Development 124, 381-389 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV4725

The zebrafish gene *cloche* acts upstream of a *flk-1* homologue to regulate endothelial cell differentiation

Wayne Liao[1], Brent W. Bisgrove[2], Holly Sawyer[1], Barbara Hug[2], Bridgit Bell[3], Kevin Peters[3], David J. Grunwald[2] and Didier Y. R. Stainier[1,*]

[1]Department of Biochemistry and Biophysics and Program in Developmental Biology, University of California, San Francisco, San Francisco, CA 94143-0554, USA
[2]Department of Human Genetics, Eccles Institute of Genetics, University of Utah, Salt Lake City, UT 84112, USA
[3]Duke University Medical Center, Box 3523, Durham, NC 27710, USA

e-mail: Didier_Stainier@biochem.ucsf.edu

SUMMARY

The zebrafish *cloche* mutation affects both the endothelial and hematopoietic lineages at a very early stage (Stainier, D. Y. R., Weinstein, B. M., Detrich, H. W., Zon, L. I. and Fishman, M. C. (1995). *Development* 121, 3141-3150). The most striking vascular phenotype is the absence of endocardial cells from the heart. Microscopic examination of mutant embryos reveals the presence of endothelial-like cells in the lower trunk and tail regions while head vessels appear to be missing, indicating a molecular diversification of the endothelial lineage. Cell transplantation experiments show that *cloche* acts cell-autonomously within the endothelial lineage. To analyze further the role of *cloche* in regulating endothelial cell differentiation, we have examined the expression of *flk-1* and *tie*, two receptor tyrosine kinase genes expressed early and sequentially in the endothelial lineage. In wild-type fish, *flk-1*-positive cells are found throughout the embryo and differentiate to form the nascent vasculature. In *cloche* mutants, *flk-1*-positive cells are found only in the lower trunk and tail regions, and this expression is delayed as compared to wild-type. Unlike the *flk-1*-positive cells in wild-type embryos, those in *cloche* mutants do not go on to express *tie*, suggesting that their differentiation is halted at an early stage. We also find that the *cloche* mutation is not linked to *flk-1*. These data indicate that *cloche* affects the differentiation of all endothelial cells and that it acts at a very early stage, either by directly regulating *flk-1* expression or by controlling the differentiation of cells that normally develop to express *flk-1*.

***cloche* mutants also have a blood deficit and their hematopoietic tissues show no expression of the hematopoietic transcription factor genes GATA-1 or GATA-2 at early stages. Because the appearance of distinct levels of *flk-1* expression is delayed in *cloche* mutants, we examined GATA-1 expression at late embryonic stages and found some blood cell differentiation that appears to be limited to the region lined by the *flk-1*-expressing cells. The spatial restriction of blood in the ventroposterior-most region of *cloche* mutant embryos may be indicative of a ventral source of signal(s) controlling hematopoietic differentiation. In addition, the restricted colocalization of blood and endothelium in *cloche* mutants suggests that important interactions occur between these two lineages during normal development.**

Key words: endothelium, receptor tyrosine kinase, *tie*, hematopoiesis, zebrafish

INTRODUCTION

Endothelial cells are the first cells to differentiate in the cardiovascular system; as such, they play an essential role in the formation and patterning of the vasculature in all vertebrates (Noden, 1991a; Poole and Coffin, 1991; Risau, 1991). The mesodermally derived endothelial cells and their progenitors, the angioblasts, are found in both extraembryonic and intraembryonic locations. On the yolk sac of most vertebrates, angioblasts in close association with hematopoietic progenitors form structures known as blood islands. Within the embryo itself, angioblasts give rise initially to the major blood vessels of the trunk, the dorsal aorta and axial vein, as well as to the endocardium of the heart. The *in situ* differentiation of angioblasts followed by their assembly into vascular channels, as observed in the trunk, is referred to as vasculogenesis.

The molecular mechanisms that regulate endothelial cell differentiation and vasculogenesis have remained largely unknown. The recent identification of two subfamilies of receptor tyrosine kinases whose expression is virtually restricted to endothelial cells and their progenitors, however, has provided an exciting entry point into these processes (reviewed by Mustonen and Alitalo, 1995). These receptor tyrosine kinases consist of the members of the vascular endothelial growth factor (VEGF) receptor family, namely Flk-1 and Flt-1, and the Tie (aka Tie-1) and Tek (aka Tie-2) orphan

receptors. Mutational studies of Flk-1, Flt-1, Tie and Tek in the mouse indicate that these receptors play critical roles in vascular development (Dumont et al., 1994; Fong et al., 1995; Puri et al., 1995; Sato et al., 1995; Shalaby et al., 1995). Analyses of homozygous mutant mice indicate that Flk-1 is required for the formation of yolk-sac blood-islands and intraembryonic blood vessels as well as for hematopoiesis (Shalaby et al., 1995), whereas Flt-1 plays an essential role in regulating the assembly of vascular endothelium (Fong et al., 1995). Tek is also expressed in angioblasts and is required for vascular formation including angiogenesis (Dumont et al., 1994; Sato et al., 1995), a process defined as the sprouting of new blood vessels from pre-existing ones. Tie, which is expressed at later stages of endothelial cell differentiation, is required for the integrity and survival of endothelial cells (Puri et al., 1995; Sato et al., 1995). However, unlike Flk-1 or Flt-1, Tie does not seem to be essential for vasculogenesis.

We have recently initiated an effort to dissect the differentiation of the cardiovascular system using a genetic approach in the zebrafish (Stainier and Fishman, 1994), and have identified several mutations that appear to affect endothelial cell differentiation (Stainier et al., 1996). The *cloche* mutation is most notable in that it affects both the endothelial and hematopoietic lineages (Stainier et al., 1995). The endocardium is missing in *cloche* mutant embryos, but a small subset of endothelial cells, i.e. those lining the lower trunk and tail vessels, appear morphologically normal under the light microscope. In order to analyze further the endothelial defect in *cloche*, we have cloned the zebrafish homologues of *flk-1* and *tie* and examined their expression in *cloche* mutants. We find that despite the similarity of the *cloche* and *flk-1* mutant phenotypes, *cloche* and *flk-1* are not linked and are thus different genes. Furthermore, we find that only cells in the lower trunk and tail regions of *cloche* mutant embryos express *flk-1*, but these cells do not go on to express *tie*. These data indicate that the *cloche* mutation deletes most endothelial cells and that the few endothelial cells that do differentiate to express *flk-1* are blocked early in their differentiation pathway. Thus, *cloche* appears to affect endothelial cell differentiation by acting upstream of *flk-1* either directly or indirectly.

MATERIALS AND METHODS

Zebrafish embryos

Zebrafish were raised and handled as described by Westerfield (1993). Developmental time at 28.5°C was determined from the morphological features of the embryo (Kimmel et al., 1995). The original *cloche* allele, clo^{m39} (Stainier et al., 1995), is a spontaneous allele identified in a semi-wild population from an Indonesian fish farm. This mutation is fully penetrant and exhibits complete and indistinguishable expressivity in a variety of genetic backgrounds tested. An ENU allele (clo^{m378}; Stainier et al., 1996), was used in this study (except for the linkage analysis where clo^{m39} was used). Like clo^{m39}, clo^{m378} is fully penetrant and exhibits complete and indistinguishable expressivity in a variety of backgrounds. In addition, clo^{m39} and clo^{m378} mutants are indistinguishable at both the cellular and molecular level further suggesting that these may represent null alleles.

