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Hhex and Scl function in parallel to regulate early endothelial and blood differentiation in zebrafish

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SUMMARY

During embryogenesis, endothelial and blood precursors are hypothesized to arise from a common progenitor, the hemangioblast. Several genes that affect the differentiation of, or are expressed early in, both the endothelial and blood lineages may in fact function at the level of the hemangioblast. For example, the zebrafish *cloche* mutation disrupts the differentiation of both endothelial and blood cells. The transcription factor gene *scl* is expressed in both endothelial and blood lineages from an early stage and can regulate their differentiation. Here we report that in zebrafish the homeobox gene *hhex* (previously called *hex*) is also expressed in endothelial and blood lineages from an early stage. We find that *hhex* expression in these lineages is significantly reduced in *cloche* mutant embryos, indicating that *hhex* functions downstream of *cloche* to regulate endothelial and blood differentiation. Ectopic expression of *hhex* through injection of a DNA construct leads to the premature and ectopic expression of early endothelial and blood differentiation genes such as *fli1*, *flk1* and *gata1*, indicating that Hhex can positively regulate endothelial and blood differentiation. However, analysis of a *hhex* deficiency allele shows that *hhex* is not essential for early endothelial and blood differentiation, suggesting that another gene, perhaps *scl*, compensates for the absence of Hhex function. Furthermore, we find that *hhex* and *scl* can induce each other's expression, suggesting that these two genes cross-regulate each other during early endothelial and blood differentiation. Together, these data provide the initial framework of a pathway that can be used to further integrate the molecular events regulating hemangioblast differentiation.

Key words: Hemangioblast, *gata1*, *flk1*, *fli1*, *cloche*, Vasculogenesis, Hematopoiesis

INTRODUCTION

During embryogenesis, blood development is closely associated with endothelial development, both temporally and spatially. For example, the blood islands on the yolk sac of avian and mammalian embryos consist of both endothelial and blood cells (reviewed by Risau and Flamme, 1995; Cleaver and Krieg, 1999). In zebrafish embryos, both endothelial and blood precursors originate from the lateral plate mesoderm and at mid-somitogenesis stages reside in a midline region known as the intermediate cell mass (ICM) (Detrich et al., 1995). Their intimate association and common origin has led to the hypothesis that a common progenitor known as the hemangioblast gives rise to both lineages (reviewed by Robb and Elefanty, 1998).

Genes that regulate the differentiation of both endothelial and blood lineages provide additional, albeit circumstantial, evidence for the existence of the hemangioblast. For example, targeted inactivation in mouse of the VEGF receptor gene *Flk1* results in the absence of both endothelial and blood cells (Shalaby et al., 1995). The zebrafish *cloche* mutation also disrupts the early differentiation of endothelial and blood cells, and in fact appears to act upstream of zebrafish *flk1* (Stainier et al., 1995; Liao et al., 1997; Thompson et al., 1998). Although these data are consistent with these genes acting at the level of the hemangioblast, cell transplantation studies indicate that they may act at multiple times and in mutliple lineages (Shalaby et al., 1997; Parker and Stainier, 1999).

It is reasonable to assume that genes regulating hemangioblast differentiation are expressed at very early developmental stages in both endothelial and blood lineages. To date, only a few genes have been clearly shown to exhibit such an expression pattern. In addition to *flk1* and *cloche*, the transcription factor gene *scl* also appears to play an important role in hemangioblast differentiation (Robb et al., 1995; Shivdasani et al., 1995; Gering et al., 1998; Liao et al., 1998; Visvader et al., 1998). In zebrafish, *scl* is first expressed in the anterior and posterior lateral plate mesoderm, regions which give rise to both endothelial and blood cells. Ectopic *scl* expression in zebrafish embryos results in the expansion of

endothelial and blood precursors at the expense of somitic and pronephric duct tissues (Gering et al., 1998). Targeted inactivation of *Scl* in mouse disrupts the formation of hematopoietic stem cells and perturbs the angiogenic remodeling of the yolk sac vasculature (reviewed by Barton et al., 1999). In addition, in *cloche* mutant embryos, *scl* expression is downregulated and forced expression of *scl* through injection of a cmv-*scl* DNA construct restores endothelial and blood differentiation, indicating that *scl* acts downstream of *cloche* to regulate both endothelial and blood differentiation (Liao et al., 1998).

The *hhex* gene (previously called *hex*, see Materials and Methods) encodes a homeobox-containing protein that is highly conserved among vertebrates (Crompton et al., 1992; Bedford et al., 1993; Newman et al., 1997; Ho et al., 1999; Yatskievych et al., 1999). Studies in *Xenopus* and zebrafish embryos have shown that Hhex can function as a transcriptional repressor and appears to be involved in anterior as well as dorsoventral patterning (Ho et al., 1999; Jones et al., 1999; Brickman et al., 2000). Embryological and genetic studies in mouse have further implicated *hhex* in forebrain, liver and thyroid formation (Thomas et al., 1998; Martinez Barbera et al., 2000). *hhex* was first identified as a hematopoietically expressed homeobox gene with expression in various hematopoietic cells and cell lines (Crompton et al., 1992; Bedford et al., 1993). Detailed studies in *Xenopus*, mouse and chick embryos have revealed that *hhex* is also expressed in angioblasts (Newman et al., 1997; Thomas et al., 1998; Yatskievych et al., 1999). In addition, overexpression of *hhex* in *Xenopus* embryos results in abnormal vascular structures due to an increase in the number of endothelial cells (Newman et al., 1997). These data suggested that *hhex* might be expressed in both endothelial and blood precursors and play a role in regulating hemangioblast differentiation. In order to test this model and the relationships between Hhex and other regulators of hemangioblast differentiation, we analyzed the expression and function of *hhex* in the endothelial and blood lineages of the zebrafish embryo.

We show that *hhex*, like *scl*, is expressed in the ICM region, which contains both endothelial and blood precursors. *hhex* expression is significantly reduced in the ICM region of *cloche* mutant embryos, indicating that *hhex* acts downstream of *cloche*. Gain- and loss-of-function analyses indicate that *hhex* is sufficient but not necessary for endothelial and blood differentiation. We further show that *hhex* and *scl* can cross-regulate each other. These and other data (Robb et al., 1995; Shivdasani et al., 1995; Liao et al., 1998) allow us to assemble the initial framework of a molecular pathway controlling hemangioblast differentiation.

MATERIALS AND METHODS

Zebrafish

Adult fish and embryos were maintained and staged as described (Westerfield, 1995). The *m378 cloche* allele (Stainier et al., 1996) and the cyclops *b16* deficiency (Hatta et al., 1991) were used in these studies.

In situ hybridization and histology

Whole-mount in situ hybridization and histology were performed as described (Alexander et al., 1998; Yelon et al., 1999). *flk1*, *fli1*, *gata1*, *hhex* and *scl* riboprobes were prepared as described (Stainier et al., 1995; Liao et al., 1997, 1998; Ho et al., 1999).

