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# Runx1 Expression Marks Long-Term Repopulating Hematopoietic Stem Cells in the Midgestation Mouse Embryo

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## Summary

**Hematopoietic stem cells (HSCs) are first found in the aorta-gonad-mesonephros region and vitelline and umbilical arteries of the midgestation mouse embryo. Runx1 (AML1), the DNA binding subunit of a core binding factor, is required for the emergence and/or subsequent function of HSCs. We show that all HSCs in the embryo express Runx1. Furthermore, HSCs in *Runx1*$^{+/-}$ embryos are heterogeneous and include CD45$^{+}$ cells, endothelial cells, and mesenchymal cells. Comparison with wild-type embryos showed that the distribution of HSCs among these various cell populations is sensitive to *Runx1* dosage. These data provide the first morphological description of embryonic HSCs and contribute new insight into their cellular origin.**

## Introduction

Hematopoietic stem cells (HSCs) are at the base of the definitive hematopoietic hierarchy and give rise to all hematopoietic lineages in the adult organism. The working definition of an HSC is a cell that can provide long-term multilineage hematopoietic reconstitution when transplanted into an adult animal and can self-renew. HSCs in the adult animal reside in the bone marrow. However, studies in the mouse embryo have shown that the first HSCs are found before bone or bone marrow is formed, in the dorsal aorta within the aorta-gonad-mesonephros (AGM) region and in two arteries that connect the dorsal aorta to the yolk sac and placenta, and the vitelline and umbilical (V+U) arteries (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996; Müller et al., 1994). Grafting experiments in frog and avian embryos likewise identified an intraembryonic region analogous to the mouse AGM as a source of the adult hematopoietic system (Cormier and Dieterlen-Lievre, 1988; Dieterlen-Lièvre, 1975; Turpen and Knudson, 1982). Also, in chick embryos, the allantois, which contributes to the formation of the umbilical artery, was shown to be a potent source of blood cell precursors (Caprioli et al., 1998). HSCs are also found in the yolk sac and in the fetal liver 1 day after they appear in the AGM region and V+U arteries. The types of cells that function as HSCs in the embryo and where these HSCs are located within the embryonic tissues from which they emerge are the questions we addressed in this study.

The formation and/or function of HSCs requires both the *Runx1* (also known as *AML1*, *Cbfa2*, and *Pebpa2b*) and *Cbfb* genes (Okuda et al., 1996; Sasaki et al., 1996; Wang et al., 1996a, 1996b). Homozygous disruption of *Runx1* or *Cbfb* does not block the development of primitive yolk sac-derived erythrocytes. However, definitive erythrocytes, myeloid cells, and lymphocytes fail to develop, indicating a role for both genes at the level of definitive hematopoietic progenitors or HSCs. *Runx1* and *Cbfb* encode the DNA binding and non-DNA binding subunits, respectively, of a transcription factor in the family of core binding factors (CBFs) (Speck and Stacy, 1995). The non-DNA binding CBFβ subunit is common to all three members of the CBF family. Therefore, the developmental specificity of the CBFs is most likely determined by differences in the expression and biochemical properties of the DNA binding subunits.

Recently, we reported on the expression pattern of *Runx1* in the embryo using mice in which *lacZ* was knocked into the *Runx1* locus (North et al., 1999). *Runx1* is expressed in all sites from which hematopoietic cells emerge, as well as in several nonhematopoietic sites. Runx1 protein is first detected in mesenchymal cells in the yolk sac at embryonic age (E) 7.5 and in both primitive erythrocytes and endothelial cells in the yolk sac blood islands at E8.0. Runx1 levels decline in primitive erythrocytes and almost all endothelial cells by E8.5 but remain high in a small number of endothelial cells and blood cells scattered throughout the yolk sac blood islands. Also at E8.5, *Runx1* expression initiates in three new sites: in mesenchymal cells at the distal tip of the allantois, in endothelial cells in the vitelline artery, and in endothelial cells in the ventral portion of the paired dorsal aortae within what will become the AGM region. Between E9.5 and E11.5, Runx1 is expressed in endothelial cells in the V+U arteries, in both endothelial cells and mesenchymal cells in the ventral portion of the AGM region, and in a small number of endothelial cells in the yolk sac, but in no other endothelial cells in the embryo. Shortly after the onset of endothelial cell expression, clusters of Runx1$^{+}$ cells can be seen inside the lumina of the dorsal aorta, the V+U arteries, and yolk sac that are closely associated with the Runx1$^{+}$ endothelium. Intraaortic clusters have been seen at a similar developmental time in the embryos of many organisms (Marshall and Thrasher, 2001). Cells in the clusters are rounded in appearance and express the pan-leukocyte marker CD45, indicating that they are hematopoietic cells (Jaffredo et al., 1998; Tavian et al., 1996). Lineage-tracing studies in chick embryos suggest that the intraaortic hematopoietic clusters form from the endothelial cells in these arteries (Jaffredo et al., 1998, 2000). Intraaortic clusters and CD45$^{+}$ cells are absent in Runx1 deficient embryos (North et al., 1999; Takakura et al., 2000; Yokomizo et al., 2001), suggesting that Runx1 specifies a subset of endothelial cells to form intraaortic clusters.

Both genetic and histological studies suggest that

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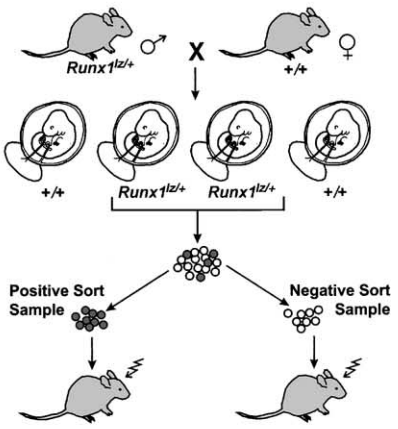


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| Embryonic Tissue | Embryonic Age | Cell Population | #Reconstituted/ #Transplanted | Range of % Reconstitution |
|---|---|---|---|---|
| AGM, V+U | 11.5 | Unfractionated | 6/10 | 5- 75% |
| AGM, V+U | 10.5 | Runx1$^+$ | 3/10 | 5- 10% |
| AGM, V+U | 10.5 | Runx1$^-$ | 0/10 | |
| AGM, V+U [1] | 11.5 | Runx1$^+$ | 14/20 | 10- 50% |
| AGM, V+U | 11.5 | Runx1$^-$ | 0/20 | |
| Fetal Liver | 12.5 | Runx1$^+$ | 18/20 | 25- 100% |
| Fetal Liver | 12.5 | Runx1$^-$ | 0/20 | |

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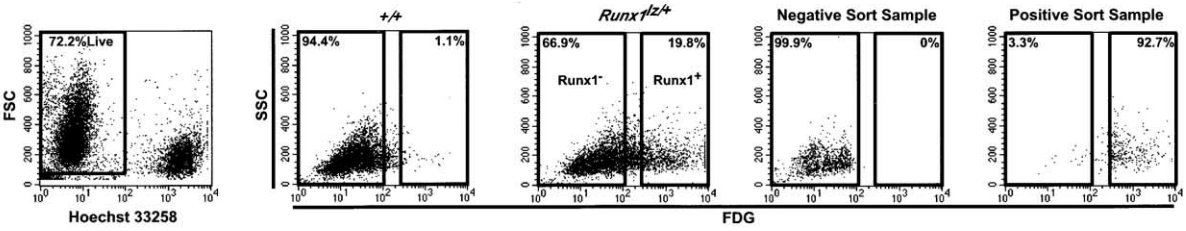


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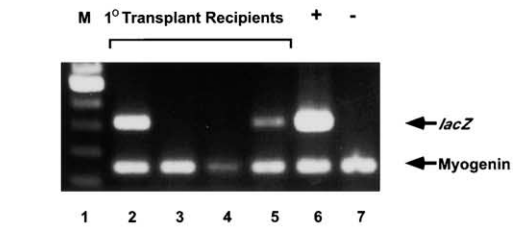


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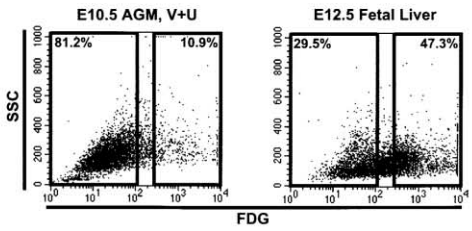


