

# Restoration of Runx1 expression in the Tie2 cell compartment rescues definitive hematopoietic stem cells and extends life of Runx1 knockout animals until birth



STEM CELLS®

TISSUE-SPECIFIC STEM CELLS

# Restoration of Runx1 Expression in the Tie2 Cell Compartment Rescues Definitive Hematopoietic Stem Cells and Extends Life of Runx1 Knockout Animals Until Birth

ANNA LIAKHOVITSKAIA,[a] RUBY GRIBI,[a] EVANGELOS STAMATERIS,[a] GAELLE VILLAIN,[b] THIERRY JAFFREDO,[b] RON WILKIE,[a] DEREK GILCHRIST,[a] JIAN YANG,[a] JANICE URE,[a] ALEXANDER MEDVINSKY[a]

[a]Ontogeny of Haematopoietic Stem Cells Group, Medical Research Council Centre for Regenerative Medicine, Institute for Stem Cell Research, University of Edinburgh, Edinburgh, United Kingdom; [b]Université Pierre et Marie Curie-Paris 6, Centre National de la Recherche Scientifique UMR7622, Laboratoire de Biologie du Développement, Paris, France

**Key Words.** HSC • Embryo • AGM region • Yolk sac • Runx1

ABSTRACT

**Mice deficient in the *runt* homology domain transcription factor Runx1/AML1 fail to generate functional clonogenic hematopoietic cells and die in utero by embryonic day 12.5. We previously generated Runx1 reversible knockout mice, in which the Runx1 locus can be restored by Cre-mediated recombination. We show here that selective restoration of the *Runx1* locus in the Tie2 cell compartment rescues clonogenic hematopoietic progenitors in early Runx1-null embryos and rescues lymphoid and myeloid lineages during fetal development. Furthermore, fetal liver cells isolated from reactivated Runx1 embryos are capable of long-term multilineage lymphomyeloid reconstitution of adult irradiated recipients, demonstrating the rescue of definitive hematopoietic stem cells. However, this rescue of the definitive hematopoietic hierarchy is not sufficient to rescue the viability of animals beyond birth, pointing to an essential role for Runx1 in other vital developmental processes.** STEM CELLS *2009;27:1616–1624*

Disclosure of potential conflicts of interest is found at the end of this article.

## INTRODUCTION

Runx1/AML1 belongs to a family of transcription factors playing important roles in various developmental processes. Runx1 is one of the most frequent targets for leukemic translocations [1–4] and plays an essential role in development of hematopoietic clonogenic activity in the mammalian embryo [5–7]. Genetic ablation of Runx1 affects early yolk sac erythropoiesis [8] and exerts severe effects on fetal liver hematopoiesis, resulting in anemia and embryonic lethality by embryonic day (E) 11.5–12.5 [5–7]. Runx1 knockout (KO) embryos contain no clonogenic hematopoietic progenitors, and Runx1$^{-/-}$ embryonic stem cells cannot contribute to adult hematopoiesis. Induced ablation of Runx1 in the adult hematopoietic system suppresses megakaryocytopoiesis and perturbs T- and B-cell differentiation, but does not deplete hematopoietic stem cells (HSCs) [9]. Runx1 deficiency in adult bone marrow results in abnormal myeloproliferation and a predisposition to lymphoma development [10–12].

During embryonic development several tissues are sequentially involved in hematopoietic activity. Soon after gastrulation, embryonic erythroid and myeloid progenitors emerge in the yolk sac (YS) [13]. The adult hematopoietic hierarchy evolves gradually from definitive HSCs localized to the aorta-gonad-mesonephros (AGM) region, umbilical cord, placenta, and YS by E10.5–E11.5 [14–20].

Here, using reversible knockout Runx1 mice [21], we show that selective restoration of Runx1 activity in the embryonic Tie2 compartment is sufficient to rescue the full spectrum of committed colony-forming progenitors (CFU-C) in the YS, as well as long-term repopulating HSCs. Once the integrity of the Runx1 locus is restored, normal expression of the endogenous Runx1 proteins is maintained and requires no further external induction [21]. Rescue of the definitive hematopoietic system prolongs the life of animals but only until birth, indicating the involvement of Runx1 in other vitally important developmental processes. Here we report that Runx1 plays a critical role in the Tie2$^{+}$ embryonic compartment and/or its downstream derivatives for the development of definitive HSCs.

Author contributions: A.L.: collection and/or assembly of data, data analysis and interpretation; manuscript writing; final approval of manuscript; R.G.: collection and assembly of data, data analysis and interpretation; E.S., R.W., D.G., and J.Y.: collection of data; G.V.: collection and assembly of data; T.J.: data analysis and interpretation; J.U.: generation of mice, support of transplantation experiments; A.M.: conception and design; financial support; data analysis and interpretation; manuscript writing; final approval of manuscript. A.L. and R.G. contributed equally to this work.