Renaming and mapping *hhex*

hex was renamed *hhex* at the recommendation of the zebrafish nomenclature committee in order to avoid confusion with *hexosaminidase* genes which are designated by *hex*. The use of *hhex* is consistent with the approved nomenclature for the human and mouse orthologues.

hhex was mapped on the MOP cross (Postlethwait et al., 1998). A pair of primers (F: 5′-CGA TCA AAC CAT CGA GCT GGA GAA GAA-3′ and R: 5′-CAT TTC GCC CGG CGG TTC TGG AAC CAT-3′) amplifies a 221-base-pair fragment, and the polymorphism was detected by SSCP analysis. To test for the presence of *hhex* in the *b16* allele, the same pair of primers was used for PCR analysis. The PCR conditions and preparation of genomic DNA from individual embryos were described by Postlethwait et al. (1994) and Johnson et al. (1996).

Mapping of *cloche*

cloche was initially mapped based on its segregation pattern with *brass*. Crossing a pair of fish heterozygous for both *brass* and *cloche* resulted in 55 out of 77 *cloche* homozygous mutants also being homozygous mutant for *brass*. As no SSR markers had yet been released in that region (Knapik et al., 1998), we used amplified fragment length polymorphism (AFLP) analysis, as described by Vos et al. (1995), to isolate closely linked polymorphic markers. We analyzed 256 primer combinations (2-base overhang) and isolated five AFLP markers within a useful genetic distance. Subsequent release of SSR markers (Shimoda et al., 1999) led to the identification of markers closer to *cloche* than our AFLP markers. Analysis of 2112 diploid and 494 haploid embryos indicates that the SSR marker z1496 maps 0.4 centimorgan (cM) proximal to *cloche*, although this number is likely to be an upper limit since we do not currently have a distal marker to help identify potential misscored embryos. The telomeric location of *cloche* complicates its isolation, as the existing large insert genomic libraries do not cover this region (W. L. and D. Y. R. S., unpublished observations).

hhex cDNA expression constructs and microinjections

The full-length *hhex* cDNA was subcloned into the *Eco*RI site of pCS2+, which contains a CMV promoter. The resulting construct, pCS2cmv-*hhex*, was used to generate pCS2cmv-*hhex*Pro, which carries a Leu154 to Pro154 mutation. Site-directed mutagenesis was performed as described in Kunkel et al. (1987) and the desired Leu to Pro mutation was verified by DNA sequencing.

Injection constructs, *hhex* or *scl* (Liao et al., 1998), were mixed with Phenol Red to monitor the injection process and approximately 100 pg of DNA was injected per embryo at the 1- to 4-cell stage. At this concentration, we observed less than 5% abnormal embryos. Embryos that needed to be genotyped for *cloche* were collected from a mapping cross (AB/SJD) and scored with z1496.

RESULTS

hhex is expressed in endothelial and blood precursors

During zebrafish embryogenesis, *hhex* is expressed in extraembryonic tissues starting at late blastula stages and in embryonic tissues starting at early somitogenesis stages. During gastrulation, *hhex* is expressed in the dorsal portion of the extraembryonic yolk syncytial layer (YSL), a pattern consistent with its proposed function in dorsoventral patterning (Ho et al., 1999). In this study, we analyze the post-gastrula

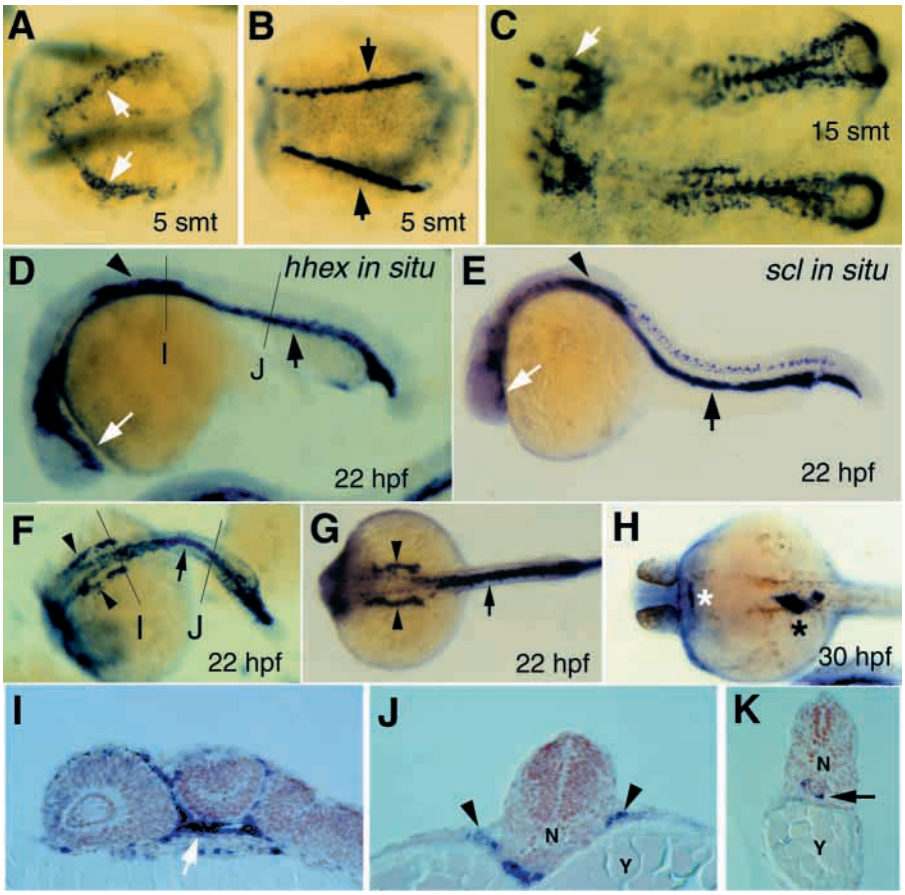


Fig. 1. Post-gastrula expression of *hhex*. (A,B) Dorsal view of anterior (A) and posterior (B) halves of a 5-somite stage (12 hpf) embryo. (C) Dorsal view of two flattened 15-somite stage (16.5 hpf) embryos. (D,F) Lateral (D) and dorsal (F) views of 22 hpf embryos. (H) Dorsal view of a 30 hpf embryo. (E,G) Lateral (E) and dorsal (G) views of *scl* expression in 22 hpf embryos. (I-K) Histological sections from a 22 hpf embryo showing *hhex* expression in the head (I, sagittal section), the midtrunk (J, transverse section), and the tail (K, transverse section) regions. The corresponding positions of the transverse sections are marked in D and F. White arrows point to the head expression. Black arrows point to the posterior ICM expression. Black arrowheads point to the *hhex* and *scl* expressing cells that do not migrate to the midline and these cells may later contribute to the endothelial cells lining the Ducts of Cuvier and/or the anterior part of the pronephros. At 22 hpf, *scl* is also expressed in primary motoneurons (E,G). In H, the white and black asterisks mark the thyroid and liver primordia, respectively. In J and K, the notochord (N) and yolk (Y) are also labeled.

expression of *hhex* and its function in endothelial and blood differentiation.

At the 5-somite stage (12 hours post fertilization, hpf), *hhex* transcripts are detected in the anterior and posterior lateral plate mesoderm (Fig. 1A,B). *hhex* expression in the posterior lateral plate mesoderm starts in the bilateral stripes of the nascent ICM, which contains both endothelial and blood precursors (Liao and Zon, 1999; Parker et al., 1999). These bilateral stripes extend both anteriorly and posteriorly as the embryo develops. At the 15-somite stage (16.5 hpf), the posterior ends of these two stripes have merged at the ventral margin of the tailbud (Fig. 1C). At this stage, some of the *hhex*-expressing cells in the mid-trunk region appear to have migrated to the ventral midline. By 22 hpf (Fig. 1D,F,K), most of the *hhex*-expressing cells in the trunk and tail have converged to the ventral midline. However, some *hhex*-expressing cells remain bilateral in the upper trunk region (arrowheads in Fig. 1D,F,J). These cells may later participate in the formation of the Ducts of Cuvier and/or the vascularization of the pronephros (Gering et al., 1998). Cells in these bilateral regions also express the hematopoietic gene *lmo2* (Thompson et al., 1998).