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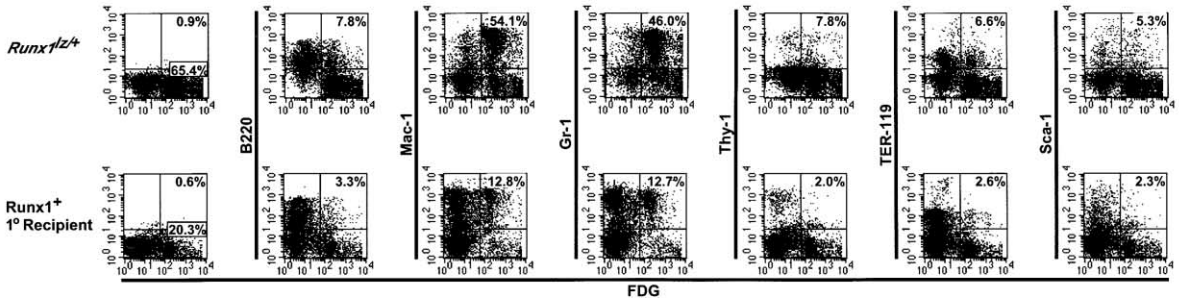


Figure 1. HSCs in the Midgestation Mouse Embryo Express Runx1

(A) Experimental design. E10.5 to 12.5 embryos were generated by crossing *Runx1*$^{lz/+}$ male mice with wild-type female mice. Tissues from *Runx1*$^{+/+}$ and *Runx1*$^{lz/+}$ embryos were isolated by dissection, pooled according to their genotype, and dispersed with collagenase to create single-cell suspensions. Cells were loaded with the β-galactosidase substrate fluorescein di-β-D-galactopyranoside (FDG). Cells from *Runx1*$^{+/+}$ embryos were used to set sort gates, and cells from *Runx1*$^{lz/+}$ embryos were separated into Runx1$^+$ and Runx1$^-$ populations on the basis of green fluorescence intensity by fluorescence-activated cell sorting (FACS) (Nolan et al., 1988). Runx1$^+$ or Runx1$^-$ cells were injected into irradiated adult recipient mice, and peripheral blood and bone marrow was assayed for the presence of donor-derived cells 4 months posttransplantation.

(B) Representative FACS profiles of E11.5 AGM region and V+U artery cells from *Runx1*$^{+/+}$ and *Runx1*$^{lz/+}$ embryos incubated with FDG. Dead cells were excluded on the basis of Hoechst 33258 uptake. Percentages of live cells in both the Runx1$^-$ and Runx1$^+$ sort regions are indicated, and samples from the purified populations obtained from *Runx1*$^{lz/+}$ embryos are shown.

(C) PCR samples of peripheral blood DNA from adult recipient mice at 4 months posttransplantation. Lanes: 1, molecular weight standard; 2–5, blood samples from two successfully repopulated (2 and 5) and nonrepopulated (3 and 4) primary (1°) transplant recipients; 6 and 7, control spleen DNA from a *Runx1*$^{lz/+}$ and *Runx1*$^{+/+}$ mouse, respectively. PCR products from the *lacZ* gene and myogenin (an internal control) are indicated.

Runx1 expression marks the first definitive hematopoietic progenitors and HSCs in the embryo, but this has not been directly demonstrated. Furthermore, Runx1 is expressed in several distinct cell types in embryonic hematopoietic tissues, but which cell types include the progenitors or HSCs is not known. Here we show by transplantation assays that all HSCs in the AGM region, V+U arteries, and fetal liver are within the Runx1$^+$ population of cells. Furthermore, we show that in embryos with only one functional *Runx1* allele, HSCs are present in the Runx1$^+$ mesenchymal, endothelial, and CD45$^+$ cell populations. In wild-type embryos, however, a mesenchymal HSC population is not detectable at the same developmental stage, and endothelial HSCs are much less prevalent. These data indicate that embryonic HSCs are phenotypically heterogeneous and that the distribution of HSCs between the CD45$^+$, endothelial, and mesenchymal cell populations is sensitive to Runx1 dosage.

## Results

### HSCs from the Midgestation Mouse Embryo Express Runx1

We previously generated a *Runx1* allele (*Runx1$^{lz}$*) in which several 3′ exons were replaced with *lacZ* sequences (North et al., 1999). The *Runx1$^{lz}$* allele encodes a Runx1-β-galactosidase fusion protein that contains an intact Runx1 DNA binding domain and nuclear localization sequences but lacks sequences required for transactivation. *Runx1$^{lz}$* is a nonfunctional *Runx1* allele, and since several stages of hematopoiesis are sensitive to Runx1 dosage (Cai et al., 2000; Hayashi et al., 2000; Mukouyama et al., 2000; Song et al., 1999; Wang et al., 1996a, 1996b), the *Runx1$^{lz/+}$* mice are haploinsufficient for Runx1.

To determine whether Runx1 is expressed in HSCs, we isolated Runx1$^+$ and Runx1$^-$ cells from the AGM region, V+U arteries, and fetal livers of *Runx1$^{lz/+}$* embryos and performed adoptive transfer experiments, as outlined in Figure 1A. We were unable to analyze Runx1$^+$ and Runx1$^-$ yolk sac cells because background fluorescence was too high in the yolk sac to detect a specific signal from the *Runx1$^{lz}$* allele (data not shown). Most of our experiments were performed at E11.5 since the frequency of HSCs is higher than at E10.5 (Müller et al., 1994). Runx1$^+$ cells constitute approximately 13% to 30% of cells isolated from the pooled AGM region and V+U arteries of E11.5 *Runx1$^{lz/+}$* embryos (Figure 1B). Upon transplantation of one embryo equivalent (ee) of Runx1$^+$ cells into individual recipient mice, donor-derived cells were detected in the peripheral blood of 14/20 transplanted mice (Figure 1F). In contrast, 0/20 mice were reconstituted with Runx1$^-$ cells. We performed the same analysis with AGM region and V+U artery cells from E10.5 *Runx1$^{lz/+}$* embryos (Figure 1D) and again observed hematopoietic reconstitution only with Runx1$^+$ cells (Figure 1F). Runx1$^+$ and Runx1$^-$ cells were also isolated from the fetal liver at E12.5 and transplanted with similar results (Figures 1D and 1F). Mice transplanted with E11.5 and E12.5 Runx1$^+$ cells showed high levels of multilineage engraftment (Figures 1E and 1F). Donor-derived bone marrow cells from primary transplant recipients were able to reconstitute hematopoiesis in secondary recipients (data not shown), demonstrating that the Runx1$^+$ cell population in the AGM region and V+U arteries contains bona fide HSCs. Thus, in each of the hematopoietic tissues we tested, all HSCs were in the Runx1$^+$ population of cells, and no HSCs were found among the Runx1$^-$ cells.

### HSCs are CD45$^+$ and CD45$^-$

To determine which of the Runx1$^+$ cells contained HSC activity, we used antibodies against cell surface markers to further segregate the Runx1$^+$ cells by FACS. Expression of the cell surface markers was also examined in histological sections with the same antibodies used for cell sorting to pinpoint the location of each cell population within the embryonic tissues.

CD45 is a pan-leukocyte marker expressed on all hematopoietic cells except erythrocytes (Ledbetter and Herzenberg, 1979; van Ewijk et al., 1981). CD45$^+$ cells were found scattered throughout E11.5 *Runx1$^{lz/+}$* embryos and in the AGM region were located primarily in the paraaortic mesenchyme (Figure 2A). CD45$^+$ cells were also found in intraaortic hematopoietic clusters (Jaffredo et al., 1998; Tavian et al., 1996) (data not shown) and occasionally in the circulation (data not shown). In general, there are fewer intraaortic hematopoietic clusters at E11.5 than at E10.5 (Garcia-Porrero et al., 1995), and *Runx1* haploinsufficiency further reduces their size and number (Cai et al., 2000). All of the CD45$^+$ cells we observed in histological sections, including CD45$^+$ cells in the paraaortic mesenchyme and scattered throughout the embryo, appear to express Runx1. However, the converse is not true, as there are Runx1$^+$ mesenchymal cells and endothelial cells that do not express CD45 (Figure 2A).