Whole-mount in situ hybridization

In situ hybridizations were performed using digoxigenin-labeled RNA probes essentially as described by Oxtoby and Jowett (1993). *clo* mutant embryos are easily distinguished from wild-type by 24 hours postfertilization (hpf). To stain for *flk-1* expression at earlier stages, pools of 20-30 embryos were examined, revealing a clear 3:1 segregation of the embryos based on their staining pattern. After 24 hpf, at least two wild-type embryos were always added to the sorted mutant embryos to serve as positive controls. Two different clones were used to probe for *flk-1* expression: the original clone covers part of the tyrosine kinase domain and extends to the end of the 3′ untranslated region (Fig. 1); a second clone was obtained from Len Zon (Harvard Medical School) and covers most of the coding region (M. A. Thompson and L. I. Zon, personal communication; manuscript in preparation). Probes derived from both clones gave exactly the same staining pattern. Embryos used for histology (Fig. 5) were overstained using a different protocol (Westerfield, 1993; M. A. Thompson and L. I. Zon, personal communication; manuscript in preparation) which calls for a 55°C hybridization (instead of the more routinely used 70°C). Histology was carried out as described (Stainier et al., 1993) and sections counterstained with nuclear fast red.

Expression patterns were documented and photographed on 160 ASA Ektachrome Tungsten film after clearing the embryos in benzylbenzoate/benzyl alcohol (2:1). Images from photographic slides were scanned on a Polaroid Sprint Scan 35 Slide Scanner. Composite figures were assembled, and contrast enhanced when necessary, using Adobe Photoshop Software (Adobe Corporation).

Linkage analysis

A *Pvu*II polymorphism in the *flk-1* gene was identified between a clo^{m39} fish and a polymorphic wild-type fish ($+^{HK}/+^{HK}$) on Southern blots using a 640 bp *Bst*XI/*Xba*I fragment of the zebrafish *flk-1* cDNA as a probe. These fish were bred, and individual F_1 fish (with the genotype $clo^{m39}/+^{HK}$) were identified as carrying the *clo* mutation and then mated to obtain mutant embryos. Four pools of 30 mutants each were then used to analyze the segregation pattern of the *Pvu*II polymorphism.

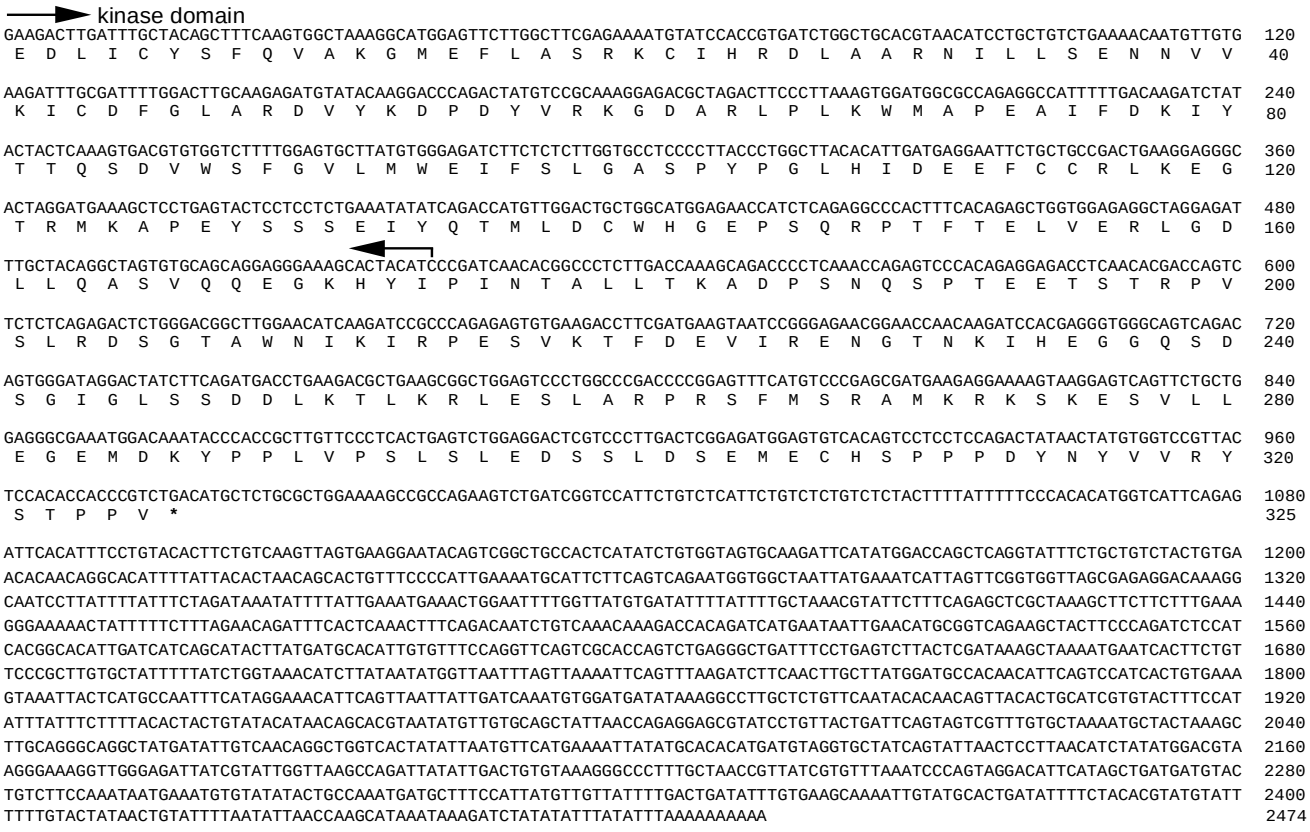
RESULTS

Isolation and expression studies of zebrafish *flk-1*

A partial *flk-1* cDNA was isolated during a PCR-based search for receptor tyrosine kinases expressed during early development (B. H., Ellen Wilson, Virginia Walter and D. J. G., unpublished). A 210 bp PCR fragment with similarity to the *flk-1* tyrosine kinase domain was used to screen a cDNA library prepared from 20-28 hpf embryos, and a 2.5 kb clone was isolated and sequenced (Fig. 1A). Sequence alignment of the protein encoded by this clone versus human and mouse Flk-1 and its close relative Flt-1 reveals several regions of high homology (Fig. 1B), closer to Flk-1 than to Flt-1 (e.g. the tyrosine kinase domain). However, this protein also contains a small carboxy-terminal domain (15 amino acids long) that is found in Flt-1 but not in Flk-1. Further molecular studies failed to reveal the existence of another closely related gene, other than *flt-4* (M. A. Thompson and L. I. Zon, personal communication; manuscript in preparation). Thus, we presume that this gene represents an ancestral gene that gave rise to both *flk-1* and *flt-1*; and rather than giving this gene a new name, we will refer to it as *flk-1*.