Correspondence: Alexander Medvinsky, Ph.D., Ontogeny of Hematopoietic Stem Cells Laboratory, Institute for Stem Cell Research, University of Edinburgh, Edinburgh EH9 3JQ, Scotland, U.K. Telephone: +44 (0)131 650 5888; Fax: +44 (0)131 650 7773; e-mail: a.medvinsky@ed.ac.uk Received January 12, 2009; accepted for publication March 15, 2009; first published online in STEM CELLS *EXPRESS* March 26, 2009.  1066-5099/2009/$30.00/0 doi: 10.1002/stem.71

## Materials and Methods

### Animals

Mice were housed and bred in animal facilities at the University of Edinburgh. Animals were kept in compliance with Home Office regulations. All transgenic mice used in these experiments were backcrossed for a minimum of 6-7 generations to the C57Bl6 background. Reactivatable Runx1 KO and silent green fluorescent protein (GFP) reporter mice have previously been described [21, 22]. Tie2-Cre deletor mice were kindly provided by M. Yanagisawa [23]. To obtain [Tie2-Cre: $Runx1^{LacZ/LacZ}$] triple-transgenic animals [Tie2-Cre: $Runx1^{LacZ/wt}$] males were crossed with $Runx1^{LacZ/wt}$ females. The day of discovery of the vaginal plug was designated as day 0.5.

### Southern Blot Analysis

To assess the efficiency of recombination and identify embryos of interest, Southern blot analysis was performed using both peripheral blood and tail samples. Genomic DNA was isolated from blood and tails of the embryos, digested with the NheI enzyme, and hybridized with the A-probe as described previously [21]. For detection of the Tie2-Cre transgene, a Cre DNA probe was used.

### Analysis of LacZ Expression and Immunohistochemistry

The X-gal staining in embryos was performed as previously described [24]. Tie2 and Cre recombinase proteins were recognized using a polyclonal anti-Tie2 antibody and a monoclonal anti-Cre antibody. Tie2 was revealed with a goat anti-rabbit antibody coupled to horseradish peroxidase (HRP) followed by a tyramide signal amplification (TSA; PerkinElmer Life and Analytical Sciences, Boston, http://www.perkinelmer.com) using cyanin 3 as a fluorescent probe. Cre was revealed with a goat anti-mouse IgG1 coupled to biotin followed by a streptavidin-HRP, a TSA amplication using cyanin 2 as a fluorescent probe. When needed, sections were counterstained with 4′,6-diamidino-2-phenylindole. Fluorescent sections were photographed with a Nikon Eclipse E800 microscope equipped with the structured Light imaging system Optigrid (Optem, Calgary, AB, Canada, http://www.optem.com). Images were acquired and merged with the Image Pro Plus software (Media Cybernetics, Crofton, MD, http://www.mediacy.com).

### Clonogenic Methylcellulose Assay

Cells from embryonic tissues were obtained after enzymatic digestion with collagenase-dispase as described previously [20] and cultured in the methylcellulose medium (M3434; Stem Cell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) according to the manufacturer's instructions. Hematopoietic colonies were scored after 8 days of culture in duplicates.

Reconstruction of the Runx1 locus in methylcellulose colonies was analyzed by polymerase chain reaction (PCR) using two sets of primers in two separate reactions for each individual colony: reaction a, for detections of $Runx1^{wt}$ and $Runx1^{Re}$; reaction b, for detection of $Runx1^{lacZ}$. Individual colonies were picked from the methylcellulose, washed in phosphate-buffered saline, boiled, and subjected to PCR reactions. The primers used were as follows: for reaction a, 5′-GGCTTCTAGGCTTTGGATGTT-3′ and 5′-TTCCCACAGCTGAGGACTCT-3′ (gives 455-bp product for wild type and 515-bp product for reactivated Runx1 allele); for reaction b, 5′-GGTTTCCATATGGGGATTGG-3′ and 5′-TTCCCACAGCTGAGGACTCT-3′ (gives 1000-bp product for the targeted Runx1/LacZ allele).

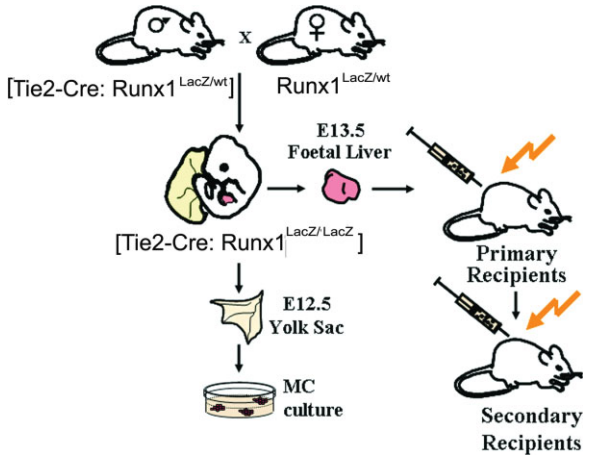


**Figure 1.** Experimental design. Crossing of [Tie2-Cre: $Runx1^{LacZ/wt}$] males with $Runx1^{LacZ/wt}$ females produced embryos of six different genotypes of which 1 of 8 is [Tie2-Cre: $Runx1^{LacZ/LacZ}$]. E12.5 yolk sacs were tested in a methylcellulose assay for the presence of hematopoietic colony-forming cells. E13.5 fetal livers from individual embryos were assayed for the presence of definitive hematopoietic stem cells using a long-term repopulating assay and subsequently by transplantation into secondary recipients. Abbreviations: E, embryonic day; MC, methylcellulose.