We transplanted CD45$^+$ and CD45$^-$ cells (Figure 2B) from the AGM region and V+U arteries of E11.5 *Runx1$^{lz/+}$* embryos into mice and found HSC activity in both populations (Figure 2D). We also isolated Runx1$^+$CD45$^+$ cells and Runx1$^+$CD45$^-$ cells (Figure 2C) and again found

(D) Runx1 (FDG) expression in E10.5 AGM region and V+U artery cells and E12.5 fetal liver cells of *Runx1$^{lz/+}$* embryos. Representative percentages in each sort window are indicated.

(E) Multilineage analysis (as described in Experimental Procedures) of bone marrow cells isolated from a *Runx1$^{lz/+}$* and a 1° transplant recipient mouse. The total percentage of donor cells in bone marrow of this particular recipient was 31.0%. Double-positive cells in the upper right quadrant document contribution to each lineage.

(F) Transplantation results. Data are the total of 13 experiments. One ee of AGM and V+U cells or 0.1 ee of fetal liver cells was transplanted per recipient. Reconstitution data were determined by PCR amplification of peripheral blood DNA at 4 months posttransplantation (C) and confirmed by FACS analysis of bone marrow cells (E). In all cases, multilineage reconstitution was demonstrated. [1]Secondary transplants were successfully performed. Relative sizes of the sorted subsets were (mean % viable cells [range]): E10.5 AGM, V+U Runx1$^+$ 10.2% (6.8–13.9), Runx1$^-$ 78.9% (71.4–88.7); E11.5 AGM, V+U Runx1$^+$ 19.8% (12.8–29.5), Runx1$^-$ 65.5% (51.6–81.3); E12.5 fetal liver Runx1$^+$ 53.5% (44.2–63.6), Runx1$^-$ 29.0% (20.5–35.5).

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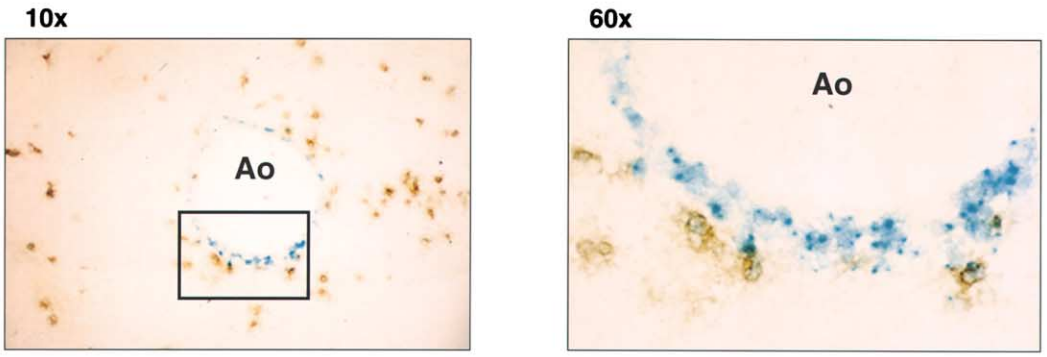


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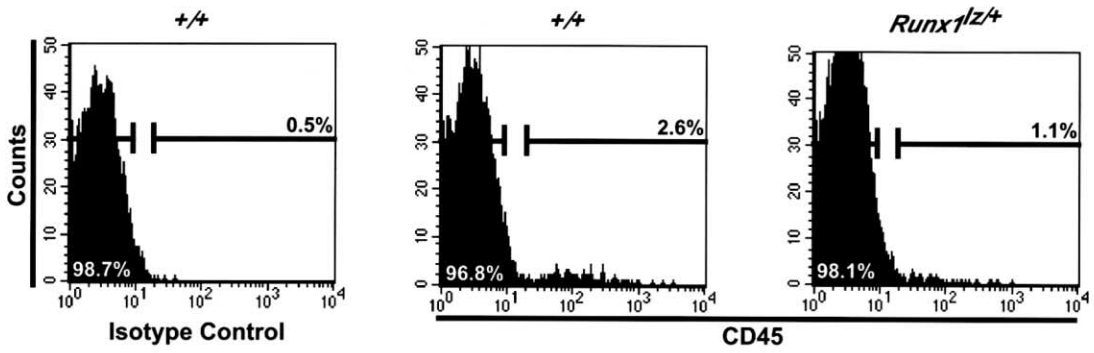


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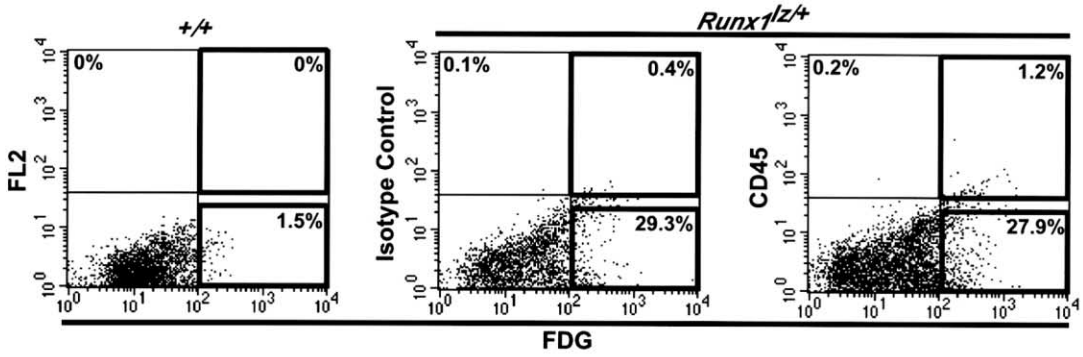


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| Embryonic Tissue | Embryonic Age | Cell Population[1] | % of Total Viable Cells [mean (range)] | | # Reconstituted[2] / # Transplanted | | |
|---|---|---|---|---|---|---|---|
| | | | *Runx1*$^{+/+}$ | *Runx1*$^{lz/+}$ | *Runx1*$^{+/+}$ | *Runx1*$^{lz/+}$ | % Reconstitution[3] |
| AGM, V+U | 10.5 | CD45$^+$ | | 1.1 (0.8-1.2) | | 0/10 | |
| AGM, V+U | 10.5 | CD45$^-$ | | 83.0 (78.8-84.7) | | 3/10 | <5% |
| AGM, V+U | 11.5 | CD45$^+$ | 2.8 (2.0-4.1) | 1.0 (0.8-1.3) | 6/19 | 5/8 | 10-55% |
| AGM, V+U | 11.5 | CD45$^-$ | 96.4 (94.1-97.7) | 96.8 (88.9-97.5) | 1/17 | 3/8 | 11-45% |
| AGM, V+U[4] | 11.5 | Runx1$^+$CD45$^+$ | | 1.1 (0.9-1.5) | | 7/8 | 11-37% |
| AGM, V+U[4] | 11.5 | Runx1$^+$CD45$^-$ | | 22.0 (19.1-28.8) | | 4/6 | 13-28% |
| Fetal Liver | 12.5 | CD45$^+$ | 9.1 (7.0-10.3) | 5.8 (5.7-5.9) | 27/27 | 6/10 | 15-74% |
| Fetal Liver | 12.5 | CD45$^-$ | 88.5 (84.6-92.0) | 83.6 (80.9-84.3) | 3/16[5] | 9/10 | 48-96% |

HSCs in both populations (Figure 2D). HSCs in fetal livers from E12.5 *Runx1*$^{lz/+}$ embryos were likewise found in both populations. In the AGM region and V+U arteries from E10.5 *Runx1*$^{lz/+}$ embryos, we found HSCs in the CD45$^-$ population only, suggesting that CD45$^-$ HSCs are more abundant than CD45$^+$ HSCs at E10.5.

We also examined the expression of CD45 on HSCs from *Runx1*$^{+/+}$ embryos. CD45$^+$ and CD45$^-$ cells were isolated from *Runx1*$^{+/+}$ embryos that carried a human β-globin transgene (Strouboulis et al., 1992) or were heterozygous for an insulin receptor substrate 3 (*Irs3*) gene deletion (Liu et al., 1999) to mark the donor-derived cells. A larger proportion of HSCs resides in the CD45$^+$ cell population of the AGM region, V+U arteries, and fetal liver of *Runx1*$^{+/+}$ embryos versus *Runx1*$^{lz/+}$ embryos (Figure 2D). Also, the percentage of CD45$^+$ cells in the E11.5 AGM region and V+U arteries was approximately 2- to 3-fold higher in *Runx1*$^{+/+}$ embryos than in *Runx1*$^{lz/+}$ embryos (Figures 2B and 2D). Thus, Runx1 dosage affects both the percentage of CD45$^+$ cells and the distribution of HSCs between the CD45$^+$ and CD45$^-$ populations.