The *flk-1* transcript was first detected by whole-mount in situ hybridization in embryos between the 5- and 7-somite stages; in the trunk region, two bilateral stripes of *flk-1*-positive cells appear in the lateral plate mesoderm flanking the embryonic axis (Fig. 2A,C). These two stripes of *flk-1*-expressing cells

A



B

Fig. 1. (A) Nucleotide and amino acid sequences of the 2.5 kb *flk-1* cDNA clone (GenBank, U75995). BLASTP sequence homology search with the coding sequence from this clone (or a nearly full-length clone; M. A. Thompson and L. I. Zon, personal communication; manuscript in preparation) reveals that it is most similar to both Flk-1 and Flt-1 and distantly related to Flt-4. (B) Amino acid sequence alignment of zebrafish Flk-1 (partial sequence) versus human and mouse Flk-1 and Flt-1. The arrow indicates the end of the tyrosine kinase domain; the shaded residues indicate those that are identical between all sequences and the boxed residues indicate those that are found in either Flk-1 or Flt-1 and the zebrafish protein. This alignment reveals that the zebrafish protein contains regions that exhibit higher identity with Flk-1 (e.g. the tyrosine kinase domain) as well as a small region in the carboxy terminal domain that is found only in Flt-1. Further molecular work has revealed no related genes other than *flt-4* (data not included; M. A. Thompson and L. I. Zon, personal communication; manuscript in preparation). Thus, we presume that this gene represents an ancestral gene that gave rise to both *flk-1* and *flt-1* sometime after the appearance of teleosts. Methods: The sequence homology search and alignments were performed using the BLASTP program available at the NCBI www site (http://www.ncbi.nlm.nih.gov/Recipon/bs_seq.html) (Altschul et al., 1990). Note that the same gene was also isolated and characterized by Fouquet et al. (1997).

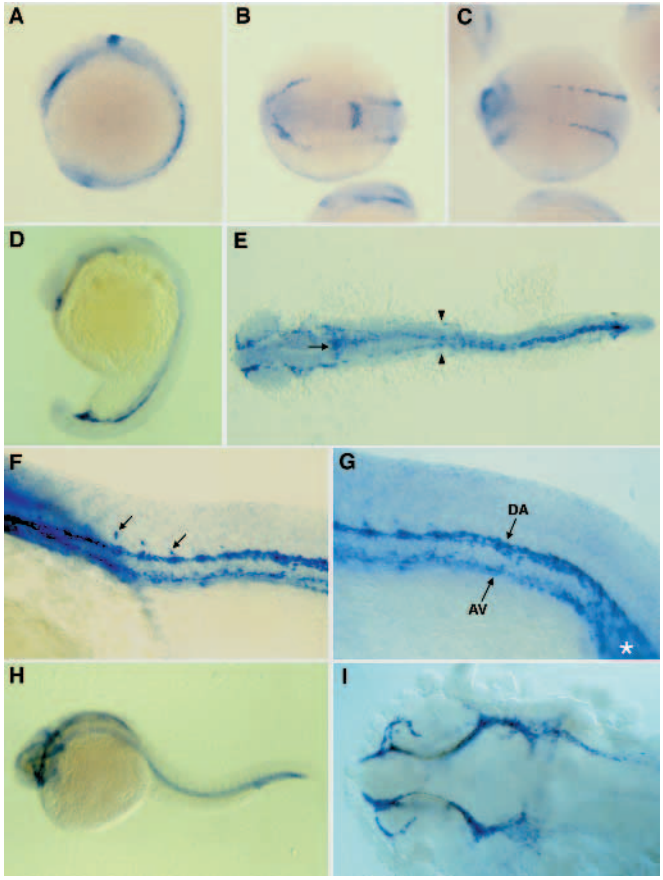


Fig. 2. Localization of *flk-1* transcripts in wild-type embryos. 7-somite stage (A-C), 20-somite stage (D,E), 24-somite stage (F,G), and 24 hpf (H,I). Embryos shown in A,D,F-H are lateral views with dorsal to the top and anterior to the left. All others are dorsal views with anterior to the left (B shows the head region, C shows the mid-trunk region; F and G show the anterior and posterior trunk regions respectively, and I shows the head region). (A-C) *flk-1*-positive cells are found in discrete bilateral stripes both anteriorly and posteriorly. There is also a transverse ectodermal stripe of staining in the hindbrain region; this staining quickly becomes weaker (see D) and although blood vessels do form there at later stages, the fate of these *flk-1*-expressing cells is not entirely clear (see also Figs 4 and 5). (D,E) *flk-1* expression appears to extend caudally from the head region as well as in both directions in the trunk region, until by the 20-somite stage there is a continuous band of *flk-1*-expressing cells from the anterior head region to the tailbud. Concurrently, *flk-1*-expressing cells in the mid- and posterior trunk regions converge medially. The site (arrowheads) where the paired lateral dorsal aortae fuse into a single medial aorta is the site of vascular breakdown in the *gridlock* mutation (Weinstein et al., 1995). At a slightly later stage, single cells (F, arrows) are starting to migrate into the intersomitic space in the upper trunk region to form the intersomitic vessels. The dorsal aorta (DA) and axial vein (AV) are clearly distinct in the trunk region at this stage, and further caudally, in the tail, there is a region of more intense staining (asterisk). [Similarly intense *flk-1* expression is also observed in the tail region of early mouse embryos (Yamaguchi et al., 1993; Shalaby et al., 1995)]. By 24 hpf (H, I), the whole vasculature including the head vessels (I) is clearly outlined by staining for *flk-1* transcript.

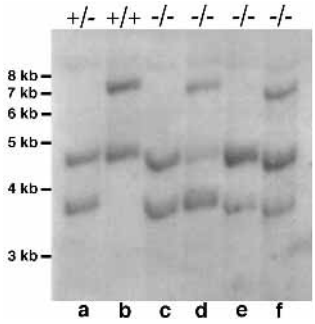


Fig. 3. Southern analysis reveals that *flk-1* and *clo* are not linked. Genomic DNA from whole fish or embryos was digested with the restriction enzyme *Pvu*II, transferred to filters and probed with a 640 bp B*st*XI/*Xba*I fragment of the zebrafish *flk-1* cDNA. The two parental fish, a clo^{m39} heterozygote (+/–) and a polymorphic wild-type fish ($+^{HK}/+^{HK}$) (+/+) display a RFLP with the wild-type fish (lane b) exhibiting a 7.4 kb band that is absent in the clo^{m39} heterozygote (lane a). The parental fish were bred and F_2 mutant embryos collected. Examination of four pools of 30 mutants each (–/–) (lanes c-f) reveals the presence of the wild-type 7.4 kb RFLP in two of the four pools, indicating that *flk-1* and *clo* are not linked.

expand rostrally and caudally as the embryo develops. In the head region, *flk-1*-positive cells appear by the 7-somite stage and align themselves in bilateral stripes which will extend caudally and presumably lay out the foundation for the head vasculature (Fig. 2B). *flk-1*-expressing cells are also found in the dorsal part of the hindbrain region (Fig. 2A,B), although this particular expression quickly weakens (Fig. 2D).

