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Interplay of Pu.1 and Gata1 Determines Myelo-Erythroid Progenitor Cell Fate in Zebrafish

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Summary

The zebrafish is a powerful model system for investigating embryonic vertebrate hematopoiesis, allowing for the critical in vivo analysis of cell lineage determination. In this study, we identify zebrafish myeloerythroid progenitor cells (MPCs) that are likely to represent the functional equivalent of mammalian common myeloid progenitors. Utilizing transgenic *pu.1*-GFP fish, real-time MPC differentiation was correlated with dynamic changes in cell motility, morphology, and gene expression. Unlike mammalian hematopoiesis, embryonic zebrafish myelopoiesis and erythropoiesis occur in anatomically separate locations. Gene knockdown experiments and transplantation assays demonstrated the reciprocal negative regulation of *pu.1* and *gata1* and their non-cell-autonomous regulation that determines myeloid versus erythroid MPC fate in the distinct blood-forming regions. Furthermore, forced expression of *pu.1* in the bloodless mutant *cloche* resulted in myelopoietic rescue, providing intriguing evidence that this gene can function in the absence of some stem cell genes, such as *scl*, in governing myelopoiesis.

Introduction

Vertebrate hematopoiesis is a multistep process involving the progressive commitment of pluripotent hematopoietic stem cells (HSCs) into lineage-restricted progenitor cells that generate the wide variety of differentiated cells of the blood system. This process involves successive waves of tissue specification regulated by the activities of critical transcription factors (Akashi et al., 2000). Zebrafish orthologs have been identified for genes expressed in nearly all blood cell types found in mammals, including stem cells, common myeloid and lymphoid progenitor cells, and mature blood cells including T and B lymphocytes, erythrocytes, and myeloid cells comprised of neutrophils and monocyte/macrophages (Bennett et al., 2001; Brownlie et al., 2003; Burns et al., 2002; Detrich et al., 1995; Gering et al., 1998; Herbomel et al., 1999; Kalev-Zylinska et al., 2002; Lieschke et al., 2002; Liu and Wen, 2002; Lyons et al., 2001; Thompson et al., 1998). The conservation of gene expression between mammals and zebrafish suggests that the regulatory mechanisms directing lineage specification are similarly conserved. However, zebrafish stem and progenitor cells have yet to be formally identified, and the genetic relationships of key regulatory factors remain poorly understood.

Studies of zebrafish hematopoiesis have identified pluripotent mesodermal cells within the gastrula hypoblast, which give rise to endothelium, erythrocytes, and cardiac tissue (Kimmel et al., 1990; Lee et al., 1994). The expression patterns of zebrafish HSC and progenitor cell genes indicate that hematopoietic tissue is derived from lateral plate mesoderm (LPM), which contributes to three distinct regions along the anterior/posterior axis, including the anterior lateral plate mesoderm (ALPM) under the head, the intermediate cell mass (ICM) in the trunk, and the posterior blood island in the ventral tail (reviewed in Hsu et al., 2001).

In mammals, HSCs give rise to common lymphoid progenitors (CLPs), which form mature B and T lymphocytes, and common myeloid progenitors (CMPs). The CMPs generate granulocytes and monocytes of the myeloid lineage, megakaryocytes of the thrombocytic lineage, and erythrocytes (Akashi et al., 2000). Unlike mammals, myeloid and erythroid populations develop in spatially distinct regions of the zebrafish embryo. Erythrocytes arise from posterior lateral plate mesoderm (PLPM) forming the ICM of the trunk and express erythroid-restricted genes including *gata1*, *band* 3, and globins (Brownlie et al., 2003; Detrich et al., 1995; Paw et al., 2003). In contrast, myeloid cells form in the ALPM beneath the embryonic head, exhibit the behaviors of functional leukocytes (Herbomel et al., 1999), and express myeloid-restricted genes, including *pu.1*, *cebp*α, *l-plastin*, and *mpo* (Bennett et al., 2001; Lieschke et al., 2001; Lyons et al., 2001). Furthermore, Lieschke et al. (2002) suggests that zebrafish myeloid and erythroid blood formation are developmentally distinct processes specified prior to gastrulation as the embryonic anterior-posterior axis is established. Thus, it has remained unclear whether CMPs exist in the zebrafish.

The zebrafish mutant *cloche* lacks both erythroid and vascular tissues (Liao et al., 1997; Thompson et al., 1998), and both can be partially rescued by overexpressing *scl*, *hex*, or *runx1* (Kalev-Zylinska et al., 2002; Liao et al., 1998, 2000). These genes are also expressed during the earliest stages of zebrafish hematopoietic development, consistent with roles in HSC development. However, the ability of these genes to rescue myelopoiesis in *cloche* mutants has not been examined.

In this study, we identify zebrafish myelo-erythroid progenitor cells (MPCs) that are likely to be the functional equivalent of mammalian CMPs. Using morpholino-mediated gene knockdown techniques, we demonstrate the in vivo roles of Pu.1 and Gata1 in directing the differentiation of MPCs into the myeloid and erythroid lineages, and we explore the autoregulation of *pu.1* and *gata1* and mechanisms of their reciprocal inhibition. Transplantation assays provide novel in vivo evidence for non-cell-autonomous signals directing the specification of MPC fate, resulting in the anatomical separation of myeloid and erythroid compartments in the zebrafish embryo. Interestingly, the forced expression of *pu.1* is

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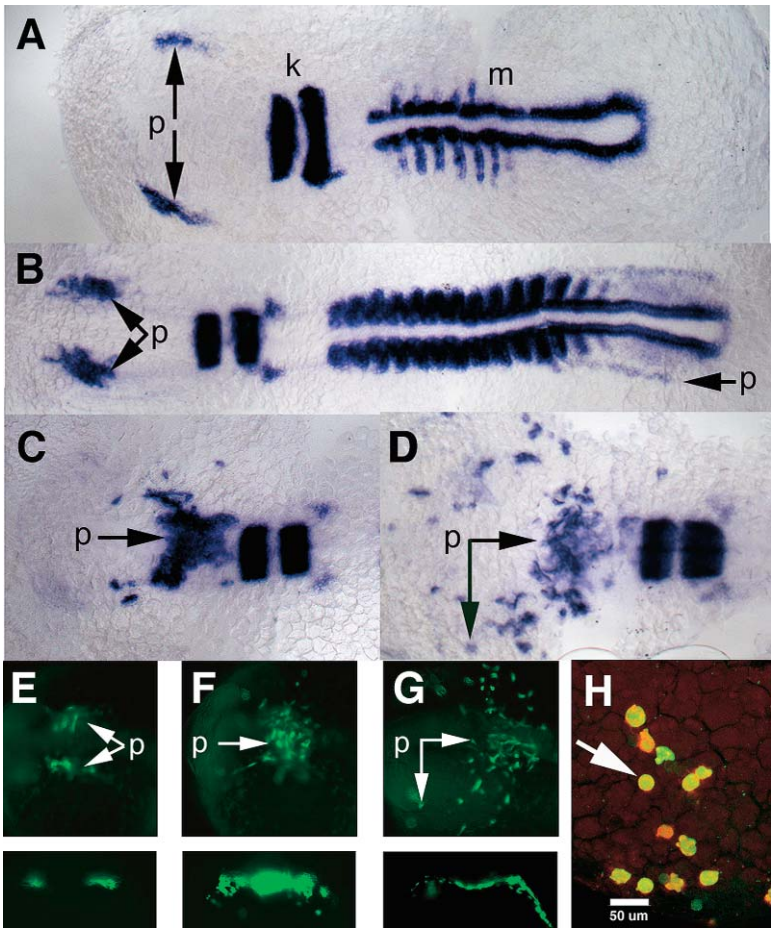

Figure 1. Movements of *pu.1*-Expressing Cells

(A–D) ISH assays for *pu.1* (p, arrow), *krox20* (k, rhombomeres 3,5), and *myoD* (m, somites) expression. Dorsal views of flattened embryos, anterior to left, at (A) 7 somites, (B) 14 somites, (C) 15 somites, and (D) 18 somites. (E–G) Upper panels, dorsal views, anterior to left: live *pu.1*-GFP transgenic embryo is shown at (E) 13 hpf, (F) 15 hpf, and (G) 20 hpf. Lower panels: corresponding anterior views. (H) Confocal image of GFP^+ cells (green) and *pu.1* mRNA (red) in a 20 hpf transgenic embryo. Arrow indicates a yellow coexpressing cell.

sufficient to rescue myeloid, but not erythroid, cell development in the *cloche* mutant, suggesting that this gene may be capable of specifically driving myelopoiesis without the expression of some upstream HSC genes. These studies demonstrate an essential role for zebrafish *pu.1* in the regulation of MPCs and the differentiation of myeloid cell lineages.

