

Cell-autonomous and non-autonomous requirements for the zebrafish gene *cloche* in hematopoiesis

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SUMMARY

Vertebrate embryonic hematopoiesis is a complex process that involves a number of cellular interactions, notably those occurring between endothelial and blood cells. The zebrafish *cloche* mutation affects both the hematopoietic and endothelial lineages from an early stage (Stainier, D. Y. R., Weinstein, B. M., Detrich, H. W. R., Zon, L. I. and Fishman, M. C. (1995) *Development* 121, 3141-3150). *cloche* mutants lack endocardium, as well as head and trunk endothelium, and nearly all blood cells. Cell transplantation studies have revealed that the endocardial defect in *cloche* is cell-autonomous: wild-type cells can form endocardium in mutant hosts, but mutant cells never contribute to the endocardium in wild-type or mutant hosts. In this paper, we analyze the cell-autonomy of the blood defect in *cloche*. The blood cell deficiency in *cloche* mutants could be an indirect effect of the endothelial defects. Alternatively, *cloche* could be required cell-autonomously in the blood cells themselves. To distinguish between these possibilities, we cotransplanted wild-type and mutant cells into a single wild-type host in order to compare their respective hematopoietic capacity. We found that transplanted wild-type cells were much more likely than mutant cells to contribute to circulating blood in a wild-type host. Furthermore, in the few cases where both wild-type and mutant donors contributed to blood in a wild-type host, the number of blood cells derived from the

wild-type donor was always much greater than the number of blood cells derived from the mutant donor. These data indicate that *cloche* is required cell-autonomously in blood cells for their differentiation and/or proliferation. When we assessed early expression of the erythropoietic gene *gata-1* in transplant recipients, we found that mutant blastomeres were as likely as wild-type blastomeres to give rise to *gata-1*-expressing cells in a wild-type host. Together, these two sets of data argue that *cloche* is not required cell-autonomously for the differentiation of red blood cells, as assayed by *gata-1* expression, but rather for their proliferation and/or survival, as assayed by their contribution to circulating blood. In addition, we found that transplanted wild-type cells were less likely to express *gata-1* in a mutant environment than in a wild-type one, suggesting that *cloche* also acts non-autonomously in red blood cell differentiation. This non-autonomous function of *cloche* in red blood cell differentiation may reflect its cell-autonomous requirement in the endothelial lineage. Thus, *cloche* appears to be required in erythropoiesis cell non-autonomously at a step prior to *gata-1* expression, and cell-autonomously subsequently.

Key words: Transplantation, *gata-1*, Blood, Erythropoiesis, Hemangioblast, Endothelium

INTRODUCTION

Cells of the vertebrate hematopoietic system arise during embryogenesis through a highly complex, multistep process that involves a number of key anatomical sites, cellular interactions, and extrinsic and intrinsic regulators. The first wave of hematopoiesis in avian and mammalian embryos originates in the yolk sac blood islands and gives rise to the primitive (i.e. embryonic) erythrocytes. In teleost fish, such as zebrafish, the Intermediate Cell Mass (ICM) appears to function in a capacity similar to the yolk sac blood islands. The ICM is derived from cells that separate from the dorso-median

border of the posterior lateral plate mesoderm by the 10-somite stage. These cells are found dorsal to and all along the primordia of the pronephric ducts and appear to have both hematopoietic and vasculogenic potential (Al-Adhami and Kunz, 1977; reviewed in Zon, 1995). In higher vertebrates, a second wave of hematopoiesis originates in a distinct, intra-embryonic domain referred to as the aorta/gonad/mesonephros (AGM) region. This domain has been shown to be the origin of the definitive blood precursors that ultimately colonize the fetal liver and the adult thymus, spleen and bone marrow (Medvinsky and Dzierzak, 1996). In zebrafish, a similar domain has been described and is associated with the ventral

wall of the developing dorsal aorta (Liao et al., 1998; Thompson et al., 1998).

Throughout development, hematopoietic precursors are found in close association with endothelial cells and their precursors. This observation has led to the hypothesis that a common progenitor, the hemangioblast, gives rise to both endothelial and hematopoietic cells (His, 1900; Sabin, 1920; Flamme and Risau, 1992). Several lines of evidence support this hypothesis. First, endothelial and blood precursors express several genes in common (Pardanaud and Dieterlen-Lièvre, 1993; Kallianpur et al., 1994) and share several surface antigens (Dieterlen-Lièvre and Martin, 1981; Peault et al., 1983; Pardanaud et al., 1987). In addition, grafting experiments have shown that avian splanchnopleural mesoderm gives rise to both angioblasts and hematopoietic precursors (Pardanaud et al., 1996; Pardanaud and Dieterlen-Lièvre, 1999). Recently and most significantly, Jaffredo and colleagues (1998) were able to show through creative lineage studies that cells in the floor of the developing chicken aorta that express an endothelial-specific marker could subsequently differentiate into cells expressing a blood-specific surface antigen. In vitro studies have also shown that certain cells can give rise to both endothelium and blood (Choi et al., 1998; Nishikawa et al., 1998). Taken together, these data strongly support the existence of the hemangioblast.

Several known extrinsic factors are involved in embryonic hematopoiesis. For example, secreted factors such as the bone morphogenetic proteins (BMPs) are required to establish ventral mesoderm, which serves as a source for blood precursors, and may also play a subsequent and more direct role in blood development. Additional extrinsic factors may come from the endodermal and endothelial microenvironments. In vitro studies have indeed shown that the visceral endoderm regulates the differentiation and proliferation of hematopoietic cells in the yolk sac (Yoder et al., 1994; Belaousoff et al., 1998). In another cell culture study, embryonic endothelial cells were shown to increase significantly the proliferation and differentiation of at least the erythroid and myeloid lineages (Fennie et al., 1995).

