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cloche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages

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SUMMARY

Endothelial and hematopoietic cells appear synchronously on the extra-embryonic membranes of amniotes in structures known as blood islands. This observation has led to the suggestion that these two ventral lineages share a common progenitor. Recently, we have shown in the zebrafish, *Danio rerio*, that a single cell in the ventral marginal zone of the early blastula can give rise to both endothelial and blood cells as well as to other mesodermal cells (Stainier, D. Y. R., Lee, R. K. and Fishman, M. C. (1993). *Development* 119, 31-40; Lee, R. K. K., Stainier, D. Y. R., Weinstein, B. M. and Fishman, M. C. (1994). *Development* 120, 3361-3366). Here we describe a zebrafish mutation, *cloche*, that affects both the endothelial and hematopoietic lineages at a very early stage. The endocardium, the endothelial lining of the heart, is missing in mutant embryos. This deletion is selective as evidenced by the presence of other endothelial cells, for example those lining the main vessels of the trunk. Early cardiac morphogenesis proceeds normally even in the absence of the endocardium. The myocardial cells form a tube that is demarcated into chambers, beats rhythmically, but exhibits a reduced contractility. This functional deficit is likely due to the absence of the endocardial cells, although it may be a direct effect of the mutation on the myocardial cells. Cell transplantation studies reveal that the endothelial defect, i.e. the endocardial deletion, is a cell-autonomous lesion, consistent with the possibility that *cloche* is part of a signal transduction pathway.

In addition, the number of blood cells is greatly reduced in *cloche* mutants and the hematopoietic tissues show no expression of GATA-1 or GATA-2, two key hematopoietic transcription factors that are first expressed during early embryogenesis. These results show that *cloche* is involved in the genesis and early diversification of the endothelial and blood lineages, possibly by affecting a common progenitor cell population.

Key words: heart, endocardium, hematopoiesis, zebrafish, *cloche*

INTRODUCTION

The molecular mechanisms that regulate the genesis and diversification of ventral mesodermal lineages are not well understood. In *Xenopus* embryos, signal(s) emanating from the vegetal hemisphere (prospective endoderm) induce the animal hemisphere (prospective ectoderm) to generate dorsal and ventral mesoderm (Nieuwkoop, 1969; Dale and Slack, 1987). Dorsal mesoderm forms head mesoderm, notochord, and some muscle, while ventral mesoderm forms blood and some posterior tissues of the embryos (Dale et al., 1985). Several models have been put forth to account for mesoderm induction and a large number of mesoderm inducing molecules identified (reviewed by Kessler and Melton, 1994).

Soon after mesoderm induction, the various mesodermal lineages differentiate. In amniotes, endothelial and hematopoietic cells appear synchronously on the extra-embryonic membranes in structures known as blood islands (Sabin, 1920; Pardanaud et al., 1989). Lineage data in the zebrafish show that individual cells in the ventral marginal zone of the early blastula can give rise to both endothelial cells and blood cells as well as to other mesodermal cells (Stainier et al., 1993; Lee et al., 1994).

The molecular mechanisms that link the early events of ventral mesoderm induction to differentiation of the endothelial and hematopoietic lineages are being actively investigated. The differentiation of blood cells has been shown to depend on transcription factors belonging to the GATA-binding protein family. GATA-1 is expressed in erythroid cells, mast cells, and megakaryocytes (Martin et al., 1990; Romeo et al., 1990). It is

involved in the transcriptional regulation of all erythroid-specific genes that have been characterized, including those encoding the globins and the heme biosynthetic proteins. Targeted gene disruption experiments in the mouse have indicated that GATA-1 is absolutely required for normal primitive and definitive erythropoiesis (Pevny et al., 1991). During development, GATA-1 expression is localized to the ventral region of the *Xenopus* embryo and precedes the expression of embryonic globins (Kelley et al., 1994). In zebrafish, GATA-1 is initially detected at the 2-somite stage in two stripes of cells that flank the paraxial mesoderm of the posterior embryo (Detrich et al., 1995). Another member of the family, GATA-2, is highly expressed in early hematopoietic progenitors, mast cells and megakaryocytes (Dorfman et al., 1992; Yamamoto et al., 1990). It is also expressed in abundance in non-hematopoietic tissues including the developing brain and mature endothelium (Wilson et al., 1990). By gene targeting experiments in the mouse, GATA-2 has been shown to play a critical role in hematopoiesis, by regulating genes controlling either growth factor responsiveness or the proliferative capacity of early hematopoietic cells (Tsai et al., 1994). In X*enopus* embryos, GATA-2 is highly expressed in the ventral region of the embryo by the end of gastrulation and later is expressed in the blood island region and the central nervous system (CNS) (Kelley et al., 1994). In zebrafish, GATA-2 is also first detected at the end of gastrulation (Detrich et al., 1995). Later, it is expressed in all hematopoietic progenitors including the presumptive stem cells found in the posterior region of the intermediate cell mass of Oellacher (ICM). Thus, GATA-1 and GATA-2 are the earliest characterized markers of blood cell differentiation.

Endothelial cell differentiation is less well understood. Three distinct mechanisms have been proposed to underlie endothelial differentiation and blood vessel formation: vasculogenesis type I refers to the endothelial cells that differentiate in situ (such as those forming the trunk vessels), while vasculogenesis type II applies to endothelial cells that differentiate after extensive migration of their progenitors (such as the endocardium and head vessels; Poole and Coffin, 1991). The third mechanism, angiogenesis (Folkman and Shing, 1992), refers to the formation of new vessels from pre-existing vessels by vascular sprouting. It is thought to be responsible for the formation of the intersomitic vessels, among others. Antibody markers (Pardanaud et al., 1987) and, more recently, genes expressed early during endothelial cell differentiation have been isolated. A receptor tyrosine kinase gene, *flk-1*, has been reported to be expressed in endothelial progenitor cells in the mouse (Millauer et al., 1993; Quinn et al., 1993; Yamaguchi et al., 1993). A close relative, *flt-1*, is expressed in a similar pattern although at a slightly later stage (Shibuya et al., 1990). Two other receptor tyrosine kinase genes, *tek* (Dumont et al., 1992; Schnurch and Risau, 1993) and *tie* (Korhonen et al., 1994), have been shown to be expressed in more mature endothelial cells. These genes are expressed in all endothelial cells. No genes have been characterized to contribute to functional diversification of endothelial cells or demarcate different vascular beds during development.