### Long-Term Repopulation Assay

To detect definitive HSCs, fetal livers were isolated from E13.5 embryos and individually transplanted into irradiated Ly5.1/1 adult recipients ($\geq$ 6 weeks of age) as described previously [20]. Donor cell contribution into the recipient blood was assessed after 6 weeks and/or longer using flow cytometry analysis upon staining with anti-Ly5.1 and -Ly5.2 monoclonal antibodies conjugated with phycoerythrin and fluorescein isothiocyanate, respectively (eBioscience, San Diego, http://www.ebioscience.com). Donor-derived lymphoid and myeloid fractions were assessed by flow cytometry in the recipient peripheral blood, bone marrow, spleen, and thymus as described previously [25]. To this end, monoclonal CD3e (PE conjugated), CD220 (biotinylated, detection by streptavidin-APC); CD8a (PE conjugated), CD4a (biotinylated, detection by streptavidin-APC), CD11b (PE conjugated), and GR1 (biotinylated, detection by streptavidin-APC) antibodies were used (BD Biosciences, San Diego, http://www.bdbiosciences.com). Appropriate isotype controls were used. Dead cells were excluded using 7-amino-actinomycin D.

## Results

### Experimental Design

Reversible Runx1 knockout mice have been described previously [21]. Briefly, the Runx1 locus in these mice is disrupted by a stop cassette containing LacZ reporter flanked by LoxP sites. $Runx1^{LacZ/LacZ}$ phenotype is embryonic lethal by E12.5. If Cre-recombinase is expressed in the $Runx1^{LacZ/LacZ}$ fertilized eggs, the Runx1 locus is restored and becomes fully functional; these mice survive and develop normally. Apart from hematopoietic cells, Runx1 is expressed in some endothelial and mesenchymal cells, motoneurons, cartilage, bone, and some other cell types [26–29]. Crossing lineage-specific Cre deletor mice with reversible knockout Runx1 mice enables lineage-specific rescue of Runx1 expression. Here we used Tie2-Cre deletor mice to test if rescue of the Runx1 locus in the Tie2 expressing cell compartment is sufficient to