A number of intrinsic factors have been determined to play important roles in the differentiation of primitive erythrocytes as well as later hematopoietic processes in mouse. Gata-1 is required for erythropoiesis (Pevny et al., 1991) and Gata-2 for the production of primitive and definitive erythroid cells, mast cells, and during T- and B-cell lymphopoiesis (Tsai et al., 1994). SCL (a.k.a. Tal-1) is required at the stem cell level for the specification of all blood lineages (Shivdasani et al., 1995). Studies in zebrafish have shown that *SCL* expression overlaps with the expression of hematopoietic and endothelial-specific markers (Liao et al., 1998; Thompson et al., 1998), and that ectopic overexpression of *SCL* results in the overproduction of both lineages (Gering et al., 1998; Liao et al., 1998). Lmo-2 (a.k.a. Rbtn2) appears to function in primitive and definitive erythropoiesis (Warren et al., 1994), while C-myb appears to function only during definitive hematopoiesis (Sitzmann et al., 1995). The receptor tyrosine kinase gene *flk-1* serves an essential function in both vasculogenesis and hematopoiesis in mouse (Shalaby et al., 1995). In vitro studies further suggest that expression of Flk-1 and exposure to vascular endothelial growth factor, a Flk-1 ligand, determine whether putative hemangioblasts differentiate into cells of the hematopoietic or endothelial lineage in culture (Eichmann et al., 1997). Studies of chimeras formed between *flk-*

1^{-/-} embryonic stem cells and wild-type mouse blastocysts show that Flk-1 is required cell-autonomously for endothelial and blood development, and further suggest that *flk-1* is involved in the movement of cells to sites of embryonic hematopoiesis (Shalaby et al., 1997). Additional potential hematopoietic regulators include Fli-1, a transcription factor whose exact role in blood development remains unclear (Mélet et al., 1996; Remy et al., 1996). Zebrafish homologues of all of the aforementioned genes and others have been used to identify likely sites of primitive and definitive hematopoiesis in the developing zebrafish embryo (reviewed in Parker et al., 1999).

The zebrafish *cloche* mutation affects both the endothelial and hematopoietic lineages (Stainier et al., 1995; Liao et al., 1997; Liao et al., 1998; Thompson et al., 1998). *cloche* mutants lack endocardium, as well as head and trunk endothelium, and nearly all blood cells. Cell transplantation studies have revealed that the endocardial defect in *cloche* is cell-autonomous: wild-type cells can form endocardium in mutant hosts, but mutant cells never contribute to the endocardium in wild-type or mutant hosts (Stainier et al., 1995). *flk-1* expression in *cloche* mutants is severely affected from its onset, indicating that *cloche* functions upstream of *flk-1* during endothelial and blood development (Liao et al., 1997). Additional studies indicate that *cloche* also functions upstream of *SCL* (Liao et al., 1998). Detailed analysis of *cloche* mutants has revealed a severe reduction or elimination of the expression of a number of genes, including *flk-1*, *fli-1*, *SCL*, *gata-1*, *gata-2*, *lmo2* and *c-myb* (Stainier et al., 1995; Liao et al., 1997; Liao et al., 1998; Thompson et al., 1998). These observations suggest that both primitive and definitive hematopoiesis, as well as endothelial development, are disrupted in *cloche* mutants. Even so, certain genes, such as *gata-1*, *fli-1* and *flk-1*, are expressed in *cloche* mutants in a very small number of cells in a posterior tail region where a small number of differentiated blood cells subsequently appears (Stainier et al., 1995; Liao et al., 1997; Liao et al., 1998; Thompson et al., 1998).

There are several possible explanations for the fact that *cloche* affects both the hematopoietic and endothelial lineages. *cloche* could act exclusively within the endothelial lineage, with blood development severely affected as a result of a defective endothelial microenvironment. Alternatively, *cloche* could function cell-autonomously in both the endothelial and blood lineages, possibly by acting at the level of the hemangioblast. We have tested these hypotheses by performing reciprocal cell transplantations between *cloche* and wild-type embryos, as well as cotransplantations of *cloche* and wild-type cells into wild-type hosts. Surprisingly, our results suggest that *cloche* functions both cell-autonomously and non-autonomously during zebrafish embryonic hematopoiesis. Wild-type donor cells are much more likely than mutant cells to contribute to circulating blood. The relative inability of *cloche* mutant cells to contribute to circulating blood does not appear to derive from an inability to populate the posterior ICM or express the erythropoietic marker *gata-1*. Instead, it appears to result from a failure of mutant cells to undergo further differentiation and/or proliferation. In addition, we found that transplanted wild-type cells were less likely to express *gata-1* in a mutant environment than in a wild-type one, suggesting that there is also a non-autonomous function for *cloche* in blood differentiation. We propose a model based on these data to explain the apparent dual function of *cloche* in zebrafish hematopoiesis.

MATERIALS AND METHODS

Zebrafish embryos

Zebrafish were maintained as described previously (Westerfield, 1995). All experiments were carried out using the *cloche*^{m378} allele (Stainier et al., 1996).

Cell transplantation

Transplantations were performed as described previously (Ho and Kane, 1990), with some minor modifications. Embryos were injected in the yolk at the 1-4 cell stage with one of the following: 5% rhodamine dextran, 2.5% lysine-fixable fluorescein dextran, or a mixture of 2.5% rhodamine dextran/0.25% lysine-fixable biotin dextran (w/v; 10,000 Da molecular mass; Molecular Probes) in 120 mM KCl. Between the high and dome stages, 5-20 cells were transplanted from each donor to a point along the margin of the host. [In the case of cotransplantations, some donor embryos were labeled with rhodamine-dextran and others with fluorescein-dextran; cells were then taken up into the transplantation pipette from one labeled embryo followed by another group of cells taken from a second, differently labeled donor embryo. Both groups of cells were then simultaneously transplanted into an unlabeled host. If needed, more details can be obtained from Ho and Kane (1990)]. Transplant recipients were permitted to develop until the 7- or 20-somite stage, or until circulation was vigorous (30 hours post-fertilisation; hpf). Donor contribution to circulating blood was assayed at 30 hpf by examining embryos under fluorescence on a Zeiss Axioplan equipped with a video camera. Images of circulating blood cells were saved on video and subsequently transferred to a computer. Individual video frames were extracted from streaming video files using Avid VideoShop 2.0 and Adobe Photoshop 4.0 software.

