells throughout the lateral mesoderm. (Aa-i,Ba-b,Ca-b) Flat mounts; anterior top. (Cc-h) Transverse sections; dorsal top. (D) Schematic of results. (A) In uninjected embryos (a-c), *lmo2*$^{end.}$ (a), *scl/tal1*$^{end.}$ (b) and *fli1* (c) expression overlap in bilateral stripes in the ALM and the PLM. *fli1* is also expressed in anterior neural crest cells (c, asterisk). [The *nkx2.5*$^{+}$ (see Fig. 2A) cardiogenic mesoderm is located in the gap between the ALM and the PLM.] (A) In *scl/tal1*-injected embryos (d-f), *lmo2*$^{end.}$ (d), *scl/tal1*$^{end.}$ (e) and *fli1* (f) are ectopically expressed in the SPM (arrows). (A) In *scl/tal1-lmo2*-injected embryos (g-i), *lmo2*$^{end.}$ (g), *scl/tal1*$^{end.}$ (h) and *fli1* (i) are, in addition, ectopically expressed in a broader domain in the head (arrowheads) and in the cardiogenic mesoderm (brackets). (B) Lineage tracing reveals that ectopic *scl/tal1*$^{end.}$ (b) is induced on the injected, β-galactosidase-positive side of the embryo (a). (C) Sections (taken at the levels indicated by c-h, shown below) confirm that *scl/tal1-lmo2* injection induced *scl/tal1*$^{end.}$ expression in the head mesoderm (c,d), in the cardiogenic mesoderm (e,f), as well as in the SPM (g,h). (D) Whereas *scl/tal1* injection (s-inj.) induces ectopic haemangioblasts (purple) in the somitic mesoderm, *scl/tal1-lmo2* injection (sl-inj.) expands haemangioblasts into the head, heart (H) and somitic mesoderm. Not, notochord.

into the *Eco*RI and *Xba*I sites of pCS2mt-SGP (Rubenstein et al., 1997) (construct made by Maz O'Reilly) and MmyoD.R1/Bam (Theze et al., 1995) were used. mRNAs were transcribed from linearised templates using mMessage-mMachine-Kits (Ambion, USA). mRNAs were characterised spectrophotometrically, on agarose gels and by in vitro translation.

Injection of fish embryos

mRNAs (100 pg of each mRNA in all experiments except the *gata1-scl/tal1-lmo2* injections where 25 pg of each mRNA were injected) were injected in 0.5 nl into one cell at the two or four cell stage using a Picospritzer II microinjector (Parker Instrumentation). *gfp* or *lacZ* mRNA (20 pg) were co-injected as tracers. The progeny of the injected cell occasionally came to lie on one side of the embryo, leaving the uninjected side as an internal control.

Whole-mount in situ hybridisation and sectioning

scl/tal1$^{end.}$ and *gata1*$^{end.}$ expression were determined with probes complementary to the 3′UTRs, which were not in the injected mRNAs. pZE62 contains 0.8 kb of the *scl/tal1* 3′UTR (Gering et al., 1998). peG1 contains the last 0.3 kb of the 3′UTR of *gata1*. It was generated by a *Hin*dIII cut-and-shut of the original plasmid, pGATA1 (Detrich et al., 1995). The zebrafish *lmo2* probe did not hybridise significantly to the injected mouse *Lmo2* mRNA and could therefore be used to detect endogenous transcripts. Antisense RNAs for in situ hybridisation were transcribed from linearised templates using Promega's T3, T7 and SP6 RNA polymerases in the presence of digoxigenin (DIG)- or fluorescein-labelled nucleotides (Roche Ltd., UK). Detection of the DIG/fluorescein antibody-alkaline phosphatase conjugate was done using BM-Purple and Fast Red (Roche Ltd., UK).

Single and double whole-mount in situ hybridisation on zebrafish

embryos was carried out as previously described (Jowett and Yan, 1996). For sectioning, embryos were transferred into ethanol, embedded in JB4 methacrylate (Agar Scientific Ltd., UK) and sectioned on a microtome (Leica Jung RM2165). All sections shown are 10 μm.

Results

Scl/Tal1 and Lmo2 are sufficient to induce haemangioblasts in non-axial mesoderm

Co-expression of *scl/tal1* and *lmo2* in blood and endothelial progenitors of the ALM and PLM (Fig. 1Aa-b) (Gering et al., 1998; Thompson et al., 1998) raises the possibility that Scl/Tal1 and Lmo2-containing transcription factor complexes may be active outside the erythroid lineage. We have shown previously that ectopic expression of *scl/tal1* alone in zebrafish induces haemangioblast formation in the SPM (Fig. 1D) (Gering et al., 1998). To determine if the restriction of this induction to the SPM correlated with expression of Lmo2, we examined *lmo2* expression in *scl/tal1*-injected zebrafish embryos. We found that *lmo2* was like endogenous *scl/tal1* (*scl/tal1*$^{end.}$) [Fig. 1Ab,e, arrow; 1D, n=15/20 (Gering et al., 1998)] and the endothelial gene *fli1* (Brown et al., 2000; Thompson et al., 1998) (Fig. 1Ac,f; arrow, n=35/55), ectopically expressed in the SPM (Fig. 1Ad; arrow; n=18/22; 1D), but not in the head paraxial mesoderm or in the *nkx2.5*$^{+}$ (Chen and Fishman, 1996) cardiogenic mesoderm (Fig. 1Ad) that lies between the ALM and the PLM (Fig. 2A). Thus, the tissue in which *scl/tal1* induces ectopic *lmo2* expression is the very tissue in which it can switch on the haematopoietic and endothelial transcription programmes. This suggests that Scl/Tal1-induced blood and endothelial development might be spatially restricted by the ability of Scl/Tal1 to activate endogenous *lmo2* expression.

To test whether providing Lmo2 exogenously through co-injection could spread the effect of Scl/Tal1 to other parts of the mesoderm, we co-injected mRNAs coding for zebrafish Scl/Tal1 and murine Lmo2 into zebrafish embryos and examined the expression of *scl/tal1*$^{end.}$, *fli1* and *lmo2*$^{end.}$. While control injections [*gfp* (n=26), *lmo2* (n=37)] had no effect and *scl/tal1* injection caused ectopic expression only in the SPM, *scl/tal1-lmo2*-co-injection had far more dramatic consequences. All three genes were now expressed laterally throughout the anteroposterior axis including the heart region (Fig. 1Ag-i, brackets; D) and in a wider domain in the head (Fig. 1Ag-i, arrowheads; D), in addition to their ectopic expression in the SPM (Fig. 1Ag-i, arrows; 1D; n_{scltal1}=15/15, n_{fli1}=34/35, n_{lmo2}=14/14). Ectopic expression coincided with maximal expression of the co-injected *lacZ* lineage marker (Fig. 1Ba,b). Transverse sections through a flat-mounted embryo confirmed that the ectopic expression of *scl/tal1*$^{end.}$ was in the mesodermal layer (Fig. 1Cc-h). This suggests that ectopic expression of *scl/tal1* and *lmo2* together significantly extends the range of mesodermal territories in which the blood and endothelial transcription programmes can be induced.