Results

Pu.1 Gene Expression and Myeloid Cell Movements

The spatiotemporal pattern of *pu.1* expression was analyzed with whole-mount mRNA in situ hybridization (ISH) assays on wild-type embryos. *pu.1* mRNA was expressed in bilateral bands of cells in the ALPM as early as the 4-somite stage (data not shown) and was readily apparent by the 7-somite stage, anterior to rhombomere 3 (Figure 1A). By the 14-somite stage, the bilateral bands of $pu.1^+$ cells appeared closer to the midline, becoming less compact, but remaining anterior to rhombomere 3 (Figure 1B). Although *pu.1* expression was observed bilaterally in PLPM cells at the 10-somite stage (data not shown), it remained weak through the 14-somite stage (Figure 1B). By the 15/16-somite stage, $pu.1^+$ cells from the ALPM were generally clustered at the midline under the developing head (Figure 1C). By the 18/20-somite stage, these cells had dispersed over the yolk cell and head (Figure 1D), while *pu.1* expression in the ICM had become weaker and restricted posteriorly (data not shown). By 24 hpf, *pu.1* expression had decreased, becoming undetectable by ISH at 35 hpf (data not shown).

To address whether dynamic changes in gene expression reflected moving cells or changing patterns in stationary cells, a stable transgenic zebrafish expressing GFP under the control of the *pu.1* promoter was analyzed (Hsu et al., 2004). Confocal microscopic analysis showed coexpression in more than 95% of cells (Figure 1H), demonstrating that GFP expression was a reliable cell lineage marker for *pu.1*-expressing cells and was never observed in developing erythroid cells. The relative stability of the GFP protein allowed some GFP^+ cells to be observed at 2 days postfertilization (dpf), a time when *pu.1* mRNA was no longer detected, thus allowing continued analysis of cells no longer expressing *pu.1* mRNA. Time-lapse analysis of living *pu.1*-GFP transgenic embryos confirmed the movement of GFP^+ cells in both ALPM and PLPM, corresponding with the dynamic spatiotemporal patterns of endogenous *pu.1* mRNA expression (Figures 1E–1G, see 1A–1D). These studies demonstrate the real-time movement of *pu.1*-expressing cells from the lateral sides of the head toward the ventral midline and their subsequent dispersion. A small subset of cells were observed to spread over the yolk without first moving ventromedially (data not shown).

GFP^+ cells also coexpressed orthologs of mammalian HSC genes, such as *scl*, *gata2*, and *lmo2*, at early times

within the bilateral clusters of the ALPM (Supplemental Figures S1A–S1C at http://www.developmentalcell.com/cgi/content/full/8/1/97/DC1/, data not shown). As GFP$^+$ cells moved ventromedially, away from bilateral cluster locations, *scl*, *gata2*, and *lmo2* gene expression was dramatically reduced or lost, though it persisted in other cells remaining in the developing brain (Supplemental Figures S1D–S1F, data not shown). As the GFP$^+$ cells became irregular in shape, exhibiting pseudopodial extensions and dispersing over the yolk, they decreased expression of *pu.1* mRNA and began expressing mature myeloid genes, such as *mpo, l-plastin*, *coronin*, and *phox47* (Supplemental Figures S1G–S1O, data not shown).

Effect of Pu.1 Loss on Myeloid and Erythroid Development

Mice deficient in *pu.1* lack monocyte/macrophages, B cells, and differentiated neutrophils (Anderson et al., 1998; McKercher et al., 1996). The functional role of Pu.1 in zebrafish myelopoiesis was assessed in gene knockdown experiments using morpholinos (Nasevicius and Ekker, 2000) designed to specifically inhibit Pu.1 protein expression. Pu.1 and control morpholino-injected animals (morphants) were analyzed for effects on myeloid differentiation and gene expression. The block in Pu.1 protein translation resulted in a dramatic decrease, or a complete loss, of detectable *pu.1* mRNA expression (Figures 2A–2D), indicating an autoregulatory function of the Pu.1 protein. The loss of Pu.1 had no apparent effect on heart development, as indicated by normal *nkx2.5* expression (Chen and Fishman, 1996), and by 28 hpf, a normal heartbeat was observed. Unlike control morphants, all Pu.1 morphants exhibited a striking decrease in granulocyte-specific *mpo* expression, although in some embryos a few cells in the posterior blood island continued to express this gene (Figures 2E–2H). The loss of *mpo* expression was rescued in 24/25 morphants by the injection of murine *pu.1* mRNA, whose translation is unaffected by the morpholino (data not shown). This control experiment demonstrated the specificity of the morpholino on the *pu.1* pathway and functional conservation of the mouse and zebrafish Pu.1 proteins. The knockdown of Pu.1 also resulted in a loss of cells expressing *l-plastin*, a marker for monocytes and macrophages (Figures 2I–2L). Other genes expressed in mature myeloid cells, including *lysozyme C*, *coronin*, and *phox 47*, were also decreased in Pu.1 morphants (data not shown). Erythroid development at 28 hpf appeared relatively normal in Pu.1 morphants, as blood circulation and abundant α-*globin* mRNA expression was observed (Figures 2E–2L). However, as α-*globin* is normally expressed at very high levels in the ICM, quantitative changes in its expression levels were difficult to assess, particularly after circulation onset. These results demonstrate that Pu.1 is required for myeloid cell differentiation.

Because Pu.1 and Gata1 crossregulate each other in mammalian CMPs, the effect of Pu.1 loss was examined further with respect to erythroid gene markers. Pu.1 morphants were assayed for *gata1* expression at 21 hpf, well before the onset of circulation. In addition to a slight increase in *gata1* expression observed in the ICM, Pu.1