The endocardium of the heart is a specialized endothelium which clearly plays specific functions in cardiac formation. In the definitive heart tube, the endocardial layer is surrounded by the myocardial layer. At the boundaries between chambers, endocardial cells migrate into the extracellular matrix under the control of signals from the myocardial cells and differentiate into prevalvular mesenchyme (Markwald et al., 1990). Whether signals also emanate from the endocardium is unknown.

We are taking a genetic approach in the zebrafish to dissect the morphogenesis of the cardiovascular system. Here we present a mutation, *cloche*, that affects the cardiovascular system in a most intriguing way. *cloche* blocks the formation of part of the vasculature, namely the endocardium of the heart. Early cardiac morphogenesis appears to proceed normally in the absence of the endocardium. *cloche* also affects blood cell differentiation at a very early stage. It appears to either act upstream of the GATA factors or affect a population of cells that differentiate to express the GATA factors.

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. The Indonesian fish were purchased through J. Sohn. The spontaneous allele *clo*^{m39} was maintained in the genetic background in which it was originally identified and also out-

Fig. 1. Live wild-type (A) and homozygous *clo*^{m39} mutant (B) zebrafish embryos at 24 hours (all times are in hours postfertilization at 28.5°C; *clo* indicates the locus of the mutation, and *m39* is the allele designation). At this time and at this magnification, a slight edema is noticeable in the pericardial region of the mutant embryo but no other abnormalities are apparent. Indeed, development appears normal until the onset of the heart beat by the 26-somite stage (22 hours). From that time onwards, the heart exhibits a weak beat and becomes abnormally enlarged, especially the atrium. Edema becomes very pronounced by day 2. There is no circulation, although cells are present in the trunk vessels (see Fig. 3). (C) In a normal heart at around 40 hours, the myocardium and endocardium (arrows) are clearly distinct. Endocardial cells are the thin elongated cells that form the inner layer of the heart. The *clo* mutant heart only has a single layer of cells (as shown in a medial view of the heart (D), see also F), determined to be the myocardium by its spontaneous contractility, and also morphologically, ultrastructurally, and biochemically, using monoclonal antibodies (mAbs) against myosin heavy chain and tropomyosin (Stainier and Fishman, 1992) (see also Figs 2 and 4). The main chambers (atrium (A) and ventricle (V)) are distinct morphologically and biochemically (using chamber specific mAbs against myosin heavy chain; data not included) (Stainier and Fishman, 1992) and contract sequentially. In the mutant heart, the chambers are dysmorphic, the atrium is abnormally enlarged and the ventricle is collapsed. Some mutant hearts undergo normal looping morphogenesis. (E) Transverse sections of the atrium of a normal heart at 3 days showing the myocardium and endocardium (arrows) as well as some blood cells (dark round cells) and of a *clo* mutant heart at 3 days (F) showing the presence of a single layer of cells, previously determined to be the myocardium. Scale bars, 200 μm (A,B); 50 μm (C-F). [In Fig. 1 C and E, a *silent heart* (*sih*) mutant embryo is shown as the wild-type control. Because there is no circulation in the *clo* mutant embryo, we wanted to distinguish the specific effects of the *clo* mutation from the indirect effects resulting from a lack of circulation. For this purpose, we examined the phenotype of another mutant, the *sih* mutant (isolated in Eugene, Oregon by C. Walker and C. Kimmel), which, as the name indicates, exhibits no heart beat, and thus no blood flow. We previously determined at the molecular level that the *sih* mutation is cardiac specific and is thus a good model to examine the indirect effects resulting from a lack of circulation (Stainier et al., in preparation). The *sih* mutant heart is morphologically similar to the wild-type heart.]

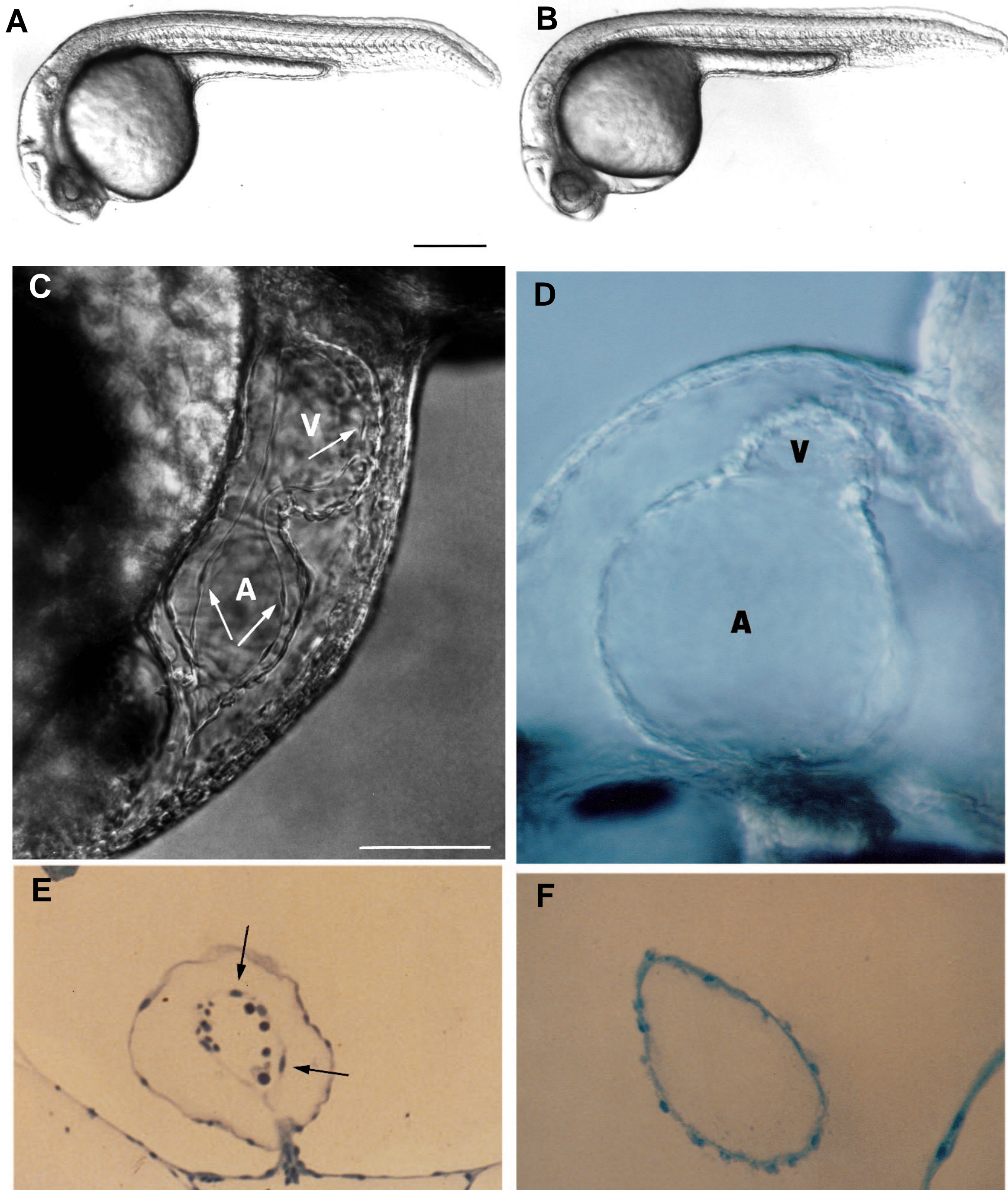
crossed to the standard wild-type (AB) laboratory strain (obtained from C. Kimmel, Eugene, Oregon). The mutation is fully penetrant and exhibits complete and indistinguishable expressivity in both backgrounds. Homozygous mutant embryos were obtained from normal crosses between identified heterozygotes carrying the clo^{m39} allele.