Whole-mount in situ hybridization, immunohistochemistry and histology

Whole-mount in situ hybridization was performed as described previously (Alexander et al., 1998) with the following modifications. Hybridized riboprobes were visualized with 4-nitro blue tetrazolium(NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) or with BCIP alone. After in situ hybridization, biotin dextran-labeled donor cells were detected in host embryos as described previously (Westerfield, 1995). Detecting fluorescein dextran-labeled donor cells following in situ hybridization was done by fixing the embryos in 4% paraformaldehyde (w/v) in phosphate-buffered saline for at least an hour, blocking a second time and detecting with alkaline phosphatase-conjugated anti-fluorescein antibodies and using di(2-amino-2-methyl-1,3-propanediol)3-indoxyl phosphate ('magenta-phos', Molecular Probes) as a substrate (burgundy). A subset of the processed hosts were embedded in JB4 (Polysciences) and sectioned at 5 μ m for further analysis.

RESULTS

The endothelial defect in *cloche* is cell-autonomous

cloche mutants lack all endocardial cells and most other endothelial cells (Stainier et al., 1995; Liao et al., 1997). Previous transplantation studies focused exclusively on the endocardial defect and revealed that it is cell-autonomous (Stainier et al., 1995). We have now analyzed the cell-autonomy of the defect in the rest of the endothelial lineage by transplanting wild-type or mutant cells into wild-type hosts. We have found that whereas wild-type cells contributed to endothelial structures (dorsal aorta, axial vein and intersomitic vessels) in 13/153 cases, mutant cells never contributed to these structures ($n=50$). These data indicate that *cloche* functions

cell-autonomously in the differentiation of all endothelial precursors.

The blood defect in *cloche* is cell-autonomous

cloche mutants also lack most blood cells (Stainier et al., 1995; Liao et al., 1997; Thompson et al., 1998). To analyze the cell-autonomy of the blood defect, we performed a large number ($n=1046$) of reciprocal cotransplantations between *cloche* and wild-type embryos at the mid- to late-blastula stage. Contribution of donor cells to the blood lineage was initially assayed by allowing wild-type host embryos to mature to 30 hpf, when blood cells are clearly circulating. Video fluorescence microscopy was used to visualize and document the donor contribution to circulating blood. Strikingly, wild-type-derived cells were more than four times (4.46) more likely to contribute to circulating blood than cotransplanted mutant cells (Table 1). These data suggest that *cloche* functions cell-autonomously in blood cell formation.

Although extremely rare, in a small number of cases ($n=3$), both wild-type and *cloche* cells contributed to circulating blood (Fig. 1). In these three cases, we observed that the number of mutant cells in circulation was always lower than the number

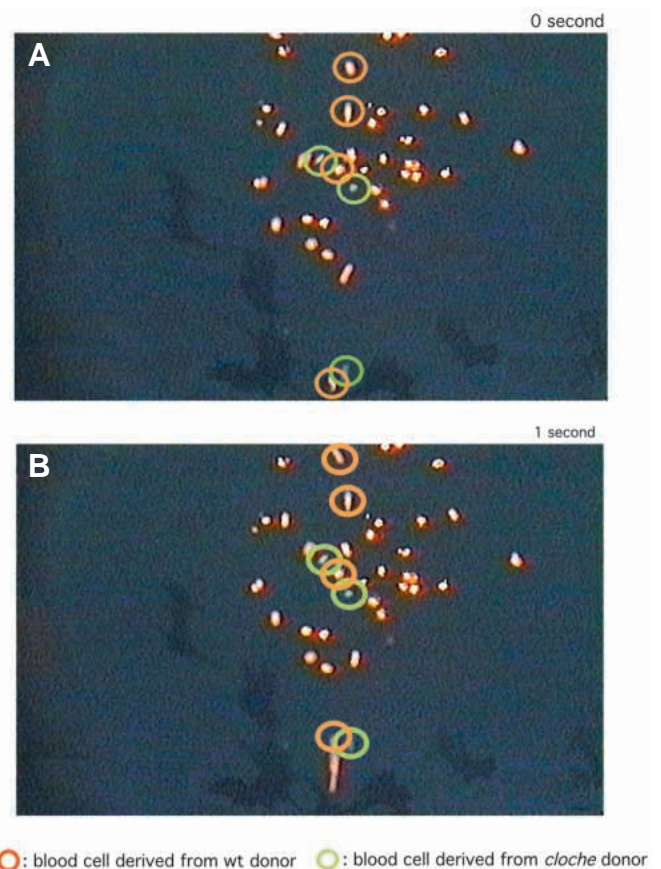


Fig. 1. Wild-type-derived (orange) and mutant-derived (green) blood cells in a 30 hpf wild-type embryo (10-7 in Table 2). Two video frames are shown, one taken at time 0 (A) and the other 1 second later (B). Donor-derived blood cells can be seen streaming across the yolk sac in a ventral (bottom) to dorsal (top) direction (compare A to B). Three green, mutant-derived cells and a subset of the orange, wild-type-derived cells are identified with green and orange circles, respectively. All the remaining fluorescent cells are wild-type-derived (i.e. orange) circulating cells.

Table 1. Frequency of donor contribution to circulating blood in wild-type hosts

Host	Donor	
	WT	Mutant
WT	98/459 (1:5)	8/167 (1:21)

Wild-type-derived cells contribute to circulating blood much more frequently than mutant cells.