The pattern of *hhex* expression in the head region expands posteriorly as the embryo develops. At the 15-somite stage, the anterior *hhex* expressing cells are restricted to two small groups of cells at the anterior margin of the head and two larger patches of cells lining the posterior eye anlagen (Fig. 1C, white arrow). These four populations of cells appear to give rise to the cranial vascular plexi at 22 hpf (Fig. 1D,I). *hhex* expression in endothelial and blood precursors diminishes after the onset of circulation (24 hpf) and disappears completely by 30 hpf. At 30 hpf, *hhex* expression is seen only in the thyroid and liver primordia (Fig. 1H).

The temporal and spatial pattern of *hhex* expression in the endothelial and blood precursors is very similar to that of *scl*. Transcripts of both genes are detected in the posterior lateral plate mesoderm at early somitogenesis stages and later remain abundant in the ICM region. At 22 hpf, the expression patterns of *hhex* and *scl* in the ICM region are essentially indistinguishable (Fig. 1D-G and data not shown). These observations suggest that *hhex* and *scl* may function at the same time during endothelial and blood development. *hhex* expression persists in vascular structures a little longer than does *scl* expression (data not shown), suggesting that *hhex* may play additional roles in blood vessel development.

hhex expression is disrupted in the ICM region of *cloche* mutants

The zebrafish *cloche* mutation affects the early differentiation of both endothelial and blood cells, suggesting that *cloche* is a regulator of hemangioblast development (Stainier et al., 1995; Liao et al., 1997, 1998; Thompson et al., 1998). To determine the molecular epistasis between *hhex* and *cloche*, we examined *hhex* expression in *cloche* mutant embryos. At the 8-somite stage (13 hpf), no *hhex* expression was detected in the head region and posterior lateral plate mesoderm of *cloche* mutants (Fig. 2A,B). However, *hhex* expression in the dorsal portion of the YSL remained unaffected. At the 15-somite stage (16.5

hpf), a small number of *hhex*-expressing cells appeared in the ventral region of the tail extension and the ventral midline of the anterior trunk region (Fig. 2C-E). The residual *hhex* expression in the ventral tail region becomes more pronounced at 22 hpf (Fig. 2F, arrowhead) and has also been observed with *flk1*, *gata1* and *scl* (Liao et al., 1997, 1998; Thompson et al., 1998). The residual *hhex* expression in the ventral midline of the anterior trunk region (Fig. 2D,F, arrows) expands into a V-shaped pattern (data not shown) and seems to be unique to *hhex* as it was not observed with *scl*, *flk1* or *gata1* (Liao et al., 1997, 1998; Thompson et al., 1998). The *hhex*-expressing cells in this region may represent hepatocyte precursors, based on their location and the fact that mouse *hhex* is expressed in the developing liver (Thomas et al., 1998).

hhex can induce the premature and ectopic expression of endothelial and blood genes in wild-type embryos

To analyze the function of *hhex* in endothelial and blood development, we first performed gain-of-function analyses. In these experiments, we injected a DNA construct containing a full-length *hhex* cDNA under the control of a CMV promoter (cmv-*hhex*) into wild-type zebrafish embryos at the 1- to 4-cell stage and subsequently examined the expression of endothelial and erythroid genes. Controlled by the CMV promoter, a mosaic and constitutive expression of exogenous *hhex* is initiated after the mid-blastula transition. Such expression limits its influence on dorsoventral patterning while still allowing its effect on endothelial and blood development to be analyzed. The injected embryos were collected at tailbud stage (10 hpf) and 24-30 hpf to assess *flk1* and *gata1* expression. At tailbud stage, *flk1* and *gata1* are normally not yet expressed (Fig. 3A,D). Overexpression of cmv-*hhex* caused premature expression of *flk1* in 47% and *gata1* in 81% of the injected embryos at tailbud stage (Fig. 3B-E; Table 1). At 24-30 hpf, we observed ectopic expression of *flk1* in 57% and *gata1* in 28% of the injected embryos (Fig. 3G-J; Table 2).

The presence of a homeodomain in Hhex suggests that it functions to regulate transcription by binding DNA. To determine whether the DNA binding activity of Hhex is required for the observed premature and ectopic expression of *flk1* and *gata1*, we constructed and injected a form of Hhex (cmv-*hhex*Pro), which contains a leucine to proline mutation between Helix 2 and Helix 3 of the homeodomain. The replacement of this highly conserved leucine disrupts the ability of the homeodomain protein to bind DNA (Le Roux et al., 1993) and has been previously used to analyze the function of Mix.1 (Mead et al., 1996) and Nkx2.3 and 2.5 (Grow and Krieg, 1998). Overexpression of cmv-*hhex*Pro did not result in significant premature or ectopic expression of *flk1* or *gata1* in the injected embryos (see Table 1). These data suggest that the DNA binding activity of Hhex is necessary to induce the premature or ectopic expression of *flk1* and *gata1*.

Because *hhex* is also expressed in the YSL at tailbud stage, it was necessary to determine whether *hhex* expression in the YSL could contribute to the induction of *flk1* and *gata1* expression. When cmv-*hhex* was injected into the yolks of 1000-cell- to sphere-stage embryos to confine its expression to the YSL, no premature expression of *flk1* was observed (data not shown). Altogether, these data show that ectopic expression of *hhex* in embryonic tissues leads to the premature or ectopic expression of endothelial and erythroid genes, suggesting that *hhex* functions in the development of these cell types.

hhex can restore the expression of endothelial and blood genes in *cloche* mutants

To further test whether *hhex* functions downstream of *cloche* as suggested by the *hhex* expression defects in *cloche* mutants, we injected cmv-*hhex* into 1- to 4-cell stage embryos collected from a *cloche* mapping cross. The injected embryos were fixed at the 5- to 7-somite stage (12-13 hpf) to examine the expression of various endothelial and blood genes (*flk1*, *fli1*, *tie1*, *gata1* and *scl*). Embryos with ectopic expression were genotyped with the SSR marker z1496, which is very tightly