Whole-mount immunohistochemistry and histology

Embryos were staged according to somite number, then stained in 0.1% saponin as described by Stainier and Gilbert (1990). Monoclonal antibodies CH1 (against tropomyosin; Lin et al., 1985) and MF20 (against myosin heavy chain; Bader et al., 1982) were obtained from the Developmental Studies Hybridoma Bank; mAb S46 (against myosin heavy chain) stains specifically the atrium in zebrafish and was obtained from Jeff Miller (Mass. Gen. Hospital). All 3 mAbs stain specifically the myocardial layer. After peroxidase staining with CH1, some of the whole mounts were postfixed in 4% formaldehyde (EM grade: Polysciences) and then embedded in JB4 (Polysciences) and sectioned at 4 µm. Sections were counterstained with methylene blue.

Cell transplantation

Transplantations were performed essentially as described previously (Ho and Kane, 1991; Halpern et al., 1993; Ho and Kimmel, 1993). Donor embryos were injected with 4% rhodamine-dextran/4% biotin-dextran (Molecular Probes) in 0.2 M KCl. Transplanted cells were removed from and transplanted to the dorsal or ventral marginal zone (this cannot be determined at the time of transplantation) of donor and host embryos. Transplantations were done this way because we had determined that the ventral marginal zone is the cardiogenic area at



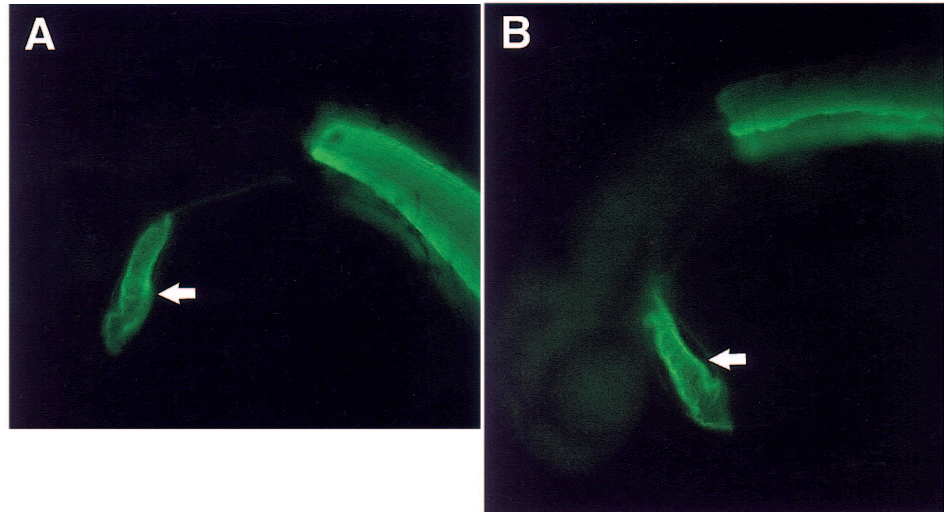


Fig. 2. The *clo* mutant heart expresses normal levels of myofibrillar proteins. A number of myofibrillar proteins were examined by immunohistochemistry, including different isoforms of myosin heavy chain, troponins I and T, and tropomyosin. Comparable levels of tropomyosin as observed by immunofluorescence are seen in wild-type (A), and *clo* mutant (B) embryos at 36 hours. Arrows point to the heart and the other staining is skeletal muscle in the trunk.

the blastula stage (Stainier et al., 1993). Ho and Kimmel (1993) recently showed that cells do not become committed to a particular germ layer until mid gastrulation. We also subsequently confirmed that cells taken from various regions of the mid blastula can form heart tissue when transplanted into the cardiogenic area, although the relative frequency of incorporation into heart tissue was not strictly analyzed with respect to the origin of the cells. At 36-48 hours, donor and host embryos were phenotyped, and host embryos were analyzed for the presence of fluorescent cells in the heart. To confirm the identity of the labeled cells, hosts were fixed and processed for HRP staining of biotin-dextran filled donor cells as described by Westerfield (1993), embedded in JB4 (Polysciences), and sectioned at 5 µm.