We performed a total of 1046 reciprocal cotransplantations and analyzed 626 transplantation events (i.e. a surviving wild-type host for which a donor had survived). Cotransplantation events in which a host received cells from two different donors were counted as two separate transplantation events. There were 459 cases in which wild-type hosts received cells from a wild-type donor ('WT to WT' hosts), and 167 cases in which wild-type hosts received cells from a mutant donor ('M to WT' hosts). Donor-derived circulating blood cells were identified microscopically by their morphology and movement through the vasculature. Wild-type-derived circulating blood cells were found at a frequency of 1 in 5 (98/459). In contrast, mutant-derived blood cells were found at the much lower frequency of 1 in 21 (8/167). Because mutant embryos lack a vasculature and therefore any circulation, it was not possible to evaluate the 'WT to M' or 'M to M' transplants using this assay.

of wild-type-derived blood cells. We attempted to quantitate the number of labeled, and thus transplant-derived, cells in the circulation of recipients of both wild-type/mutant cells and wild-type/wild-type cells. The number of fluorescent cells passing across a specific point on the yolk ball in a given period of time was used as an approximate measure of the number of a given type of cells in circulation. Table 2A details the results from the three independent cotransplantation events in which both wild-type and mutant donors contributed to circulating blood. In all three of these cases, wild-type-derived cells outnumbered mutant-derived cells by a factor of at least four. In contrast, in the 14 cases in which both wild-type donors were observed to contribute to circulating blood, only occasionally did any one donor contribute to four times or more the number of circulating blood cells than the other wild-type donor (Table 2B). Taking these wild-type cotransplantation events as reference, the likelihood that all three of the aforementioned cases, in which wild-type-derived cells outnumbered mutant cells, occurred by chance is quite small ($P=0.06$, $x=3$). These experiments show that mutant cells are less likely to contribute to circulating blood than wild-type cells (Table 1), and when they do they appear to contribute to fewer circulating blood cells (Table 2). These data indicate that the blood defect in *cloche* is cell-autonomous, and that *cloche* is required for the early differentiation, proliferation and/or maintenance of blood precursors.

***cloche* cells migrate normally to the posterior ICM**

We have previously shown that *cloche* acts upstream of a zebrafish *flk-1* homologue to regulate endothelial cell differentiation (Liao et al., 1997). In mouse, *flk-1* has been implicated in both primitive and definitive hematopoiesis, as well as in vasculogenesis. Shalaby and colleagues (1997) observed in chimeric studies that *flk-1*^{-/-} embryonic stem cells fail to contribute to either hematopoietic or endothelial lineages, and instead are found in other tissues, indicating that cells lacking Flk-1 cannot reach sites of embryonic hematopoiesis. Because *cloche* mutants lack most sites of *flk-1* expression, we investigated whether *cloche* cells fail to home

Table 2. Number of donor-derived circulating blood cells/min in individual wild-type hosts

A Experiment no.	Genotype of cotransplantation donor		Ratio (high/low)
	WT	Mutant	
9-30	154	21	7.3
10-7	168	8	21.0
11-1	4	1	4.0

B Experiment no.	Wild-type cotransplantation donor		Ratio (high/low)
	Donor no. 1	Donor no. 2	
9-24	~200	~200	~1.0
10-1A	159	248	1.6
10-1B	275	6	45.8
10-1C	30	257	8.6
10-1D	30	13	2.3
10-8A	171	130	1.3
10-8B	16	36	2.3
10-15A	40	60	1.5
10-23A	96	8	12.0
10-23B	60	318	5.3
10-28A	20	4	5.0
10-28B	40	104	2.6
10-28C	132	68	1.9

Mutant blastomeres contribute fewer cells to circulating blood than wild-type ones.

In the three cases where both the mutant and wild-type donors contributed to circulating blood in a wild-type host, the number of mutant cells in circulation was much lower than the number of wild-type-derived cells. Mutant cells could be distinguished from wild-type cells because different fluorescent lineage tracer dyes were used to inject each donor (i.e. fluorescein dextran versus rhodamine dextran; see Materials and methods). The results in A contrast those observed in the 14 cases shown in B, in which both wild-type donors contributed to circulating blood in a wild-type host. In 5 out of the 14 cases (shown in bold) the number of circulating blood cells derived from one wild-type donor exceeded the number of blood cells derived from the other wild-type donor by a factor of 4 or greater. If this frequency is taken as a reference for the frequency in which cotransplantation donors randomly contribute disproportionately to the blood lineage, then the Poisson distribution predicts that the probability of observing 3 such differences in 3 independent events, as seen in A, is relatively low ($P=0.06$; $x=3$). Counting the number of labeled cells that passed by a given point on the yolk sac of the host embryo during a 60-second interval was used to quantitate the number of circulating, donor-derived blood cells. Numbers are an average of three independent counts. Ratio indicates the ratio of the number of cells derived from the donor with the largest contribution versus the number of cells derived from the donor with the smallest contribution. Assignment to the 'Donor #1' or 'Donor #2' category was arbitrary and does not reflect the order of transplantation into the host.

to the appropriate hematopoietic compartments during zebrafish embryogenesis. Such a homing defect could explain our earlier observation that mutant cells are less able to contribute to circulating blood.

To test this hypothesis, wild-type and *cloche* blastomeres or wild-type and wild-type blastomeres were cotransplanted into wild-type hosts. At the 20-somite stage, the ICM, the primitive hematopoietic compartment of the developing zebrafish embryo and the equivalent of the mammalian and avian yolk sac, has begun to form. [As temporal reference points, *gata-1* is first expressed at the 2-somite stage (10.5 hpf), *flk-1* at the 7-somite stage (12.5 hpf), *hemoglobin* at the 12-somite stage (15 hpf), the main vessels of the trunk assemble around the 24-somite stage (21 hpf) and circulation begins at about the 30-somite stage (24 hpf) (reviewed in Zon, 1995; Parker et al.,

Table 3. Frequency of donor contribution to the posterior ICM following cotransplantation

Cotransplantation		Contribution by each donor to the posterior ICM	Number of events
Donors	Host		
WT/WT	→ WT	WT ⁺ WT ⁺	12
		WT ⁻ WT ⁺	4
WT/Mutant	→ WT	WT ⁺ Mutant ⁺	14
		WT ⁺ Mutant ⁻	3
		WT ⁻ Mutant ⁺	5

Mutant blastomeres contribute to the posterior ICM as frequently as wild-type blastomeres do.