### Runx1$^+$ Endothelial and Mesenchymal Cells Are HSCs

The data suggest that some HSCs in the AGM region and V+U arteries of *Runx1*$^{lz/+}$ embryos are CD45$^-$ endothelial cells or mesenchymal cells; thus, we used antibodies recognizing several different endothelial cell surface markers, including CD31, Flk-1, and VE-cadherin, to determine if cells expressing these markers have HSC activity. CD31 (or platelet/endothelial cell adhesion molecule, PECAM-1) is reportedly expressed on all endothelial cells in the embryo but not on angioblasts (Drake and Fleming, 2000). CD31 is also found on bone marrow HSCs (van der Loo et al., 1995) and on intraaortic clusters (Garcia-Porrero et al., 1998). Flk-1 was cloned from fetal liver hematopoietic cells and is expressed on endothelial cell progenitors and endothelial cells (Drake and Fleming, 2000; Matthews et al., 1991; Shalaby et al., 1997; Yamaguchi et al., 1993). Flk-1 is expressed in intraaortic hematopoietic clusters in mouse embryos, as visualized by intracellular X-gal staining in *Flk-1*$^{lz/+}$ embryos (Shalaby et al., 1997) but not in chick embryos as detected by immunohistology (Jaffredo et al., 1998). VE-cadherin is expressed on endothelial cells in the dorsal aorta and major blood vessels in a pattern similar to CD31 (Drake and Fleming, 2000).

Antibodies to CD31, Flk1, and VE-cadherin appeared to uniformly label all endothelial cells in the AGM region (Figure 3A) and V+U arteries (data not shown) but did not label the Runx1$^+$ mesenchymal cells. Five to twelve percent of AGM region and V+U cells were positive for CD31, Flk-1, or VE-cadherin by FACS (Figure 3A and Table 1), and on average 27% of CD31$^+$, Flk1$^+$, or VE-Cad$^+$ cells also expressed Runx1 (data not shown). In all cases, we found HSCs in both positive (CD31$^+$, Flk1$^+$, or VE-Cad$^+$) and negative populations of cells from the AGM region and V+U arteries of *Runx1*$^{lz/+}$ embryos (Table 1). Fractionation of Runx1$^+$ cells into CD31, Flk-1, or VE-cadherin positive and negative populations also revealed HSCs in both populations (Table 1).

As many endothelial cell markers are also found on cells within the intraaortic clusters (Marshall and Thrasher, 2001), we used antibodies to CD31 or VE-cadherin in conjunction with antibodies to CD45 to separate endothelial cells from the hematopoietic clusters (Figures 3B and 3C). We found that CD31$^+$CD45$^-$ and VE-Cad$^+$CD45$^-$ cells from the AGM region and V+U arteries of *Runx1*$^{lz/+}$ embryos contained HSCs (Table 2), indicating that some HSCs in *Runx1*$^{lz/+}$ embryos are CD45$^-$ endothelial cells.

Since some HSCs in *Runx1*$^{lz/+}$ embryos were CD45$^-$ and some did not express endothelial cell markers, we determined whether these HSCs were mesenchymal cells. We gated on the Runx1$^+$ fraction of AGM region and V+U artery cells and separated the cells that expressed VE-cadherin alone, CD45 alone, or coexpressed both markers from Runx1$^+$ cells that expressed neither VE-cadherin nor CD45 (Figure 3D), which should represent Runx1$^+$ mesenchymal cells. We found that hematopoiesis in 6/9 mice transplanted with Runx1$^+$VE-Cad$^-$CD45$^-$ cells was reconstituted with donor-derived cells (Table 2), leading us to conclude that at least some HSCs in *Runx1*$^{lz/+}$ embryos are mesenchymal cells. In contrast, we found no hematopoietic reconstitution with VE-Cad$^-$, CD31$^-$ (Table 1), or VE-Cad$^-$CD45$^-$ (Table 2) populations of cells from the AGM region and V+U arteries of E11.5 *Runx1*$^{+/+}$ embryos. Therefore, E11.5 *Runx1*$^{+/+}$ embryos do not appear to contain detectable mesenchymal HSCs, although we cannot rule out the possibility that very low numbers of these HSCs are present. Most, if not all, HSCs in E11.5 *Runx1*$^{+/+}$ embryos expressed the endothelial markers VE-cadherin and CD31, in addition to CD45, and we conclude that most HSCs in E11.5 *Runx1*$^{+/+}$ embryos are within the hematopoietic clusters. We did find hematopoietic reconstitution with the Flk-1$^-$ population from *Runx1*$^{+/+}$ embryos (Table 1). However, cell surface Flk-1 is downregulated on the CD45$^+$ intraaortic clusters in chick em-

Figure 2. HSCs Are CD45$^+$ and CD45$^-$

(A) Transverse sections (10× and 60× magnification) through the AGM regions of E11.5 *Runx1*$^{lz/+}$ embryos incubated with X-gal (blue) to visualize Runx1 expression and stained with an antibody to CD45 to detect hematopoietic cells (brown). Region in right panel is outlined in left panel. Ao, aortic lumen. Note in 60× panel that CD45$^+$ cells are also Runx1$^+$.

(B) Comparison of CD45 expression on E11.5 AGM region and V+U artery cells from *Runx1*$^{+/+}$ and *Runx1*$^{lz/+}$ embryos. Representative percentages and sort regions used in transplantation studies are indicated.

(C) Dot plot of CD45 and Runx1 expression of AGM region and V+U artery cells with sort gates and relevant controls.

(D) Transplantation results: [1]one ee of AGM region and V+U cells and 0.1 ee of fetal liver cells was transplanted per recipient; [2]reconstitution data were determined by PCR amplification of peripheral blood DNA at 1 and 4 months posttransplantation; data are the total of 15 experiments; [3]the extent of reconstitution for mice transplanted with *Runx1*$^{lz/+}$ donor cells was determined by FACS analysis of bone marrow; in all cases multilineage reconstitution was demonstrated; [4]secondary transplants were performed and reconstitution demonstrated; [5]although the purity of the CD45$^-$ population was ≥95%, repopulation by contaminating CD45$^+$ cells cannot be ruled out.