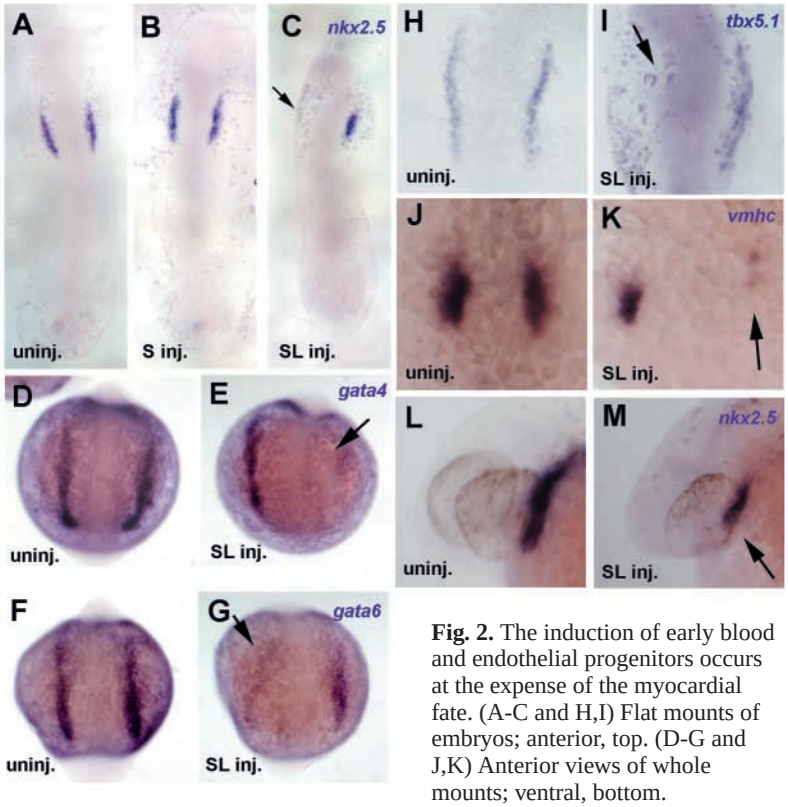

Fig. 2. The induction of early blood and endothelial progenitors occurs at the expense of the myocardial fate. (A-C and H,I) Flat mounts of embryos; anterior, top. (D-G and J,K) Anterior views of whole mounts; ventral, bottom. (L,M) Lateral views, anterior to the left. (A-C) At the 10-somite stage, *nkx2.5* expression is reduced on the injected side in *scl/tal1-lmo2* (SL)-injected embryos (C, arrow) but is perfectly normal in *scl/tal1* (S)-injected (B) embryos. (D-I) Likewise, *gata4* (D,E), *gata6* (F,G) and *tbx5.1* (H,I) were reduced (E,G,I, arrows) on the side injected with *scl/tal1-lmo2*. (J-M) *vmhc*$^{+}$ (Yelon et al., 1999) ventricular tissue was reduced at the 18-somite stage (J,K) and the *nkx2.5*$^{+}$ heart tissue was reduced by 34 hpf (L,M) by injection with *scl/tal1-lmo2*.

This phenotype was specific to the injection of *scl/tal1* and *lmo2* mRNAs. Replacing Scl/Tal1 with the murine myogenic bHLH protein MyoD [which induced ectopic anterior expression of the muscle marker *desmin* (Xu et al., 2000)] in 12-somite stage embryos (n=13/15) induced no ectopic *scl/tal1*$^{end.}$ expression (n=12) and reduced normal expression in 5/12 embryos. Likewise, embryos co-injected with Scl/Tal1 and another LIM-only protein, murine Lmo4 (Grutz et al., 1998; Kenny et al., 1998), did not display widespread ectopic expression of *scl/tal1*$^{end.}$ (n=20). In contrast, Lmo1, a Lim-only protein that, like Lmo2 and Scl/Tal1, is involved in chromosomal translocations in leukaemic T-cells (Rabbitts, 1998) and that like Lmo2 has been reported to interact with Scl/Tal1 in vitro (Valge-Archer et al., 1994), could replace Lmo2 in our assay (n=17). Thus, Lmo2 and Lmo1 are functionally equivalent in this assay as well as during tumorigenesis (Rabbitts, 1998).

Ectopic expression of *scl/tal1* and *lmo2* abolished normal cardiac gene expression. At early somite stages, *nkx2.5* (n=17/19), *gata4* (Reiter et al., 1999) (n=10/16), *gata6* (n=12/22) and *tbx5.1* (Begemann and Ingham, 2000) (n=6/12) were down-regulated in *scl/tal1-lmo2*-injected embryos (Fig. 2A,C-I, arrows). Only *gata5* (Brown et al., 2000; Reiter et al., 1999) expression appeared unchanged (weak reduction in 2/18; data not shown). Control embryos injected with *gfp*, *lmo2* or

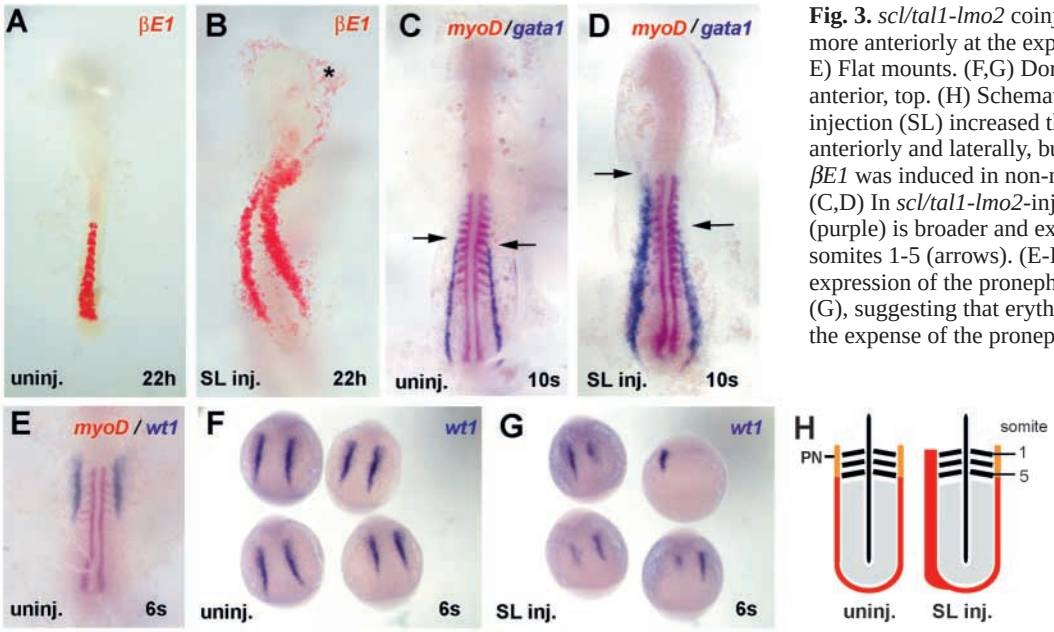

Fig. 3. *scl/tal1-lmo2* coinjection induces erythropoiesis more anteriorly at the expense of the pronephros. (A-E) Flat mounts. (F,G) Dorsal views of whole mounts; anterior, top. (H) Schematic of results. (A,B) *scl/tal1-lmo2* injection (SL) increased the number of *βE1*$^+$ erythroid cells anteriorly and laterally, but they did not fuse at the midline. *βE1* was induced in non-neural ectoderm (B, asterisk). (C,D) In *scl/tal1-lmo2*-injected embryos, *gata1* expression (purple) is broader and expanded anteriorly adjacent to somites 1-5 (arrows). (E-H) Scl/Tal1 and Lmo2 reduced expression of the pronephros (PN) marker *wt1* (G), suggesting that erythroid development is expanded at the expense of the pronephros as shown in H.

scl/tal1 displayed very little reduction in *nkx2.5* (0/12, 0/20, 0/21) or *gata4* (1/16, 0/20, 1/15) expression at this time. Consistent with the loss of early heart gene expression, we found a reduction in ventricular tissue just prior to the fusion of the bilateral heart fields (Fig. 2J,K, arrow; *n*=8/17) and a reduction in the overall size of the myocardium by 34 hpf (Fig. 2L,M; *n*=14/17). These results suggest that blood and endothelial development occurred at the expense of the myocardial fate.