Blastomeres from two different donors were cotransplanted into a wild-type host and contribution of donor cells was assessed microscopically. We analyzed 16 cases in which cells from two different wild-type donors were cotransplanted into a single wild-type host (WT/WT to WT) and one or both donors contributed to the posterior ICM. Of these 16 cases, there were 12 in which both wild-type donors contributed cells to the posterior ICM and 4 in which only one of the donors contributed to the posterior ICM. We analyzed 22 cases in which cells from one wild-type donor and one mutant donor were cotransplanted into a single wild-type host (WT/Mutant to WT) and one or both donors contributed to the posterior ICM. Of these 22 cases, there were 14 in which both the wild-type and the mutant donors contributed to the posterior ICM, 3 in which the wild-type donor but not the mutant donor contributed to the posterior ICM, and 5 in which the opposite was observed. Thus, the frequency at which mutant blastomeres were capable of contributing to the posterior ICM did not differ significantly from the frequency at which wild-type blastomeres contributed to the same region.

1999).] We identified cases where either one or both types of blastomeres contributed to the posterior ICM by looking for fluorescent, donor-derived cells in the region posterior to the yolk extension, using a combination of Nomarski and fluorescence microscopy. We chose to restrict our analysis to the posterior ICM because it can be clearly defined spatially at the 20-somite stage. [The term 'posterior ICM' has also been used to refer to the *gata-1*⁻ *gata-2*⁺ group of cells found in the posterior tail region, although the origin of these cells is not clear. In this paper, we define the anterior and posterior parts of the ICM as the *gata-1*⁺ regions anterior and posterior to the caudal end of the yolk extension, respectively.] We found that in wild-type hosts *cloche* and wild-type blastomeres were equally likely to contribute to the posterior ICM (Table 3). We also examined the subsequent fate of mutant-derived and wild-type-derived cells that were observed to contribute to the posterior ICM. Contribution to the posterior ICM does not, a priori, indicate that a given cell is a hematopoietic precursor. Such a cell could instead contribute to neighboring tissues such as the vasculature. In the 48 cases in which wild-type blastomeres were observed to contribute to the posterior ICM, nearly half (23/48) went on to show donor contribution to circulating blood. In contrast, in the 20 cases in which *cloche* blastomeres were observed to contribute to the posterior ICM, only one quarter (5/20) went on to show donor contribution to circulating blood. Together, these data show that while mutant blastomeres are just as likely as wild-type blastomeres to contribute to the posterior ICM, once in the posterior ICM they are half as likely to contribute to circulating blood. Thus, *cloche* does not appear to play an essential role in the initial migration of blood precursors to the posterior ICM, a site of embryonic hematopoiesis, but instead it appears to affect subsequent proliferation or maturation of hematopoietic cells.

cloche is not required cell-autonomously for the initial differentiation of erythropoietic precursors

The above results suggest that *cloche* plays a cell-autonomous role in the proliferation or maturation of hematopoietic cells. To examine whether *cloche* also plays a role in earlier events in blood development, we examined whether *cloche* mutant cells can express *gata-1* when transplanted into a wild-type host. Reciprocal transplantations were performed between *cloche* and wild-type embryos and the host embryos were permitted to develop until the 7-somite stage. Successful transplant recipients were identified by fluorescence microscopy, fixed, analyzed for *gata-1* expression, and donor-derived cells were subsequently identified by immunohistochemistry. We found that in wild-type hosts, *cloche* cells contributed to the posterior domain of *gata-1* expression as frequently as wild-type cells (1:6, Table 4; Fig. 2J, arrowhead). Interestingly, *cloche* cells contributed to the anterior domain of *gata-1* expression only half as frequently as wild-type cells (1:12 versus 1:6, Table 4; Fig. 2I, arrowhead). These data further support a model whereby *cloche* is not required cell-autonomously in the early stages of red blood cell differentiation (i.e. the initiation of *gata-1* expression), but instead for blood cell proliferation or survival. These data also suggest a cellular or molecular difference between the anterior and posterior domains of *gata-1* expression.

cloche also appears to have a non-autonomous function in blood development

The data so far indicate that *cloche* functions cell-autonomously in blood development. We wanted to analyze the behavior of wild-type cells in a mutant environment but these experiments