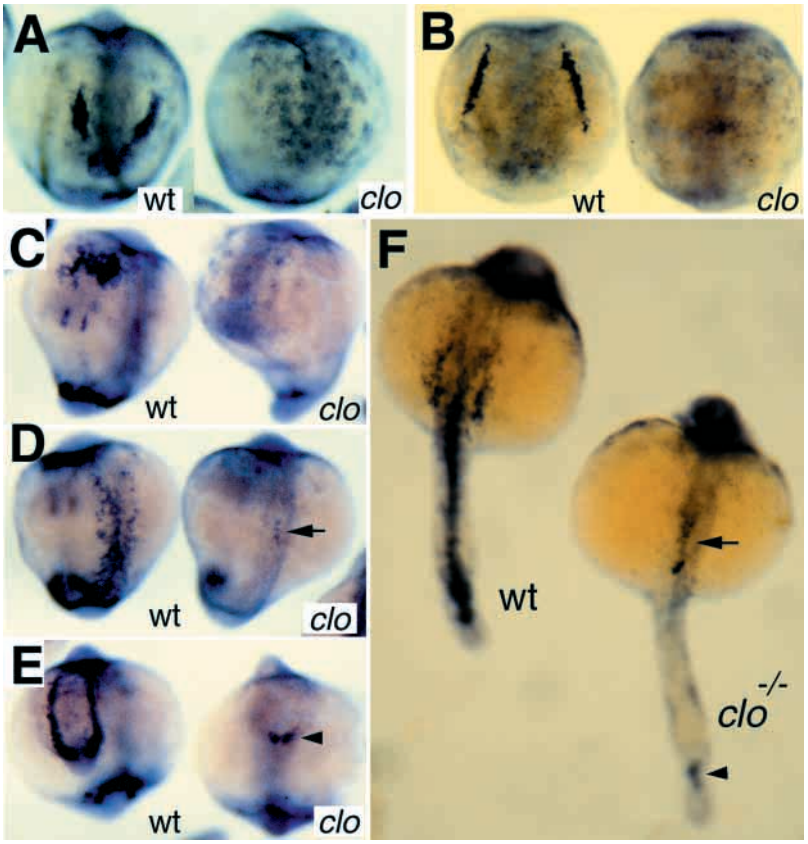


Fig. 2. *hhex* expression is disrupted in the endothelial and blood lineages of *cloche* mutants. *hhex* expression in wild-type (left) and *cloche* mutant (right) embryos at the 8-somite stage (13 hpf) (A,B), the 15-somite stage (16.5 hpf) (C,D,E), and 22 hpf (F). (A,C) Dorsoanterior views. (B,D,F) Dorsomedial views. (E) Dorsoposterior view. Black arrows (D,F) mark the *hhex*-expressing cells in the mid-trunk region of *cloche* mutants that on a lateral view appear to lie most ventrally (data not shown) and are thought to contribute to the liver. Black arrowheads (E,F) point to the residual *hhex* expression in the ventral region of the tail in *cloche* mutants. *hhex* continues to be expressed in the dorsal YSL during early somitogenesis stages (A,B).

Table 1. *hhex* gain-of-function analysis

DNA injected	Stage analyzed	In situ probe	Number of injected embryos	Number of embryos with premature or ectopic expression	% of embryos with premature or ectopic expression
Uninjected	Tailbud	*flk1*	61	0	0
cmv-*hhex*	Tailbud	*flk1*	60	28	46.6
cmv-*hhex*Pro	Tailbud	*flk1*	65	6*	9.2*
Uninjected	Tailbud	*gata1*	47	0	0
cmv-*hhex*	Tailbud	*gata1*	112	91	81.2
cmv-*hhex*Pro	Tailbud	*gata1*	83	8*	9.6*
Uninjected	24 hpf	*flk1*	45	0	0
cmv-*hhex*	24 hpf	*flk1*	118	67	56.8
cmv-*hhex*Pro	24 hpf	*flk1*	110	6*	5.5*
Uninjected	24 hpf	*gata1*	52	0	0
cmv-*hhex*	24 hpf	*gata1*	89	25	28.1
cmv-*hhex*Pro	24 hpf	*gata1*	107	9*	8.4*

*The staining intensity of the premature and ectopic expression in these embryos was significantly weaker than that seen in embryos injected with cmv-*hhex* (data not shown).

linked to *cloche* (see below). A relatively equal number of homozygous wild-type and homozygous *cloche* mutant embryos were found with ectopic expression of endothelial and blood genes (see Table 2), indicating that *hhex* overexpression can induce the expression of endothelial and blood genes in the absence of *cloche* function.

hhex and *cloche* map to the lower telomeric regions of LG 12 and LG 13, respectively

hhex expression is downregulated in *cloche* mutants. In addition, *hhex* overexpression can restore endothelial and blood gene expression in *cloche* mutants. These data indicate that *hhex* functions downstream of *cloche*, or in fact that *cloche* encodes Hhex. In order to test this latter possibility, we mapped both *cloche* and *hhex*. Using half-tetrad analysis (Johnson et al., 1995), we estimated the Gene to Centromere Distance for *cloche* to be 65.8 cM (179 wild-type and 24 mutant embryos were generated after blocking the second meiotic division of gynogenotes), suggesting that *cloche* is located in a very distal region. We subsequently found that *cloche* is linked to the pigmentation mutation *brass*, which was previously mapped to LG 13. The estimated genetic distance between *brass* and *cloche* is 28.5 cM (see Materials and Methods). These data place *cloche* to the lower telomeric region of LG 13. A bulk segregant analysis screening for AFLP markers identified several closely linked markers, including one (ctgt) that maps 1.1 cM proximal to *cloche*. Upon release, we also tested a cluster of SSR markers that map at the distal end of LG13. From the 1999 version of the MGH map (Shimoda et al., 1999), the closest marker to *cloche* is z1496 (Fig. 4A). Analysis of 4718 meioses indicates that z1496 lies 0.4 cM proximal to *cloche*.

hhex was mapped on the MOP cross (Postlethwait et al., 1998) to the lower end of LG12 near z1141 (Fig. 4B), placing it at about position 77.5 cM on the MGH map. Table 3 shows the mapping statistics. Zebrafish