Shortly after the 13-somite stage, the trunk *flk-1* staining reaches the tailbud region. In the mid-trunk lateral plate mesoderm, the *flk-1*-positive cells have begun to converge towards the ventral midline (data not included). The convergence of these cells extends caudally as the tail structure develops and the cells eventually merge into a single stripe which terminates at the ventral region of the tail (Fig. 2E). The *flk-1*-positive cells in the ventral midline later form the dorsal aorta and axial vein (see below). In the anterior region of the trunk lateral plate mesoderm, staining continues to extend rostrally (Fig. 2E). Further rostrally, in the heart region, where the primitive myocardial tubes are being assembled, staining appears between the bilateral stripes of *flk-1-expressing* cells at the 15-somite stage (data not included). By the 18-somite stage, a small but dense group of *flk-1*-positive cells is found in the midline region (Fig. 2E, arrow). We presume that this group of cells gives rise to the endocardial cells (Stainier et al., 1993), while the transverse stripes of *flk-1*-positive cells on either side of the endocardial progenitors likely form the endothelium lining the first aortic arches, which connect the outflow tract of the heart to the arterial vasculature (Rieb, 1973).

At the 20-somite stage, the *flk-1*-positive cells from the anterior trunk region meet the *flk-1*-positive cells from the head (Fig. 2E). By the 24-somite stage, the dorsal aorta (DA) and axial vein (AV) are clearly distinct in the trunk region (Fig. 2F,G). Some *flk-1*-positive cells emerge between the somites, apparently sprouting from the dorsal aorta (Fig. 2F,G). These intersomitic vessels are being formed by angiogenesis. At 24 hpf, when the basic vascular system of the whole embryo has been laid out and circulation can be observed, *flk-1* clearly labels all the endothelial cells lining the vasculature (Fig. 2H,I). On the basis of these staining patterns it appears that, at least until 36 hpf (data not included), all endothelial cells express *flk-1*.

The *clo* gene does not encode zebrafish Flk-1

Because of the similarity between Flk-1 deficient mice and

zebrafish *clo* mutants, we wanted to analyze linkage between zebrafish *flk-1* and *clo*. We initially identified a *Pvu*II polymorphism in the *flk-1* gene between a clo^{m39} fish and a polymorphic wild-type fish ($+^{HK}/+^{HK}$) and followed the segregation of this polymorphism in a mapping cross. Fig. 3 shows that the 7.4 kb wild-type allele is present in two of the four batches of mutant embryos (30 embryos per batch), thus demonstrating that *flk-1* and *clo* are not linked.

The *clo* mutation affects the generation of most endothelial cells

We used *flk-1* as a molecular marker for endothelial cells and their progenitors to characterize further the *clo* mutant phenotype. Up to the 20-somite stage, the *flk-1*-positive cells found in the anterior head region and trunk lateral plate mesoderm of wild-type embryos appear to be missing in *clo* mutants (Fig. 4A). At the 20-somite stage, faint staining is observed in the tail region, near the caudal end of the yolk extension (Fig. 4B, large arrow). This staining becomes more distinct with time yet is always much weaker than that seen in wild-type siblings (Fig. 4C-G). In 30 hpf wild-type embryos, the endothelial cells of the entire vasculature, including the blood vessels in the head, dorsal aorta and axial vein in the trunk, clearly express *flk-1* (Fig. 4D,E). In *clo* mutant embryos, however, *flk-1* message is detected only in the lower trunk and tail regions (Fig. 4F,G). Examination of 36 hpf living mutant embryos with Nomarski optics reveals the presence of endothelial-like cells exclusively in this region (see Fig. 3B in Stainier et al., 1995). Thus, in *clo* mutant embryos, extensive aspects of *flk-1* expression are missing; distinct levels of endothelial expression appear around the 20-somite stage only in cells of the lower trunk and tail regions. Faint expression is also seen in the dorsal part of the hindbrain (small arrows) and the tailbud (arrowheads) in both wild-type and mutant embryos (Fig. 4A-C). The exact nature of the *flk-1*-expressing cells in these regions is not clear, especially as *flk-1* has recently been reported to be expressed outside the vasculature (e.g. in retinal progenitor cells; Yang and Cepko, personal communication).

Wild-type and *clo* mutant embryos were also processed for histology after overstaining for *flk-1* expression. Examination of serial sections confirms that only cells in the lower trunk and tail regions of *clo* mutant embryos express *flk-1* (Fig. 5). In the mid-trunk region for example, while vessels are clearly delineated in wild-type embryos (Fig. 5D), no *flk-1* expression is seen in the mutants (Fig. 5G). In fact, somitic tissue appears to occupy the space where the dorsal aorta and axial vein usually form. Altogether, these data indicate that most endothelial cells are missing from *clo* mutant embryos.

The *clo* mutation affects the differentiation of all endothelial cells

To characterize further the *flk-1*-positive cells found in the lower trunk and tail regions of *clo* mutant embryos and to determine whether *clo* also plays a role in the differentiation of these cells, we examined the expression of *tie* (B. Bell, D. Y. R. Stainier and K. Peters, unpublished data). *tie* encodes an endothelial-specific receptor tyrosine kinase whose expression in mouse marks later stages of endothelial cell differentiation (Korhonen et al., 1994; Dumont et al., 1995). Similarly, in zebrafish, *tie* expression marks later stages of endothelial cell differentiation, first appearing around the 18-somite stage (data not included). In 30 hpf wild-type embryos, *tie* expression is detected in all endothelial cells, and similar to *flk-1* expression, high levels of *tie* expression are found in the lower trunk and tail regions (Fig. 6A,B). In *clo* mutants, however, there are no *tie*-positive cells at this or later stages (Fig. 6A,C). Thus, the *flk-1*-positive cells found in the lower trunk and tail regions of *clo* mutant embryos do not go on to express *tie-1*, indicating a block early in their differentiation. On the basis of these data, we conclude that *clo* also functions in the endothelial cells of the lower trunk and tail regions.

Residual hematopoietic activity is detected in *clo* mutants in the lower trunk and tail regions

We previously reported that the *clo* mutation affects blood cell differentiation (Stainier et al., 1995): The number of blood cells is greatly reduced in *clo* mutant embryos and, at early stages, the hematopoietic tissues show no expression of GATA-1 and GATA-2, two key hematopoietic transcription factor genes first expressed at the end of gastrulation. Because *clo* mutants display a delayed onset of *flk-1* expression in a region where blood cell differentiation is known to occur (Detrich et al., 1995; Stainier et al., 1995), we wished to examine whether hematopoiesis was also delayed. In 30 hpf wild-type embryos, GATA-1 is expressed mainly in erythroid cells accumulated on the yolk sac, in the heart, and in a small group of cells in the lower trunk and tail regions just posterior to the caudal end of the yolk extension (Fig. 7A). (The location of this small group of GATA-1-positive cells within the stem cell population of the posterior intermediate cell mass (ICM) indicates that they are newly differentiated blood cells, not yet recruited into the circulation; Detrich et al., 1995; Stainier et al., 1995). In *clo* mutants, no GATA-1-positive cells are found on the yolk sac or in the heart; however, there are a few GATA-1-expressing cells in the ventral region of the tail (Fig. 7A-C). These GATA-1-positive cells are always within the region outlined by the *flk-1*-expressing cells (Fig. 7C,D).