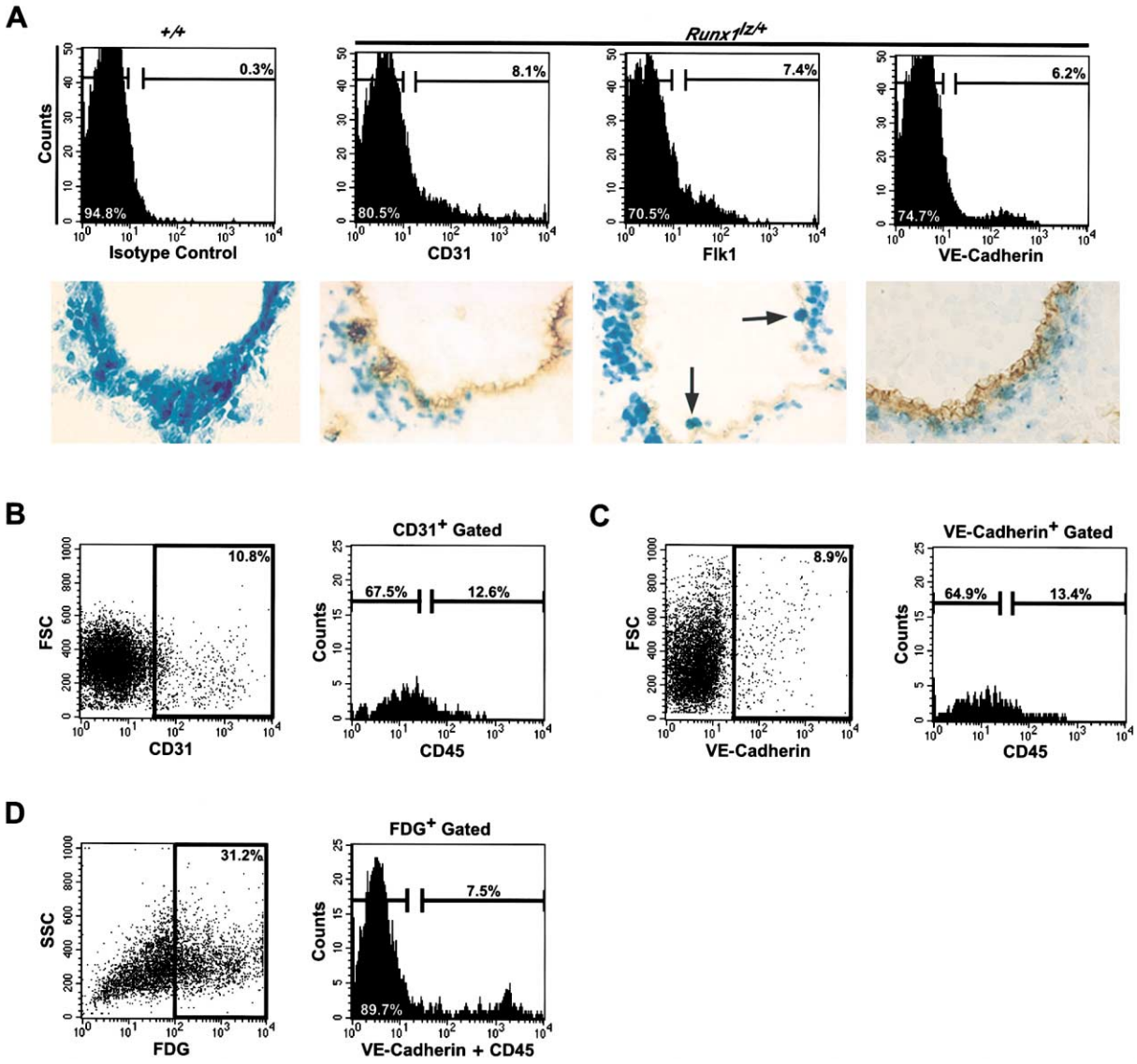



Figure 3. Endothelial Markers on HSCs

(A) (Top) FACS profiles of E11.5 *Runx1*$^{lz/+}$ AGM region and V+U artery cells incubated with antibodies to CD31, Flk1, and VE-cadherin. Representative cell distribution and sort regions are shown. (Bottom) Transverse sections of the AGM region from an E11.5 *Runx1*$^{lz/+}$ embryo incubated with X-gal alone (blue), or double-stained for Runx1 (blue) and CD31, Flk-1, or VE-cadherin (brown). Arrows indicate hematopoietic clusters. Note that in Flk-1-stained sections the hematopoietic clusters do not appear to express Flk-1.

(B and C) Fractionation of CD31 positive (B) or VE-cadherin (C) positive *Runx1*$^{lz/+}$ endothelial cells based on CD45 expression. Sort gates are indicated.

(D) Isolation of Runx1$^{+}$ mesenchymal cells based on the absence of cell surface VE-cadherin and CD45, measured in the same channel.

bryos (Jaffredo et al., 1998), and this also appears to be the case in mouse (Figure 3A). This suggests that the Flk-1$^{-}$ population of HSCs is localized to the hematopoietic clusters.

We also examined whether CD31, Flk-1, and VE-cadherin are expressed on E12.5 fetal liver HSCs. CD31 was found on all E12.5 fetal liver HSCs in both *Runx1*$^{lz/+}$ and *Runx1*$^{+/+}$ embryos (Table 1). Flk-1 and VE-cadherin, on the other hand, were found on only a small proportion of fetal liver HSCs. Thus, HSCs appear to retain CD31 (a finding that is in agreement with CD31 expression by adult bone marrow HSCs) but to downregulate cell surface VE-cadherin soon after they emerge.

**Expression of the Adult HSC Markers Sca-1 and c-kit**

Finally, we examined the expression of two other markers found on HSCs in adult bone marrow, Sca-1 and c-kit (Morrison et al., 1995; Spangrude et al., 1988). Cell surface Sca-1 marks a subset of HSCs from the AGM regions of E11.5 wild-type embryos (de Bruijn et al., 2002 [this issue of *Immunity*]), and c-kit was shown to mark all HSCs in both the AGM region and fetal liver (Sanchez et al., 1996). Approximately 16% of AGM region and V+U artery cells was Sca-1$^{+}$, and Runx1 was expressed in approximately one-third of Sca-1$^{+}$ cells (Figure 4A). We found HSCs in both the Sca-1$^{+}$ and Sca-1$^{-}$ populations of the AGM region and V+U artery cells

Table 1. Expression of Endothelial Cell Markers on HSCs

| Embryonic Tissue | Embryonic Age | Cell Population[1] | % of Total Viable Cells (Range) | | # Reconstituted[2] / #Transplanted | | |
|---|---|---|---|---|---|---|---|
| | | | *Runx1*$^{+/+}$ | *Runx1*$^{lz/+}$ | *Runx1*$^{+/+}$ | *Runx1*$^{lz/+}$ | % Reconstitution[3] |
| AGM, V+U | 10.5 | CD31$^+$ | | 8.8 (6.9–9.9) | | 1/10 | <5% |
| AGM, V+U | 10.5 | CD31$^-$ | | 74.4 (69.7–79.7) | | 2/10 | <5% |
| AGM, V+U | 11.5 | CD31$^+$ | 17.3 (15.6–19.2) | 8.5 (6.6–12.1) | 5/17 | 2/8 | 10%–11% |
| AGM, V+U | 11.5 | CD31$^-$ | 81.0 (78.7–82.9) | 75.5 (71.2–80.5) | 0/14 | 6/8 | 12%–33% |
| AGM, V+U[4] | 11.5 | Runx1$^+$CD31$^+$ | | 2.5 (1.7–2.8) | | 3/9 | 10%–14% |
| AGM, V+U[4] | 11.5 | Runx1$^+$CD31$^-$ | | 22.0 (18.9–26.1) | | 4/9 | 10%–16% |
| AGM, V+U | 11.5 | Flk1$^+$ | 10.0 (7.0–13.0) | 8.8 (5.6–12.5) | 1/8 | 3/9 | 13%–25% |
| AGM, V+U | 11.5 | Flk1$^-$ | 85.1 (81.8–88.3) | 74.0 (69.8–76.1) | 3/7 | 4/9 | 12%–24% |
| AGM, V+U[4] | 11.5 | Runx1$^+$Flk1$^+$ | | 3.8 (2.9–5.8) | | 2/10 | 13%–21% |
| AGM, V+U[4] | 11.5 | Runx1$^+$Flk1$^-$ | | 19.8 (15.5–21.4) | | 2/8 | 14%–23% |
| AGM, V+U | 11.5 | VE-Cad$^+$ | 2.7 (2.5–3.0) | 7.4 (5.3–10.8) | 4/17 | 6/10 | 9.6%–73.2% |
| AGM, V+U | 11.5 | VE-Cad$^-$ | 95.6 (93.3–96.7) | 82.1 (74.6–84.4) | 0/17 | 4/10 | 8.3%–46.2% |
| AGM, V+U[4] | 11.5 | Runx1$^+$ VE-Cad$^+$ | | 2.9 (2.4–3.5) | | 6/7 | 11%–21% |
| AGM, V+U[4] | 11.5 | Runx1$^+$ VE-Cad$^-$ | | 20.8 (19.2) | | 5/6 | 13%–22% |
| Fetal Liver | 12.5 | CD31$^+$ | 17.3 (15.5–19.0) | 18.1 (10.5–24.6) | 13/15 | 10/10 | 48%–90% |
| Fetal Liver | 12.5 | CD31$^-$ | 81.1 (79.6–82.5) | 67.9 (59.2–80.0) | 0/9 | 0/10 | |
| Fetal Liver | 12.5 | Flk1$^+$ | 2.4 (2.2–2.5) | | 1/8[5] | | |
| Fetal Liver | 12.5 | Flk1$^-$ | 97.2 | | 6/6 | | |
| Fetal Liver | 12.5 | VE-Cad$^+$ | 2.3 (1.5–3.0) | 7.8 (5.8–9.6) | 1/10[5] | 0/10 | |
| Fetal Liver | 12.5 | VE-Cad$^-$ | 97.3 (96.3–98.0) | 83.3 (82.2–84.3) | 10/10 | 8/10 | 27%–100% |