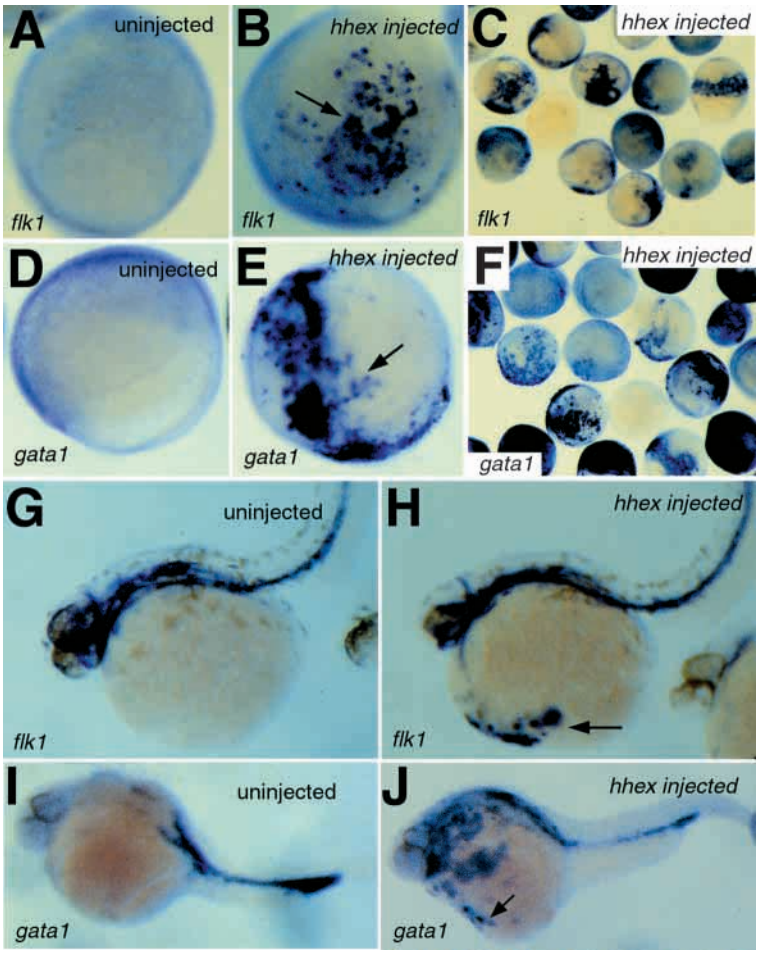


Fig. 3. *hhex* can induce ectopic and premature *flk1* and *gata1* expression in wild-type embryos. (A-C) *flk1* expression in uninjected control (A) and cmv-*hhex* injected (B,C) embryos at tailbud stage (10 hpf). (D-F) *gata1* expression in uninjected control (D) and cmv-*hhex* injected (E,F) embryos at tailbud stage. (G,H) *flk1* expression in uninjected control (G) and cmv-*hhex* injected (H) embryos at 24 hpf. (I,J) *gata1* expression in uninjected control (I) and cmv-*hhex* injected (J) embryos at 24 hpf. Arrows in B,E,H and J point to premature or ectopic expression in the injected embryos.

Table 2. Genotype of cmv-*hhex* injected embryos showing ectopic expression

In situ probe	Genotyping results		
	wt/wt	wt/*clo*	*clo/clo*
scl	14	43	13
gata1	18	35	20
flk1	18	53	25
fli1	8	14	14
tie1	13	14	12

cmv-*hhex* was injected into 1- to 4-cell stage embryos collected from a *cloche* (*clo*) mapping cross. The expression of endothelial and blood genes was examined in the injected embryos at the 5- to 7-somite stage (12-13 hpf) or at tailbud stage (10 hpf) (*fli1*). The embryos showing ectopic (*scl*, *gata1*, *flk1*, *fli1*) or premature (*tie1)* expression were collected and genotyped using the closely linked SSR marker z1496.

Wt, wild type.

Table 3. Map statistics for *hhex*

Marker	Pat	Mat	R	N	Distance (cM±s.e.m.)	95%	LOD
z1400	14	13					
			1	22	4.54±4.44	0.1, 22.8	4.9
STS.90.79	45	25					
			3	30	10.00±5.48	2.1, 26.5	4.8
z1141	18	19					
			0	35	0	0, 10.0	10.5
hhex	37	54					

Marker, marker name; Pat, number of segregants with paternal genotype; Mat, number of segregants with maternal genotype; R, recombinants; N, total segregants scored for both markers; Distance, distance between the two markers; 95%, 95% confidence interval in cM; LOD, logarithms of the odds score.

Hhex shares very high sequence identity with its *Xenopus*, chick, mouse and human homologues (Ho et al., 1999). Comparative mapping provides additional evidence that *hhex* is the orthologue of human *HHEX* and mouse *Hhex*. Table 4 shows conserved syntenies with human and mouse chromosomes. The lower end of zebrafish LG12 contains several genes that are apparent orthologues of human genes lying in the middle of the long arm of human chromosome 10, between 10q23 and 10q24, and others from human chromosome 17. This group of genes occupies a single chromosome in zebrafish, two in humans and five in mouse, suggesting that the mouse genome (which separated from the human lineage about 100 million years ago; Kumar and Hedges, 1998) has suffered more chromosome rearrangements in this region than either the human or zebrafish genomes in the 430 million years since their divergence. In summary, these data show that *cloche* does not encode zebrafish Hhex.

The expression of endothelial and blood genes is not affected by a loss of Hhex function

Previous studies have shown that the gamma-ray induced mutation *b16* is a deletion in the lower telomeric region of LG12 (Talbot et al., 1998). We found that *hhex* is also deleted in the *b16* allele (Fig. 5A), allowing us to examine the consequences of the loss of Hhex function during embryogenesis. The expression patterns of several blood (*scl* and *gata1*) and endothelial (*flk1*, *tie1*, and *tie2*) genes were analyzed in *b16* mutant embryos. Since the *cyclops* gene is also deleted in *b16*, we were able to identify *b16* homozygotes by their cyclopia. We did not observe any significant difference in the expression pattern of endothelial and blood genes between wild-type and *b16* mutant embryos (Fig. 5B). These results suggest that Hhex function is not essential for early endothelial and blood development. Loss of *hhex* function may be compensated in this process by another gene such as *scl*, whose expression pattern and function upon overexpression appears to be similar to that of *hhex* (Gering et al., 1998; Liao et al., 1998).

hhex and *scl* can regulate each other

hhex and *scl* can both regulate endothelial and blood gene expression and exhibit similar expression patterns in endothelial and blood precursors, suggesting that both genes function at the same time during hemangioblast development. To investigate the relationship between *hhex* and *scl*, we overexpressed one of the two genes and examined the expression of the other at the 5- to 7-somite stage (12-13 hpf).

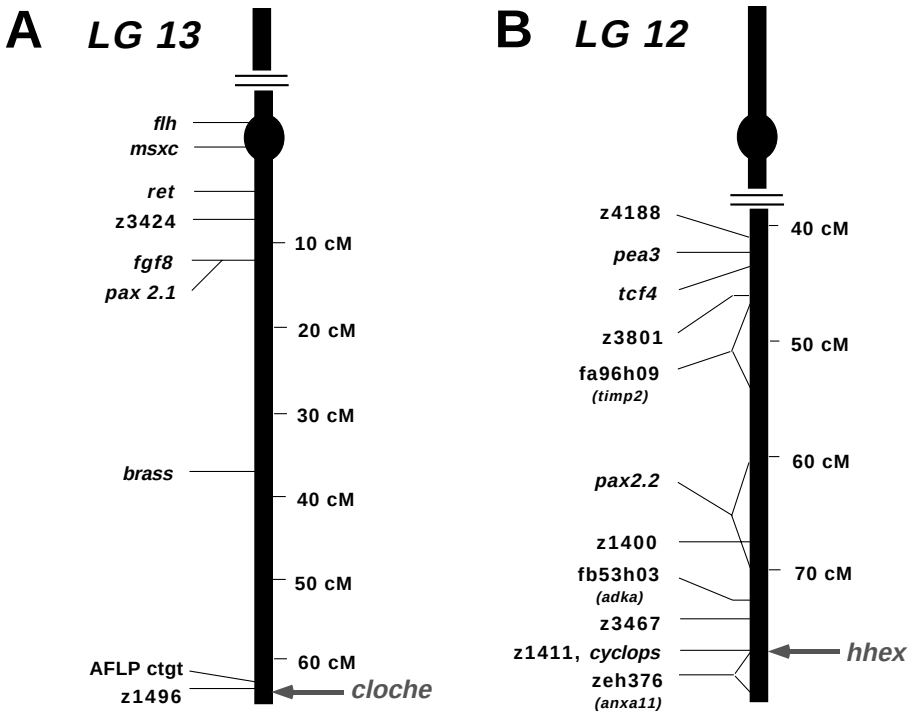


Fig. 4. *cloche* and *hhex* map to different linkage groups. (A) Genetic map of the lower half of LG13, based on the map published by Postlethwait et al. (1998). The grey arrow points to the region where *cloche* maps. The SSR marker z1496 used for genotyping the injected embryos maps 0.4 cM proximal to *cloche*. (B) Genetic map of the lower half of LG12 showing the relative position of the genes listed in Table 4. *hhex* (grey arrow) maps near *cyclops* and the SSR marker z1411.