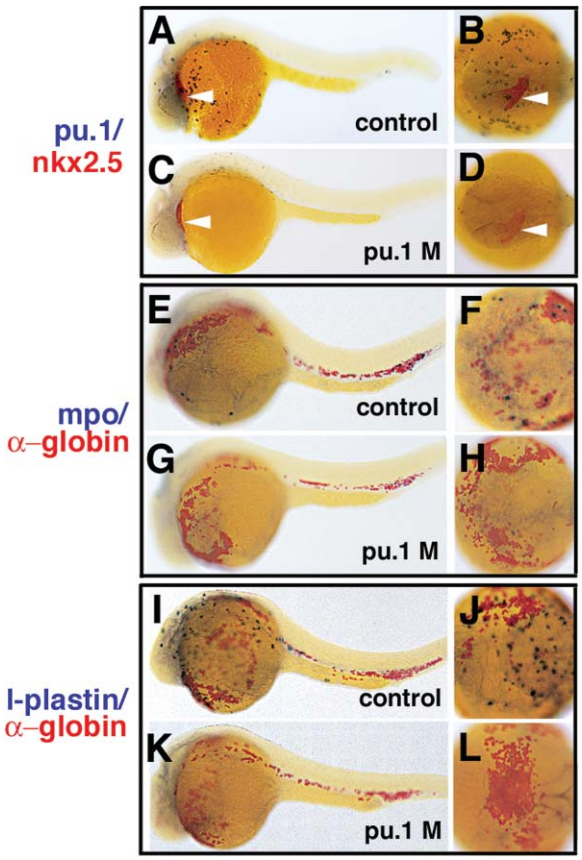

Figure 2. Pu.1 Is Required for Myelopoiesis

(A, B, E, F, I, J) Control or (C, D, G, H, K, L) Pu.1 morphants at 28 hpf. Double ISH assays for (A–D) *pu.1* and *nkx2.5* (arrowheads), (E–H) *mpo* and α-*globin*, (I–L) *l-plastin* and α-*globin* (blue and red, as indicated). Left panels: lateral views; right panels: dorsal views; anterior left.

morphants exhibited an ectopic cluster of *gata1*$^+$ cells in the ALPM under the head, a region that normally never expresses *gata1* (Figures 3A–3D). The ectopic anterior expression of *gata1* could be observed as early as 15 hpf (data not shown) and was never observed in control morphants at these stages. These *gata1*$^+$ cells did not originate from the posterior ICM, since the circulation of blood begins after 24 hpf. All Pu.1 morphants expressed α-*globin* in these ALPM cells (n = 87, Figures 3E–3H), indicating that these cells differentiated further into mature erythrocytes.

The transgenic expression of GFP in Pu.1 morpholino-injected *pu.1*-GFP embryos (Pu.1-GFP morphants) was unaffected (data not shown) because the morpholino specifically binds to and blocks the translation of endogenous *pu.1*, but not GFP, mRNA. Combined with the stability of the GFP protein (Zernicka-Goetz et al., 1996), these factors allowed the continued visualization of GFP$^+$ cells in Pu.1 morphants despite the decrease in endogenous *pu.1* mRNA levels. We exploited these features of the *pu.1*-GFP transgenic line, using the persistent expression of the GFP protein as a cell-specific lineage marker.

In Pu.1-GFP morphants, GFP$^+$ cells formed normally

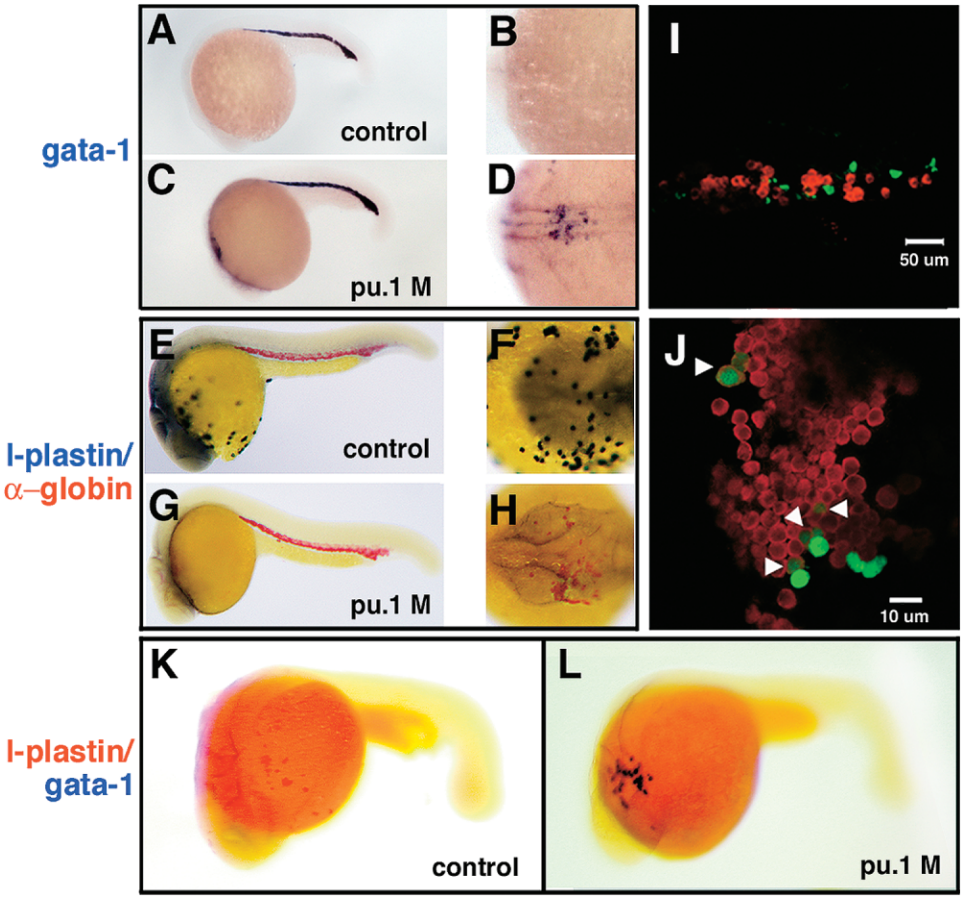

Figure 3. Pu.1 Suppresses Erythroid Development

(A, B, E, F, I, and K) Control morphants or (C, D, G, H, J, and L) Pu.1 morphants at (A–D) 21 hpf or (E–H, K, L) 24 hpf. Expression of (A–D) *gata1* (blue), (E–H) *l-plastin* (blue), and (I–L) α-*globin* (red). Left panels, lateral views; right panels, dorsal views; anterior left. Confocal images of 28 hpf *pu.1*-GFP transgenic embryos injected with (I) control or (J) Pu.1 morpholinos showing GFP (green) and α-*globin* (red) expression. Arrowheads indicate coexpressing cells. *spadetail* mutants injected with (K) control or (L) Pu.1 morpholinos showing *gata1* (blue) and *l-plastin* (red).

in the LPM and moved ventrally, clustering at the midline. However, these cells showed a distinct decrease in subsequent motility and there was only limited dispersion away from this region, with few cells migrating over the yolk cell by 24 hpf (data not shown). This loss of dynamic motility is consistent with a transition toward a nonmotile, erythroid fate. To determine whether ectopic erythrocytes in the ALPM of Pu.1 morphants were derived from cells that normally express *pu.1*, Pu.1-GFP morphant embryos were analyzed for the coexpression of GFP with α-*globin*. In control morphants at 28 hpf, confocal microscopic analysis showed that GFP protein and α-*globin* mRNA were never coexpressed (Figure 3I). However, in addition to the loss of motility, a subset of cells in Pu.1-GFP morphants also expressed α-*globin* mRNA (Figure 3J, arrowheads), demonstrating that *pu.1*-expressing cells are not restricted to a myeloid cell fate.

The zebrafish mutant *spadetail* (*spt*) fails to form erythrocytes in the posterior blood compartment, hence its classification as a "bloodless" mutant (Thompson et al., 1998). However, myeloid cell development in the anterior mesoderm compartment of this animal is unaffected (Figure 3K; Lieschke et al., 2002). In Pu.1-*spt* morphants, erythropoiesis was observed in the anterior myeloid compartment (Figure 3L), indicating the presence of cells in the *spt* ALPM that are competent to form either myeloid or erythroid cells depending upon the expression of *pu.1*. Altogether, these experiments demonstrate that *pu.1* is required for myelopoiesis and negatively regulates the expression of erythroid genes in *pu.1*-expressing cells, a function most evident in the anterior blood compartment.