DISCUSSION

flk-1 expression and the formation of blood vessels

Expression of *flk-1* is, to date, the earliest marker for endothelial cells and their progenitors. In the mouse, *flk-1* expression can be detected in presumptive mesodermal yolk-sac blood-island progenitors as early as 7.0 days postcoitum (i.e. early to mid-gastrulation) (Shalaby et al., 1995), and in avians, *flk-1* (also called *Quek1*) is expressed in the mesoderm from the onset of gastrulation (Eichmann et al., 1993). In zebrafish, *flk-1* expression is first detected during early somitogenesis (a process which initiates much earlier in fish than in amniotes), at a time when differentiation markers for other cardiovascular cell types are also appearing, e.g. Nkx-2.5 in myocardial cells (Lee et al., 1996; Jon Alexander and D.Y.R.S., unpublished) and GATA-1 in blood cells (Detrich et al., 1995; Stainier et al., 1995). *flk-1* expression identifies two physically separate populations of endothelial progenitor cells: one in the head and another in the trunk. In agreement with this pattern of *flk-1* expression, lineage data indicate that endothelial progenitors are located throughout the marginal zone at the late blastula stage (Warga, 1996): trunk and tail endothelial prog-

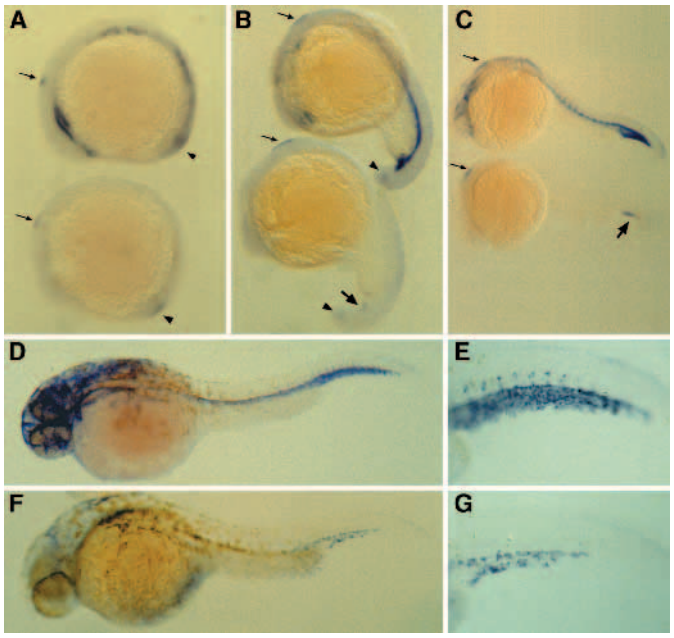


Fig. 4. *flk-1* is expressed only in cells of the lower trunk and tail regions in *clo* mutant embryos. *flk-1* expression in wild-type and *clo* mutant embryos. Lateral view of 10-somite stage (A), 20-somite stage (B), 24 hpf (C) and 30 hpf embryos (D-G). (A-C) Wild-type above and *clo* mutant below. Until the 20-somite stage, the only *flk-1* expression observed in mutant embryos is in the ectodermal region of the hindbrain (small arrows); there is also a small area of faint and hazy expression in the tailbud (A, B, arrowheads). At the 20-somite stage (B), staining is observed in the tail region (large arrow) (the tail begins at the level of the anus, which itself is located at the caudal end of the yolk extension). This staining is more noticeable by 24 hpf (C, large arrow) although it is very much weaker than in wild-type. By 30 hpf, the extent of *flk-1* staining in mutant embryos (F,G) can be fully appreciated as compared to wild-type (D,E): only cells of the lower trunk and tail regions express *flk-1* and this staining is much less intense than in wild-type.

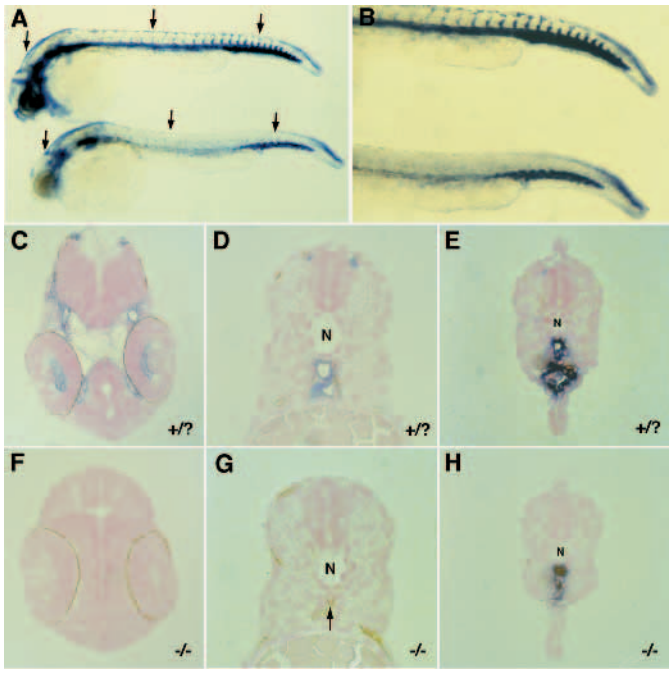


Fig. 5. Histological examination of wild-type and *clo* mutant embryos overstained with *flk-1* (see Methods). Lateral view of the 36 hpf embryos used for histology (A,B), wild-type above and mutant below; transverse sections of the head, trunk and tail regions of wild-type (C,D,E) and *clo* mutants (F,G,H) respectively. Arrows in A indicate the A-P position of the sections shown. (A,B) *flk-1* expression is seen in the lower trunk and tail regions of mutant embryos but is missing from the rest of the trunk. In the head region, *flk-1* expression is observed in intracranial vessels in wild-type (C) but not in mutants (F); there is also staining on the dorsal aspect of the hindbrain region which is seen in both wild-type and mutant embryos (and can be observed at earlier stages (Fig. 2A-C)), as well as staining on the ventrolateral aspects of the caudal head region which appears on sections as hazy background on the outside of overstained embryos (data not included). (D,G) In the the trunk region, while vessels are clearly stained in wild-type embryos, no *flk-1* expression is seen in mutants and in fact, somitic tissue appears to occupy the space where the dorsal aorta and axial vein usually form (G, arrow). (E,H) In the tail region, *flk-1*-expressing cells are present in both wild-type and *clo* mutant embryos although at an apparently reduced number in the mutants. N, notochord.

enitors are concentrated in the ventral half of the marginal zone whereas head endothelial progenitors span the entire marginal zone. Endocardial progenitors are themselves located in the ventral marginal zone (Lee et al., 1994; Warga, 1996).