Table 4. Conserved syntenies of loci around *hhex*

Zebrafish marker	Zebrafish location[a]	Zebrafish cross[b]	Human marker	Human location[c]	Expect (E) value[d]	Mouse marker	Mouse location[e]
z4188	LG12_42.1	MOP					
pea3	LG12_42.1-43.4	Tub	*E1AF*	17_320.39 cR_{3000}	e-152	*Pea3*	11_60.0
tcf4	LG12_44.5	HS	*TCF-4*	10_517.39 cR_{3000}	5e-45	*Tcf4*	2_94.0
z3801	LG12_46.8	MOP					
fa96h09	LG12_46.8-53.8	WU	*TIMP2*	17_512.35 cR_{3000}	e-29	*Timp2*	11_72.0
z1400	LG12_67.9	MOP					
pax2.2	LG12_60.8-71.4	OR-LN54	*PAX2*	10_477.24 cR_{3000}	e-180	*Pax2*	19_43.0
fb53h03	LG12_74.6	HS	*ADKA*	10_381.07 cR_{3000}	2e-74	*Adk*	14_A2-B
z1141	LG12_77.5	MOP	*KCNMA1*	10_388.77 cR_{3000}	0.74	*Kcnma1*	Unknown
hhex	LG12_77.5	MOP	*HHEX*	10_370.6 cR_{3000}	4e-75	*Hhex*	19_47.5
cyclops (ndr2)	LG12_77.5	MOP	*NODAL (oy47b06)*	10_370.67 cR_{3000}	2e-23	*Nodal*	10_31.5
zeh376	LG12_>77.5	Tub	*ANXA11*	10_401.27 cR_{3000}	4e-40	*Anxa11*	14_3.3v

[a]Locations were determined with respect to the position of nearby SSR markers mapped on the MGH mapping cross (http://zebrafish.mgh.harvard.edu/mapping/ssr_map_index.html).

[b]Zebrafish map locations were combined from several mapping crosses. MOP (MOP cross, Johnson et al., 1996; Postlethwait et al., 1998), Tub (Tübingen radiation hybrid panel; Geisler et al., 1999; http://wwwmap.tuebingen.mpg.de/), HS (Stanford/Oregon Heat Shock Cross; http://zebrafish.stanford.edu/genome/Frontpage.html), WU (Washington University scoring on the LN54 radiation hybrid panel; Huckriede et al., 1999; http://zfish.wustl.edu/rh.html), OR-LN54 (Oregon scoring on the LN54 radiation hybrid panel; this work).

[c]Putative orthologues were identified as the reciprocally closest blast hits in the human and zebrafish databases for the loci shown. Locations of the putative human orthologues are given in centiRay (cR_{3000}) as located on the GB4 radiation hybrid panel (GeneMap'99 (http://www.ncbi.nlm.nih.gov/genemap/query.cgi), and confirmed as a cytogenetic location as given in OMIM (Online Mendelian Inheritance in Man (http://www3.ncbi.nlm.nih.gov/Omim/searchomim.html).

[d]The nucleotide sequence of the zebrafish locus was used in a blastx search against the human non-redundant database (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) and the table shows the Expect value, a measure of the likelihood that a match this good would occur simply by chance when searching a database of a given size. The lower the value, the more similar the two sequences.

[e]The location of putative mouse orthologues were obtained from Mouse Genome Database (http://www.informatics.jax.org/searches/marker_form.shtml).

We found that *hhex* could induce the ectopic expression of *scl* and *scl* could induce the ectopic expression of *hhex*, although it appeared that *hhex* was a more potent inducer of *scl* expression than *scl* was of *hhex* (Fig. 6A,B and data not shown). To further investigate this cross-regulation, we performed similar experiments in embryos collected from a *cloche* mapping cross. Similar results were observed in wild-type and *cloche* mutant embryos as confirmed by genotyping with z1496 (attached table in Fig. 6). These data indicate that *hhex* and *scl* can cross-regulate each other positively and that this cross-regulation does not require *cloche* function.

A subset of *fli1* expressing cells are affected by the *cloche* mutation at early somitogenesis stages

In order to differentiate between the activities of Hhex and Scl, we investigated genes that, like *hhex*, are expressed in the forming vasculature and thus might be regulated by Hhex. *fli1* is an ETS-domain transcription factor gene that is expressed in angioblasts as well as in hematopoietic and neural crest cells in mouse and *Xenopus* embryos (Meyer et al., 1995; Melet et al., 1996; reviewed by Sharrocks et al., 1997). In zebrafish, *fli1* is first expressed in the posterior lateral plate and intermediate mesoderm and at late somitogenesis stages in endothelium, cranial neural crest, pharyngeal pouches and the pronephric primordia (Thompson et al., 1998; Brown et al., 2000).

The defects of *fli1* expression in *cloche* mutants can be clearly observed at late stages (Thompson et al., 1998; Brown et al., 2000). At early somitogenesis stages, a detailed examination reveals that a subset of the *fli1* expression pattern is missing in *cloche* mutants. At the 3-somite stage, *fli1* is expressed in the posterior lateral plate and adjacent intermediate mesoderm and no obvious difference is observed between wild-type and *cloche* mutant embryos at this stage (data not shown). At the 7-somite stage, *fli1* expression starts to appear in the head region and is missing in approximately a quarter of the embryos collected from a cross of *cloche*

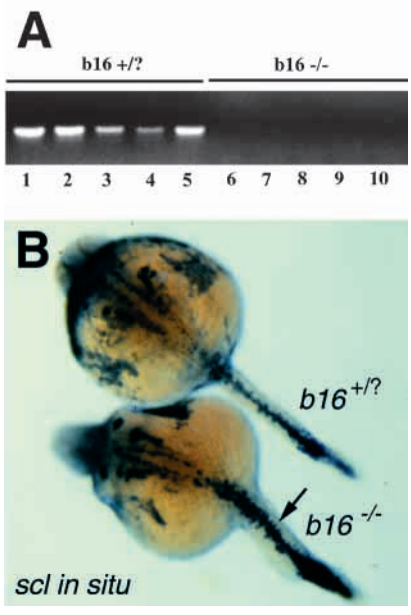
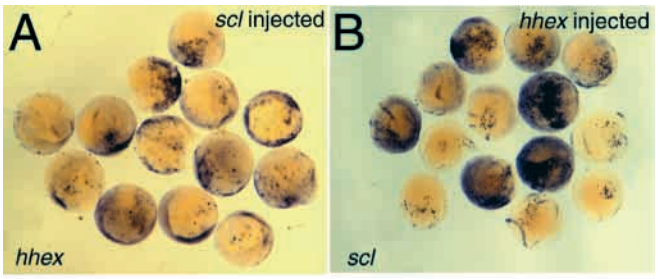


Fig. 5. *b16* mutant embryos do not contain *hhex* but show apparently normal endothelial and blood gene expression. (A) PCR amplification of an intron in *hhex* shows that it is present in wild-type embryos (lanes 1 to 5) but absent in *b16* mutant embryos (lanes 6-10), indicating that *hhex* is deleted in the *b16* deficiency. (B) *scl* expression in wild-type and *b16* mutant embryos at 24 hpf. *scl* expression appears unaffected in *b16* mutants, which were identified by their cyclopia. The increase in *scl* expression in the trunk of *b16* mutants (arrow) is at least partly due to the reduced circulation, which leads to the accumulation of blood cells in this region.