Table 4. Frequency of donor contribution to the *gata-1*-positive domain

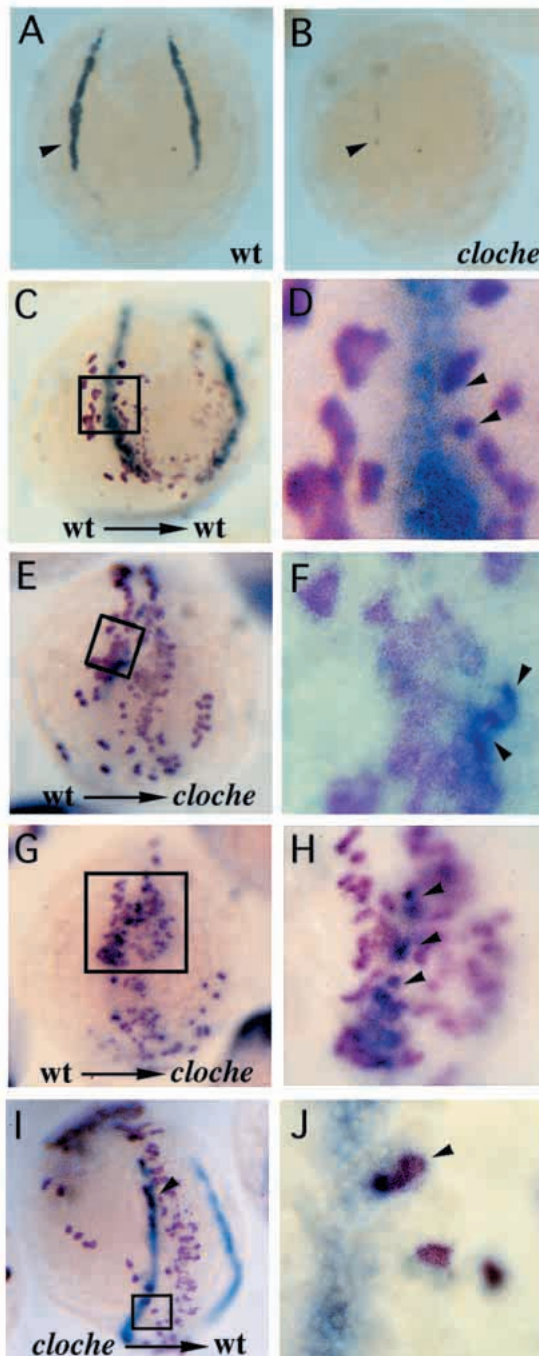
Transplant	<i>gata-1</i> positive domain contribution	
	Anterior	Posterior
WT→WT	28/168 (1:6)	27/168 (1:6)
WT→Mutant	4/49 (1:12)	4/49 (1:12)
Mutant→WT	6/70 (1:12)	12/70 (1:6)
Mutant→Mutant	0/9	0/9

Analysis of *gata-1* expression in host embryos.

Reciprocal transplantations between wild-type and *cloche* mutant embryos were performed. Host embryos were analyzed for *gata-1* expression at the 7-somite stage. Following in situ hybridization analysis, donor-derived cells were identified in host embryos by staining for biotin or fluorescein. The contribution of donor-derived cells was then evaluated, and transplant recipients were categorized according to whether donor-derived *gata-1*-positive cells were present in the anterior or posterior half of the *gata-1*-positive domain. A total of 498 transplantations were performed and 321 analyzed. Wild-type-derived cells contributed to the anterior and posterior domains of *gata-1* expression in wild-type hosts with equal frequency (1 in 6). Mutant-derived cells contributed to the posterior domain of *gata-1* expression in wild-type hosts at a frequency equivalent to that seen in the WT to WT transplants (1:6) but to the anterior domain of *gata-1* expression less frequently (1:12). Surprisingly, wild-type-derived *gata-1*-positive cells were found in mutant hosts at a frequency lower than that seen in the WT to WT transplants (1:12 versus 1:6). Analysis of these differences by the χ^2 test indicates that they are significant. Note that very few of the host embryos (1/70 in Mutant to WT transplants and 5/168 in WT to WT transplants) showed contribution to both the anterior and posterior domains of *gata-1* expression. Also note that the relationship, if any, between the anterior and posterior domains of *gata-1* expression at the 7-somite stage and the anterior and posterior ICM at the 20-somite stage is not clear at this point.

are not possible using a simple visualization assay, due to the lack of vasculature and circulation in *cloche* mutants. To begin to analyze this problem we assayed the ability of wild-type cells to express *gata-1* in a mutant host. *gata-1* expression is much reduced in *cloche* mutants, although residual expression can be detected in posterior regions even at early segmentation stages (Fig. 2B, arrow). In contrast, no anterior *gata-1* expression has been detected in *cloche* mutants at any stage, thus any anterior *gata-1*-positive cells in mutant hosts must derive from the wild-type donor. Surprisingly, we found only a small number of cases where wild-type-derived, *gata-1*-positive cells were present in *cloche* hosts (1:12; Table 4). The frequency of these events was twofold lower than the frequency with which wild-type cells contributed to the *gata-1*-positive cell population in wild-type

hosts (1:6; Table 4). Furthermore, in the wild-type to wild-type transplants we frequently observed lone donor-derived *gata-1*-expressing cells (Fig. 2D, arrowheads). By contrast, in the few cases where wild-type-derived cells expressed *gata-1* in mutant hosts the clone containing *gata-1*-expressing cells always consisted of a large aggregate of donor cells (Fig. 2E-H). Histological analysis of these embryos confirmed the close proximity of wild-type-derived *gata-1*-positive cells to other wild-type-derived cells (data not shown), suggesting that the presence of a wild-type microenvironment facilitates *gata-1* expression. These data show that the *cloche* environment is less conducive to *gata-1* expression than the wild-type one, suggesting that *cloche* also plays a non-autonomous role in an early step of blood development.



DISCUSSION

cloche mutants lack most endothelial and blood cells. This combined defect could indicate that *cloche* functions cell-autonomously in both the endothelial and blood lineages, possibly by acting at the level of the hemangioblast. Alternatively, some aspect of early blood development may be dependent upon a functional endothelial microenvironment for which *cloche* is required. The colocalized residual expression of both *flk-1* and *gata-1* in the posterior tail region of *cloche* mutants indicates that blood cells are differentiating exclusively in the immediate proximity of the few differentiating endothelial cells and could be argued to support the latter, non-autonomy hypothesis (Liao et al., 1997). The data presented in this report, however, support a model in which *cloche* acts both cell-autonomously and non-autonomously in blood development.

cloche acts cell-autonomously in blood development

Cotransplantation experiments into wild-type hosts show that mutant-derived cells are much less likely to give rise to circulating blood than wild-type-derived cells. Furthermore, in