Careful examination of *flk-1* expression in embryos between the 14- to 18-somite stages reveals that the endocardial progenitor cells appear to originate from the anterior group of angioblasts (i.e. those that populate the head region). Indeed, several hours before the head and trunk populations of angioblasts join, *flk-1* staining appears in the presumptive heart region between the bilateral stripes of *flk-1*-expressing cells. The subsequent aggregation of *flk-1*-positive cells in the midline region occurs at the precise location where we have previously observed and described the endocardial progenitor cells at later stages (Stainier et al., 1993). These data indicate that the endocardium originates from the cephalic paraxial and/or anterior lateral plate mesoderm, and undergoes minimal medial migration to reach its final destination. This interpretation is in agreement with previous studies in avian embryos which concluded that the endocardium arises from the head mesoderm (Noden, 1991b; Coffin and Poole, 1991). Thus, the endocardium does not appear to originate from the trunk lateral plate mesoderm as previously suggested (reviewed by Noden, 1991a). These data and the lineage data from Warga and Nüsslein-Volhard also indicate that, based on their migration pattern, there are at least two populations of cells in the ventral marginal zone of zebrafish blastulae. Myocardial and endocardial progenitors migrate dorso-anteriorly towards the head region during gastrulation whereas blood as well as trunk and tail endothelial progenitors take a more posterior route. Determining the exact migration pathways of these cells and proving unequivocally that endocardial cells indeed come from the head region will require careful lineage and cell tracking analyses.

Finally, these data also shed some light on the postulated existence of the hemangioblast, defined as the common mesodermal precursor for both endothelial and hematopoietic cells (Sabin, 1920). In zebrafish blastulae, blood progenitors lie in

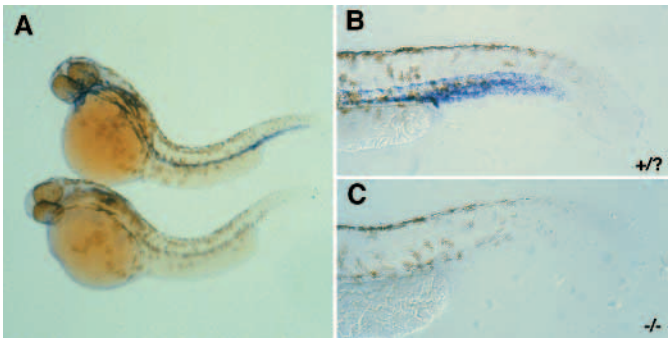


Fig. 6. *tie* is not expressed in *clo* mutant embryos. *tie* expression in wild-type and *clo* mutant embryos at 30 hpf. (A) Lateral view of wild-type above and *clo* mutant below. (B,C) Higher magnification view of the lower trunk and tail regions of wild-type (B) and *clo* mutant (C). At this stage, *tie* is strongly expressed throughout the wild-type vasculature. In *clo* mutants, *tie* expression is not detected at this or later stages.

the ventral marginal zone together with trunk and tail endothelial progenitors while head endothelial progenitors are found throughout the marginal zone. Thus, while trunk and tail endothelial cells may share part of their lineage with blood cells, it is clear that some head endothelium is already distinct from the blood lineage at the blastula stage. These data indicate that some, but not all, endothelial cells may originate from hemangioblasts and that there may in fact be two distinct endothelial lineages, only one of them related to hematopoiesis. Recent transplantation experiments in chick also indicate the presence of two distinct endothelial lineages, only one of them related to hematopoiesis (Pardanaud et al., 1996).

Role of *cloche* in endothelial cell differentiation

The *clo* mutation affects the differentiation of both the endothelial and blood cell lineages. Similarly, Flk-1 deficient mice lack most endothelial and blood cells (Shalaby et al., 1995). We therefore analyzed linkage between zebrafish *flk-1* and *clo* and found no linkage between these two genes, indicating that *clo* does not encode zebrafish Flk-1.

As assessed by *flk-1* expression, the *clo* mutation appears to block the generation of most endothelial cells. In *clo* mutants, *flk-1* expression is not detected in regions of the early embryo where endothelial cells and their progenitors normally reside. Around the 20-somite stage, a few cells in the tail region are clearly-expressing *flk-1*, although at a much reduced level as compared to wild-type (Fig. 4B). This staining becomes more pronounced by 24 hpf (Fig. 4C) and at 30 hpf, the extent of *flk-1* staining in mutant embryos (Fig. 4F,G) can be fully appreciated as compared to wild-type (Fig. 4D,E). *flk-1*-positive cells are only present in the lower trunk and tail regions and this staining is weaker than in wild-type, apparently because there are fewer *flk-1*-expressing cells in mutant embryos (Fig. 5E,H). Examination of serial sections of *flk-1*-stained embryos indicates that all endothelial cells in both wild-types and mutants express *flk-1*. Thus, most endothelial cells appear to be missing in *clo* mutant embryos confirming the original Nomarski optics observations in live embryos (Stainier et al., 1995).

In the lower trunk and tail regions of *clo* mutant embryos, a few cells express *flk-1* (Fig. 3D,E). These cells do not go on to express *tie* (Fig. 5A,C) indicating that they are blocked at an early stage of their differentiation. Thus, *clo* appears to affect the differentiation of all endothelial cells, although the (presumed) absence of its function has different effects in the head and trunk versus the tail endothelial progenitor populations. The molecular basis for this spatial distinction is not clear at this point. It is possible, for example, that *clo* acts in conjunction with one or more genes to regulate directly or indirectly the expression of *flk-1*, and that in the absence of *clo* function these other genes are capable of inducing limited *flk-1* expression in only the lower trunk and tail regions of the embryo. It is also interesting to note that in *clo* mutant embryos endothelial cells differentiate mostly in the tail region, and that tail formation has been hypothesized to involve mechanisms distinct from primary body formation (which gives rise to the head and trunk regions) (Holmdahl, 1925; Griffith et al., 1992).