Genotype of injected embryos

Injected cDNA	*in situ* probe	Genotype results wt/wt	wt/*clo*	*clo*/*clo*
cmv-*scl*	*hhex*	18	18	12
cmv-*hhex*	*scl*	14	43	13

Fig. 6. *hhex* and *scl* can cross-regulate each other. (A) *hhex* expression in cmv-*scl* injected embryos at the 5- to 7-somite stage (12-13 hpf). (B) *scl* expression in cmv-*hhex* injected embryos at the 5- to 7-somite stage. The table shows genotyping data with z1496 of the injected embryos that showed ectopic *hhex* or *scl* expression.

heterozygous fish (Fig. 7A,B). At the 10-somite stage, some *fli1* expressing cells in the lateral plate mesoderm appear to migrate toward the ventral midline in wild-type embryos. This expression is absent in a quarter of the embryos from a *cloche* cross (Fig. 7C,D), and genotyping with z1496 showed that all the embryos with defective *fli1* expression were *cloche* mutants (data not shown). At the 20-somite stage (Fig. 7E-H), *fli1* expression is normal in the cranial neural crest, pharyngeal pouches and pronephric primordia but absent in the endothelial lineage of *cloche* mutants, except at the ventral region of the tail as previously reported (Thompson et al., 1998; Brown et al., 2000). These data suggest that *fli1* expression in the endothelial lineage is defective in *cloche* mutants, a defect obscured at early stages by *fli1* expression in the adjacent intermediate mesoderm (Fig. 7B).

Overexpression of *hhex* can induce ectopic *fli1* expression

To position *fli1* relative to *hhex* and *scl*, we injected cmv-*hhex* or cmv-*scl* into embryos collected from a *cloche* mapping cross at the 1- to 4-cell stage. Ectopic expression of *fli1* was observed at tailbud stage in approximately 20% of the *hhex* injected embryos but, at the dose used, not in the *scl* injected embryos (Fig. 7I and data not shown). (Although *scl* overexpression can induce ectopic *hhex* expression, it is possible that the level of ectopic *hhex* expression following *scl* overexpression is not sufficient to induce ectopic *fli1* expression. In addition, it is possible that higher effective concentrations of Scl would lead to ectopic *fli1* expression.) Genotyping revealed that a quarter of the cmv-*hhex* injected embryos showing ectopic *fli1* expression were *cloche* mutants (Table 2). These data indicate that Hhex can also positively regulate the expression of *fli1* in the absence of *cloche* function.

DISCUSSION

hhex expression in endothelial and blood precursors

Zebrafish Hhex shares very high sequence identity with its homologues in other vertebrates both inside and outside the homeodomain (Ho et al., 1999). Our comparative mapping data indicate that zebrafish *hhex* is the orthologue of human *HHEX* and mouse *Hhex* (Table 4). *hhex* is expressed in the developing cardiovascular system in a broadly conserved temporal and spatial pattern. In *Xenopus*, *hhex* expression in the angioblasts has been reported to initiate several hours after the onset of *flk1* expression and does not appear to be associated with the developing hematopoietic system (Newman et al., 1997). In chick embryos, *hhex* is expressed in blood islands starting at mid-gastrulation stages and in

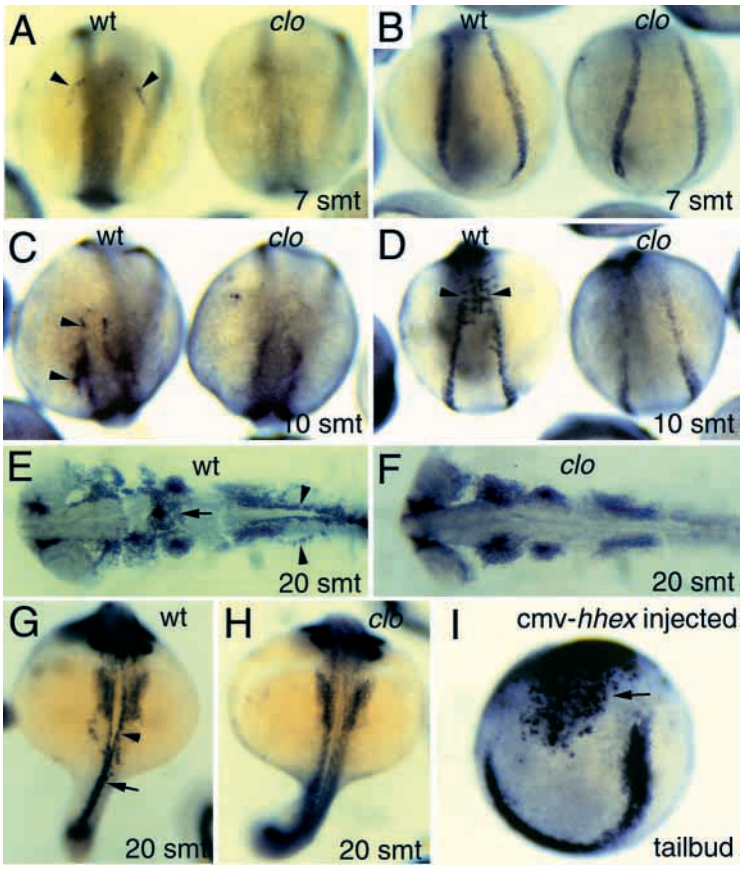


Fig. 7. Endothelial *fli1* expression is disrupted in *cloche* mutants. (A-H) *fli1* expression in wild-type (E,G, and left embryo in A-D) and *cloche* mutant (F,H, and right embryo in A-D) embryos at the 7-somite stage (13 hpf) (A,B), 10-somite stage (14 hpf) (C,D) and 20-somite stage (19 hpf) (E-H). (A,C) Dorsoanterior views. (B,D) Dorsoposterior views. (E,F) Dorsal views of the anterior portion of flattened 20-somite stage embryos. (G,H) Mid-dorsal views of 20-somite stage embryos. Black arrowheads in A,C,D,E,G point to endothelial *fli1* expression that is missing in *cloche* mutants. *fli1* expression in the endocardium (black arrow in E) and ICM (black arrow in G) is clearly missing in *cloche* mutants. (I) Ectopic expression of *fli1* (black arrow) is found in embryos injected with cmv-*hhex* (tailbud stage).

extraembryonic and intraembryonic vascular endothelial cells as vessels form (Yatskievych et al., 1999). The most detailed expression information is available in mouse: *hhex* is first expressed in the nascent blood islands of the visceral yolk sac at the same stage that *flk1* is first expressed in this population of cells (Thomas et al., 1998). *hhex* is subsequently expressed in the embryonic angioblasts and endocardium and then rapidly turned down as these endothelial cells differentiate. Similarly, in zebrafish, *hhex* expression parallels that of *flk1* (Fouquet et al., 1997; Liao et al., 1997; Sumoy et al., 1997). It first appears both anteriorly and posteriorly by the 3-somite stage. Posteriorly, it is expressed in the nascent ICM, which contains both endothelial and blood precursors. This expression pattern is reminiscent of that exhibited by *fli1*, *gata2* and *scl* (Liao et al., 1998; Thompson et al., 1998; Brown et al., 2000). Subsequently, *hhex* expression is observed in the developing endothelial cells including the endocardium until about the onset of circulation. This evolutionarily conserved expression pattern suggests that *hhex* plays an important role in regulating the differentiation of both endothelial and blood cells.

hhex and *scl* expression in the ICM region parallel each other at early stages. At later stages, *hhex* is transiently expressed in the forming blood vessels while *scl* is expressed in circulating blood cells (Liao et al., 1998). These data suggest that *hhex* and *scl* both function in controlling early endothelial and blood differentiation and that *hhex* and *scl* play additional roles in later stages of endothelial and blood formation, respectively.