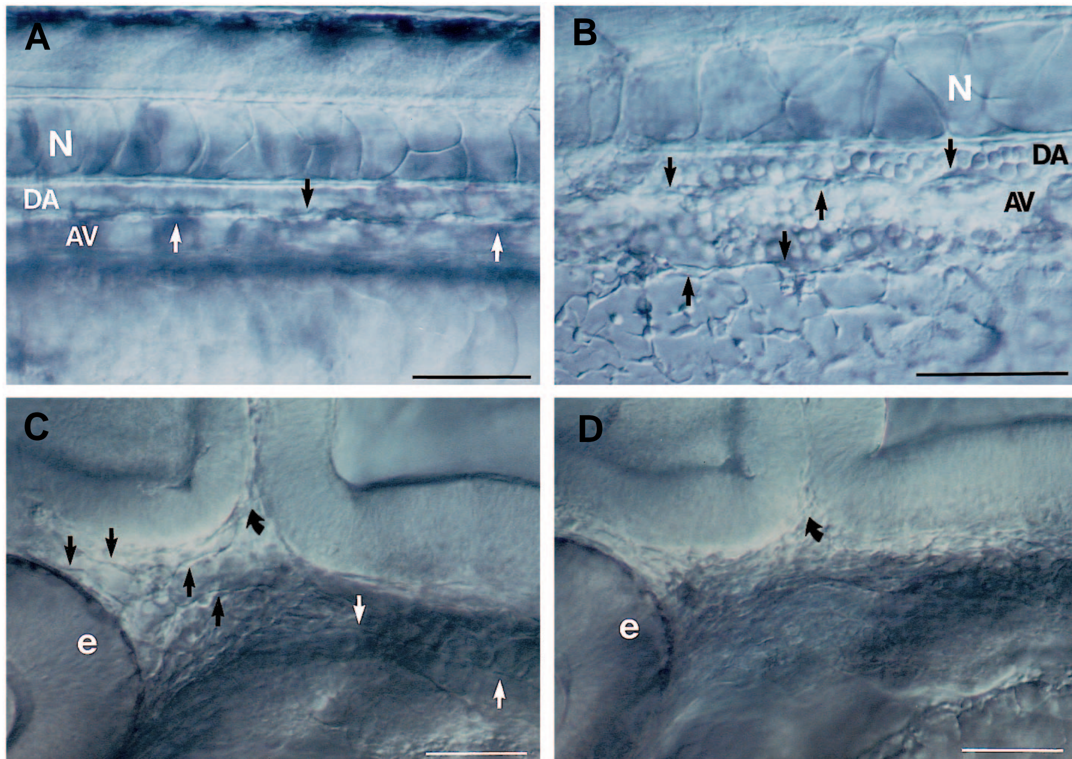


Fig. 3. (A,B) Live embryos at 36 hours. As in wild type (A), the clo mutant (B) has blood vessels in the trunk. A shows the central vessels in the mid-trunk region; B shows the central vessels more caudally where they are easier to photograph in the mutant. Arrows point to endothelial cells. The dorsal aorta (DA) sits between the notochord (N) and the axial vein (AV). (Cells fill the trunk vessels in wild-type and mutant embryos but because they don't circulate in the mutants, they are more clearly distinguished.) In the head, at 28 hours (which is after the initiation of the heart beat but before circulation is established in the head; Stainier et al., 1993), blood vessels are clearly seen in wild-type (C) but not in clo (D). Straight arrows point to endothelial cells; curved arrow points to the midbrain-hindbrain boundary. We also examined plastic sections throughout the head region and confirmed the Nomarski optics observations. At earlier times (and also here at 28 hours), the head mesoderm in clo appears more condensed than in wild-type, suggesting that the migrating angioblasts that normally form part of the loose head mesenchyme may in fact be missing. e, eye. Scale bars, 50 µm.

In situ hybridization and probes

Synthesis of digoxigenin-labeled GATA-1 and GATA-2 sense and antisense RNA probes and wholemount *in situ* hybridizations were performed as described previously (Hemmati-Brivanlou et al., 1990; Detrich et al., 1995).

RESULTS

We discovered the mutation clo^{m39} (named *cloche* for its bell-shaped heart) in a search for spontaneous mutations in a semi-wild population from an Indonesian fish farm. Mutants were originally detected by the swollen appearance of the heart as well as by its weak contractility at 36 h (hours post fertilization at 28.5°C). The phenotype is zygotic recessive, and the mutant embryos survive for one week of development. The mutation segregates as a single locus and is fully penetrant; approximately one-quarter (400 from 1572) of embryos obtained from crosses between heterozygotes were mutant. Development appears normal until the onset of the heart beat by the 26-somite stage (22 hours) (Fig. 1A,B). From that time onwards, the heart exhibits a weak contractility, the atrium becomes abnormally enlarged, and the ventricle appears collapsed (Fig. 1D). The embryonic heart is normally composed of two concentric layers of cells (Fig. 1C, E), the outer myocardium and inner endocardium. In the mutant heart, there is only a single layer of cells (Fig. 1D, F) that contracts rhythmically and spontaneously. By morphological, ultrastructural, and biochemical criteria (using monoclonal antibodies (mAbs) against myosin heavy chain (MF20) and tropomyosin (CH1; Fig. 2)), we determined this layer to be the myocardial layer, or muscular component of the heart. The endocardium, the endothelial layer of the heart, is completely absent. Cardiac cushions, which are endocardial in origin (Markwald et al., 1990), are also missing. Early cardiac morphogenesis appears to proceed normally even in the absence of the endocardial layer. The myocardial tube forms and becomes divided into chambers which can be distinguished morphologically and biochemically, using chamber-specific mAbs against myosin heavy chain (MF20, S46) (Stainier and Fishman, 1992) (data not included). Some mutant hearts undergo normal looping morphogenesis with the ventricle looping to the right. In summary, the *clo* mutation deletes the endocardial cells from the definitive heart tube.

The *clo* mutation affects endothelial cell differentiation

Because the endocardium is the endothelial lining of the heart, it was important to determine the extent of the endothelial deletion in *clo* mutant embryos. Microscopic examination of living mutant embryos using Nomarski optics, as well as histology on semi-thin sections, revealed that endothelial cells are present and form the dorsal aorta and the axial vein, which are the central vessels of the trunk (Fig. 3B). In the head, where later forming vessels are clearly visible in wild-type embryos (straight arrows: Fig. 3C), no endothelial vessels are seen in the mutant (Fig. 3D). Currently, there are no molecular markers available for zebrafish endothelial cells that would allow us to confirm the observation that endothelial vessels are missing from the head region in mutant embryos. Nevertheless, it is clear that the endocardium is missing while the trunk vessels are lined by morphologically normal endothelial cells. Thus, the *clo* mutation specifically deletes a subset of endothelial cells.