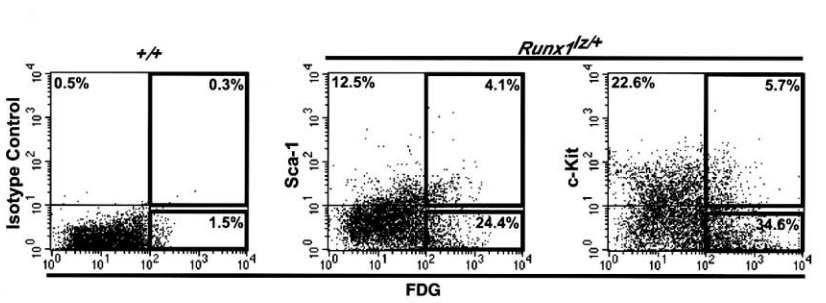
[1] One ee of AGM region and V+U cells, or 0.1 ee of fetal liver cells (0.1–0.2 ee for Flk-1) was transplanted per recipient.
[2] Reconstitution data were determined by PCR amplification of peripheral blood DNA at 1 and 4 months posttransplantation. Data are the total of 41 experiments.
[3] The extent of reconstitution for mice transplanted with *Runx1*$^{lz/+}$ donor cells was determined by FACS analysis of bone marrow. In all cases multilineage reconstitution was also demonstrated.
[4] Secondary transplants were performed, and reconstitution was demonstrated.
[5] Although the purity of the Flk-1$^+$ and VE-Cad$^+$ population was ≥98%, repopulation by contaminating Flk-1$^-$ and VE-Cad$^-$ cells cannot be ruled out.

in both *Runx1*$^{lz/+}$ and *Runx1*$^{+/+}$ embryos (Figure 4C). c-kit was expressed on approximately 14% of Runx1$^+$ cells in the AGM region and V+U arteries, and conversely, Runx1 was expressed in 20% of c-kit$^+$ cells (Figure 4A). The Runx1$^+$c-kit$^+$ cells represented 4%–6% of the total cells in these tissues. When we transplanted

Table 2. HSCs in *Runx1*$^{lz/+}$ Embryos Include Endothelial Cells and Mesenchymal Cells

| Embryonic Tissue | Embryonic Age | Cell Population[1] | % of Total Viable Cells (Range) | | # Reconstituted[2] / # Transplanted | | |
|---|---|---|---|---|---|---|---|
| | | | *Runx1*$^{+/+}$ | *Runx1*$^{lz/+}$ | *Runx1*$^{+/+}$ | *Runx1*$^{lz/+}$ | % Reconstitution[3] |
| AGM, V+U | 11.5 | CD31$^+$CD45$^+$ | | 1.5 (1.2–1.8) | | 4/10 | 10%–18% |
| AGM, V+U | 11.5 | CD31$^+$CD45$^-$ | | 7.4 (6.8–8.1) | | 4/10 | 10%–14% |
| AGM, V+U | 11.5 | VE-Cad$^+$CD45$^+$ | 1.5 | 1.1 (1.0–1.3) | 4/10 | 5/9 | 10%–13% |
| AGM, V+U | 11.5 | VE-Cad$^+$CD45$^-$ | 1.0 | 1.8 (1.4–2.4) | 0/10 | 3/9 | 10%–22% |
| AGM, V+U[4] | 10.5 | Runx1$^+$VE-Cad$^+$ and/or CD45$^+$ | | 2.2 | | 0/4 | |
| AGM, V+U[4] | 10.5 | Runx1$^+$VE-Cad$^-$CD45$^-$ | | 14.9 | | 2/4 | <5% |
| AGM, V+U[4] | 11.5 | Runx1$^+$VE-Cad$^+$ and/or CD45$^+$ | | 3.2 (1.9–3.9) | | 6/9 | 10%–14.5% |
| AGM, V+U[4] | 11.5 | Runx1$^+$VE-Cad$^-$CD45$^-$ | | 19.0 (15.0–24.3) | | 6.9 | 10%–18% |
| AGM, V+U | 11.5 | VE-Cad$^+$ and/or CD45$^+$ | 3.4 | | 5/7 | | |
| AGM, V+U | 11.5 | VE-Cad$^-$CD45$^-$ | 95.9 | | 0/5 | | |

[1] One ee of AGM region and V+U cells was transplanted per recipient.
[2] Reconstitution data were determined by PCR amplification of peripheral blood DNA at 1 and 4 months posttransplantation. Data are the total of 10 experiments.
[3] The extent of reconstitution for mice transplanted with *Runx1*$^{lz/+}$ donor cells was determined by FACS analysis of bone marrow. In all cases multilineage reconstitution was also demonstrated.
[4] Secondary transplants were performed and reconstitution demonstrated.




C

| Embryonic Tissue | Embryonic Age | Cell Population[1] | % of Total Viable Cells [mean (range)] | # Reconstituted[2] / # Transplanted | | |
|---|---|---|---|---|---|---|
| | | | *Runx1*$^{lz/+}$ | *Runx1*$^{+/+}$ | *Runx1*$^{lz/+}$ | % Reconstitution[3] |
| AGM, V+U | 11.5 | Sca-1$^+$ | 6.3 (4.2-9.0) | | 4/10 | 15-38% |
| AGM, V+U | 11.5 | Sca-1$^-$ | 85.2 (81.6-88.2) | | 3/10 | 16-36% |
| AGM, V+U[4] | 11.5 | Runx1$^+$Sca-1$^+$ | 4.4 (3.7-5.5) | | 2/10 | 19-30% |
| AGM, V+U[4] | 11.5 | Runx1$^+$Sca-1$^-$ | 21.0 (15.9-23.1) | | 2/10 | 15-32% |
| AGM | 11.5 | c-kit$^+$ | | 12/20[5] | | |
| AGM | 11.5 | c-kit$^-$ | | 0/18[5] | | |
| AGM, V+U[4] | 11.5 | Runx1$^+$c-kit$^+$ | 5.0 (4.0-5.7) | | 7/13 | 13.5-28% |
| AGM, V+U | 11.5 | Runx1$^+$c-kit$^-$ | 29.1 (23.8-33.3) | | 0/13 | |

Figure 4. Sca-1 and c-kit Expression on Runx1$^+$ HSCs

(A) Representative FACS profiles for E11.5 AGM region and V+U artery cells from a *Runx1*$^{lz/+}$ embryo incubated with FDG and stained for Sca-I or c-kit. Representative percentages of single-positive and double-positive cell types and sort regions are shown.

(B) Transverse section through the AGM region of an E11.5 *Runx1*$^{lz/+}$ embryo showing colocalization of c-kit (brown) and Runx1 (blue) in endothelial (arrow) and mesenchymal (arrowhead) cells. Asterisks indicate two Runx1$^+$c-kit$^-$ cells.

(C) Transplantation results: [1]one ee of *Runx1*$^{lz/+}$ AGM region and V+U cells was transplanted per recipient; [2]reconstitution data were determined by PCR amplification of peripheral blood DNA at 1 and 4 months posttransplantation; [3]the extent of reconstitution for mice transplanted with *Runx1*$^{lz/+}$ donor cells was determined by FACS analysis of bone marrow; in all cases multilineage reconstitution was demonstrated; [4]secondary transplants were performed and reconstitution demonstrated; [5]data from Sanchez et al. (1996).

the Runx1$^+$c-kit$^+$ and the Runx1$^+$c-kit$^-$ cells, we found HSCs only in the double-positive population (Figure 4C). Most of the Runx1$^+$c-kit$^+$ cells in the AGM region of *Runx1*$^{lz/+}$ embryos were located in the endothelium, in mesenchymal cells directly under the endothelium (Figure 4B), and, based on individual staining patterns, in intraaortic hematopoietic clusters (North et al., 1999; Takakura et al., 1998; Yokomizo et al., 2001), thus defining the location of HSCs in this tissue.

## Discussion

Runx1 is required for definitive hematopoiesis (Okuda et al., 1996; Sasaki et al., 1996; Wang et al., 1996a, 1996b), and here we show that all HSCs in the AGM region, V+U arteries, and the fetal liver express Runx1. We also found that the first HSCs that emerge during embryonic development are heterogeneous. Some HSCs in the AGM region and V+U arteries of *Runx1*$^{lz/+}$ embryos express cell surface CD45, some express only endothelial markers, and some express neither hematopoietic nor endothelial markers. These conclusions are based on transplantation into adult mice and do not address whether these or other cells contribute to HSC populations in the developing embryo.