Hhex can function as a positive regulator of endothelial and blood gene expression

Gain-of-function analyses show that *hhex* is capable of inducing premature and ectopic expression of *flk1* and *gata1* (Fig. 3). It is unclear whether the cells expressing premature or ectopic *flk1* or *gata1* would eventually differentiate into endothelial or blood cells. Even though we did observe ectopic expression of late endothelial genes such as *tie1* and *tie2* upon *hhex* overexpression, the cells expressing these genes did not appear elongated as most differentiated endothelial cells do. Moreover, the ectopic expression of *flk1* and *gata1* did not appear to be limited to the mesodermal germ layer, as some of the *flk1* and *gata1* expressing cells were observed in dorsal (i.e. ectodermal) regions of the embryo (data not shown). This situation is reminiscent of that seen with *gata5* overexpression where ectopic expression of myocardial genes is observed much more frequently than the formation of ectopic beating tissue (Reiter et al., 1999). Thus, while some of the cells ectopically expressing *flk1* or *gata1* may be ectopic endothelial or blood cells, most of them probably represent other cell types in which only part of the endothelial or blood transcription program has been activated.

Extra-embryonic tissues have been implicated in endothelial and blood formation in mouse (Belaoussof et al., 1998). Zebrafish *hhex*, like mouse *hhex*, is expressed in extraembryonic tissues, but this expression does not appear to play a direct role in endothelial or blood formation: overexpression of *hhex* in the YSL did not cause ectopic *flk1* or *gata1* expression. Thus, by virtue of its expression in endothelial and blood precursors, we propose that Hhex functions cell-autonomously in the differentiation of these cell types.

Overexpression of *hhex* mRNA in *Xenopus* embryos led to vascular malformations but no ectopic expression of the endothelial marker *Xmsr* was observed (Newman et al., 1997). These results and the fact that *flk1* expression appears to precede *hhex* expression in *Xenopus* embryos led Newman et al. (1997) to propose a role for Hhex in regulating endothelial cell proliferation. Thus, Hhex may have both an inductive and proliferative role in the endothelial lineage.

In various settings, Hhex has been shown to function as a transcriptional repressor (Brickman et al., 2000; Tanaka et al., 1999), suggesting that the ectopic activation of gene expression reported here is not a direct effect. Functional dissection of Scl has shown that its DNA binding domain is not required for its activity in vasculogenesis and hematopoiesis (Porcher et al., 1999). One of the models suggested to explain this observation is that Scl sequesters an unidentified repressor of hemangioblast development through the formation of Scl-Repressor heterodimers. Our data indicate that the DNA binding domain of Hhex is required for its activity. One model consistent with these data is that Hhex negatively regulates the aforementioned repressor of hemangioblast development at the transcriptional level. Alternatively, we have previously proposed that Hhex downregulates *bmp* expression in the gastrulating zebrafish embryo (Ho et al., 1999). Hhex may function similarly in the endothelial lineage and modulate *bmp* expression. Experiments in chick embryos indicate that Bmp inhibits the appearance of QH1 positive (i.e. endothelial) cells in cardiogenic explants (Yukiko Sugi and Roger Markwald, personal communication), suggesting that regulating the level of Bmp signaling may be critical to endothelial cell differentiation. In this regard, it is interesting to note that tissues directly adjacent to the forming dorsal aorta such as the hypochord and notochord express high levels of collagen, which can bind Bmp through its cysteine-rich domain (Zhu et al., 1999; Larrain et al., 2000). Further analysis should help define the role of Bmp signaling in endothelial cell differentiation and blood vessel formation.

Hhex function does not appear to be required for early endothelial and blood development

hhex is deleted in the gamma-ray induced deficiency *b16*, but the expression of various endothelial and blood genes in *b16* mutant embryos appears normal. It is formally possible that a second *hhex* gene exists in zebrafish although early endothelial differentiation also appears normal in mouse *hhex* mutants (Martinez Barbera et al., 2000; blood differentiation has not yet been analyzed in these mutants). These data indicate that in zebrafish, as in mouse, Hhex is not required for hemangioblast development and suggest the involvement of another regulator that can compensate for the loss of Hhex function. Scl is potentially this other regulator as it is expressed in a pattern similar to that of Hhex in the endothelial and blood lineages and appears to function similarly based on overexpression experiments in zebrafish. In mouse, Scl is required for normal blood development (reviewed by Orkin and Zon, 1997; Barton et al., 1999) and also for the remodeling of the yolk sac vasculature (Visvader et al., 1998). It will be interesting to analyze embryos mutant for both *hhex* and *scl* and thereby test whether *scl* can indeed compensate for the absence of Hhex function during early endothelial and blood differentiation.

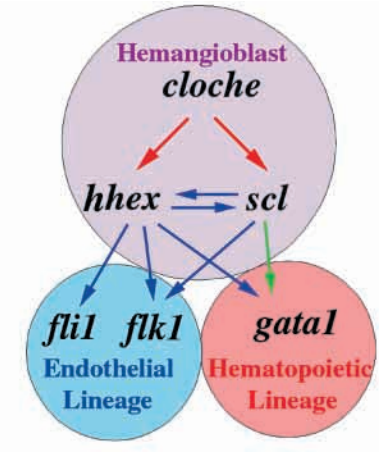


Fig. 8. Model of hemangioblast development and early endothelial and blood cell differentiation. The cell lineages are shown as colored circles. In this model, *hhex* and *scl* function downstream of *cloche* and cross-regulate each other during hemangioblast development. *hhex* and *scl* can induce the expression of the endothelial gene *flk1* as well as the erythroid gene *gata1*, suggesting that they positively regulate the differentiation of both endothelial and blood cells. Integrating data obtained in zebrafish and in mouse, the color of the arrows represents results obtained from either gain-of-function (blue), loss-of-function (red) or both types of analyses (green).

A pathway regulating early endothelial and blood differentiation

Similar to all endothelial and blood genes published to date, *hhex* expression in endothelial and blood precursors is severely reduced in *cloche* mutants (Fig. 2). In addition, *hhex* overexpression can induce ectopic expression of endothelial and blood genes in *cloche* mutants (Table 2). These observations place *hhex* downstream of *cloche*. We further show that *hhex* and *scl* can cross-regulate each other in a way that does not require *cloche* function. In addition, the endothelial expression of *fli1* is disrupted in *cloche* mutants and *hhex* but apparently not *scl* can lead to the ectopic activation of *fli1*. Taken together, these results combined with those obtained by Liao et al. (1998) suggest a molecular framework for hemangioblast development as shown in Fig. 8. *hhex* and *scl* cross-regulate each other and function downstream of *cloche* to activate the endothelial and blood genes *fli1*, *flk1* and *gata1*. This framework should facilitate the testing and integration of additional factors thought to regulate hemangioblast development.

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