Heart tube formation in zebrafish occurs essentially as in other vertebrates. From bilateral regions of precardiac mesoderm, two primitive myocardial tubes form on either side of the midline (Stainier et al., 1993). A distinct group of cells,

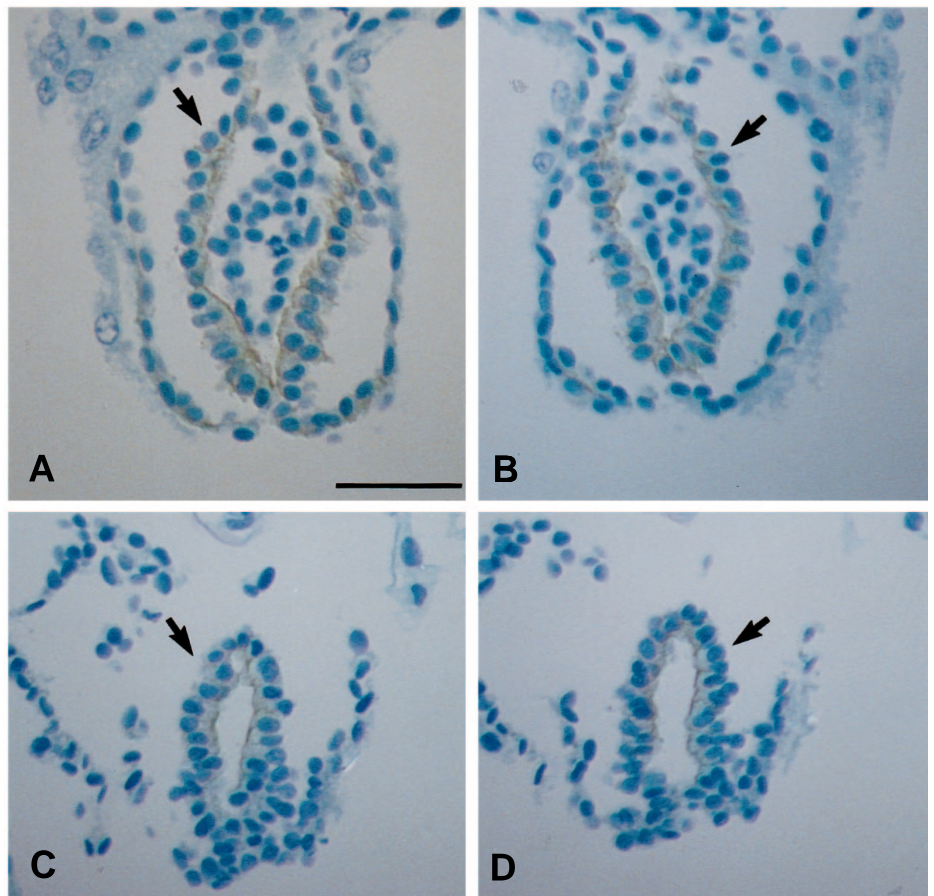


Fig. 4. The endocardial progenitor cells are absent from the heart region in *clo* mutant embryos at the time of heart tube formation. Horizontal sections of a wild-type (A,B) and a *clo* mutant embryo (C,D) at the 24-somite stage. In wild type, tropomyosin immunoreactive myocardial cells (arrows) surround the endocardial progenitor cells as the two primitive myocardial tubes fuse to form the definitive heart tube (Stainier et al., 1993). In *clo*, tropomyosin immunoreactive myocardial cells (arrows) surround a space which is conspicuously empty and somewhat collapsed. (Serial sections were examined throughout this region in several mutant embryos and no endocardial progenitor cells were seen). In C and D, a more apical region of the heart cone (i.e. a more arterial region of the heart) is shown which explains why the mutant heart appears smaller than wild type. Scale bar, 50 μm.

Table 1. Mosaic analysis of the endocardial defect in *clo* embryos

Host	Donor	
	WT (Donor/Host)	M (Donor/Host)
A. Endocardial cells		
WT	9/208	0/64
M	10/65	0/28
B. Myocardial cells		
WT	36/208	14/64
M	25/65	6/26

Number of cases where donor-derived endocardial (A) and myocardial (B) cells were found in host embryos following cell transplantation.

A total of 600 transplants were performed, at a ratio of one donor to four hosts. We analyzed the 365 surviving hosts for which there was also a surviving donor; there were 208 wild-type hosts transplanted with cells from a wild-type donor ('WT to WT' hosts), 65 mutant hosts transplanted with cells from a wild-type donor ('WT to M' hosts), 64 wild-type hosts transplanted with cells from a mutant donor ('M to WT' hosts), and 28 mutant hosts transplanted with cells from a mutant donor ('M to M' hosts). Donor-derived endocardial cells were found in both WT to WT hosts (9/208, 4.3%) and WT to M hosts (10/65, 15.4%), but not in M to WT hosts (0/64) or M to M hosts (0/28). (Because we never observed complete rescue of the endocardial defect, mutant host embryos were easily identified). In contrast and as a control, donor-derived myocardial cells were found with similar frequencies (approximately 20% of hosts) in all 4 classes of transplants. The probability of not finding donor-derived endocardial cells in all 64 M to WT transplants is $P<0.06$ if we take the WT to WT frequency as reference, and $P<0.01$ if we pool the WT to WT and WT to M frequency to use as reference.

the endocardial progenitor cells, sits medially between them. The myocardial tubes then fuse to enclose the endocardial cells and form the definitive heart tube. In this context, we examined the developmental events leading to the absence of the endocardium in *clo* mutant embryos. One possible explanation was that the endocardial progenitor cells were absent, a second was that they might reach the region of the cardiac primordia but fail to differentiate. In the normal zebrafish embryo, at the early stages of heart tube formation, the endocardial progenitor cells reside between the fusing left and right primitive myocardial tubes (Fig. 4A, B). In the mutant, these progenitor cells are absent, leaving the fusing heart tube empty and somewhat collapsed (Fig. 4C, D). Thus, the *clo* mutation seems to affect the generation of the endocardial progenitor cells rather than their final differentiation.

The endocardial defect in *clo* mutants is cell-autonomous

Inductive interactions between the precardiac mesoderm and neighbouring tissues appear to be necessary for myocardial cell differentiation (Jacobson and Sater, 1988; Nascone and Mercola, 1995), and possibly also for endocardial formation. To examine the cell-autonomy of the endocardial defect in *clo*, we performed reciprocal cell transplantations between *clo* and wild-type embryos at the mid to late blastula stage. Transplantations were performed essentially as described previously (Ho and Kane, 1991; Lee et al., 1994). We found that wild-type cells can form endocardium in mutant embryos as shown