### Partitioning of HSCs between CD45$^+$ and CD45$^-$ Cells Is Sensitive to Runx1 Dosage

The proportion of CD45$^+$ HSCs is higher in *Runx1*$^{+/+}$ than in *Runx1*$^{lz/+}$ embryos, especially in the fetal liver. The reason for this is not known; it is possible that Runx1 dosage affects the number of CD45 molecules on the

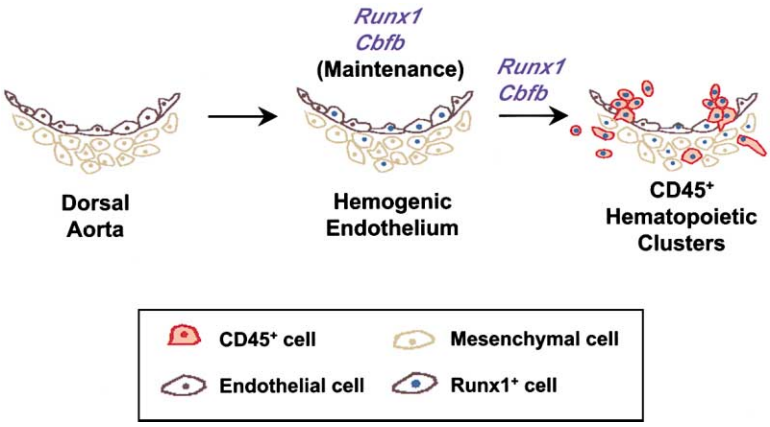



Figure 5. Model for Runx1 Function in HSC Emergence

Runx1 expression (blue nuclei) is initiated in endothelial cells and mesenchymal cells in the ventral aspect of the dorsal aorta soon after it forms and later in CD45$^+$ intraaortic clusters that differentiate from the Runx1$^+$ endothelium. HSCs are found among the Runx1$^+$ endothelial, mesenchymal, and CD45$^+$ cell populations in *Runx1*$^{lz/+}$ embryos. The Runx1$^+$ endothelial and mesenchymal cells are present in the absence of functional Runx1, but intraaortic clusters fail to form. Runx1 function is also required to maintain Runx1 expression in the hemogenic endothelium (North et al., 1999).

cell surface, causing CD45$^{lo}$ cells to fall into the CD45$^-$ gate. Alternatively, Runx1 dosage could affect the number of cells that express CD45. Since the size and number of intraaortic hematopoietic clusters is also reportedly smaller in *Runx1*$^{lz/+}$ embryos (Cai et al., 2000), we believe the latter explanation is more likely. This interpretation is consistent with our CD45 FACS profiles (Figure 2B) and also with a report by Mukouyama et al. (2000) that in vitro cultures of dispersed AGM regions from *Runx1*$^{+/-}$ embryos contained significantly fewer CD45$^+$ cells than those from *Runx1*$^{+/+}$ embryos. Our data do not address whether the CD45$^+$ HSCs in *Runx1*$^{lz/+}$ embryos are all located within hematopoietic clusters or whether some are CD45$^+$CD31$^-$ cells scattered throughout the paraaortic mesenchyme, since we did not directly test the activity of CD45$^+$CD31$^-$ cells. In contrast, in E11.5 *Runx1*$^{+/+}$ embryos, the majority of HSCs in the AGM region and V+U arteries are CD45$^+$ and also express the endothelial markers CD31 and VE-cadherin, suggesting that most HSCs are located within the hematopoietic clusters.

### Runx1$^+$ Endothelial Cells Are HSCs

It has long been suggested that endothelial cells and hematopoietic cells share a common precursor, the bipotential hemangioblast. In support of this concept, single "blast colony" cells isolated from embryonic stem cell cultures gave rise to both endothelial cells and primitive erythrocytes in vitro (Choi et al., 1998). The emerging view for definitive hematopoiesis is somewhat different. It has been proposed that definitive hematopoietic cells may develop from a cell that is incorporated into the endothelium of a blood vessel, as opposed to a bipotential hemangioblast precursor presumably of mesenchymal-like morphology (Jaffredo et al., 1998; Nishikawa et al., 1998). The data presented here demonstrate that *Runx1*$^{lz/+}$ embryos contain a population of HSCs that express cell surface VE-cadherin or CD31 but not CD45. VE-cadherin is a particularly good marker for endothelium, as it is concentrated at the adherens junctions of endothelial cells and is not found on mesenchymal cells or round cells in the embryonic circulation (Breier et al., 1996; Nishikawa et al., 1998). Indeed, we showed that most HSCs have downregulated cell surface VE-cadherin once they reach the fetal liver. We conclude, therefore, that some of the HSC activity in the AGM regions and V+U arteries of E11.5 *Runx1*$^{lz/+}$ embryos is derived from cells in the endothelium that do not express CD45 but do express Runx1.

Runx1 dosage affects the number of endothelial HSCs (defined as a CD31$^+$ or VE-Cad$^+$ cell that is CD45$^-$), as CD45$^-$ HSCs were found in E11.5 *Runx1*$^{+/+}$ embryos at a lower frequency than in *Runx1*$^{lz/+}$ embryos. In addition, *Runx1*$^{lz/+}$ embryos contain a population of mesenchymal HSCs that were not detected in E11.5 *Runx1*$^{+/+}$ embryos. One possible explanation for the presence of mesenchymal HSCs in *Runx1*$^{lz/+}$ embryos is that the expression of endothelial markers may be lower than in wild-type embryos, causing Runx1$^+$ endothelial cells to fall into the CD31$^-$ or VE-Cad$^-$. We cannot rule out this possibility. However, we favor a model that reduced Runx1 dosage affects the distribution of HSCs into the CD45$^+$, endothelial, and mesenchymal cell pools. We hypothesize that it may do so by delaying the transition of the hemogenic endothelium to CD45$^+$ hematopoietic clusters and possibly the differentiation of mesenchymal cells into hemogenic endothelium. We speculate that the Runx1$^+$ endothelial cells that might rapidly contribute to the formation of a CD45$^+$ hematopoietic cluster by E11.5 in a normal embryo may do so more slowly in a *Runx1*$^{lz/+}$ embryo, and as a result, these endothelial HSCs accumulate in greater numbers. Similarly, the impairment in intraaortic cluster formation seen in *Runx1*$^{lz/+}$ embryos (Cai et al., 2000) might delay the differentiation of Runx1$^+$ mesenchymal cells into endothelial cells and/or their incorporation into the endothelium of the aortic wall, resulting in a detectable pool of mesenchymal Runx1$^+$ HSCs at E11.5. We speculate that reduced Runx1 dosage might also delay the differentiation of an HSC into a hematopoietic progenitor cell. Such a delay would explain the results of Cai et al. (2000), who reported that HSC emergence in the yolk sac is precocious (E10.5) and that HSC activity is higher in the AGM region at E10.5 in *Runx1*$^{+/-}$ embryos than in wild-type embryos. We similarly detected more HSC activity in E10.5 *Runx1*$^{lz/+}$ embryos than was originally reported in +/+ embryos (Müller et al., 1994) and estimate, based on the frequency of repopulation, that at E11.5 HSCs are ≥2-fold more abundant in the AGM region and V+U arteries in *Runx1*$^{lz/+}$ versus +/+ embryos. The concomitant decrease in committed progenitors in *Runx1*$^{lz/+}$ embryos (Cai et al., 2000; Mukouyama et al., 2000; Wang et al., 1996a, 1996b) is consistent with a model that

reduced Runx1 dosage causes a delay in the HSC to progenitor cell transition.

## The Role of Runx1 in HSC Emergence

We previously reported that Runx1 is expressed in endothelial cells and mesenchymal cells before hematopoietic clusters are visible (North et al., 1999). In Runx1-deficient embryos, a population of Runx1$^+$ endothelial cells and mesenchymal cells appear, but intraaortic hematopoietic clusters fail to form, and endothelial cell expression of Runx1 is prematurely extinguished. We concluded that Runx1 is required for the formation of intraaortic clusters and may act as a molecular switch, specifying the conversion from an endothelial to hematopoietic cell fate. Here, however, we show that Runx1$^+$ endothelial cells and mesenchymal cells also have HSC activity. This indicates that a hematopoietic cell fate is already specified in a Runx1$^+$ mesenchymal or endothelial cell. These Runx1$^+$ mesenchymal and endothelial cells emerge in Runx1-deficient animals but are unable to progress to a CD45$^+$ hematopoietic cell (Figure 5). The manifestation of this failure in situ is the lack of cluster formation (North et al., 1999; Yokomizo et al., 2001) and the inability to provide long-term hematopoietic reconstitution upon transplantation (Cai et al., 2000).