Scl/Tal1 and Lmo2-induced haemangioblasts differentiate into erythrocytes in a regionally restricted manner

To see whether the induced progenitors develop into erythrocytes, we examined the expression of two erythroid genes, *beta embryonic globin 1* (*βE1*) (Quinkertz and Campos-Ortega, 1999) and *alas2* (Brownlie et al., 1998), in *scl/tal1-lmo2*-injected embryos. At 22 hpf, erythrocytes are usually located in the ICM in the posterior midline of the embryo (Fig. 3A). In *scl/tal1-lmo2*-injected embryos, mesodermal cells expressing *βE1* and *alas2* were still restricted to the posterior part of the embryo, albeit remaining in more lateral positions (Fig. 3B, 10/10, 13/13). However, the numbers of *βE1*$^+$ cells were increased compared to uninjected embryos. This expansion of erythroid cells requires the co-injection of *lmo2* because there was only minimal expansion with *scl/tal1* alone (Gering et al., 1998) (data not shown). Therefore, although many of the early blood and endothelial progenitors induced in *scl/tal1-lmo2*-injected embryos did not become erythrocytes, some local expansion was apparent.

To characterise this expansion more precisely, we analysed the expression of the early erythroid gene *gata1* (Detrich et al., 1995), which at the 10-somite stage (14 hpf) is expressed in the lateral cells that later give rise to erythocytes (Fig. 3C, blue stain, 3 hours). Its expression normally reaches as far anterior as somite 5 or 6 (Fig. 3C, arrows), however, in *scl/tal1-lmo2*-injected embryos, *gata1* expression was expanded to somite 1 (Fig. 3D arrows; H; *n*=32/36). The lateral mesoderm adjacent to somites 1 to 5 usually expresses the pronephric gene *wt1* at the 6 somite stage (13 hpf; Fig. 3E,H) (Drummond et al., 1998; Weidinger et al., 2002). In *scl/tal1-lmo2*-injected embryos, *wt1* expression was reduced at the same stage (Fig. 3F,G,H; *n*=8/14) suggesting that erythropoiesis had been induced at the expense of pronephros development. As seen for the later erythroid markers, *gata1* expression was excluded from ectopic *fli1*$^+$ cells in the head (Fig. 4D, green arrow), the heart (Fig. 4D, bracket) and the SPM (Fig. 4D, black arrow) (*n*=12).

The expression pattern of *gata1* in *scl/tal1-lmo2*-injected embryos was also 5-7 cells wide instead of 2-4 cells wide (Fig. 3C,D,H; *n*=33/36). *gata1* expression usually only overlaps the medial aspect of the *fli1* expression pattern (Fig. 4B, blue arrow), but in injected embryos it was expanded into the lateral aspect (Fig. 4D, blue arrows). This lateral aspect of the *fli1* expression domain normally coincides with expression of the pronephric duct (PND) marker *pax2.1* (Fig. 4E,F) (Krauss et al., 1991), suggesting that these lateral *fli1*$^+$ cells are PND progenitors and that *gata1* expression had been induced in them by *scl/tal1-lmo2*-injection (Fig. 4I). Indeed two out of three PND markers were affected in *scl/tal1-lmo2*-injected embryos, with *pax2.1* and *pax8* (Pfeffer et al., 1998) expression reduced or missing at 14 hpf (*pax2.1*: Fig. 4G,H; *n*=15/20; *pax8*: *n*=13/19) but *lim1* appeared to be unaffected (*n*=12). These results suggest that ectopic expression of *scl/tal1* and *lmo2* in PND progenitors induces ectopic expression of erythroid genes while compromising early PND gene expression. In contrast, *scl/tal1* alone had no effect on early *pax2.1* expression, only on its maintenance in differentiating PND cells (Gering et al., 1998).

In summary, our data show that ectopic haemangioblasts induced by Scl/Tal1 and Lmo2 only undergo erythroid differentiation in pronephric mesoderm and that this correlates with the induction of *gata1* expression. Importantly, the timing of both the haemangioblast and erythroid gene expression programmes corresponds to the naturally induced cells.