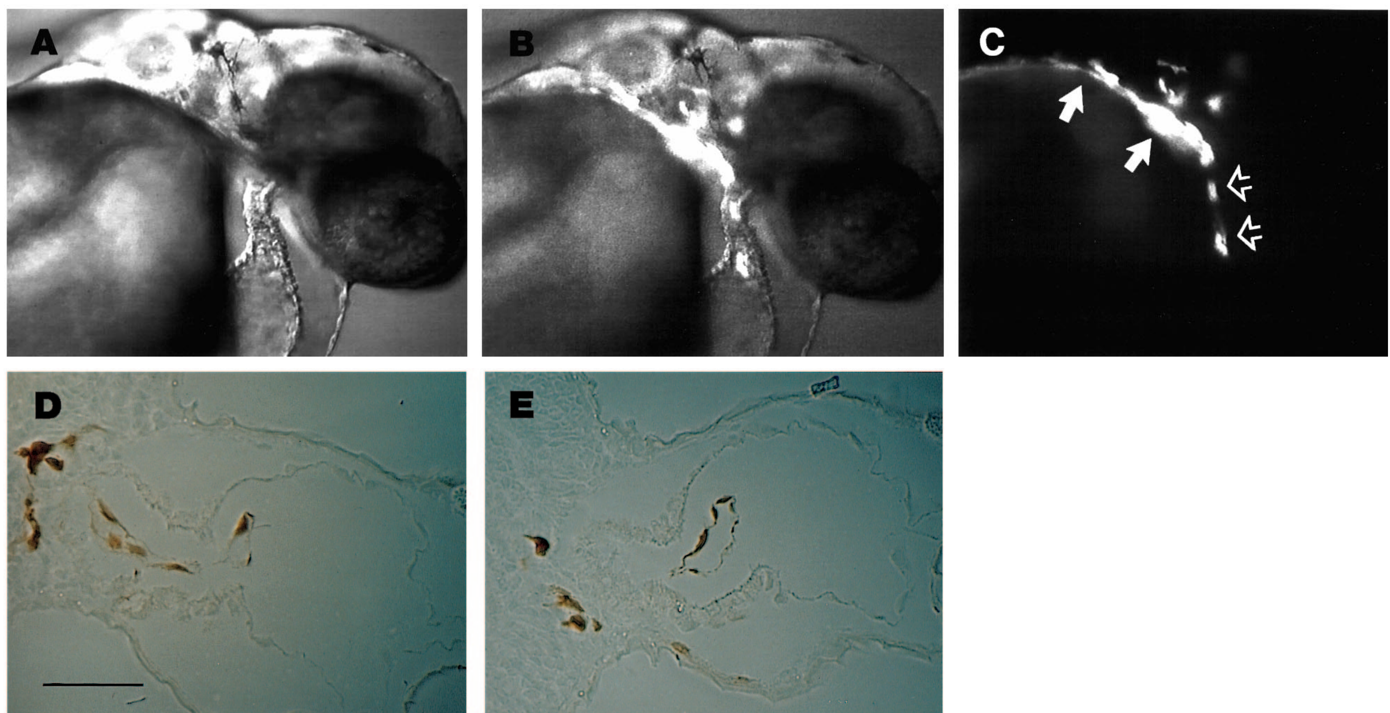


Fig. 5. Wild-type cells can form endocardium in *clo* mutant embryos. Nomarski (A), Nomarski + fluorescence (B), and fluorescence (C) micrographs of the same living 36 hour mutant host embryo. Labeled cells, derived from a wild-type donor embryo, populate the outflow tract (filled arrows) and form an elongated mass of tissue in the ventricular chamber of the myocardial tube (open arrows). Transverse sections through the heart of another mutant host after horseradish peroxidase staining of the biotin dextran labeled wild-type cells (D,E) reveal that transplanted wild-type cells form a distinctly elongated cell layer, the endocardial layer, inside the heart of the host embryo. When endocardium was observed in wild-type (WT) to mutant (M) transplants, all the endocardial cells were derived from WT as assessed by immunoreactivity to biotin (n=10). Scale bar 50 μm (D,E).

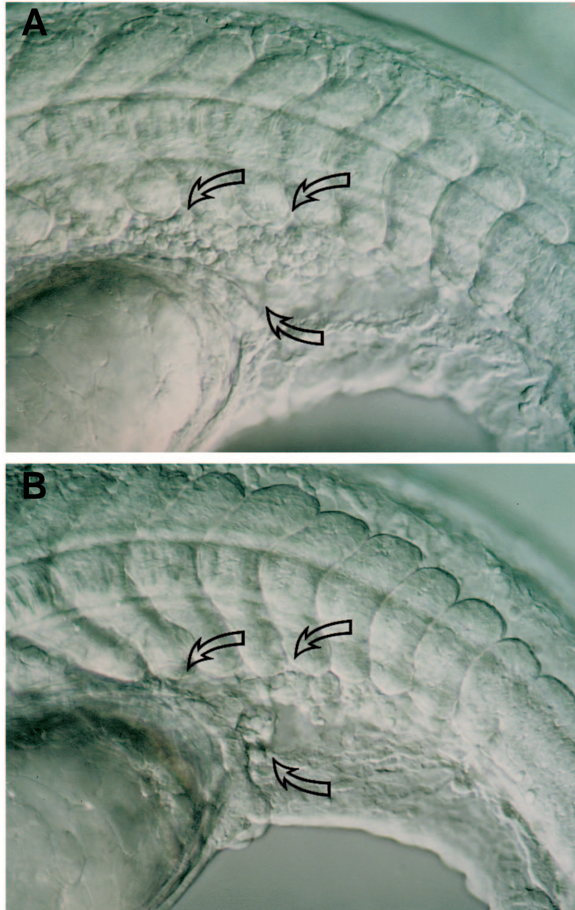


Fig. 6. The number of presumptive hematopoietic stem cells is reduced in *clo* mutant embryos. Nomarski micrographs of living wild-type (A), and *clo* mutant (B) embryo around the 20-somite stage. Hematopoietic stem cells are thought to come from the posterior region of the ICM. Wild-type embryos contain a large number of round, undifferentiated cells in this region (open arrows) while *clo* mutants exhibit a marked reduction in the number of these cells. These presumptive stem cells are further identified as they express GATA-2 but not GATA-1 (see Fig. 7). This reduction of cells in the posterior region of the ICM is the earliest morphological phenotype observed during development and was used reliably to sort mutant embryos at an early stage.

in Table 1A and Fig. 5. Conversely, we never observed mutant cells contributing to the endocardium in wild-type or mutant embryos, although mutant cells contribute to wild-type and mutant myocardium with normal frequency (approximately 20% of the transplants; Table 1B). These results indicate that the *clo* mutation acts in a cell-autonomous fashion in the generation of the endocardial cells and are consistent with the possibility that *clo* participates in a receptor signalling pathway.