Effect of Gata1 Loss on Erythroid and Myeloid Development

In murine models, *gata1* is crucial for erythrocyte and thrombocyte development (Pevny et al., 1991; Shivdasani et al., 1997). The functional role of Gata1 in zebrafish erythroid and myeloid cell development was analyzed in Gata1 and control morphants. The block in Gata1 translation resulted in a reduction in detectable *gata1* mRNA expression at 20 hpf, indicating an autoregulatory function of the Gata1 protein (Figures 4A–4F). By 30 hpf, Gata1 morphants had failed to express α-*globin* (Figures 4G–4N) or exhibit circulating erythrocytes. However,

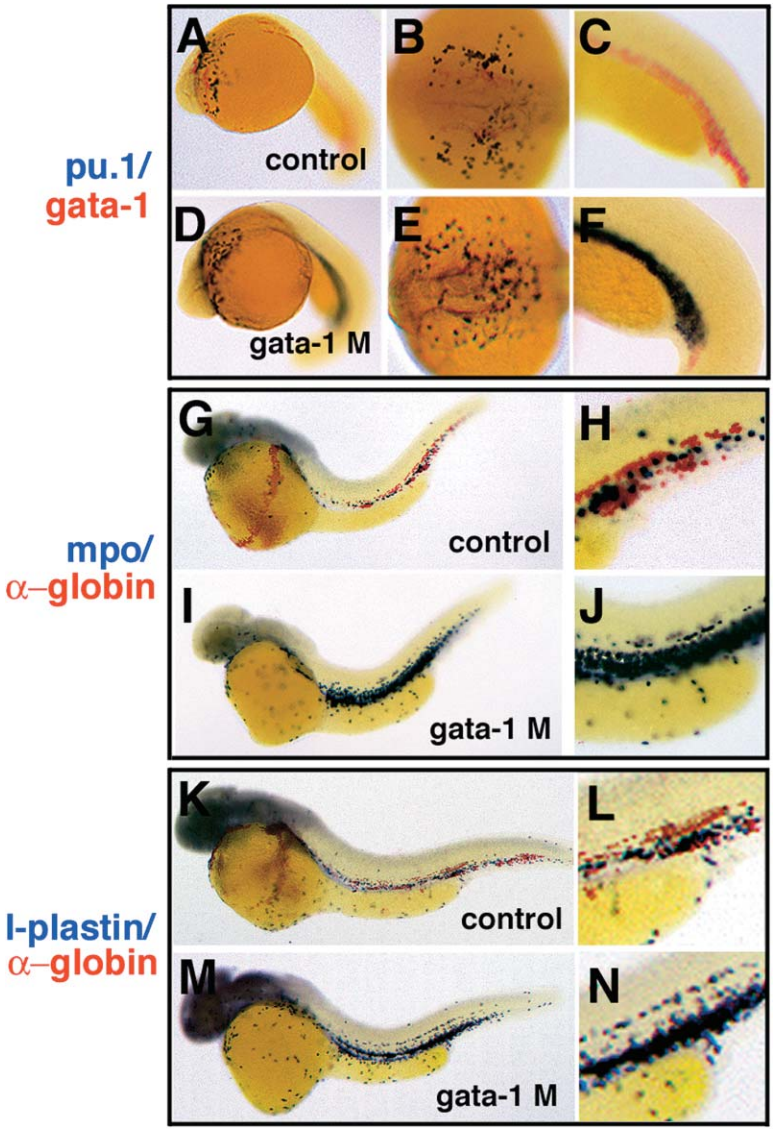

Figure 4. Gata1 Suppresses Myelopoiesis

(A–C, G, H, K, and L) Control morphants and (D–F, I, J, M, and N) Gata1 morphants were analyzed by double ISH assays for (A–F) *pu.1* and *gata1,* (G–J) *mpo* and α-*globin,* and (K–N) *l-plastin* and α-*globin* (blue and red, as indicated). Embryos at (A–F) 20 hpf or (G–N) 32 hpf. Left panels, lateral views; middle panels, dorsal views; right panels, magnified lateral views of the posterior ICM; anterior left.

cells expressing *pu.1* were slightly increased in the ALPM and dramatically increased in the ICM by 20 hpf (Figures 4A–4F). *pu.1* is normally expressed in the PLPM at the 14-somite stage and is downregulated by 18 hpf; however, in Gata1 morphants, *pu.1* expression remained elevated through 24 hpf (data not shown). At later stages, robust ectopic *mpo* and *l-plastin* expression was observed in ICM of Gata1 morphants, demonstrating a marked expansion of granulocyte and monocyte gene expression (Figures 4G–4N). Interestingly, Gata1-*spt* morphants did not express myeloid genes in the trunk (data not shown).

Consistent results were observed in the *vlad tepes* mutant, a zebrafish line harboring an inactivating mutation in the *gata1* gene that blocks erythropoiesis (Lyons et al., 2002). A consistent increase in *pu.1*, *mpo*, and *l-plastin* expression was observed in the ICM of *vlad* mutants, though not to the extent observed in the Gata1 morphants (Supplemental Figures S2A–S2F). These experiments demonstrate that Gata1 is required for erythroid development and negatively regulates myelopoiesis within the ICM, a compartment normally restricted to erythropoiesis.

Genetic Relationship between *pu.1*, *gata1*, and Earlier Hematopoietic Genes

To examine the effect of Pu.1 or Gata1 loss on early hematopoietic cell gene expression, Pu.1 and Gata1 morphants were analyzed for the expression of *gata2*, *scl*, and *lmo2*. In Pu.1 morphants, a marked change in the expression patterns of both *scl* and *lmo2* was observed in the anterior of the embryo. In wild-type embryos, both of these genes are coexpressed with *pu.1* in a subset of cells residing within bilateral clusters along the head, and their expression is dramatically reduced as *pu.1*-expressing cells move ventromedially. In Pu.1 morphants, the expression of *scl* and *lmo2* is maintained in cells as they move ventromedially and in their limited dispersion over the yolk cell (Figures 5A–5F and Supplemental Figures S3A–S3F). The Pu.1 or Gata1 morpholino did not appear to affect *gata2* expression,

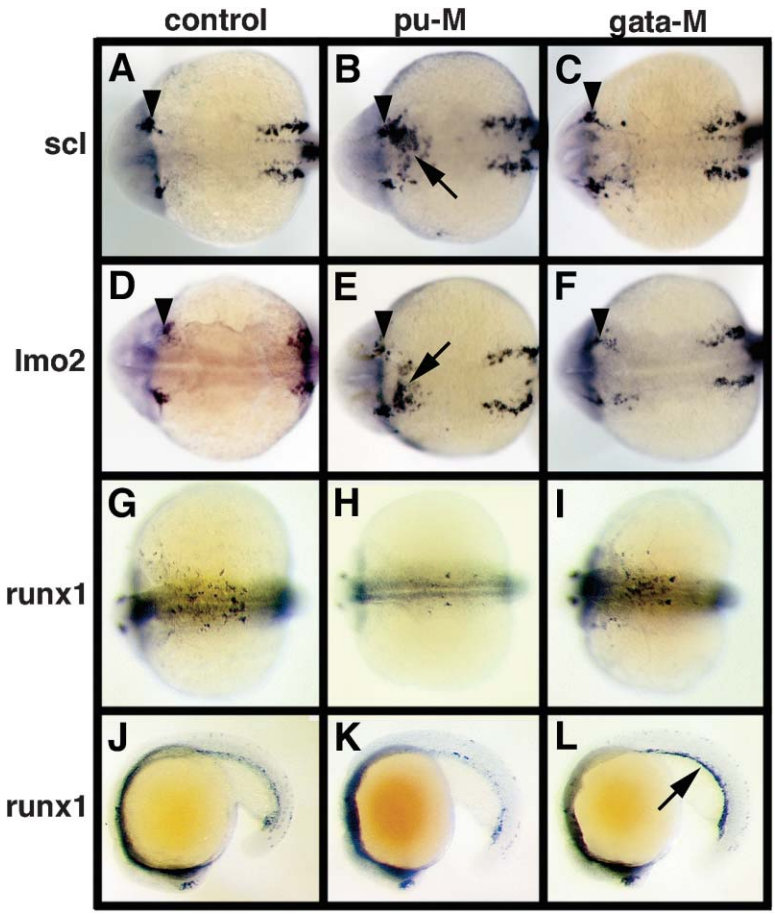

Figure 5. Early Hematopoietic Gene Regulation by Pu.1 and Gata1

Embryos at (A–F) 22 hpf or (G–L) 20 hpf are oriented anterior left in (A–I) dorsal and (J–L) lateral views. Control (left), Pu.1 (middle), and Gata1 (right) morphants were analyzed for the expression of (A–C) *scl*, (D–F) *lmo2,* and (G–L) *runx1*. Arrows indicate ectopic gene expression and arrowheads indicate neural expression.