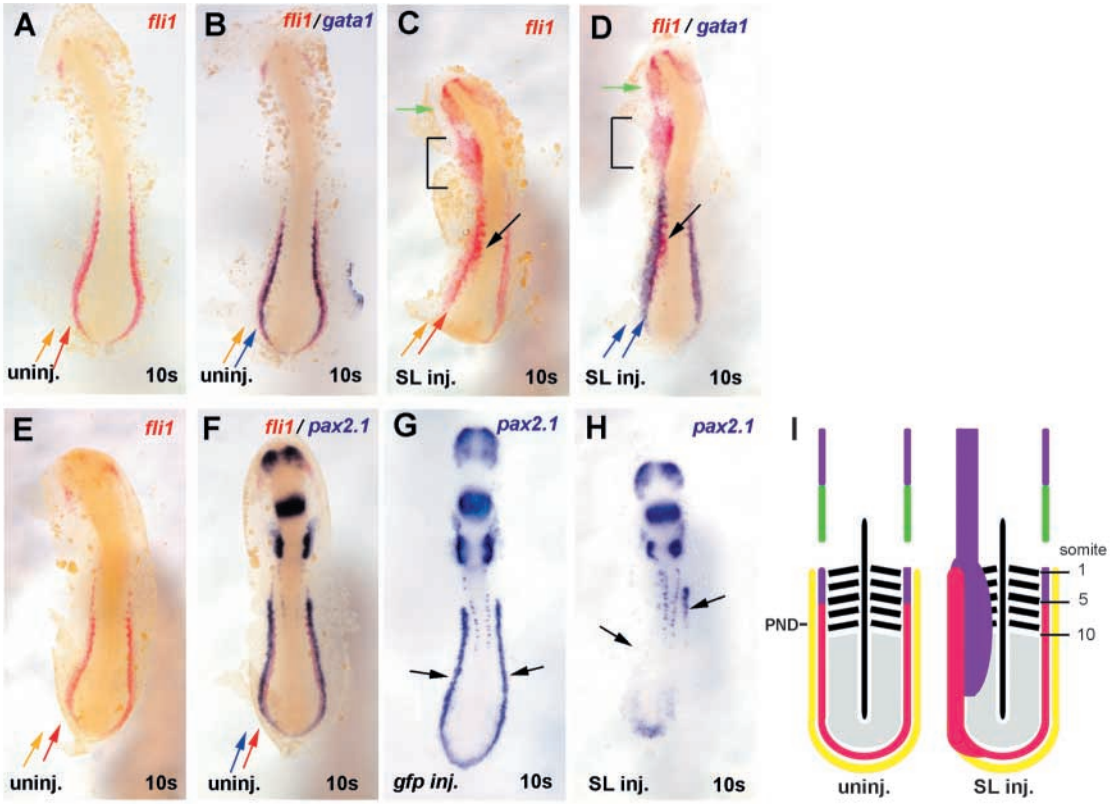

Fig. 4. Lateral expansion of erythroid development at the expense of the pronephric duct. All embryos flat mounted; anterior, top. (A,B) In uninjected embryos, *gata1* expression pattern (purple) overlapped with the stronger medial aspect of *fli1* expression (red) in the PLM (blue arrow in b). (C,D) In *scl/tal1-lmo2*-injected embryos (SL), *gata1* was excluded from the *fli1*$^{+}$ cells in the head (green arrow), heart (bracket) and somitic mesoderm (black arrow) but completely overlapped with *fli1* expression in the PLM (blue arrows), demonstrating expansion into the weaker, lateral aspect of the *fli1* domain. (E,F) In uninjected embryos, the weaker lateral aspect of the *fli1* domain overlaps with expression of the PND marker *pax2.1*. Yellow arrows in A,B,C,E indicate *fli1*$^{+}$ PND progenitors. (G,H) *pax2.1* expression was reduced in the PLM of *scl/tal1-lmo2*-injected embryos (H). (I) Schematic showing that in *scl/tal1-lmo2*-injected embryos, *gata1*$^{+}$ erythroid cells (red) form ectopically from haemangioblasts developing from PLM that normally gives rise to the PND (yellow). Ectopic haemangioblasts induced in the head, heart and somitic mesoderm (purple) do not develop into erythrocytes, showing that erythropoiesis is regionally restricted.

Gata1, Scl/Tal1 and Lmo2 can induce ectopic anterior erythropoiesis without overt ventralisation of the embryo

To determine if the addition of *gata1* to *scl/tal1* and *lmo2* could overcome the tissue restrictions to erythroid differentiation seen in our system, we co-injected mRNAs for all three transcription factors into zebrafish embryos. By 24 hpf, widespread development of round, *βE1*$^{+}$ (data not shown; *n*=13/13), *alas2*$^{+}$ (Fig. 5B, black arrowhead; *n*=13/13) and haemoglobin-containing (diaminofluorene^{+}; data not shown; *n*=10/10) cells likely to be erythrocytes was seen in the heart and head regions. Earlier, at the 10 somite stage (14 hpf), endogenous *gata1* (*gata1*$^{end.}$) also displayed ectopic anterior expression (Fig. 5E,F; *n*=9/9). Ectopic expression of *alas2* and *gata1*$^{end.}$ was not observed after injection of either *gata1* alone (n_{alas2}=20, n_{gata1}=19) or with *scl/tal1* (Fig. 5C; n_{alas2}=20, n_{gata1}=35). In fact, *gata1* injection causes morphologic malformations (Lyons et al., 2002) and often led to reduced blood marker expression (Fig. 5C). The induction of the erythroid programme is Gata1-specific because Gata1 could not be replaced by Gata2 (Fig. 5D, *n*=23) or Gata5 (data not shown; *n*=19) in this assay. The *gata2* and *gata5* mRNAs were active as *gata2* reduced *chordin* expression in the embryonic shield (*n*=7/29) (Sykes et al., 1998) and *gata5* induced a slight increase in endodermal *sox17*$^{+}$ cells at 90% epiboly (*n*=11/20) (Reiter et al., 2001; Weber et al., 2000) (data not shown).

Mead et al. (Mead et al., 2001) suggested that in *Xenopus* embryos *gata1-scl/tal1-lmo2* injection induces erythroid development through the induction of *bmp4* expression and ventralisation of the embryo. In zebrafish, however, injection of 25 pg of each mRNA neither activated ectopic *bmp4* expression at tailbud stage (10 hpf; Fig. 5G,H; *n*=20) nor obviously ventralised/posteriorised the embryo, yet was sufficient to cause anterior erythropoiesis (Fig. 5B). Head structures, as illustrated by the *pax2.1* expression pattern in the brain (Fig. 5B, red arrows), were clearly present. Only injection of 100 pg of each mRNA caused wider *bmp4* expression in half of the embryos (*n*=10/20) at the same stage. However, this expression was ventral not dorsal (Fig. 5I,J, arrowheads mark ventral expression), suggesting that embryos were retarded and displayed earlier normal expression rather than ectopic expression. Since *eve1* (Joly et al., 1993) expression in ventroposterior mesoderm was also normal (data not shown, *n*=11), we conclude that even the 100 pg-injected

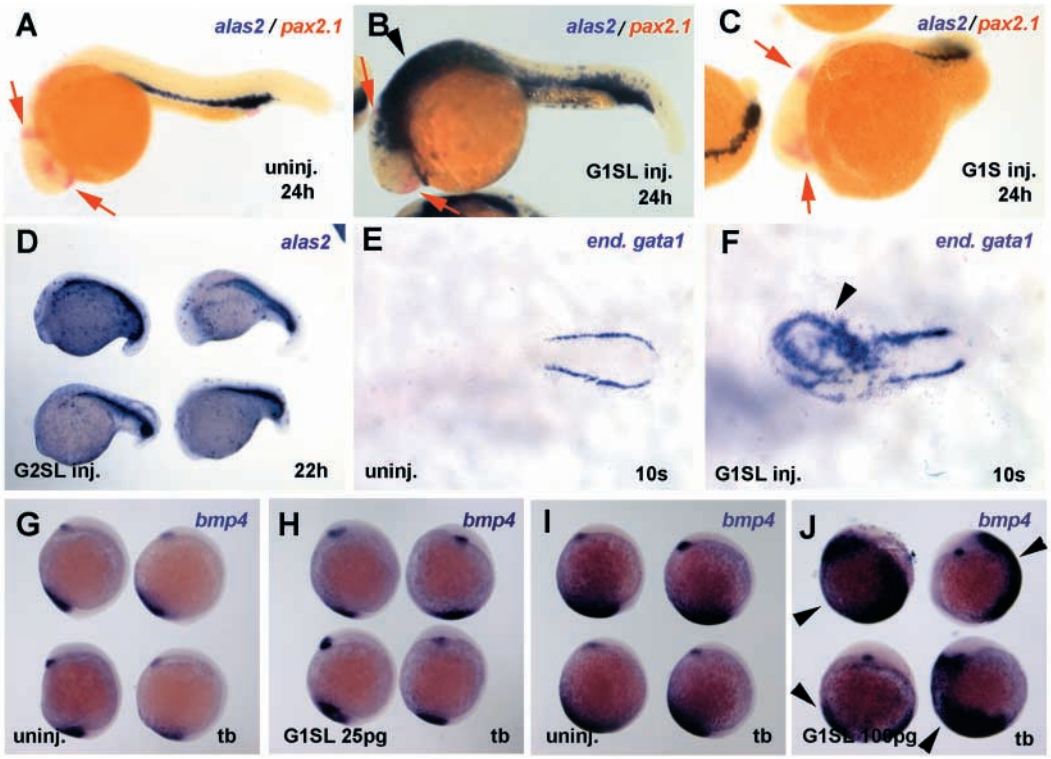

Fig. 5. Gata1-scl/tal1-lmo2 injection induced anterior erythropoiesis without causing overt ventralisation/posteriorisation. (A-D,G-J) Whole mounts; anterior, left in A-D, top in G-J. (E,F) Flat mounts; anterior, left. (A-D) *alas2* expression was restricted to posterior regions in uninjected (A) and *gata1-scl/tal1*-injected (G1S, C) embryos. *gata1-scl/tal1-lmo2* injection (G1SL, B) induced *alas2* expression in the head (black arrowheads) (A-C) Red arrows mark *pax2.1* expression in the optic stalk and in the midbrain-hindbrain boundary in the head. (D) Equimolar amounts of *gata2* mRNA (G2SL) could not replace *gata1*. (E,F) *gata1-scl/tal1-lmo2* injection (F) induced anterior expression of *gata1*$^{end.}$. (G,H) *gata1-scl/tal1-lmo2* injection (25 pg of each mRNA) induced anterior erythropoiesis without inducing *bmp4*. (I,J) At 100 pg, the three mRNAs delayed development and *bmp4* expression was maintained longer ventrally but not induced dorsally. Arrowheads in J indicate the ventral side of the embryo.

