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

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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<sup>m39</sup> (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<sup>m378</sup>; Stainier et al., 1996), was used in this study (except for the linkage analysis where clo<sup>m39</sup> was used). Like clo<sup>m39</sup>, clo<sup>m378</sup> is fully penetrant and exhibits complete and indistinguishable expressivity in a variety of backgrounds. In addition, clo<sup>m39</sup> and clo<sup>m378</sup> mutants are indistinguishable at both the cellular and molecular level further suggesting that these mutations 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 P<sup>v</sup>uI polymorphism in the flk-1 gene was identified between a clo<sup>m39</sup> fish and a polymorphic wild-type fish (+<sup>flk-1/</sup><sup>flk-1K</sup>) on Southern blots using a 640 bp BstXI/XbaI fragment of the zebrafish flk-1 cDNA as a probe. These fish were bred, and individual F<sub>1</sub> fish (with the genotype clo<sup>m39</sup><sup>+/</sup><sup>flk-1K</sup>) 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 P<sup>v</sup>uI 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 flt-1 revealed high homology (Fig. 1B), closer to Flt-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

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kinase domain

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/Reciprocal bs_seq.html) (Altschul et al., 1990). Note that the same gene was also isolated and characterized by Fouquet et al. (1997).

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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 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 PvuII polymorphism in the flk-1 gene between a clo<sup>mut</sup> fish and a polymorphic wild-type fish (+<sup>HK</sup>/<sup>HK</sup>) 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 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-islnd 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-
Endocardial progenitors 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

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**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 it 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 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.
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).

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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.
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; Walsmey 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.

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