The *clo* mutation affects blood cell differentiation

In addition to the endothelial defect, the other detectable phenotype we observed in *clo* mutant embryos is a blood deficit. The first identifiable hematopoietic cells in the zebrafish arise in the intermediate cell mass (ICM), and the hematopoietic stem cells concentrate in the posterior region of the ICM (Detrich et al., 1995). Using Nomarski optics, we found the posterior region of the ICM to be severely depleted of cells in mutant embryos (Fig. 6). This reduction of hematopoietic stem cells is intriguing because, as mentioned earlier, single cells in the ventral marginal zone of the early blastula can give rise to both endothelial (including endocardial) and blood cells (Stainier et al., 1993; Lee et al., 1994), suggesting that endothelial and blood cells derive from a common lineage.

To analyze this blood deficit further, we examined the expression pattern of GATA-1 and GATA-2, two key hematopoietic transcription factors expressed early during development. Starting at the 2-somite stage in zebrafish, GATA-1 is expressed specifically in a subset of hematopoietic progenitors which does not include the presumptive stem cells of the posterior ICM (Fig. 7A, C) (Detrich et al., 1995). We found no GATA-1 expression in *clo* mutant embryos (Fig. 7B, D). GATA-2 is expressed in both hematopoietic and mature endothelial cells (as well as in certain neural tissues) and regulates the transcription of many cell-specific genes. In zebrafish, it is expressed from an early stage in all hematopoietic progenitors including the stem cells of the posterior ICM (Fig. 7E) (Detrich et al., 1995). We found no GATA-2 expression in *clo* hematopoietic tissues (Fig. 7F). Neural expression was normal (data not included). The *clo* mutation therefore acts at a very early stage to perturb the differentiation of components of both the endothelial and hematopoietic lineages.

DISCUSSION

We are taking a genetic approach in the zebrafish to dissect the molecular mechanisms underlying cardiovascular formation and function. In collaboration with W. Driever (Driever et al., 1994), we have recently completed a large-scale mutagenesis screen of the zebrafish genome to identify single gene mutations that affect the formation of the cardiovascular system (Stainier et al., in preparation). The identification and analysis of mutations such as *cloche*, which exhibits a fairly specific and informative phenotype, should further our understanding of cardiovascular development. The *clo* mutation affects the endothelial lineage: it deletes a subset of the endothelial cells such as those inside the heart tube while others, such as those lining the trunk vessels, appear morphologically normal. This is the first report of a genetic locus controlling endothelial cell differentiation. It is also the first locus observed to affect both the endothelial and hematopoietic lineages. Furthermore, the selective endothelial deletion observed in *clo* mutant embryos provides support for a mechanistic distinction for blood vessel formation such as the one based on endothelial progenitor cell migration (Poole and Coffin, 1991). The isolation and use of molecular markers for endothelial cells and their progenitors, such as *flk-1*, *flt-1*, *tek* and *tie*, should help us better define the state of endothelial cell differentiation in the various regions of the mutant embryo. This analysis will determine the extent of the endothelial deletion caused by *clo* and may help us assess the validity of the various models of blood vessel formation.

Cardiac morphogenesis

The endocardial layer is present in the heart of all vertebrates.

It is continuous with the endothelium of the major blood vessels, the axial vein and the dorsal aorta, and as such, it ensures the smooth flow of blood through the cardiac pump. In the lamprey, the most primitive living vertebrate, although the endocardial layer is absent from the mature heart, it is present in the developing heart (Percy and Potter, 1991). In *clo* mutant embryos, early cardiac morphogenesis is relatively normal. The definitive myocardial tube forms in the absence of the endocardial cells and is demarcated into chambers that beat rhythmically and sequentially. Some mutant hearts even undergo looping morphogenesis. Yet, the *clo* heart is dysmorphic, the walls of the atrium are distended, and the ventricle is reduced in size. These observations suggest that the endocardium may be necessary for a late step in the differentiation of the myocardium. Additionally, the *clo* heart exhibits a clearly reduced contractility which may be a direct effect of the mutation, or more likely be due to the absence of the endocardial cells (wild-type cells transplanted into mutant myocardium also exhibit a diminished contractility). Several studies have indicated that there is communication between the endocardium and myocardium in the adult heart and that endocardial cells may modulate the contractility of mature myocardial cells (reviewed by Brutsaert and Andries, 1992; and by Shah and Lewis, 1993). The contractile deficit of the *clo* heart suggests that the endocardial cells may modulate myocardial function in the embryonic heart as well. Thus, while myocardial cells have clearly been shown to induce endocardial cells to differentiate into prevalvular mesenchyme (Markwald et al., 1990), our data suggest that the endocardial cells themselves play a role in myocardial cell maturation and function.

Hematopoiesis

In *clo* mutant embryos, there is a reduction in the number of 'stem' cells in the posterior region of the ICM. The hematopoietic tissues show no expression of GATA-1 or GATA-2 as assessed by whole-mount in situ hybridization. Thus, *clo* appears to act upstream of both GATA-1 and GATA-2 and exhibits the earliest hematopoietic defect that has been reported in any vertebrate species and the only one combined with a specific endothelial deficit. We have previously characterized two zebrafish mutations, *bloodless* and *spadetail*^{b104} that have a blood phenotype (Detrich et al., 1995). *bloodless* embryos have no mature circulating erythroid cells; they lack GATA-1