embryos were not ventralised/posteriorised, suggesting that anterior erythropoiesis was not a consequence of gross ventralisation/posteriorisation of the embryo, but rather a specific induction of the erythroid programme by Gata1 in haemangioblasts induced by Scl/Tal1 and Lmo2.

No ectopic myeloid development in scl/tal1-lmo2-injected embryos

To determine the potential of the induced haemangioblasts to make blood lineages other than erythroid, expression of early myeloid genes was examined in *scl/tal1-lmo2*-injected embryos at the 10-12 somite stage (14-14.5 hpf). The four early myeloid genes analysed, *pu.1* (Lieschke et al., 2002), *runx1* (Kalev-Zylinska et al., 2002), *c-myb* (Thompson et al., 1998) and *dra* (Herbomel et al., 1999), were expressed in the ALM that gives rise to myeloid cells (Fig. 6A,C,E,G, arrowheads). They are also expressed in the PLM caudal to somite 5, which mainly gives rise to red blood cells (Fig. 6A,C,E,G, black arrows). In addition, *dra* is expressed in early ventral mesodermal progenitors around the tail bud (Fig. 6G, green arrow). In *scl/tal1-lmo2*-injected embryos, the expression of these four myeloid genes was not dramatically expanded in the head (Fig. 6,B,D,F,H, arrowheads). *pu.1*$^{+}$ and *c-myb*$^{+}$ cells appeared disorganised (Fig. 6B,F, arrowheads; *n*=23, *n*=17), while the expression of *runx1* and *dra* was unaffected or even reduced compared to uninjected embryos (Fig. 6D,H; *n*=19, *n*=15). *c-myb* and *dra* were induced in the cardiogenic mesoderm (Fig. 6F,H, brackets; *n*=14/17, *n*=11/15), however, *runx1* and *pu.1* were not expressed there (Fig. 6B,D; *n*=0/19, *n*=0/23). In addition, the number of *L-plastin*$^{+}$ (Herbomel et al., 1999) macrophages that develop from the ALM was either unchanged or slightly reduced but never increased in 20 hpf *scl/tal1-lmo2*-injected embryos (Fig. 6I,J; *n*=18).

We also studied myeloid gene expression in the PLM. The posterior expression of *runx1*, *c-myb* and *dra* was expanded anteriorly (Fig. 6D,F,H, blue arrows; *n*=10/19, *n*=14/17, *n*=11/15) and laterally (Fig. 6D,F,H, purple arrows; *n*=13/19, *n*=13/17, *n*=11/15), but these cells also expressed *gata1*, suggesting that they were undergoing erythroid rather than myeloid differentiation. Furthermore, Pu.1, a transcription factor known to interfere with erythropoiesis (Rekhtman et al., 1999; Zhang et al., 2000), did not display a similar expansion of its expression pattern (Fig. 6B, black arrow). Interestingly, *dra* expression was also expanded in the posterior SPM (Fig. 6H, green arrow; *n*=11/15), suggesting an expansion of early ventral progenitors that have not yet switched on early blood and endothelial genes like *scl/tal1*, *lmo2* and *fli1* (Fig. 6H and Fig. 1Ad-i). We conclude that regional controls over myeloid differentiation are not over-ridden by expression of *scl/tal1* and *lmo2*.

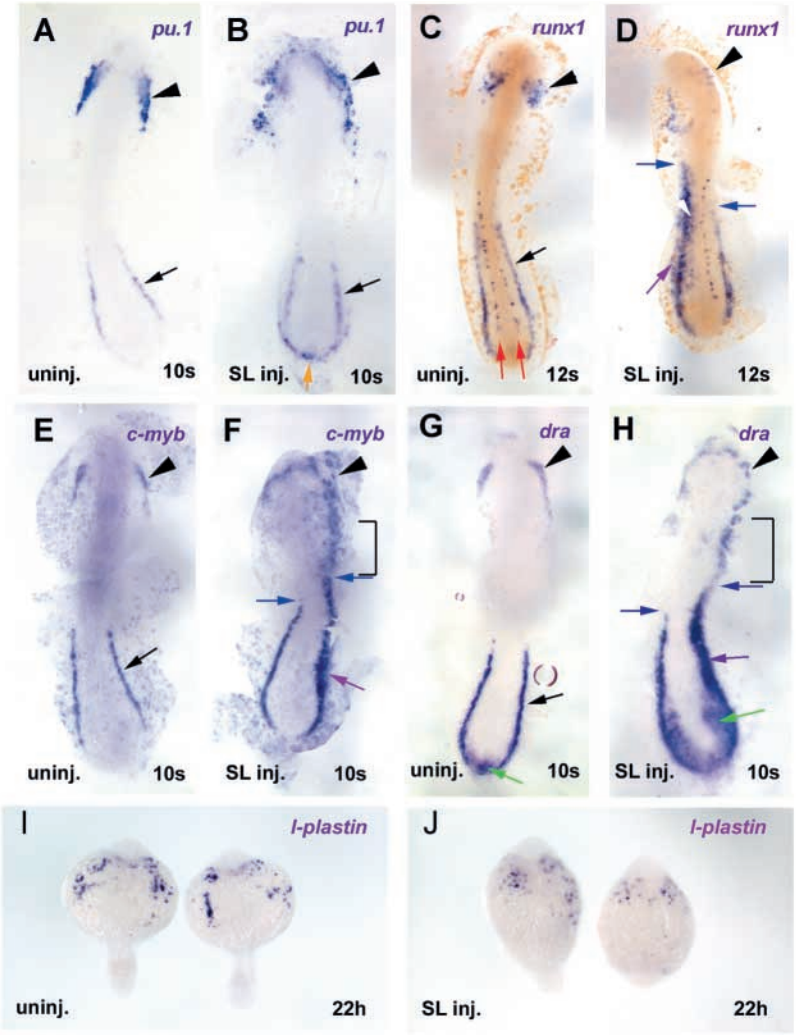

Fig. 6. The number of myeloid cells is not increased in *scl/tal1-lmo2*-injected embryos. (A-H) Flat mounts; anterior, top. (I,J) Anterior views of whole mounts with dorsal towards the top. (A-H) Some but not all myeloid genes were ectopically activated in *scl/tal1-lmo2*-injected (SL inj.) embryos. Arrowheads indicate gene expression in anterior myeloid progenitors (A-H). The orange arrow indicates ectopic *pu.1* expression around the tailbud (B). Red arrows indicate *runx1*$^{+}$ Rohon-Beard sensory neurons (C). Brackets indicate ectopic gene expression in the heart region (F,H). Black arrows indicate normal gene expression in the PLM (A-C,E,G). Blue arrows indicate the anterior gene expression limit in the PLM in *scl/tal1-lmo2*-injected embryos (D,F,H). Purple arrows indicate laterally expanded gene expression in the PLM (D,F,H). White arrow indicates ectopic *runx1* expression in the anterior SPM (D). Green arrows indicate the expansion of *dra*$^{+}$ cells in the posterior SPM (G,H). (I,J) The number of mature *L-plastin*$^{+}$ myeloid cells was not increased in *scl/tal1-lmo2*-injected embryos.