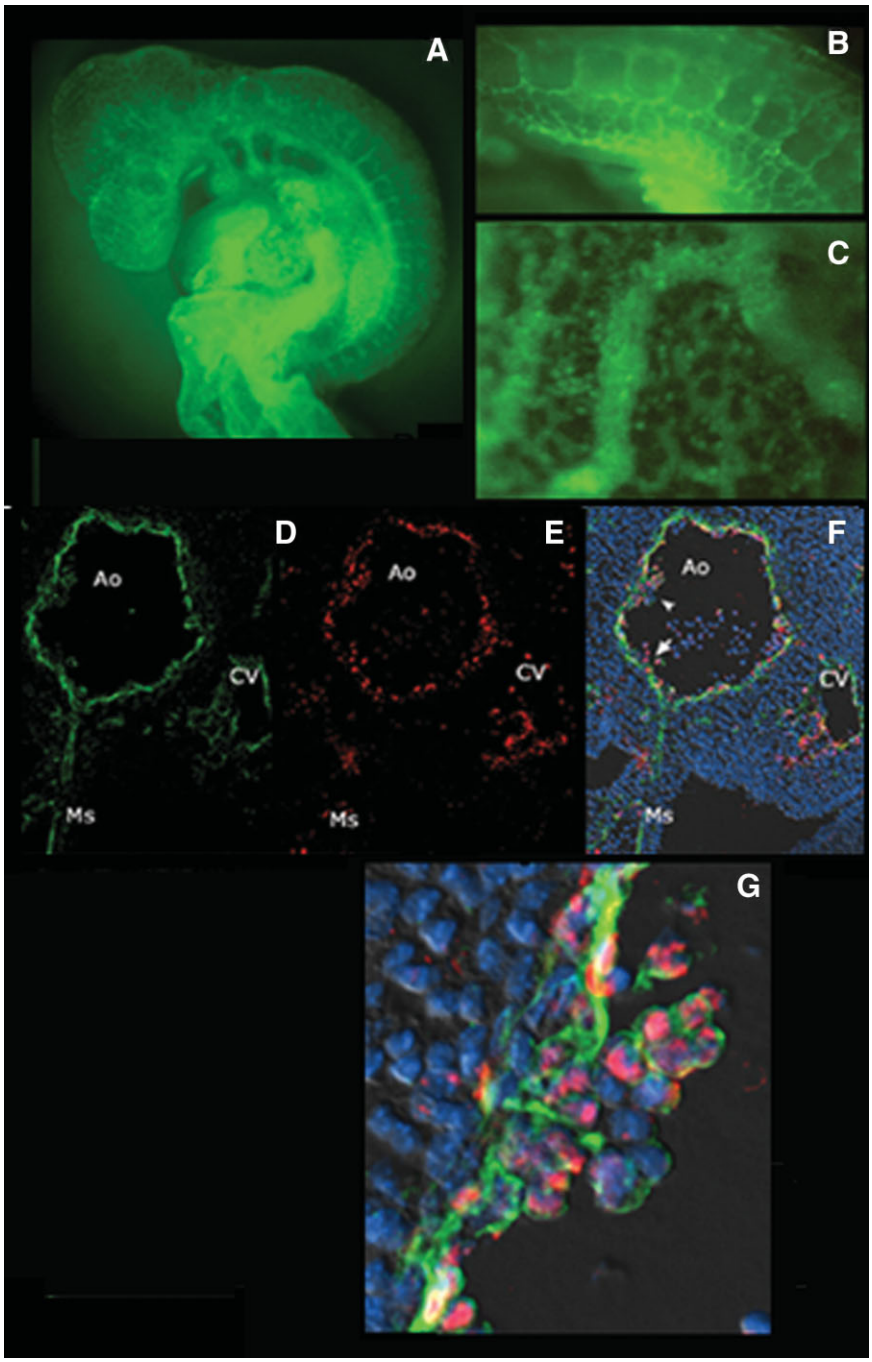


**Figure 2.** Cre-mediated recombination in [Tie2-Cre: sGFP] embryos. **(A):** Activation of green fluorescent protein in the vasculature network of the E9.5 embryo. **(B, C):** Magnified images of intersomitic vascular network and yolk sac vascular network. **(D–F):** Transverse sections of E10.5 embryo through the aorta-gonad-mesonephros region. **(D):** Anti-Tie2 antibody staining (green); **(E)** anti-Cre-recombinase antibody staining (red); **(F)** merged **(D)** and **(E)** and 4′,6-diamidino-2-phenylindole staining (blue, not shown) images. Note lateral (arrowhead) and ventral (arrow) intra-aortic clusters. **(G):** Magnified image of the lateral intra-aortic cluster shown in **(F)** by arrowhead. Abbreviations: Ao: dorsal aorta; CV: cardinal veins; Ms: mesenterium.

rescue HSCs. Tie2 receptor tyrosine kinase marks vascular endothelium [30] and some hematopoietic cells, including HSCs [31, 32]. By a two-step breeding, litters were produced that contained compound transgenic [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryos (about 1/8 of all littermates). The rescue of clonogenic hematopoietic progenitors was tested by an in vitro methylcellulose assay and rescue of definitive HSCs was tested by an in vivo long-term repopulation assay (Fig. 1).

## Cre-Mediated Recombination in [Tie2-Cre: sGFP] Embryos

To assess the reactivation pattern of Runx1 in [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryos, control crossing of Tie2-Cre males with silent reporter sGFP females [22] was set up. Tie2-Cre mice have been previously characterized [23] and used in a number of publications [33–41]. As expected, GFP expression in transgenic [Tie2-Cre: sGFP] embryos highlighted the developing vasculature (Fig. 2A–2C). The endothelial lining of the dorsal aorta coexpressed Tie2 and Cre-recombinase (Fig. 2D–2G). Some double-positive circulating cells were also observed (not shown). On rare occasions we observed single positive cells, which could be a reflection of differing rates of maturation and/or stability of Cre and Tie2 proteins (not shown).

Tie2 was coexpressed with Flk1 and VE-cadherin, traditionally used as endothelial markers, as indicated by the dynamics of development of the Flk1$^{+}$Tie2$^{+}$ and VE-cad$^{+}$Tie2$^{+}$ populations during E8.5–E10.5 (supporting information Fig.