nor posterior *scl* and *lmo2* gene expression in the ICM of morphants (data not shown).

Pu.1 and Gata1 regulated the expression of *runx1* and *cebp*α. Pu.1 morphants exhibited a reduction in *runx1* expression in both the anterior blood compartment and the ICM at 20 hpf (Figures 5G, 5H, 5J, and 5K). Conversely, Gata1 morphants exhibited a dramatic increase in *runx1* expression in the ICM and possibly a slight increase in the anterior myelopoietic compartment (Figures 5G, 5I, 5J, and 5L). Similar morpholino effects were observed for the *cebp*α gene (data not shown). These experiments indicate that the maintenance of *runx1* and *cebp*α gene expression is dependent upon Pu.1 and negatively regulated by Gata1.

Analysis of the Zebrafish Myelo-Erythroid Progenitor Cell

Results of the Pu.1 and Gata1 morpholino experiments provide compelling evidence for the presence of myelo-erythroid progenitor cells (MPCs) in both the anterior and posterior, capable of forming either myeloid or erythroid derivatives. These findings are consistent with the region-specific regulation and reciprocal inhibition of these two genes within the embryo, leading to the anatomically discrete regions of myelopoietic and erythropoietic development. To test this hypothesis, cell sorting and transplantation experiments were performed, assaying MPC cytology and cell-autonomous fate determination.

Transgenic *pu.1*-GFP zebrafish were disaggregated at the 6- to 7-somite stage (12 hpf), when GFP$^+$ cells are restricted to the ALPM and myelopoietic development. Cytological examination of GFP-sorted cells showed a relatively homogeneous population of intermediate-sized cells with round, slightly irregular, nuclei containing dispersed chromatin and cytoplasm lacking distinct granularity (Figure 6A). At later stages (22 hpf), these cells became heterogeneous in size, with some having condensed nuclei and others exhibiting nuclear indentation, morphologically resembling promyelocytes and myeloid cells at different stages of early maturation (Figure 6B). For comparison, GFP$^+$ erythroid cells from a *gata1*-GFP transgenic line were also analyzed (Long et al., 1997). *gata1*-GFP cells were isolated at 14 hpf, when they were first evident in the PLPM, which formed a population of homogeneous intermediate-sized cells with slightly irregular-shaped nuclei (Figure 6C). At 22 hpf, *gata1*-GFP cells remain homogeneous, possessing coarsely textured chromatin, abundant, deeply basophilic agranular cytoplasm, and paranuclear pallor (Figure 6D). These are cytological features characteristic of early erythroid precursors that clearly distinguish them from *pu.1*-GFP cells at the same developmental stage (Figure 6B; Fleming et al., 2003).

While early *pu.1*-GFP cells and *gata1*-GFP cells (12–14 hpf) both showed immature blast cell morphologies, consistent with being hematopoietic progenitor cells,

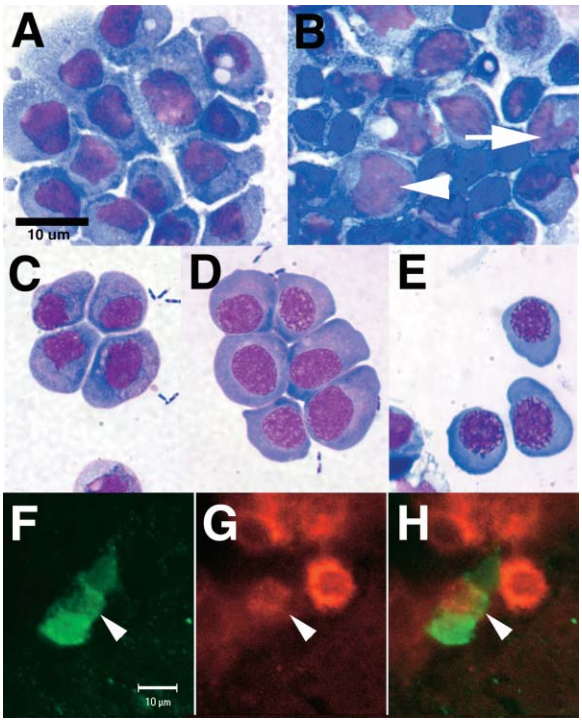

Figure 6. Cytological Analysis and Transplantation Assays

(A–D) Cytospins of sorted GFP$^+$ cells from *pu.1*-GFP transgenic embryos at (A) 12 hpf and (B) 22 hpf and from *gata1*-GFP transgenic embryos at (C) 14 hpf and (D) 22 hpf. A promyelocyte (arrowhead) and characteristic myeloid nuclear indentation (arrow) are indicated.

(E) Cells from *pu.1*-GFP embryos injected with the Pu.1 morpholino.

(F–H) Magnified confocal images of transplanted anterior-derived *pu.1*-GFP cells expressing (F) GFP (green), (G) *gata1* (red), and (H) GFP with *gata1*. Arrowhead indicates a coexpressing cell.

by 22 hpf *pu.1*- and *gata1*-GFP cells had differentiated further, acquiring morphologies representative of maturing myeloid and erythroid cells, respectively. The GFP$^+$ cells isolated from 22 hpf Pu.1-GFP morphant embryos (Figure 6E) included those with erythroid morphologies, unlike cells isolated from control *pu.1*-GFP embryos (Figure 6B). These results are consistent with the shift from myeloid to erythroid gene expression in Pu.1 morphants, demonstrating that the loss of *pu.1* expression also causes a switch toward erythroid cell morphologies.

To test whether the myelopoietic development of *pu.1*-GFP cells is cell autonomous, GFP-sorted cells, from *pu.1*-GFP transgenic zebrafish at the 5- to 7-somite stage, were transplanted into the ICM and/or surrounding lateral mesoderm trunk region of nonfluorescent wild-type host embryos at the 9- to 14-somite stage. At the time of cell sorting, GFP$^+$ cells were only present in the ALPM of donor embryos; however, to unequivocally demonstrate that the transplanted cells originated from the ALPM, embryos were also dissected and only the anterior halves of the embryos were used for cell sorting and subsequent transplantation assays. The host embryos developed to 22–24 hpf and were assayed by confocal microscopy for the coexpression of GFP with erythroid genes. From 18 host embryos, each with 1 to 10 GFP$^+$ cells, we observed that 10/43 individual cells and a cluster containing 8–10 cells coexpressed GFP and *gata1* mRNA. These cells were distributed in positions along the length of the hindyolk and were generally in mesoderm among, or adjacent to, *gata1*$^+$ cells in the ICM (Figures 6F–6H). These experiments indicate that MPCs from anterior myelopoietic regions are capable of expressing erythroid genes when placed within the region of the posterior erythrocyte-forming compartment. The expression of *gata1* was not observed in control transplantation experiments, in which the cells were placed back into the anterior blood compartment (data not shown). These experiments demonstrate the presence of uncommitted MPCs capable of forming either myeloid or erythroid derivatives, depending on non-cell-autonomous signals that act in anterior and posterior blood-forming regions as late as somitogenesis stages of zebrafish development.