Widespread endothelial differentiation in *scl/tal1-lmo2*-injected embryos

In the absence of erythroid or expanded myeloid differentiation in the head and heart mesoderm, and in the SPM in *scl/tal1-lmo2*-injected embryos, we found that the expression of the early endothelial genes *flk1* (Fouquet et al., 1997; Liao et al., 1997; Sumoy et al., 1997) and *flt4* (Thompson et al., 1998) was expanded in the head (Fig. 7C,F, arrowheads; *n*=20/20, *n*=15/19), into the heart mesoderm (Fig. 7C,F, brackets; *n*=20/20, *n*=6/19) and into the SPM (Fig. 7C,F; arrows; *n*=14/20, *n*=15/19) at 10 somites (14 hpf) (Fig. 7N). While the expansion into the head and heart mesoderm required the co-injection of *lmo2* with *scl/tal1*, the latter alone was sufficient to induce *flk1* and *flt4* in the SPM (Fig. 7B,E, arrows; N).

The endothelial nature of these cells was further substantiated by two findings. Firstly, we found ectopic expression of the late endothelial genes *tie1* (data not shown) and *tie2* (Lyons et al., 1998) in the somitic mesoderm of *scl/tal1*-injected embryos (Fig. 7G,H; *n*=11/15) and all along the anteroposterior axis of *scl/tal1-lmo2*-injected embryos (Fig. 7G,I-K; *n*=17/17) at 22 hpf. This suggests that many of the ectopic *flk1*$^{+}$ and *flt4*$^{+}$ cells identified in injected embryos at 14 hpf differentiated into *tie1*- and *tie2*-expressing ECs by 22 hpf. Secondly, these cells appeared to have lost their haemangioblast character in that they no longer expressed *scl/tal1*$^{end.}$ (Fig. 7K,M). While anterior ectopic *tie2* expression was prominent (Fig. 7K; arrowhead), ectopic expression of *scl/tal1*$^{end.}$ was very limited (Fig. 7L,M). The posterior expression pattern of *scl/tal1*$^{end.}$ closely resembled that of *βE1* (Fig. 3B) in that it marked ventrolateral cells that had not migrated towards the midline (Fig. 7M, red arrows). By contrast, in the same anteroposterior position, *tie2* expression was found in the midline (Fig. 7K, red arrow). The only region of the injected embryo where *scl/tal1*$^{end.}$ and *tie2* appeared to be co-expressed ectopically was in the posterior SPM (Fig. 7K,M, brackets). We assume that these cells represent the less mature descendants of the expanded posterior, *dra*$^{+}$, ventral mesodermal progenitors that we identified at 14 hpf (Fig. 6H, green arrow). Thus, we conclude that many of the early progenitors identified at 14 hpf develop into ECs. These results suggest that in the absence of haematopoietic induction cues, the endothelial transcription programme matures and becomes stabilised in Scl/Tal1-Lmo2-induced haemangioblasts.

Discussion

In this report, we show that the transcription factors Scl/Tal1 and Lmo2 can synergistically act to specify haemangioblasts from non-axial mesoderm in zebrafish embryos. Our data thereby identify a potential novel role for Lmo2-Scl/Tal1-containing transcription factor complexes in early post-gastrula mesoderm, additional to their already established functions in erythropoiesis and leukaemogenesis. We show that while ectopic expression of *scl/tal1* is sufficient to induce ectopic expression of early blood and endothelial genes in the somitic paraxial mesoderm (SPM), induction in other parts of the mesoderm requires the co-injection of *lmo2* mRNA. In the presence of Lmo2, Scl/Tal1 induces early haematopoietic and endothelial transcription programmes (including the genes *fli1*, *scl/tal1*$^{end.}$ and *lmo2*$^{end.}$) throughout the lateral mesoderm, in a