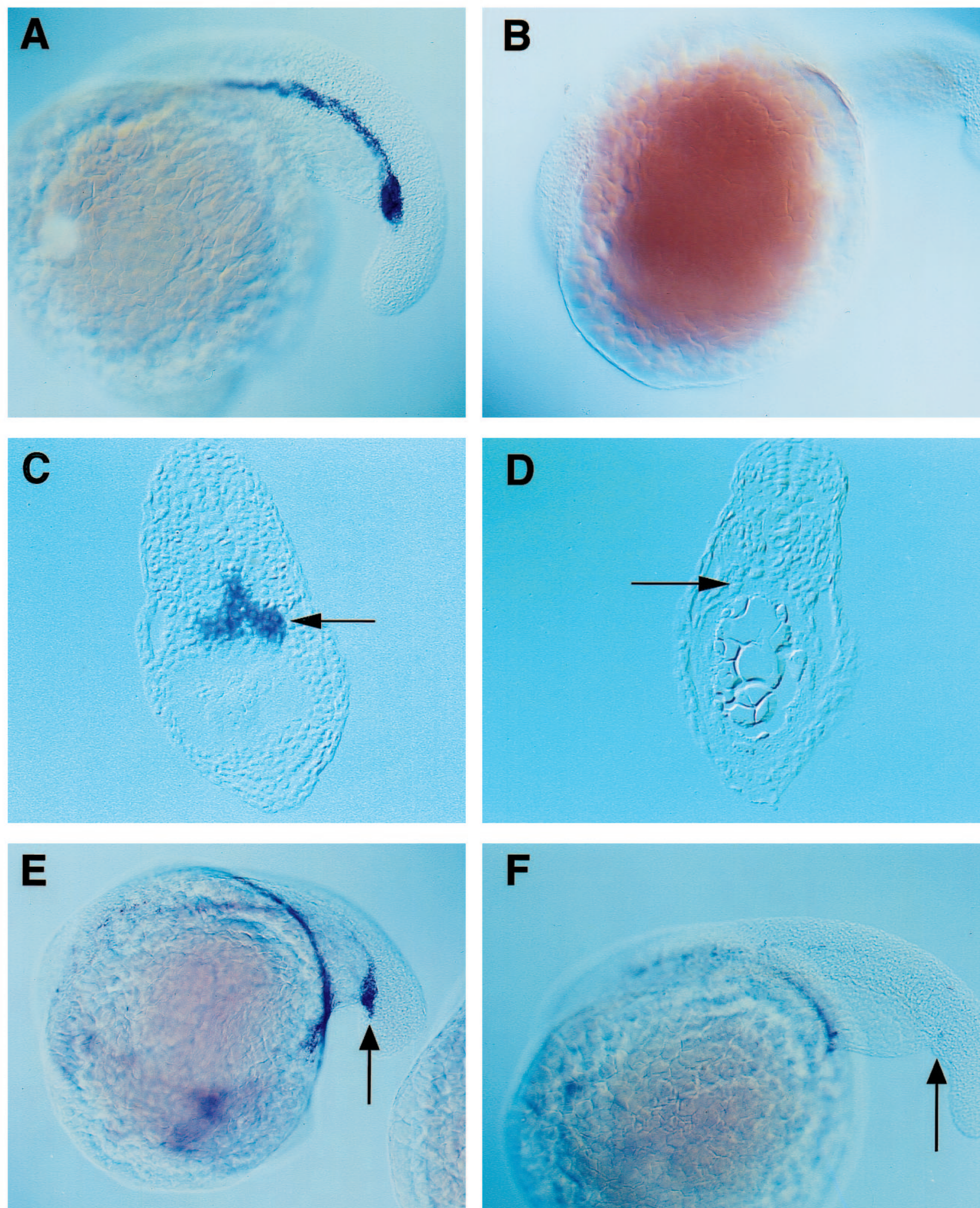


Fig. 7. *clo* mutant embryos do not express GATA-1 or GATA-2 in their hematopoietic tissues. In situ hybridization with a GATA-1 or GATA-2 antisense RNA probe was performed on wild-type embryos (A,C,E) and on *clo* mutants (B,D,F). (A-D) GATA-1 staining at the 22-somite stage. In wild-type embryos, GATA-1 expression is originally found in two longitudinal chords of cells that extend from the mid-trunk region to just beyond the yolk tube and pronephric ducts. These cell populations will fuse at the midline to form the ICM as seen in whole mounts (A) and transverse sections of the tail region (C). *clo* mutants fail to express GATA-1 (B,D). (E,F) GATA-2 staining of 20-somite stage embryos. GATA-2 is normally expressed from an early stage in all hematopoietic progenitors including the presumptive stem cells of the posterior ICM (E, arrow). In *clo* mutants, the hematopoietic tissues do not express GATA-2 (F). GATA-2 is also expressed in certain neural tissues (Detrich et al., 1995) and this expression is normal in mutant embryos (data not included).

expression yet express GATA-2 in the stem cell population of the posterior ICM. *spadetail* mutants have general deficiencies in mesodermal tissues that stem from a defect in the convergence movements that take place during gastrulation (Kimmel et al., 1989); they exhibit decreased GATA-1 expression yet also express GATA-2 in the stem cell population. *clo* mutants do not express GATA-2 in the ICM region suggesting that *clo*, unlike *bloodless* or *spadetail*, affects the generation of the stem cell population. The hematopoietic defect of the *clo* mutation appears similar to that resulting from mutations in GATA-2 (Tsai et al., 1994), or rhombotin (Warren et al., 1994), although *clo* appears to affect an earlier step. Embryos homozygous for a loss-of-function mutation in either GATA-2 or rhombotin exhibit a profound deficit in hematopoietic stem cells, but in contrast to *clo*, have no gross vascular abnormalities.

In *clo* mutant embryos, because of the lack of circulation, cells accumulate in the trunk vessels. These cells exhibit a large, round morphology typical of blood cells and a few of them appear to contain heme based on DAF staining (data not included). The number of these potential red blood cells is markedly reduced compared to the level of circulating erythrocytes in wild type. Gene targeting experiments in the mouse indicated that GATA-1 is necessary for erythrocyte differentiation (Pevny et al., 1991), yet our data might suggest that GATA-1 can be partially circumvented. However, it remains to be determined whether the heme detected by DAF staining is incorporated into hemoglobin, whether these cells are viable, and whether very low levels of GATA-1 may be expressed.

clo affects the differentiation of both the endothelial and hematopoietic lineages at a very early stage. At least three models may explain this phenotype. First, *clo* may affect a common progenitor cell population that gives rise to both endothelial and blood cells. Our lineage data show that endothelial and blood cells can derive from a single cell at the blastula stage although always accompanied by other mesodermal derivatives. Careful lineage analysis should establish whether a population of 'hemangioblasts' exists (cells that give rise exclusively to endothelial and blood cells) and whether all endothelial and blood cells come from these hemangioblasts. *clo* might thus act on these presumptive hemangioblasts, thereby affecting both lineages. Second, *clo* might be expressed in the endothelial and blood lineages after they diverge. Third, *clo* might affect the endothelial lineage cell-autonomously (as our data indicate) and the blood lineage in a non-cell autonomous manner. Endothelial and hematopoietic cells generally appear synchronously in adjoining regions. In this model, the endothelial cells or their progenitors induce the differentiation of the hematopoietic progenitors. In *clo* mutants, endothelial cells would be defective in inducing the hematopoietic progenitors resulting in the blood phenotype observed. Testing the validity of this last model by cell transplantation would be a formidable task mainly because of the very low frequency with which one can transplant cells that give rise to either endothelial cells or blood cells (due to their close lineal relationship). The isolation of the *cloche* gene, and the analysis of its expression pattern might allow us to look more closely at this intriguing model and investigate further the molecular mechanisms that regulate the genesis and diversification of the endothelial and blood lineages.

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