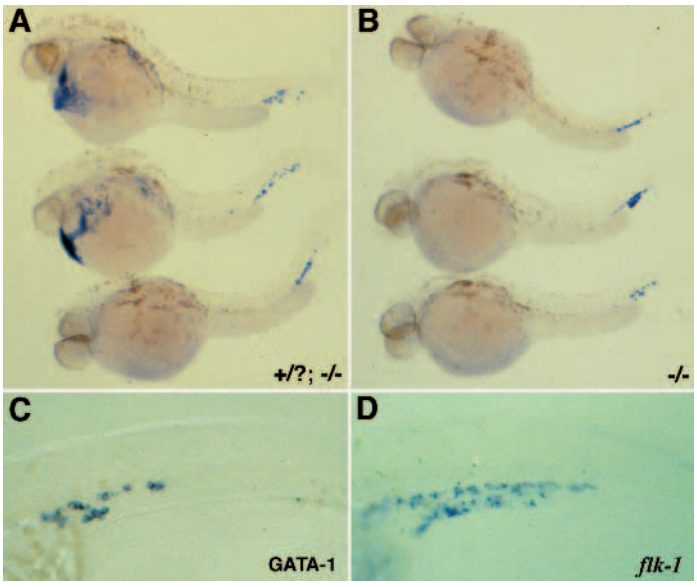


Fig. 7. GATA-1 is expressed in *clo* mutant embryos only in the region outlined by the *flk-1*-expressing cells. GATA-1 expression in wild-type and *clo* mutant embryos at 30 hpf. (A) Lateral view of 2 wild-type (top) and one *clo* mutant (bottom) embryos. (B) *clo* mutant embryos. (C,D) Higher magnification view of cells expressing GATA-1 (C) and *flk-1* (D) in the lower trunk and tail regions of *clo* mutant embryos. GATA-1 expression is not detected at early stages in *clo* mutant embryos. Expression appears as *flk-1* is being detected and only in the region lined by the *flk-1*-positive cells. Additional analysis reveals that although GATA-2 does not seem to be expressed in *clo* hematopoietic tissues, the GATA-1-expressing cells appear to mature normally as assessed by positive diaminofluorene staining used to detect heme (data not included).

In Flk-1 deficient mice as in *clo* mutants, *tie* is not expressed, indicating the absence of more mature endothelial cells (Shalaby et al., 1995). The absence of *tie* expression is not an indirect consequence of the lack of circulation seen in these mutants; indeed, we find normal *tie* expression in *silent heart* mutants (a mutation originally identified in Eugene, Oregon by Walker and Kimmel, which blocks the initiation of the heart beat and causes a lack of circulation). Furthermore, in Flk-1 deficient mice, *flk-1* expression is activated appropriately in the developing mesoderm, in contrast to the lack of early *flk-1* expression observed in *clo* mutants. Finally, the necrosis present in Flk-1 deficient mice prevents analysis of older mutant embryos, but it is possible that in these mutants, as in *clo* homozygotes, a small subset of vessels would form in the tail region at late stages.

In *clo* mutants, early *flk-1* expression is mainly absent. One model is that *clo* plays a role within the endothelial lineage in the transduction of the signal that induces *flk-1* expression, or in the differentiation of the cells that normally go on to express *flk-1*. Several additional lines of evidence support such a model. First, *clo* acts cell-autonomously within the endothelial lineage (Stainier et al., 1995). Second, using GATA-1 and -2 as probes, a molecular defect can be detected in *clo* mutant embryos by the end of gastrulation (i.e. several hours before *flk-1* expression is first detected), arguing that the *clo* gene product first functions before *flk-1* is normally expressed. We therefore propose that *clo* acts upstream of *flk-1*, directly or indirectly, to regulate endothelial cell differentiation.

Role of *cloche* in blood cell differentiation

Analysis of *clo* mutant embryos at early stages revealed no GATA-1 or -2 expression, indicating that *clo* affects hematopoietic differentiation (Stainier et al., 1995). Because *clo* also affects endothelial cell differentiation, we presented several models to explain this phenotype. In one of these models, we hypothesized that endothelial cells or their progenitors induce the differentiation and/or maturation of the physically adjacent hematopoietic progenitors. The observation of *flk-1*-positive cells in 24 hpf *clo* mutant embryos led us to examine GATA-1 expression at these late stages. In agreement with an induction model, we find some GATA-1-expressing cells within the region lined by the *flk-1*-positive endothelial cells. Additional experiments, however, must be performed to test the validity of this model. For example, cell transplantation experiments to analyze the cell-autonomy of the blood defect in *clo* mutants would be useful, but the very low frequency with which one can transplant cells that give rise to either trunk endothelial cells or blood cells (due to their close lineal relationship) makes this approach impractical.

The restricted differentiation of blood in the ventroposterior-most region of *clo* mutant embryos also argues for a ventral source of blood inducing signal(s) as has been previously proposed in *Xenopus* (Kelley et al., 1994; Walmsley et al., 1994). In this model, blood cell differentiation would occur in *clo* mutants only in the region (or derivatives of the region) exposed to the highest concentration of inducing signal(s).

We must also consider the possibility that our *clo* mutations are weak alleles, and that residual *clo* function leads to limited endothelial and blood cell differentiation and/or maturation. Arguing against this possibility is the fact that the two existing *clo* alleles are phenotypically indistinguishable at the cellular and molecular levels, even when outcrossed into widely polymorphic strains, suggesting that they represent loss-of-function mutations.

In summary, we have shown that *clo* is an early regulator of endothelial cell differentiation and that it is likely to function upstream of *flk-1*, the earliest endothelial marker isolated to date. We have also collected additional data consistent with a model in which endothelial cells play a role in blood cell differentiation and/or maturation. The isolation and characterization of the *clo* gene as well as the analysis of its expression pattern will shed further light on the mechanisms regulating the differentiation of the endothelial and hematopoietic lineages.

We thank Marina Gerson and Virginia Walter for excellent technical help, Jon Alexander, Leon Parker, Debbie Yelon and other members of the lab for comments on the manuscript, Rachel Warga and Len Zon and his group for sharing unpublished data and discussions. This work was supported in part by the NIHLB Program of Excellence in Molecular Biology (W. L.), a Postdoctoral Fellowship from NSERC Canada (B. W. B.), a James S. McDonnell Foundation Scholarship and NIH HL55265 (K. P.), the Primary Children's Medical Center Foundation (Utah) and NSF (D. J. G.), a University of California CRCC grant, the UCSF Simon Fund, the March of Dimes, the David and Lucile Packard Foundation, and NIH HL54737 (D. Y. R. S.).

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403-410.

Coffin, J. D. and Poole, T. J. (1988). Embryonic vascular development: immunohistochemical identification of the origin and subsequent morphogenesis of the major vessel primordia in quail embryos. *Development* **102**, 735-748.

Coffin, J. D. and Poole, T. J. (1991). Endothelial cell origin and migration in embryonic heart and cranial blood vessel development. *Anatomical Record* **231**, 383-395.

Detrich III, H. W., Kieran, M. W., Chan, F. Y., Barone, L. M., Yee, K., Rundstadler, J. A., Pratt, S., Ransom, D. and Zon, L. I. (1995). Intraembryonic hematopoietic cell migration during vertebrate development. *Proc. Natl. Acad. Sci. USA* **92**, 10713-10717.

Dumont, D. J., Gradwohl, G., Fong, G.-H., Puri, M. C., Gertsenstein, M., Auerbach, A. and Breitman, M. L. (1994). Dominant-negative and targeted null-mutations in the endothelial receptor tyrosine kinase *tek*, reveal a critical role in vasculogenesis of the embryo. *Genes Dev.* **8**, 1897-1909.

Dumont, D. G., Fong, G., Puri, M. C., Gradwohl, G., Alitalo, K. and Breitman, M. L. (1995). Vascularization of the mouse embryo: a study of *flk-1, tek, tie* and VEGF expression during development. *Dev. Dynam.* **203**, 80-92.

Eichmann, A., Marcelle, C., Breant, C. and Le Douarin, N. M. (1993). Two molecules related to the VEGF receptor are expressed in early endothelial cells during avian embryonic development. *Mech. Dev.* **42**, 33-48.