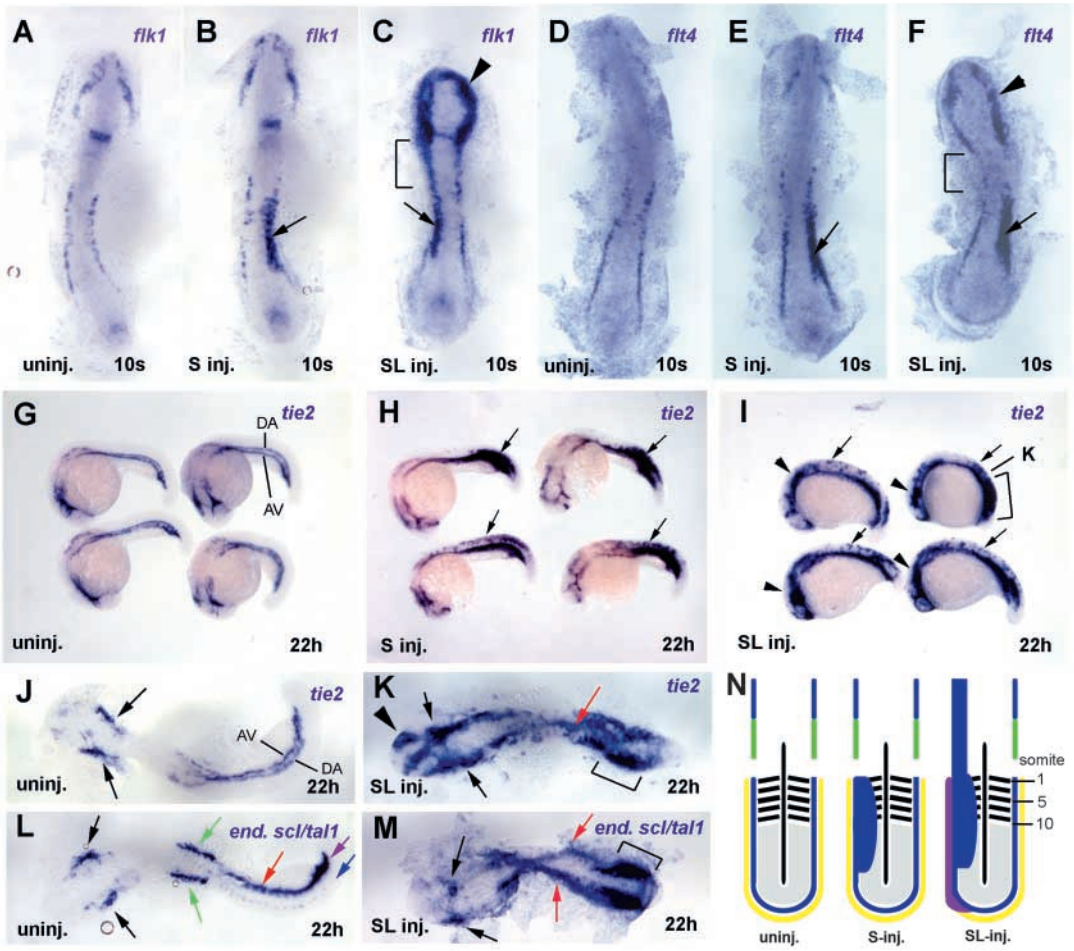

Fig. 7. The number of endothelial progenitors is increased in *scl/tal1-lmo2*-injected embryos. (A-F,J-M) flat mounts. Anterior, top in A-F, left in J-K. (G-I) Whole mounts; anterior, left. (N) Schematic of results. (A-F) In *scl/tal1*-injected (S inj.) 10 somite (14 hpf) embryos, the endothelial genes *flk1* and *flt4* were ectopically expressed only in the SPM (B,E; arrows). In *scl/tal1-lmo2*-injected (SL inj.) embryos, *flk1* showed strong ectopic expression in the head (C, arrowhead) and in the heart region (C, bracket), while *flt4* was expressed in a wider domain in the head (F, arrowhead) that was expanded posteriorly into the heart region, leaving a smaller gap between the anterior and posterior *flt4*$^{+}$ domains (F, bracket). (G-I) At 22 hpf, the late endothelial marker *tie2* was expressed in all ECs including the cells of the dorsal aorta (DA) and axial vein (AV, G). *Tie2* was ectopically expressed only in the posterior of *scl/tal1*-injected (S) embryos (H, arrows), but all along the anteroposterior axis in *scl/tal1-lmo2*-injected embryos (I, arrows and arrowheads). (J-M) Most of the *tie2*$^{+}$ cells were *scl/tal1*$^{end.}$ negative, suggesting that they were differentiating ECs. *tie2* (J) was normally expressed in all ECs of the axial vessels (DA and AV) and the head (black arrows), *scl/tal1* (L) is expressed in erythrocytes (red arrow), blood and endothelial progenitors of the tail (purple arrow), ECs of the head (black arrows) and the cardinal veins (green arrows), as well as in ventral neurons of the spinal cord (blue arrow). Ectopic expression of *tie2* was widespread anteriorly (K, arrowhead), but *scl/tal1*$^{end.}$ expression was limited to its normal expression in bilateral patches in the head mesoderm (L,M, black arrows). In the trunk, *scl/tal1*$^{end.}$ was expressed in ventrolateral cells (M, red arrows) similar to the *βE1*$^{+}$ blood cells. By contrast, *tie2* expression was found medially (K, red arrow) as well as dorsally (I, black arrows). Only posteriorly, did co-expression mark early blood and endothelial progenitors (I,K,M, brackets). (N) In the absence of a blood-inducing intrinsic/extrinsic factor, Scl/Tal1- and Scl/Tal1-Lmo2-induced ectopic haemangioblasts in the head, heart and somitic mesoderm express *flk1* and *flt4* (blue) and develop into ECs, suggesting that endothelial development is the default state.

wider domain in the head and in the SPM. Such a role is consistent with the co-expression of these two genes in the ALM and the PLM which are thought to harbour haemangioblasts.

Differential competence of mesodermal tissues

In *scl/tal1-lmo2*-injected embryos, ectopic blood and endothelial development occurs at the expense of other mesodermal tissues, in particular the somitic, pronephric and cardiogenic mesoderm. Scl/Tal1 and Lmo2 appear to interfere with the endogenous transcription programmes, activating early blood and endothelial genes instead. Since Scl/Tal1 and Lmo2 activate these genes synergistically, and since they are known to form transcription factor complexes (Rabbitts, 1998), we assume that gene activation involves recruitment of components of the complex endogenously. Absence of such endogenous transcription factors is likely to render ectopic Scl/Tal1 and Lmo2 ineffective. Obvious candidates are proteins already known to bind Scl/Tal1 and Lmo2, such as the E proteins and Gata factors, respectively. Their absence might explain why the notochord is always unaffected (data not shown).

Repression of endogenous transcription programmes could result from activation of repressors or direct repression by Scl/Tal1-Lmo2 complexes. Alternatively, Scl/Tal1 and Lmo2 could scavenge transcription factors that are essential for an alternative transcription programme. Scl/Tal1 is believed to scavenge E proteins normally required to interact with myogenic bHLH proteins (Goldfarb and Lewandowska, 1995; Hofmann and Cole, 1996) or for the expression of E protein target genes in pre-leukaemic T-cells, requiring Lmo1/2 in the latter context (Herblot et al., 2000; Larson et al., 1996). Gata factors are known to interact with Lmo2 and other Lim-only proteins (Chang et al., 2003; Osada et al., 1995). In the PLM, *gata1* and *gata2* are expressed. In the SPM, *gata2* is induced ectopically by injection *of scl/tal1* and *scl/tal1-lmo2* (M.G. and R.K.P., unpublished). In the ALM, *gata2* is expressed together with *gata4*, *5* and *6*, which are also expressed in the cardiogenic mesoderm (Reiter et al., 1999; Rodaway et al., 1999) (A. Gibson and R.K.P., unpublished). Although *scl/tal1-lmo2* injection down-regulates *gata4* and *6* in the heart region, *gata5* expression is almost unchanged. It is possible that Scl/Tal1 and Lmo2 recruit heart Gata factors, leading not only to induction of the early blood and endothelial transcription programmes but also to a blockade of heart-specific gene expression. It remains to be seen whether Gata4, 5 and 6 can interact with Lmo2 and which interaction partners Scl/Tal1 and Lmo2 require in the various mesodermal tissues in which they can ectopically switch on blood and endothelial genes.

While induction of the haemangioblast programme requires both Scl/Tal1 and Lmo2 in the head and the heart mesoderm, injection of *scl/tal1* mRNA is sufficient to induce it in the SPM. We show here that only in this tissue can Scl/Tal1 induce ectopic *lmo2* expression. This suggests that once *lmo2* is activated, the two partners can synergistically drive the expression of genes like *fli1*, *flk1*, *flt4*, *tie1* and *tie2*. It does, however, not explain why Scl/Tal1 can induce *lmo2* in the SPM in the first place. It is possible that a Lmo2 paralogue is expressed in the SPM and assists Scl/Tal1. Alternatively, Scl/Tal1 could simply scavenge E proteins. This might be enough to abrogate somitic development given the vast array of bHLH proteins it depends on and, in the absence of other developmental options, SPM cells may undergo endothelial development. It should be noted that in mammalian and avian embryos endothelial development occurs from somites after the onset of blood circulation (Ambler et al., 2001; Beddington and Martin, 1989; Pardanaud et al., 1996; Wilting et al., 1995). It is therefore possible that ectopic *scl/tal1* expression induces earlier expression of the intrinsic endothelial programme.