Fig. 2. Analysis of *gata-1* expression in donors and hosts. (A,B) Comparison of the patterns of *gata-1* expression in a wild-type (A) and a *cloche*^{m378} mutant (B) just prior to the 7-somite stage; arrowhead points to *gata-1* expression in the *cloche* mutant (B), which is always confined to the posterior region, and to the corresponding area in wild-type (A). (C,D) Wild-type-derived cells in the posterior domain of *gata-1* expression in a 7-somite stage wild-type host. In these and all subsequent panels, the donor-derived *gata-1*-positive cells are both burgundy-colored (as they contain fluorescein dextran) and turquoise-colored (as they are *gata-1*-positive) to yield an overall dark blue color. D corresponds to the boxed area in (C) and shows some lone donor-derived *gata-1*-positive cells (arrowheads). (E-H) Wild-type-derived cells in the anterior domain of *gata-1* expression in 7-somite stage mutant hosts. (F and H) correspond to boxed areas in E and G, respectively. Arrowheads point to donor-derived *gata-1*-positive cells. Note that in E-H, the clones of donor-derived cells that surround the *gata-1*-expressing cells are always quite large. (I,J) Mutant cells present in the *gata-1* expression domain of a 7-somite stage wild-type host. Arrowhead in I points to donor-derived *gata-1*-positive cells in the anterior domain of *gata-1* expression. J corresponds to the boxed area in I and shows donor-derived *gata-1*-positive cells (arrowhead) in the posterior domain of *gata-1* expression.

the few cases where both wild-type and mutant donors contributed to circulating blood in a wild-type host, the number of blood cells derived from the wild-type donor was always much greater than the number of blood cells derived from the mutant donor. These data clearly indicate that even in a wild-type endothelial microenvironment, *cloche* cells are at a disadvantage, as compared to wild-type cells, in forming blood. Thus, *cloche* is required cell-autonomously for blood development.

The blood defect in *cloche* does not appear to stem from an inability of the mutant cells to move to the posterior ICM. Indeed, mutant-derived cells are as likely to be found within the posterior ICM as wild-type-derived cells. Furthermore, mutant-derived cells are as likely to contribute to the posterior domain of *gata-1* expression in wild-type hosts as are wild-type-derived cells. These data argue that the cell-autonomous requirement for *cloche* in blood development must then occur after the initiation of *gata-1* expression. For example, *cloche* may be involved in the intrinsic control of blood cell proliferation. Recent work examining the expression pattern of *jak2a*, a gene associated with proliferation in mouse hematopoiesis (Zhuang et al., 1994; Neubauer et al., 1998; Parganas et al., 1998), supports such a model. In zebrafish embryos, *jak2a* is normally expressed at high levels in the developing hematopoietic system and at a lower level in the central nervous system (CNS) and developing fin buds. In *cloche* mutants, *jak2a* expression appears normal in the CNS and fin buds but is not detectable in the hematopoietic system (Oates et al., 1999). *cloche* could also be required for stem cell survival or maintenance of the hematopoietic fate; further experiments are needed to investigate these possibilities.

It is also interesting to note that mutant-derived cells are less likely to contribute to the anterior versus the posterior domain of *gata-1* expression in wild-type hosts (1:12 versus 1:6, Table 4). Early markers of both endothelial and blood differentiation are also expressed at very low levels only in the posterior region of *cloche* mutants. This distinction between anterior and posterior regions may indicate the existence of factors involved in endothelial and blood differentiation that are present at a higher concentration in the posterior region of the embryo. Other models are possible: for example, migration or homing to the anterior domain of *gata-1* expression may depend on *cloche* function.

***cloche* also appears to act cell non-autonomously in blood development**

Typically, reciprocal transplantations are performed to confirm that a given gene functions solely in a cell-autonomous or non-autonomous fashion. Such an approach was not possible in this case

because *cloche* mutants lack a functional vasculature, thereby preventing the assessment of wild-type contribution to circulating blood in mutant hosts. We partially circumvented this problem by analyzing transplant recipients for *gata-1* expression. This approach revealed a potential non-cell-autonomous function for *cloche* in blood development.

Wild-type-derived cells in mutant hosts did not express *gata-1* with the frequency expected from the wild-type to wild-type transplant controls (1:12 versus 1:6; Table 4). Furthermore, in the rare cases where wild-type-derived cells were seen to express *gata-1* in a mutant host the size of the wild-type-derived clone was always quite large: it appeared that the wild-type-derived *gata-1*-positive cells were surrounded by a large number of wild-type-derived *gata-1*-negative cells (Fig. 2E-H). This apparent need for large aggregates of wild-type-derived cells in mutant hosts suggests that a wild-type microenvironment (possibly in the form of differentiating endothelial cells) helps wild-type donor cells express *gata-1*. Thus, *cloche* appears to function in blood development cell non-autonomously prior to *gata-1* expression. The fact that the mutant environment is less conducive to *gata-1* expression also explains why little *gata-1* expression is seen in *cloche* mutants even though mutant cells are as likely as wild-type cells to express *gata-1* in a wild-type environment (i.e. the posterior