Pu.1 Drives Myeloid Cell Differentiation in the *cloche* Mutant

The *cloche* mutant displays a lack of both hematopoietic and vascular tissues, suggesting that the affected gene functions during early stem/progenitor cell stages. While the injection of mRNAs encoding zebrafish orthologs of mammalian stem cell genes, such as *scl,* has been shown to partially rescue erythroid and endothelial cell development in *cloche*, the recovery of myelopoiesis has not been studied. When embryos from heterozygous *cloche* crosses were injected with zebrafish *scl* mRNA, *l-plastin* (Figure 7G), *pu.1*, and *mpo* expression (data not shown) was observed in genotyped *cloche* mutant embryos. These results indicate that, like erythropoiesis and vasculogenesis, myelopoiesis can be rescued by the expression of *scl*.

While *pu.1* is required for zebrafish myelopoiesis, to determine if it is sufficient to drive myeloid cell differentiation, we tested whether *pu.1* mRNA expression in *cloche* could rescue myelopoiesis. The *pu.1* mRNA-injected wild-type embryos exhibited relatively normal myeloid and erythroid gene expression patterns (Figures 7A and 7B). However, the forced expression of *pu.1* in genotyped *cloche* mutants resulted in a partial rescue of both *l-plastin* and *mpo* expression (Figures 7C–7F and 7H) but not *gata1* or α-*globin* (compare with Figure 7G), indicating that *pu.1* is unable to rescue erythropoiesis. These experiments indicate that *pu.1* expression is both necessary and sufficient to specifically drive myeloid gene expression in an early stem/progenitor cell in *cloche* that is competent to differentiate along the myeloid pathway.

Discussion

We are only beginning to understand the molecular mechanisms regulating the hierarchical progression of hematopoietic development, and the zebrafish has become a powerful vertebrate model system for analyzing this complex process in vivo. We present novel evidence identifying the zebrafish MPC, a critical component of myelopoiesis that is likely to represent the functional equivalent of the mammalian CMP cell. We demonstrate the in vivo capacity of MPCs to form myeloid or erythroid cells and their fate determination by the Pu.1 and Gata1 transcription factors.

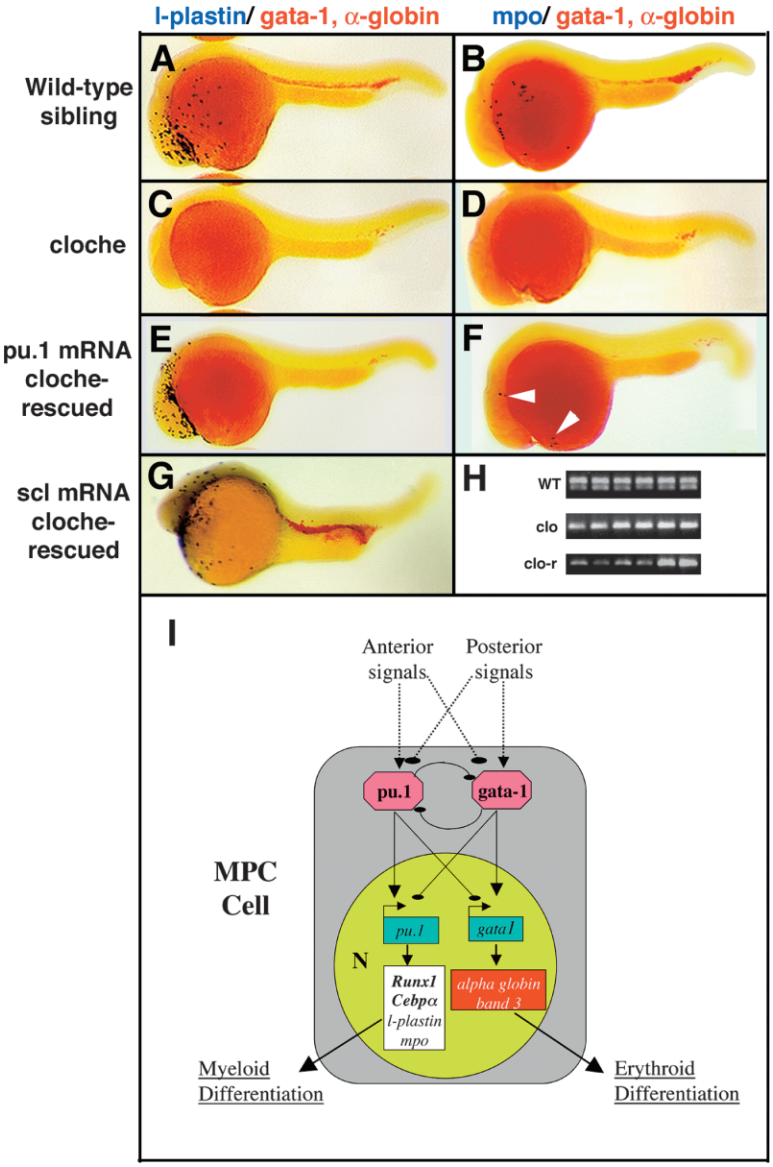

Figure 7. Pu.1 Drives Myelopoiesis in *cloche* Mutants

(A–G) Double ISH assays for (A, C, E, and G) *l-plastin* or (B, D, and F) *mpo* with *gata1* and α-*globin* (blue and red as indicated), in (A and B) wild-type siblings or (C–G) *cloche* mutants at 24 hpf. Lateral views, anterior left. (E and F) *pu.1* mRNA-injected *cloche* embryos. (G) *Scl* mRNA-injected *cloche* embryo.

(H) Genotyping of representative individual embryos (from [E]) by PCR. Wild-type siblings (wt), *cloche* mutants (clo), or rescued *cloche* mutants expressing *l-plastin* (clo-r).

(I) Model of MPC regulation. Region-specific, extracellular signals regulate the expression of *pu.1* or *gata1* and their effects in MPCs. The Pu.1 and Gata1 proteins reciprocally antagonize expression of each other and positively autoregulate their own. Expression of *pu.1* or *gata1* activates gene expression, indicative of lineage-specific differentiation. Octagons represent proteins, boxes represent genes. Putative positive (arrows) and negative (oval-heads) regulatory effects are indicated.

Myelopoiesis and Dynamic Patterns of Gene Expression

Dynamic changes in gene expression are often difficult to interpret because changes in the spatial pattern of gene expression may represent cell movement or adjacent cell populations transiently expressing a gene at different times. The stable GFP protein expressed in *pu.1*-GFP transgenic embryos served as a cell lineage marker, allowing cell movements and dynamic changes in gene expression to be determined even after endogenous *pu.1* expression was downregulated. The *pu.1* gene was first expressed in bilateral clusters of cells in the ALPM, where some cells coexpressed early hematopoietic genes including *scl*, *gata2*, and *lmo2*. All cells expressing *pu.1* migrated ventromedially, losing the expression of these early hematopoietic genes. At this stage, these cells morphologically resemble early immature myeloblasts, similar to those observed in mammals. These cells were also identified in the zebrafish kidney, the adult hematopoietic organ in teleosts, which may represent MPCs in mature zebrafish (Hsu et al., 2004). Later, these cells exhibit increased motility, begin to lose *pu.1* expression, and start expressing mature myeloid genes. These cells exhibit cytological morphologies ranging between myeloblast and band cells, resembling the different stages of mammalian CMP cell maturation. Taken together, these data establish the developmental progression in the gene expression, cellular morphology, cell movements, and motile behavior of *pu.1*-expressing cells, demonstrating the in vivo transition of these cells from MPCs into maturing myeloid cells.