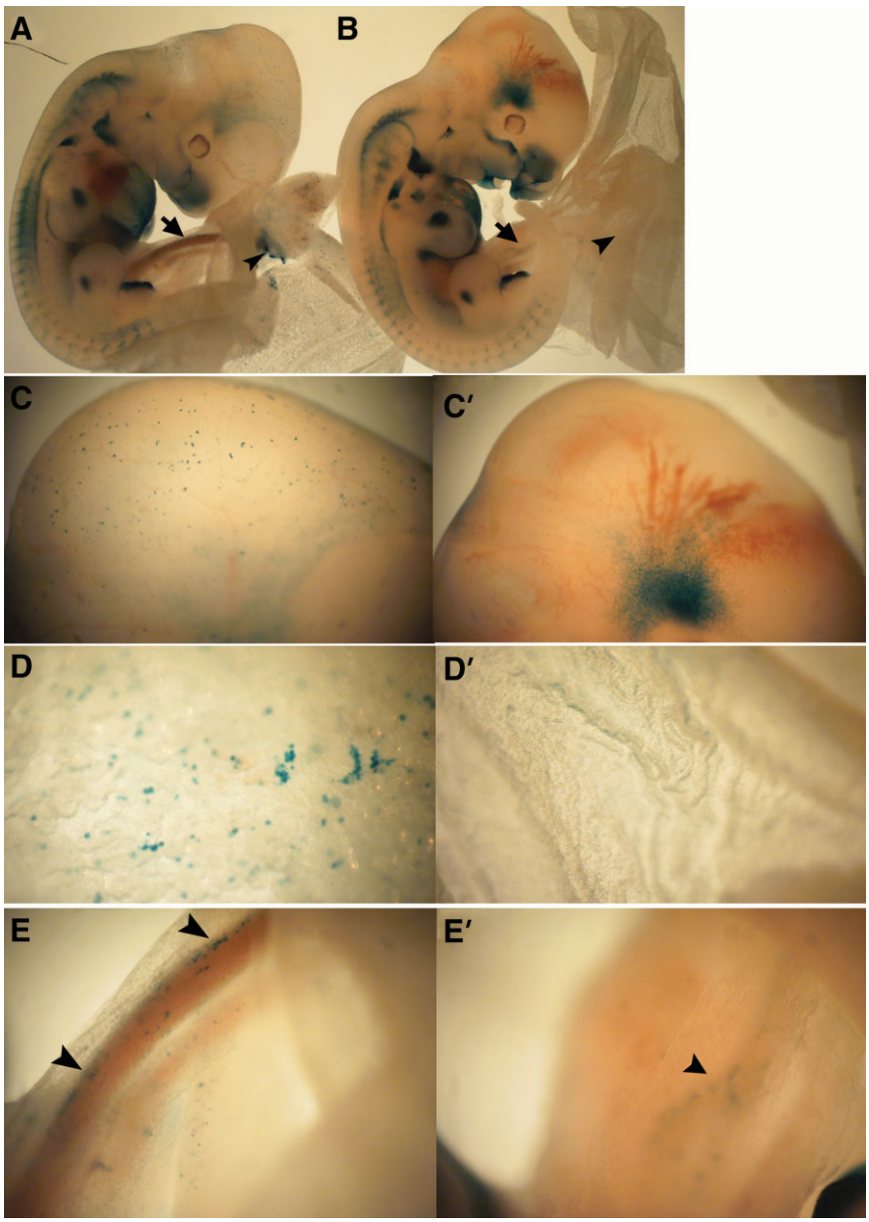


**Figure 3.** Recombination in E11.5 [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryos. **(A, C, D, E):** Heterozygous Runx1$^{LacZ/wt}$ embryo; **(B, C′, D′, E′)** [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryo; **(A, B)** general view (arrows: umbilical cords; arrowheads: placentas); **(C, C′)** head; **(D, D′)** yolk sac; **(E, E′)** cord. LacZ expression marks a number of nonhematopoietic sites (such as motoneurons, somites, limbs) in all embryos. Note that the LacZ expression in hematopoietic sites of [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryos is either fully absent (placenta) or significantly attenuated (umbilical cord). Clusters inside the cord of the Runx1$^{LacZ/wt}$ embryo (**E**, arrowhead) are readily observed; compare with weak LacZ staining in [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryos (**E′**, arrowhead). (This observation is supported by histological analysis shown in Fig. 4.) Note that LacZ$^{+}$-stained hematopoietic cells, typical for heterozygous Runx1$^{LacZ/wt}$ embryos, are not seen in the head or the yolk sac of the [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryo (compare **D** with **D′** and **C** with **C′**, respectively). Note slight hemorrhage in the head of the [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryo.

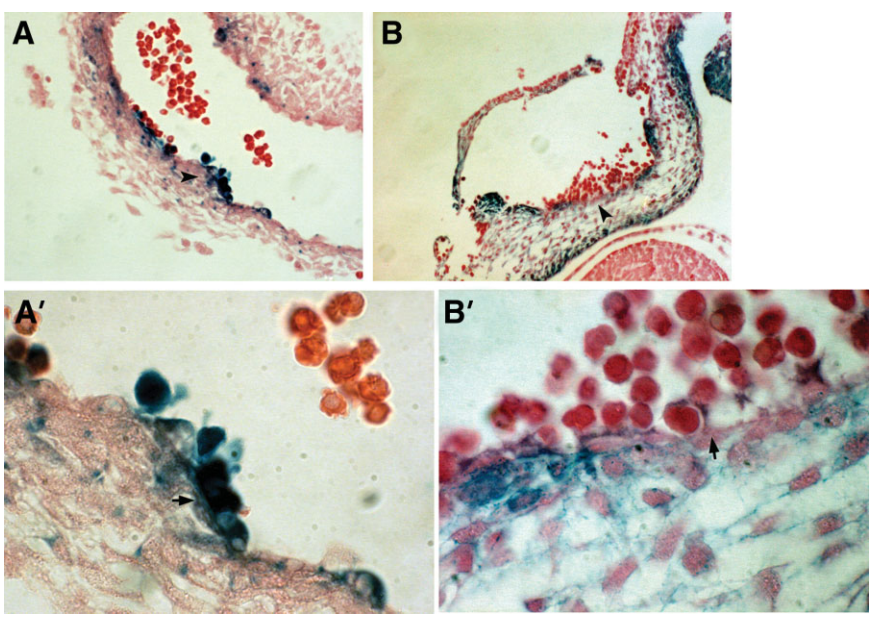


**Figure 4.** Rescue of intravascular hematopoietic cell clusters in the umbilical cord of E11.5 [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryos. **(A, A′):** Runx1$^{LacZ/wt}$ embryos: typical cell clusters adjacent to the endothelial lining of the umbilical artery contain Runx1/LacZ-positive cells (**A**, arrowhead). Note that some endothelial cells underlying these clusters are LacZ$^{+}$ (**A′**, arrow). In addition, many cells in the surrounding mesenchyme are LacZ$^{+}$. **(B, B′):** [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryos: rescue of intravascular cell clusters due to reconstitution of targeted Runx1 alleles as a result of Cre-mediated recombination. Hematopoietic cluster (arrowhead) is LacZ-negative due to loss of the LacZ stop cassette. Endothelial cells underlying the cluster are also LacZ-negative, whereas surrounding mesenchyme remains LacZ$^{+}$ in line with weak staining shown in Fig. 3E′.

1A). However, the Tie2$^+$ population also included Flk1- and VE-cadherin-negative subsets. Thus, Tie2 labels both mesoderm and the endothelial compartment, both implicated in development of primitive and adult-type hematopoietic cells [42–47]. This explains why GFP labeling occurs in all three hematopoietic cell populations (Ter119, CD45, and CD41) despite the fact that only the CD41$^+$ population expresses Tie2 in the early embryo (supporting information Fig. 1B). The CD45 fraction becomes GFP$^+$ with delay compared with the CD41$^+$ and Ter119$^+$ fractions. This is in line with the maternal origin of the earliest CD45$^+$ population [48] and the notion that embryo-derived CD45$^+$ population develops later from embryonic CD41$^+$ progenitors [49, 50]. Embryonic definitive HSCs are also Tie2$^+$ [30–32], which results in complete labeling of the hematopoietic system in the adult (supporting information Fig. 1C).