## Implications for Identifying HSCs in Nonhematopoietic Tissues

Runx1 expression marks all HSCs in the embryonic hematopoietic tissues and in the adult mouse bone marrow (our unpublished data). One implication of our data is that all HSCs must express Runx1 at the time of transplantation in order for hematopoietic reconstitution to occur. A corollary hypothesis is that a Runx1$^-$ cell cannot be induced to express Runx1 and acquire HSC activity following transplantation. If both are true, then Runx1 may be a useful marker for locating HSCs in adult, nonhematopoietic tissues. Recent experiments showed that cells isolated from muscle or brain could contribute to the formation of blood (Bjornson et al., 1999; Jackson et al., 1999). We predict that the cells in nonhematopoietic tissues with HSC activity will be expressing Runx1 at the time of isolation, and that Runx1, in combination with c-kit, will be a useful marker for locating these cells in muscle, brain, and potentially other tissues.

## Experimental Procedures

### Embryo Generation

Embryos were generated from timed matings between genetically marked male mice and wild-type female mice. Male mice used in this study were either *Runx1$^{lz/+}$* (North et al., 1999), Ln72 (homozygous for a human β-globin transgene) (Strouboulis et al., 1992), or insulin receptor substrate 3-deficient (*Irs3$^{-/-}$*) (Liu et al., 1999). Both *Runx1$^{lz/+}$* and *Irs3$^{-/-}$* mice are of mixed 129S3/SvImJ and C57BL/6J backgrounds and were bred to129S3/SvImJ x C57BL/6J F1 females. Ln72 mice are maintained on a mixed C57BL/10 and CBA background and bred to C57BL/10 x CBA F1 female mice. Detection of the vaginal plug was designated as E0.5. Embryos were isolated from the uterus at E10.5, E11.5, or E12.5 into phosphate-buffered saline supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (PBS/FCS). *Runx1$^{+/+}$* and *Runx1$^{lz/+}$* embryos were identified at the time of dissection by staining severed heads with X-gal (Sigma) as described previously (North et al., 1999).

### Cell Preparations

AGM regions, vitelline, and umbilical arteries or fetal livers were dissected from embryos. AGM regions and V+U arteries were pooled. The tissues were disaggregated with collagenase, as described (de Bruijn et al., 2000, 2002). Tissues from *Runx1$^{+/+}$* and *Runx1$^{lz/+}$* embryos were pooled according to genotype.

### Fluorescent Antibody Staining and Flow Cytometry

Dispersed cells from *Runx1$^{lz/+}$* embryos were loaded with fluorescein di-β-D-galactopyranoside (FDG, Molecular Probes) by hypotonic shock and allowed to develop on ice, as described previously (North et al., 1999). FDG was detected in the FITC channel of a FACStarPlus (Becton-Dickinson). Background levels of fluorescence were determined from FDG-loaded *Runx1$^{+/+}$* cells.

All antibodies used in this study were from Pharmingen. Each antibody was either conjugated directly to PE (Phycoerythrin) or Cy (CyChrome), or purified and detected with a PE- or Cy-labeled anti-rat antibody. Background levels of fluorescence were determined by incubation with PE or Cy isotype control antibodies (Pharmingen). Antibodies used in the sorts include the following: c-kit (CD117; 2B8), CD31 (PECAM-1; MEC13.3), CD45 (30-F11), Flk-1 (VEGF-R2; Avas 12α1), VE-Cadherin (CD144; 11D4.1), and Sca-1 (Ly6A/E; D7 and E13-161.7). Antibody staining and sorting was performed both alone and in conjunction with FDG loading for Runx1 expression. Single-cell suspensions were incubated with the appropriate antibody or combinations of antibodies for 20 min on ice. Cells were washed twice and incubated with a secondary antibody when required. Labeled cells were resuspended in PBS/FCS and run through the FACS sorter (FACStarPlus or FACS Vantage SE, Benton-Dickinson) using Hank's Balanced Salt Solution (Gibco) in the sheath fluid, a 100 μm nozzle, at a pressure of 9 or 14 psi with a flow rate of 1000 cells/second. Dead cells were excluded by Hoechst 33258 staining (Molecular Probes). Cells were sorted into 100% FCS; purity ranged from 95%–99% for antibody sorts and 90%–95% for FDG sorts.

### Reconstitution Analysis

Two- to four-month-old 129S3/SvImJ x C57BL/6J F1 mice or C57BL/10 x CBA F1 mice were used as recipients for transplantation. Mice were sublethally irradiated with a split dose of 900 rads from a $^{60}$Co or $^{137}$Cs source. Mice were maintained on sterile water supplemented with antibiotics following irradiation. Sorted cells were injected into the tail vein of recipients along with $2 \times 10^5$ 129S3/SvImJ x C57BL/6J F1 or C57BL/10 x CBA F1 spleen cells to aid short-term survival. The number of cells in an embryo equivalent (ee) was determined by multiplying the number of cells per tissue, per embryo by the fraction of positive or negative cells for each marker. Cell counts per tissue (mean ± SD, $\times 10^4$) were 42.2 ± 2.0, 53.7 ± 3.9, and 270.2 ± 66.4 for *Runx1$^{lz/+}$* E10.5 AGM+VU, E11.5 AGM+VU, and E12.5 fetal liver, respectively, and 46.8 ± 9.5 and 250.1 ± 38.1 for *Runx1$^{+/+}$* E11.5 AGM+VU and E12.5 fetal liver, respectively. The relative sizes of the sorted subsets are presented in the tables. Blood was collected at 1 and ≥ 4 months posttransplantation. Donor cell contribution was determined by PCR of peripheral blood genomic DNA using primers specific for *lacZ* (395bp), 5′GCAGATGCACG GTTACGATGC3′ and 5′GTGGCAACATGGAAATCGCTG3′; human β-*globin* (428bp), 5′CTTCAGGTTCCCAGTGAGGATG3′ and 5′GCTC CCTAAGGGGTAAAGAGTG3′; or neomycin resistance gene (*neo*) sequences (880bp), 5′GCTCAGAAGCAAAGACACAAA3′ and 5′GCC TGCTTGCCGAATATCATG3′. PCR for *myogenin* sequences (245bp), 5′TTACGTCCATCGTGGACAGC3′ and 5′TGGGCTGGGTGTTAGTC TTA3′, was used to normalize DNA amounts between blood samples. The PCR products were visualized by ethidium bromide staining of agarose gels.

### Multilineage Analysis and Secondary Transplantation

The total contribution of *Runx1$^{lz/+}$* cells to repopulated bone marrow was calculated based on the percentage of Runx1$^+$ (FDG$^+$) cells in the bone marrow of transplant recipients compared to the percentage of Runx1$^+$ bone marrow cells in a *Runx1$^{lz/+}$* mouse. Bone marrow was also examined for multilineage engraftment using FDG in combination with each of the following PE-conjugated lineage markers (Pharmingen): T cells, Thy-1.2 (53-2.1); B-cells, CD45R/B220 (RA3-

6B2); myeloid cells, CD11b (Mac-1; M1/70); granulocytes, Gr-1 (Ly-6G; RB6-8C5); stem and progenitor cells, Sca-1 (Ly6A/E; D7). Incubations and washes were performed as described for embryonic cells.

### Immunohistology

E11.5 embryos were harvested and genotyped as described. The caudal half of each embryo, including the vitelline and umbilical arteries, was either prepared for paraffin sectioning, as described previously (North et al., 1999) or immersed in O.C.T. compound (Tissue Tek) and flash frozen on dry ice with 95% ethanol. Specimen blocks were serially sectioned at 8 μm. Frozen sections were placed on Superfrost slides (Fisher) and stored at −80°C. Immunohistochemistry with antibodies utilized in the sorts was performed on appropriate sections using the Vector Elite ABC kit (Vector labs). Following antibody incubation and detection with DAB, the slides were stained overnight at 37°C with X-gal and in some cases counterstained with methyl-green (Vector labs).

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