MPCs Require Pu.1 for Myeloid Cell Differentiation

The targeted knockdown of Pu.1 demonstrates its very early requirement for activating downstream target genes necessary for subsequent myeloid cell differentiation. Analysis of Pu.1-GFP morphants further demonstrated that the initial ventromedial movement of *pu.1*$^{+}$

cells is independent of *pu.1* expression, while subsequent myeloid motility requires Pu.1. It is possible that the ventromedial cell movement may reflect the continuation of convergent gastrulation movements that contribute to other anterior mesodermal derivatives, such as the cardiac anlage, which develops normally in the Pu.1 morphant. The limited spread of these cells over the yolk may be due to vestigial myeloid motility or possibly to a transient elongation of an uncommitted MPC state that maintains their limited motility exhibited at the end of gastrulation. This explanation may be consistent with the prolonged expression of early hematopoietic genes, *scl* and *lmo2,* that is also observed during early erythropoiesis in the ICM (Figures 5A–5F and Supplemental Figures S3A–S3F). The eventual loss of motility in these *pu.1*$^{+}$ cells is consistent with their adopting mature erythroid fates.

The Pu.1 morpholino specifically blocks the translation of the Pu.1 protein, and thus the loss of *pu.1* mRNA expression indicates that the Pu.1 protein contributes to the autoregulation of its own gene transcription. In vitro studies have indicated that the mammalian Pu.1 protein can bind and activate its own promoter, consistent with a positive autoregulatory function or transcriptional maintenance (Chen et al., 1995). Alternatively, the lack of Pu.1 protein could lead to the negative regulation of its own transcription indirectly through its effects on other genes such as *gata1*. Evidence from studies in mammals and *Xenopus* show that Pu.1 and Gata1 regulate the respective development of myeloid and erythroid cell lineages and that these two proteins negatively crossregulate each other (reviewed in Cantor and Orkin, 2002). Thus, the subsequent increase in *gata1* expression due to the loss of *pu.1* repression, including its ectopic expression in the ALPM, could also contribute to the downregulation of endogenous *pu.1* transcription.

Since the Pu.1 morpholino can exert its inhibitory effects only following the onset of Pu.1 translation, the ectopic expression of *gata1* in the ALPM resulting from Pu.1 loss demonstrates the lack of commitment of MPCs to myeloid cell fates at this developmental time. Importantly, while the Pu.1 and Gata1 morpholinos have robust effects on endogenous *pu.1* transcription, they do not block transgenic GFP expression in the Pu.1-GFP morphants (Figure 3J). In the absence of endogenous *pu.1* expression, the GFP^{+} cells lose their ability to migrate over the yolk, begin to express mature erythroid genes, and acquire erythroid cell morphologies. Thus, MPCs are competent to differentiate down either the myeloid or erythroid pathway, and the expression of *pu.1* during somitogenesis stages promotes myelopoiesis and inhibits erythropoiesis in the ALPM. As in other species, zebrafish *pu.1* plays a critical role in determining the fate of all myeloid cells, highlighting the evolutionary conservation of *pu.1* function.

MPCs Require Gata1 for Erythroid Cell Differentiation

The evidence supporting the reciprocal negative regulation of *pu.1* and *gata1* prompted the investigation of zebrafish *gata1* function. The morpholino-induced knockdown of Gata1 protein resulted in a dramatic loss of erythrocyte formation, consistent with the *vlad* mutant phenotype. The loss of *gata1* mRNA in the Gata1 morphant indicates a positive autoregulatory function (Kobayashi et al., 2001), although this loss can also be indirectly due to inhibition caused by the elevated activity of *pu.1*. While it was difficult to discern whether there was a consistent quantitative increase in the expression of myeloid genes in the ALPM, there was a robust increase in *pu.1, mpo*, and *l-plastin* expression in the ICM, the normal site of erythropoiesis. This increase in myeloid gene expression, in the absence of *gata1*, was confirmed in the *vlad* mutant, although the *vlad* mutant was reported to have normal myeloid and lymphoid cells (Lyons et al., 2002). While compelling evidence suggests that the point mutation in *vlad* should result in an inactive Gata1 protein, our data suggest that this mutant may exhibit a hypomorphic phenotype relative to Gata1 morphants.

Regulation of Early Hematopoietic Genes in MPCs

The Pu.1 morpholino blocked the normal suppression of both *scl* and *lmo2* as MPCs move ventromedially, while *gata2* expression was unaffected. The normal expression of *pu.1* may be needed to turn off these genes as MPCs move ventromedially. However, Gata1 morphants, which substantially increase *pu.1* expression in the ICM, did not exhibit a corresponding decrease in *scl* and *lmo2* gene expression in that region (data not shown). Thus, it is unlikely that *pu.1* expression is directly responsible for the suppression of either of these genes in the ALPM. Alternatively, there is a relatively longer period of coexpression of these two genes with *gata1* during erythropoiesis in the ICM (data not shown). Thus, the lack of *scl* and *lmo2* suppression may correlate with the change in MPC cell fate toward the erythroid lineage and/or a delay in differentiation.

Zebrafish *runx1* and *pu.1* are expressed at similar developmental times, and the suppression of *runx1* in the Pu.1 morphant indicates its regulation by *pu.1*. The increased *runx1* expression in the ICM of the Gata1 morphant suggests that Gata1 may normally inhibit the expression of *runx1*. However, since the overexpression of *runx1* has been reported to drive erythropoiesis and vasculogenesis in the *cloche* mutant (Kalev-Zylinska et al., 2002), the simplest explanation may be that the loss of Gata1 prevents these cells from continued erythropoiesis and blocks cells in a *runx1*-expressing state. Alternatively, zebrafish *runx1* may also serve a necessary function in myelopoiesis, and the increased *runx1* expression in the ICM of Gata1 morphants may correlate with the observed shift of these MPCs toward myelopoietic fates.

The Determination of MPC Fate by Pu.1 and Gata1

In vitro studies have demonstrated that forced *gata1* expression can reprogram myelomonocytic cells into erythroid cells by downregulating *pu.1*, while forced expression of *pu.1* can drive myeloid differentiation by downregulating *gata1* (Kulessa et al., 1995; Nerlov and Graf, 1998). Our data establish the presence of MPCs in both blood-forming regions of the zebrafish and support the reciprocal antagonistic gene regulation of *pu.1* and *gata1*. While Pu.1 repression of *gata1* appears to be

complete in the anterior, *pu.1* is transiently expressed in the PLPM, suggesting that the normal transcriptional repression of *pu.1* by Gata1 may be relatively less complete in MPCs within this region. Presumably, once the levels of these two transcription factors reach the point at which the cumulative autoregulatory and antagonistic effects of one protein become dominant, only the activity of that transcription factor will be maintained (Figure 7I). While this mechanism can explain the formation of anatomically distinct myelopoietic and erythropoietic domains in the zebrafish embryo, it does not address how the dominance of either factor is established. Transplantation assays demonstrated that non-cell-autonomous signals could redirect or favor the expression of *gata1* over *pu.1* in MPCs derived from the anterior myeloid compartment. These results, along with the capacity of MPCs to respond to Pu.1 and Gata1 morpholinos, suggest that these progenitors are not committed to either a myelopoietic or erythropoietic fate in the pregastrula embryo (Lieschke et al., 2002), but remain responsive to extrinsic signals into somitogenesis stages.