## Selective Reactivation of Runx1 in the Tie2 Compartment of [Tie2-Cre: Runx1$^{LacZ/LacZ}$] Embryos: Phenotypic Analysis

As described previously, LacZ expression is observed in various tissues of Runx1$^{LacZ/wt}$ heterozygous embryos, such as motoneurons, limbs, olfactory placodes, somitic regions, and branchial arches (Fig. 3A). The AGM region, YS, umbilical cord, and placenta are implicated in the development of definitive HSCs and contain Runx1-expressing cells. Intra-aortic cell clusters are associated with the development of clonogenic progenitors and definitive HSCs [7, 51–53]. Similar clusters can be readily observed on whole mount preparations of, and sections through, the umbilical cord (Figs. 3E and 4A, 4A′). Inside the umbilical cord, large clusters are often localized to the anterior domain of the umbilical cord artery, topographically corresponding to the ventral domain of the dorsal aorta (Fig. 3E). Knockout Runx1$^{LacZ/LacZ}$ embryos do not form intra-aortic clusters, although the embryonic vasculature contains yolk sac-derived erythroid cells (supporting information Fig. 2 and [5]). In [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryos, motoneurons and olfactory placodes remain LacZ$^+$, indicating that Cre-mediated recombination does not extend to tissues unrelated to the Tie2 lineage (Fig. 3B). In contrast to Runx1$^{LacZ/LacZ}$ embryos, [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryos contained rescued clusters that were LacZ-negative due to excision of the LacZ reporter (Figs. 3E, 3E′ and 4B, 4B′). Although rescued clusters were observed in umbilical cord vessels, it was difficult to detect them with certainty in the dorsal aorta, presumably due to their small size. One explanation for this could be that the subendothelial mesenchyme within the AGM was not rescued.

## Reconstitution of Hematopoiesis in [Tie2-Cre: Runx1$^{LacZ/LacZ}$] Embryos: Functional Analysis

Genotypes of individual embryos were determined by Southern blot using DNA prepared from tails and peripheral blood (supporting information online Fig. 3). [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryos showed almost entire recombination in blood, and had very little recombination in tail specimens (~5%), consistent with minor endothelial/hematopoietic-specific recombination caused by the Tie2-Cre. In contrast to Runx1$^{LacZ/LacZ}$ embryos, which die by E12.5 of severe hematopoietic deficiency, [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryos survive, but only until birth. E16.5–E18.5 litters cumulatively contained 11.6% of rescued [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryos (supporting information Table 1). Extensive hemorrhaging was observed in the neural system of Runx1$^{LacZ/LacZ}$ embryos by E11.5–E12.5, which may also be a contributing factor to the early lethality [5, 6, 21]. This is either abolished or significantly attenuated in [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryos (Fig. 3C′). The number of CFU-C and morphological types of hematopoietic colonies formed by E12.5 yolk sacs of [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryos was similar to those in Runx1$^{LacZ/wt}$ embryos (Fig. 5A). The restoration of the Runx1 locus in hematopoietic colonies was confirmed by PCR analysis (Fig. 5B).

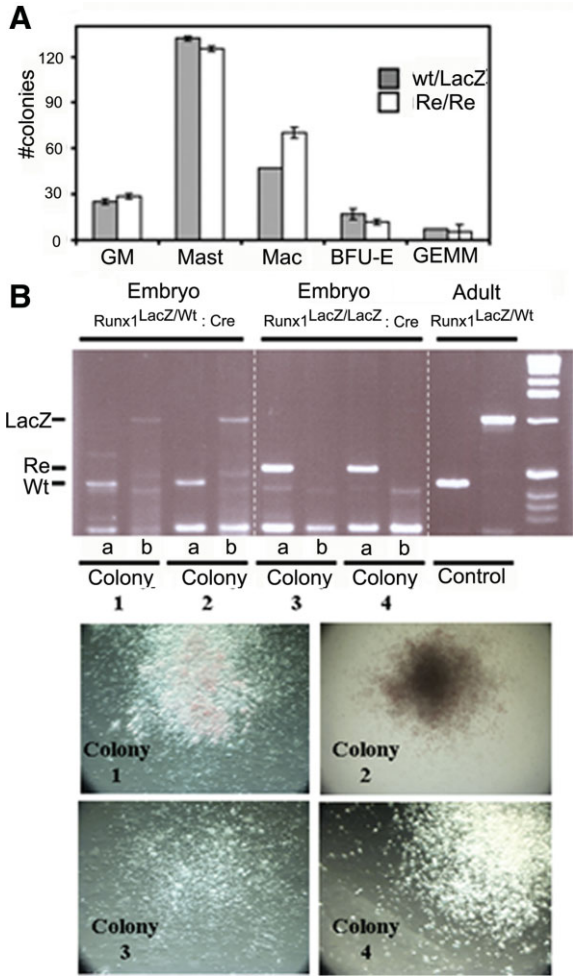


**Figure 5.** Rescue of hematopoiesis in [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryos. **(A):** The number of clonogenic myeloid progenitors in E12.5 yolk sac was assessed in comparison with Runx1$^{LacZ/wt}$ embryos using a standard methylcellulose assay. Three embryos of each genotype were used. (Total number of colonies per one yolk sac are shown.)([Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryo is designated Re/Re.) **(B):** Restoration of the Runx1 locus was analyzed in individual methylcellulose colonies by polymerase chain reaction using two separate reactions (a and b) with two sets of primers (see "Materials and Methods"). Four representative colonies are shown. Abbreviations: BFU-E, blast-forming unit-erythroid; GEMM, granulocyte, erythrocyte, monocyte, macrophage; GM, granulocyte-macrophage; Mac, macrophage; Mast, mast cell.