Gata1, Scl/Tal1 and Lmo2 can induce anterior erythropoiesis without overt ventralisation of the embryo

Erythropoiesis only proceeds from Scl/Tal1-Lmo2-induced haemangioblasts located in the PLM, not from the heart, head or somitic mesoderm. This correlates with where *gata1* expression was induced. Consistent with a previous study (Mead et al., 2001), we show that co-injection of Scl/Tal1, Lmo2 and Gata1 causes widespread induction of erythropoiesis. However, we also demonstrate that this is due to relief of the posterior restriction, leading to induction of *gata1*$^{end.}$ expression and erythropoiesis anteriorly. We additionally show that other Gata factors, in particular Gata2, cannot replace Gata1. Furthermore, *gata1-scl/tal1-lmo2*-injected embryos are not ventralised/posteriorised as *eve1* expression is unchanged and *bmp4* expression is not obviously activated, suggesting that *gata1-scl/tal-lmo2*-injection can induce anterior erythropoiesis downstream of the dorsal-ventral patterning of the mesoderm.

In our hands, injection of *gata1* alone was not sufficient to induce either anterior *gata1*$^{end.}$ expression or head erythropoiesis. This result conflicts with a recent paper showing that injected *gata1* mRNA can induce expression of a *gfp* reporter gene under the control of the *gata1* promoter in transgenic zebrafish (Kobayashi et al., 2001). We assume that the difference lies with the transgenic reporter construct used or its chromosomal integration site. Nevertheless, our data confirm that Gata1 can auto-regulate its own expression, albeit with the help of Scl/Tal1 and Lmo2.

We observed no increase in the number of myeloid cells in *scl/tal1-lmo2*-injected embryos, whereas erythropoiesis was expanded laterally and anteriorly. The anterior expansion occurred into the region of the pronephros adjacent to somites 1-5. This is surprising given that *scl/tal1* and *lmo2* are expressed in this region at the 10 somite stage (14 hpf). However, at the 6 somite stage (13 hpf), they are not expressed in this part of the PLM but are restricted to the PLM posterior to somite 6 (data not shown). Whether their more anterior expression at the 10-somite stage is a result of cell migration or de novo expression of *scl/tal1* and *lmo2* is currently unknown. Nevertheless, our data argue that premature/ectopic expression of *scl/tal1* and *lmo2* in the PLM adjacent to somites 1-5 is sufficient to induce ectopic erythropoiesis in a region of the embryo that normally forms the pronephros.

The lateral expansion occurred at the expense of the PND. We showed previously that PND development was compromised in *scl/tal1*-injected embryos, however, initial expression of the PND gene *pax2.1* was normal at the 10-somite stage and only displayed gaps by 22 hpf (Gering et al., 1998). In contrast, in *scl/tal1-lmo2*-injected embryos, *pax2.1* and *pax8* expression was already lost at the 10-somite stage. Thus, Scl/Tal1 and Lmo2 together are affecting the PND-specific transcription programme at a much earlier stage than Scl/Tal1 on its own. Loss of *pax2* and *pax8* in the mouse completely abrogates pronephric development (Bouchard et al., 2002). Interestingly, *lim1* expression was not lost in the PLM of *scl/tal1-lmo2*-injected embryos. In the mouse embryo, *lim1* appears to be a competence factor that marks the territory competent for pronephric induction. Its expression is initially independent of *pax2/8* which is consistent with its continued expression in *scl/tal1-lmo2*-injected zebrafish embryos. The continued expression of *lim1* further suggests that Scl/Tal1 and Lmo2 have no negative influence on its expression and that *lim1* expression does not interfere with erythropoiesis.

Is endothelial differentiation the default fate of haemangioblasts?

Scl/Tal1 and Lmo2 activated some but not all blood genes in the heart, head and somitic mesoderm. In the absence of induction of the complete myeloid or erythroid transcription programmes, blood cells did not develop from these tissues. Instead, the cells first switched on *flk1* and *flt4*, later expressed *tie1* and *tie2* and eventually down-regulated *scl/tal1*^{end}. Thus, many Scl/Tal1-Lmo2-induced progenitors appeared to

differentiate into ECs, suggesting that endothelial development may be the default fate of the haemangioblast.

A role for Lmo2 and Scl/Tal1 in specifying normal haemangioblasts

Since yolk sac ECs develop in *lmo2*$^{-/-}$ (Warren et al., 1994; Yamada et al., 2000) and *scl/tal1*$^{-/-}$ (Elefanty et al., 1999; Visvader et al., 1998) mice, it would appear that Lmo2 and Scl/Tal1 are dispensible for angioblast formation and only confer haematopoietic competence to the mesodermal tissue they are expressed in. However, results from studies on *scl/tal1*$^{-/-}$ ES cells (Chung et al., 2002; Faloon et al., 2000; Robertson et al., 2000) and the absence of contributions to adult haematopoiesis or angiogenesis by *lmo2*-null ES cells in chimeric mice (Yamada et al., 1998; Yamada et al., 2000), suggest that these proteins are needed for correctly programmed haemangioblast formation. Haemangioblast-like BL-CFCs derived from ES cells are highly enriched in the *scl/tal1*-positive fraction of Flk1^{+} cells (Chung et al., 2002), and BL-CFCs do not form in the absence of a functional *scl/tal1* gene (Faloon et al., 2000; Robertson et al., 2000). Ubiquitous *scl/tal1* expression in differentiating ES cells increases the number of Flk1^{+} cells and subsequently the number of primitive blood progenitors among the Flk1^{+} cells as well as the number of VE-Cadherin^{+} definitive blood progenitors (haemogenic ECs) (Endoh et al., 2002). *scl/tal1* also negatively influences smooth muscle cell differentiation from early ES cell-derived Flk1^{+} cells in favour of more haemangioblasts (Ema et al., 2003). However, ECs do develop from *scl/tal1*$^{-/-}$ ES cells (Faloon et al., 2000; Robertson et al., 2000) and from *lmo2*$^{-/-}$ ES cells (M. M. McCormack and T.H.R., unpublished) indicating that endothelial development from ES cells can occur independently of haemangioblast formation and of *lmo2* or *scl/tal1* expression. Yet, ECs formed in the absence of *lmo2* or *scl/tal1* are not normal as they fail to form a primary plexus or undergo vascular remodelling in the embryo (Elefanty et al., 1999; Visvader et al., 1998; Yamada et al., 2000; Yamada et al., 2002). The molecular basis of these defects is not known. Interestingly, rescue experiments show that primitive as well as definitive HCs only form from *scl/tal1*$^{-/-}$ ES cells if Scl/Tal1 function is restored at the stage when the cells are still of mesodermal character, suggesting that even the haemogenic ECs that give rise to definitive HCs need to have experienced *scl/tal1* expression long before they give rise to HCs (Endoh et al., 2002). These results suggest that the early lack of Lmo2 or Scl/Tal1 in the mesoderm can have very late consequences in the endothelial lineage and the data presented here support a model whereby these two factors cooperate to specify normal haemangioblasts from early mesodermal progenitors.

R.K.P., M.G., T.H.R. and Y.Y. were supported by the Medical Research Council and Y.Y. also by the National Foundation for Cancer Research. We would like to thank A. Forster for his help in this work and R. F. Bachvarova, A. Ciau-Uitz, A. D. Johnson, M. Walmsley and the reviewers for critical comments on the manuscript. We are grateful to T. Mohun, A. R. F. Rodaway, M. O'Reilly and A. Gibson for expression constructs. We would also like to thank L. Zon, M. Hammerschmidt, B. Thisse, M. Fishman, D. Stainier, G. Lieschke, P. Ingham, M. Busslinger, I. Drummond, S. Wilson, J. Campos-Ortega and K. Crosier for probes.

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