Fong, G.-H., Rossant, J., Gertsenstein, M. and Breitman, M. L. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* **376**, 66-70.

Fouquet, B., Weinstein, B. M., Serluca, F. C. and Fishman, M. C. (1997). Vessel Patterning in the embryo of the zebrafish: guidance by notochord. *Dev. Biol.*, in press.

Griffith, C. M., Wiley, M. J. and Sanders, E. J. (1992). The vertebrate tail bud: three germ layers from one tissue. *Anat. Embryol.* 185, 101-113.

Holmdahl, D. E. (1925). Experimentelle Untersuchungen uber die Lage ser Drenze zwischen primarer und sekundarer Korperentwicklung beim Hunh. *Anat. Anz.* **59**, 393-396.

Kelley, C., Yee, K., Harland, R. and Zon, L. I. (1994). Ventral expression of GATA-1 and GATA-2 in the Xenopus embryo defines induction of hematopoietic mesoderm. *Dev. Biol.* **165**, 193-205.

Kimmel, C.B., Kane, D.A., Walker, C., Warga, R. M. and Rothman, M. B. (1989). A mutation that changes cell movement and cell fate in the zebrafish embryo. *Nature* 337, 358-362.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullman, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev.l Dynam.* **203**, 253-310.

Korhonen, J., Polvi, A., Partanen, J. and Alitalo, K. (1994). The mouse *tie* receptor tyrosine kinase gene: expression during embryonic angiogenesis. *Oncogene* **9**, 395-403.

Lee, K.-H., Xu, Q. and Breitbart, R. (1996). A new tinman-related gene, Nkx-2.7, anticipates the expression of Nkx-2.5 and Nkx-2.3 in zebrafish heart and pharyngeal endoderm. *Dev. Biol.* (in press).

Lee, R. K., Stainier, D. Y., Weinstein, B. M. and Fishman, M. C. (1994). Cardiovascular development in the zebrafish. II. Endocardial progenitors are sequestered within the heart field. *Development* **120**, 3361-3366.

Mustonen, T. and Alitalo, K. (1995). Endothelial receptor tyrosine kinases involved in angiogenesis. *J. Cell Biol.* **129**, 895-898.

Noden, D. M. (1991a). Origins and assembly of avian embryonic blood vessels. *Ann. NY Acad. Sci.* **1**, 236-249.

Noden, D. M. (1991b). Origins and patterning of avian outflow tract endocardium. *Development* **111**, 867-876

Oxtoby, E. and Jowett, T. (1993). Cloning of the zebrafish *krox-20* gene (*krx-20*) and its expression during hindbrain development. *Nucl. Acids Res.* **21**, 1087-1095.

Pardanaud, L., Altmann, C., Kitos, P., Dieterlen-Lievre, F. and Buck, C. A. (1987). Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. *Development* **100**, 339-349.

Pardanaud, L., Luton, D., Prigent, M., Bourcheix, L.-M., Catala, M. and Dieterlen-Lievre, F. (1996). Two distinct endothelial lineages in ontogeny, one of them related to hemopoiesis. *Development* **122**, 1363-1371.

Pardanaud, L., Yassine, F. and Dieterlen-Lievre, F. (1989). Relationship between vasculogenesis, angiogenesis and haemopoiesis during avian ontogeny. *Development* **105**, 473-485.

Poole, T. J. and Coffin, J. D. (1991). Morphogenetic mechanisms in avian vascular development. In: *The Development of the Vascular System* (eds. R. N. Feinberg, G. K. Sherer and R. Auerbach), pp. 25-36. Basel: Karger.

Puri, M. D., Rossant, J., Alitalo, K., Bernstein, A. and Partanen, J. (1995). The receptor tyrosine kinase Tie is required for integrity and survival of vascular endothelial cells. *EMBO J.* **14**, 5884-5891.

Rieb, J.-P. (1973). La circulation sanguine chez l'embryon de *Brachydanio rerio*. *Ann. Embryol. Morphol.* **6**, 43-54.

Risau, W. (1991). Vasculogenesis, angiogenesis and endothelial cell differentiation during embryonic development. In: *The Development of the Vascular System* (eds. R. N. Feinberg, G. K. Sherer and R. Auerbach), pp. 58-68. Basel: Karger.

Sabin, F. (1920). Studies on the origin of blood vessels and of red blood corpuscules as seen in the living bastoderm of chicks during the second day of incubation. *Contrib. Embryol. Carnegie Inst. Washington* **9**, 214-262.

Sato, T. N., Tozawa, Y., Deutch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W. and Qin, Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* **376**, 70.

Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X.-F., Breitman, M. and Schuh, A. C. (1995). Failure of blood island formation and vasculogenesis in Flk-1 deficient mice. *Nature* **376**, 62-66.

Stainier, D. Y., Lee, R. K. and Fishman, M. C. (1993). Cardiovascular development in the zebrafish. I. Myocardial fate map and heart tube formation. *Development* **119**, 31-40.

Stainier, D. Y. R. and Fishman, M. C. (1994). The zebrafish as a model system to study cardiovascular development. *Trends Cardiovas. Med.* **4**, 207-212.

Stainier, D. Y. R., Weinstein, B. M., Detrich, H. W., Zon, L. I. and Fishman, M. C. (1995). *cloche*, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. *Development* **121**, 3141-3150.

Stainier, D. Y. R., Fouquet, B., Chen, J.-N., Warren, K. S., Weinstein, B. M., Meiler, S., Mohideen, M.-A. P. K., Neuhauss, S. C. F., Solnica-Krezel, L., Schier, A. F., Zwartkruis, F., Stemple, D. L., Malicki, J., Driever, W. and Fishman, M. C. (1996). Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. *Development* **12**3, 285-292.

Walmsley, M. E., Guille, M. J., Bertwistle, D., Smith, J. C., Pizzey, J. A. and Patient, R. K. (1994). Negative control of Xenopus GATA-2 by activin and noggin with eventual expression in precursors of the ventral blood islands. *Development* **120**, 2519-2529.

Warga, R. M. (1996). Origin and specification of the endoderm in the zebrafish, *Danio rerio*. Dissertation zur Erlangung des Grades eines Doktors der Naturwissenschaften der Fakultat fur Biologie der Eberhard-Karls-University of Tubingen.

Weinstein, B. M., Stemple, D. L., Driever, W. and Fishman, M. C. (1995). *gridlock*, a localized heritable vascular patterning defect in the zebrafish. *Nature Medicine* **1**, 1143-1147.

Westerfield, M. (1993). *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Brachydanio rerio)*, Eugene: University of Oregon Press.

Yamaguchi, T. P., Dumont, D. J., Conlon, R. A., Breitman, M. L. and Rossant J. (1993). flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. *Development* **118**, 489-498.

Yang, X. and Cepko, C. L. (1996). Flk-1, a receptor for vascular endothelial growth factor (VEGF), is expressed by retinal progenitor cells. *J. Neurosci.* **16**, 6089-6099.

(Accepted 25 October 1996)