By E18.5, the cellularity of the fetal liver in [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryos was decreased on average by 25% (Fig. 6A). The average cellularity of fetal spleens was only $0.5 \times 10^6$ compared with $1.4 \times 10^6$ in wild-type embryos, which is approximately 30% of the wild-type spleen size. The cellularity of E18.5 thymi was similar to their wild-type counterparts (Fig. 6A), and all major myeloid and lymphoid cell populations in the peripheral blood were present in proportions similar to those found in wild-type embryos (Fig. 6B–6E). The decreased cellularity found in the fetal liver and

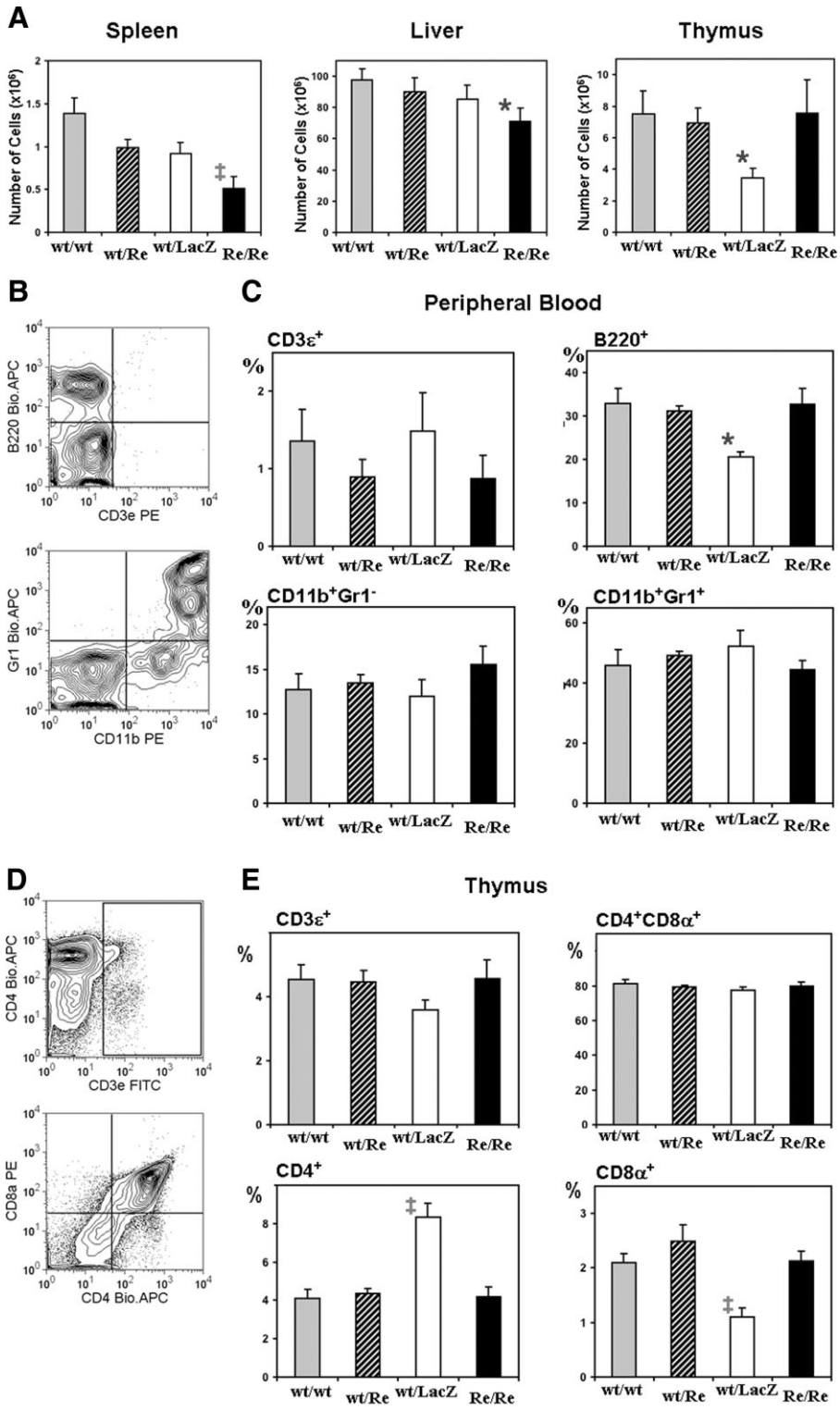


**Figure 6.** Hematopoietic characteristics of E18.5 [Tie2-Cre: $Runx1^{LacZ/LacZ}$] embryos. **(A):** Cellularity of spleen, liver, and thymus. In [Tie2-Cre: $Runx1^{LacZ/LacZ}$] embryos, we observed a slight but statistically significant reduction in liver cellularity; the spleen cellularity is reduced by 2/3; thymus cellularity is similar to wild-type embryos. **(B, C):** Lymphoid and myeloid populations in the peripheral blood. **(D, E):** Lymphoid populations in the thymus. Note that whereas [Tie2-Cre: $Runx1^{LacZ/LacZ}$] embryos (denoted as Re/Re) show normal distribution of myeloid and lymphoid populations, heterozygous $Runx1^{LacZ/wt}$ embryos show significant reduction in the number of $B220^+$ cells in the peripheral blood and increased ratio between $CD4^+$ and $CD8^+$ populations in the thymus. Abbreviations: APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; Re/Re, [Tie2-Cre: $Runx1^{LacZ/LacZ}$] embryos; wt/LacZ, $Runx1^{LacZ/wt}$ embryos; wt/Re, [Tie2-Cre: $Runx1^{LacZ/wt}$] embryos; wt/wt, $Runx1^{wt/wt}$ embryos. (*$p < .05$; ± $p < .01$)

spleen of E18.5 [Tie2-Cre: $Runx1^{LacZ/LacZ}$] embryos may be a result of the Runx1 deficiency of stromal components that are not rescued in these tissues.