There are many developmental signaling pathways that may regulate anterior versus posterior hematopoietic gene activation. Both Bmp and Wnt signaling pathways regulate dorsal-ventral patterning and hematopoiesis. Other pathways affecting patterning of the ALPM include retinoic acid receptor and notochord signaling factors. Clearly, the extrinsic signals responsible for the regulation of progenitor cell fate remain to be determined. The use of in vivo assays exploiting the discrete blood compartments of the zebrafish model may provide a valuable means of identifying and analyzing MPC-specific signaling molecules relevant to higher vertebrates.

Pu.1 and MPCs in Bloodless Zebrafish Mutants

The spadetail (*spt*) mutant exhibits defects in trunk mesoderm formation and lacks erythrocytes, leading to its "bloodless" classification (Ho and Kane, 1990; Thompson et al., 1998). Recently, the *spt* mutant was reported to exhibit normal myelopoiesis (Lieschke et al., 2002), and the ability to induce erythropoiesis in the anterior of Pu.1-*spt* morphants indicates the presence of normal MPCs in the *spt* ALPM. The inability to induce myelopoiesis in the trunk of Gata1-*spt* morphants suggests the lack of functional MPCs in this region. The simplest interpretation of these findings is that the *spt* mutation affects the general formation of trunk mesodermal derivatives, including the ICM, rather than having a hematopoietic-specific role.

The *cloche* mutant exhibits abnormal heart development and lacks all blood and vasculature (Stainier et al., 1995). The *cloche* mutation acts upstream of several early hematopoietic genes including *scl*, *lmo2, runx1, hex*, and *gata2*, and the ectopic expression of zebrafish orthologs of mammalian HSC genes, such as *scl*, *runx1*, and *hex*, have been shown to partially rescue erythropoiesis and vascular development in this mutant. While we show that overexpressing *scl* in *cloche* rescues both myelopoiesis and erythropoiesis (Figure 7G), the overexpression of *pu.1* rescues only myelopoiesis. The lack of concurrent erythropoietic rescue by *pu.1* in *cloche* suggests that myelopoietic rescue is not due to the activation of earlier genes, such as *scl*, that also rescue erythropoiesis. It remains possible that the overexpression of *pu.1* could suppress erythropoietic rescue in the rescued *cloche* mutants; however, erythropoietic inhibition was not readily observed in injected wild-type *cloche* siblings. Thus, while *pu.1* expression normally occurs downstream of *scl*, it may not require the expression of some of these earlier hematopoietic genes to drive myelopoiesis.

It is interesting that while the loss of *pu.1* leads to ectopic erythropoiesis, its overexpression does not lead to consistent excessive myelopoiesis in the anterior of rescued *cloche* mutants or wild-type siblings, nor does it induce dramatic ectopic myelopoiesis or erythropoietic inhibition in the posterior (Figure 7; data not shown). These observations may reflect how tightly MPC differentiation is controlled by autoregulatory and non-cell-autonomous mechanisms (Figure 7I). It is possible that excessive anterior myelopoiesis is kept in check by inhibitory autoregulatory mechanisms, even when *pu.1* is overexpressed. Furthermore, non-cell-autonomous, or other unknown, mechanisms may contribute to preventing the overexpression of *pu.1* from causing an inhibition of *gata1* and erythropoiesis, or an expansion of myelopoiesis, in posterior blood regions.

In summary, we have identified MPCs in the zebrafish and demonstrated the respective specification of myeloid and erythroid cell lineages by the negative cross-regulation of Pu.1 and Gata1 transcription factors. How MPCs are initially established and the genetic identification of the non-cell-autonomous signaling mechanisms controlling MPC regulation remain for further investigation.

Experimental Procedures

Zebrafish

Wild-type AB stocks of *Danio rerio*, transgenic, and mutant lines were maintained by standard methods (Kimmel et al., 1995; Westerfield, 1995). The transgenic lines, *pu.1*-GFP and *gata1*-GFP, and *spadetail* (*spt*^{b104}) and *cloche* (*clo*^{m39}) mutants have been previously described.

Morpholinos

Morpholinos (Gene Tools) were designed to target the translational start (ATG) of the *pu.1* and *gata1* genes. The Pu.1 morpholino sequence is 5′-GATATACTGATACTCCATTGGTGGT-3′ and its mismatch control sequence is 5′-GATA**A**ACTG**T**TACTC**G**ATTG**C**T GGT-3′. The Gata1 morpholino sequence is 5′-CTGCAAGTGTAG GTATTGAAGATGTC-3′ and its mismatch control sequence is 5′-CTGC**T**AGTG**A**AGTATA**G**AAG**T**TGTC-3′.

Microinjections

Morpholinos and mRNAs were injected into the yolk of 1- to 4-cell stage embryos. Morpholino stocks (1 mM) were diluted 1:1 with 2% phenol red dye. mRNAs were transcribed from linearized pCS2 plasmid, purified, and diluted to 50 ng/μl (mMessage Machine, Ambion).

Whole-Mount In Situ Hybridization and Genotyping

Digoxogenin- or fluorescein-labeled antisense mRNA probes for zebrafish *myoD*, *krox20*, α-*globin*, *cebp*α, *pu.1*, *mpo*, *l-plastin*, *lysozyme C*, *gata1*, *nkx2.5*, and *runx1* were synthesized according to published literature. Whole-mount single or double mRNA in situ hybridization (ISH) assays were performed as described (Bennett et al., 2001). In double ISH assays, the NBT/BCIP staining was performed first, inactivated, and processed further for the second fast red stain. Double mRNA ISH/GFP immunohistochemical assays

using rabbit anti-GFP (1:100; Molecular Probes) and anti-rabbit Alexa 488-conjugated secondary antibodies (1:1000; Molecular Probes) were performed as described (Novak and Ribera, 2003).

Confocal images were captured with a Zeiss LSM 510 META NLO laser-scanning microscope and a Zeiss LD 40× 0.6NA Achroplan objective lens. A multitrack line scan configuration allowed line-by-line pseudo-simultaneous capture of red and green channels while eliminating most bleed-through. The excitation sources for GFP and Fast Red were Argon2 488 nm and HeNe1 543 nm laser lines, respectively. A UV/488/543/633 primary dichroic beam-splitter provided the excitation reflector. BP500–550 nm and LP560 emission filters were used for GFP and Fast Red detection, respectively.

Following ISH analysis, individual *cloche* embryos were genotyped by PCR for the genetic marker Z1496 (Liao et al., 1998). Standard PCR reactions included an internal control for the α-actin gene.

Cell Sorting, Cytospin, and Transplantation Assays

Cells were sorted from transgenic GFP-expressing embryos. For cytospins, embryos (100–150) were collected, disaggregated into a single cell suspension, spun at 200 rpm for 5 min, resuspended in cold 0.9× PBS + 5% FBS (1 ml), and passed through a 4 μm filter. FACS analysis was performed with a Mo-Flo flow cytometer (DakoCytomation) based on propidium iodide (1 mg/ml, Sigma) exclusion and GFP fluorescence. GFP^+ cells were spun down at 200 rpm for 5 min and resuspended in 2–10 μl of 0.9× PBS + 5% FBS. GFP^+ cells (1–2 × 10^5 obtained as above) were cytocentrifuged at 340 rpm for 3 min onto glass slides (Shandon) and processed for May-Grünwald and Giemsa staining (Fluka). For transplantation assays, GFP^+ embryos at 5–7 somites were sorted as above or were dissected to isolate the anterior half of the embryos prior to sorting. GFP^+ cells were back-loaded into a pulled glass needle and transplanted as described (Westerfield, 1995). Cells were transplanted into wild-type hosts (9- to 14-somite stage) that developed to 22–24 hpf prior to being processed for GFP and *gata1* expression, as described above.

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