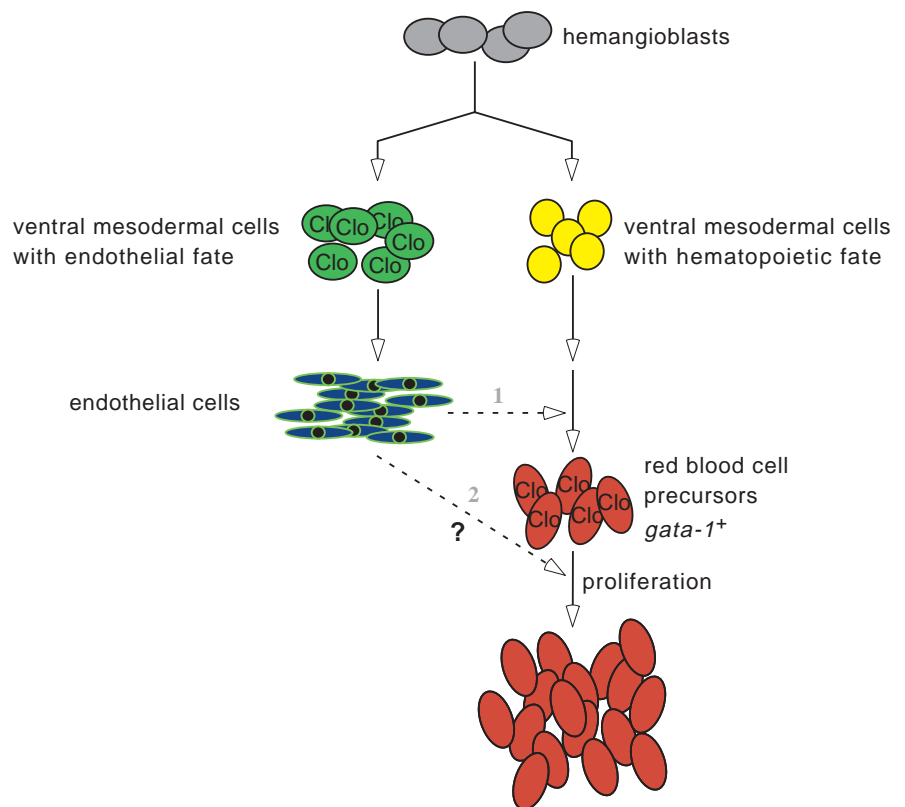


Fig. 3. Model depicting the possible roles for *cloche* during vasculogenesis and hematopoiesis. Hemangioblasts give rise to both endothelial (green) and hematopoietic cells (yellow). *cloche* is required cell-autonomously for endothelial precursors to differentiate and initiate vasculogenesis. Some hematopoietic precursors differentiate into *gata-1*-expressing cells, in a process that appears to require *cloche* function cell non-autonomously (dashed arrow 1). *cloche* is also required cell-autonomously in red blood cells for their proliferation or maturation. *cloche* may also be required non-autonomously in this latter step (dashed arrow 2).

ICM; Table 4). Our studies address very specific aspects of hematopoiesis since *gata-1* is an erythropoietic specific marker and most circulating blood cells at 30 hpf are red blood cells (Zon, 1995; Parker et al., 1999). Additional transplantation studies using molecular markers associated with other aspects of blood development such as stem cell generation, proliferation and differentiation into non-erythropoietic lineages will be required to analyze the role of the endothelial microenvironment in these other processes.

A model for *cloche* function in vasculogenesis and hematopoiesis

How does one explain the dual cell-autonomous and non-autonomous function of *cloche* in hematopoiesis? What is the role of *cloche* in endothelial versus blood differentiation? The model outlined in Fig. 3 proposes one possible answer. In this model, hemangioblasts give rise to both endothelial and blood precursors. *cloche* is required intrinsically for the differentiation of endothelial cells. The initiation of *gata-1* expression in the blood lineage appears to be independent of *cloche* function in the blood lineage but dependent on *cloche* function in the microenvironment. Further maturation of these blood cells, or perhaps their proliferation or survival, requires *cloche* function intrinsically. It is of course possible that these later aspects of blood development, proliferation and survival also require *cloche* function in the microenvironment. [Expanding upon this model with a concrete example, *cloche* could encode a transcription factor or be part of the signalling machinery in angioblasts as well as in *gata-1*-expressing blood cells. The differentiating endothelial cells would secrete factors necessary for the differentiation of blood precursors into *gata-1*-expressing cells. The absence of *cloche* function could directly prevent the expression of these secreted factors or block angioblast differentiation in an immature state. *cloche* function in *gata-1*-expressing cells would be required for their proliferation. Other models are of course possible.] To summarize, *cloche* appears to be required for erythropoiesis cell non-autonomously prior to *gata-1* expression and cell-autonomously subsequent to *gata-1* expression.

Regarding the role of *cloche* in vasculogenesis, we and others have reported that a small number of cells in the posterior tail region of *cloche* mutants express *flk-1* but do not appear to express later markers of endothelial differentiation such as *tie-1* and *tie-2* (Liao et al., 1997; Thompson et al., 1998; Wayne Liao and D. Y. R. S., unpublished). In the current studies we did not observe mutant contribution to vascular structures in wild-type embryos, suggesting that *flk-1* expression per se is not sufficient for incorporation into the vasculature, although admittedly the number of transplants analyzed is not high enough to support this statement conclusively. It is possible that mutant cells can contribute to the vasculature but at a much reduced frequency as compared to wild-type cells, as is the case with their contribution to circulating blood.

Our studies also provide new evidence for the requirement of the endothelial microenvironment in blood development. Our in vivo observations support prior observations made in cell culture systems (Fennie et al., 1995). In those studies, endothelial cells from murine yolk sacs were purified and cocultured with hematopoietic cells. The addition of appropriate supplemental factors led to a significant expansion

of the erythroid and myeloid lineages. More recently, Ohneda et al. (1998) conducted additional in vitro studies and showed that different AGM-derived endothelial cell lines could support the maintenance or differentiation of hematopoietic stem cells. *cloche* appears to have a non-cell-autonomous role in red blood cell development. As *cloche* acts cell-autonomously in the endothelial lineage, our studies suggest that *cloche* function in the endothelial lineage regulates erythropoiesis. It is possible that the non-autonomous function of *cloche* in erythropoiesis reflects its requirement in a tissue other than the endothelium. Previous work has pointed to the importance of the endoderm (Yoder et al., 1994; Belaoussoff et al., 1998), but there is no evidence thus far that endoderm development is affected in *cloche* mutants. Furthermore, analysis of *casanova*, a mutant which appears to lack gut endoderm from an early stage (J. Alexander and D. Y. R. Stainier, unpublished), shows that *gata-1* expression is initiated normally in these mutants, suggesting that gut endoderm is not required for red blood cell differentiation per se.

The model presented here provides a framework for further analysis of the apparent dual role of *cloche* in blood development. *cloche* appears to play a critical cell-autonomous role in both vasculogenesis and erythropoiesis. Furthermore, the endothelial microenvironment whose differentiation is under the control of *cloche* function also appears to play an important role in erythropoiesis. Additional transplantation studies using appropriate markers may further illuminate the role of the endothelial microenvironment in the formation and differentiation of hematopoietic stem cells into non-erythropoietic lineages.

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