Interestingly, thymic cellularity in heterozygous $Runx1^{LacZ/wt}$ embryos was significantly reduced and contained enhanced numbers of CD4 single-positive (SP) and decreased number of CD8 SP thymocytes (Fig. 6E). A previous publication reported normal size of thymi and partial suppression of both SP populations concurrent with reduced CD4 SP and enhanced CD8 SP populations in the periphery of $Runx1^{+/-}$ adult animals [54].

## Definitive HSCs Are Rescued in the [Tie2-Cre: $Runx1^{LacZ/LacZ}$] Embryo

To determine whether the development of HSCs was rescued in [Tie2-Cre: $Runx1^{LacZ/LacZ}$] embryos, fetal livers were

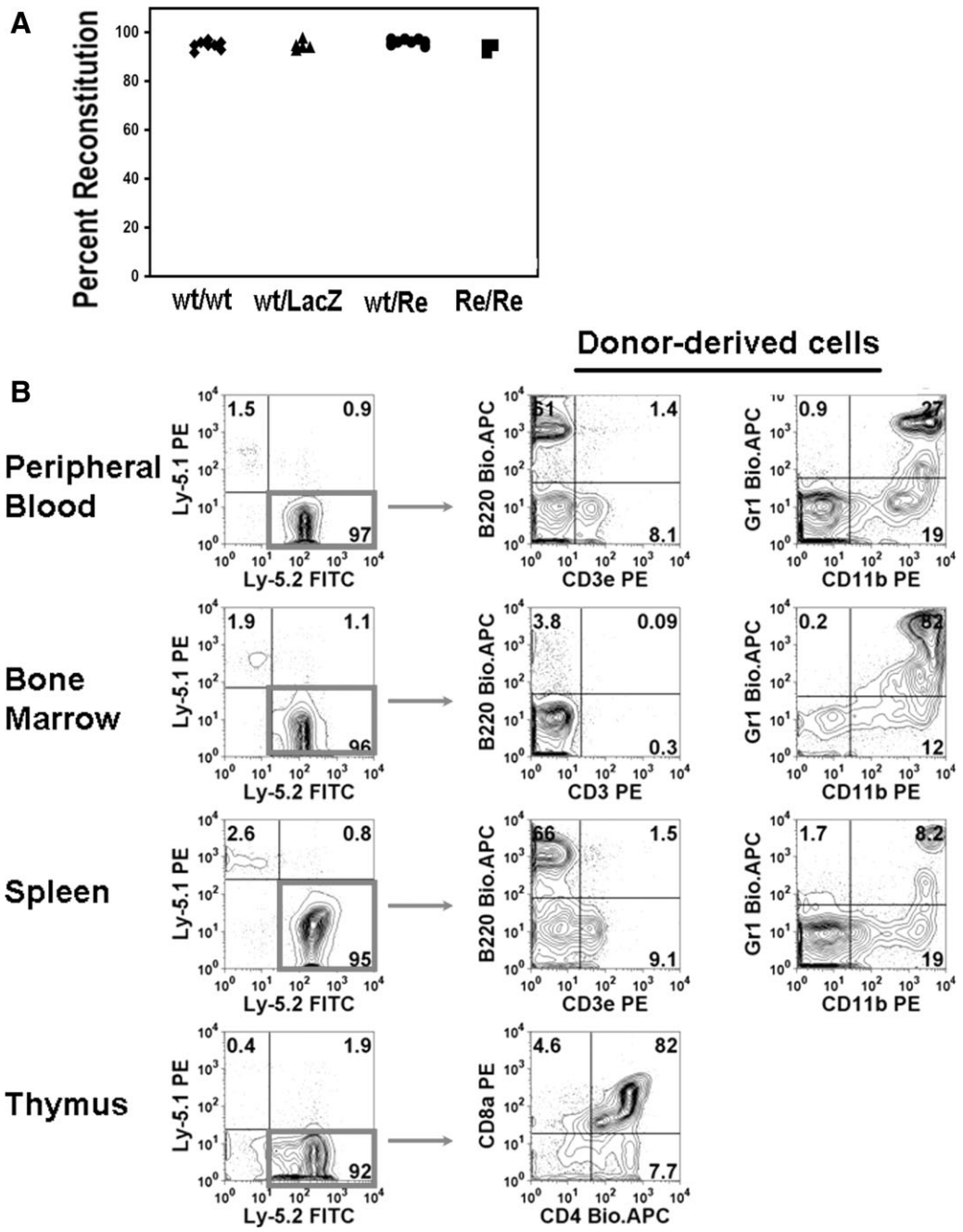


**Figure 7.** Rescue of definitive hematopoietic stem cells in [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryos. **(A):** E13.5 fetal livers were individually transplanted into irradiated adult Ly5.1/1 recipients, and their contribution in adult hematopoiesis was assessed using fluorescence-activated cell sorting analysis. Each symbol represents an individual recipient. **(B):** Primary recipients of wild-type and [Tie2-Cre: Runx1$^{LacZ/LacZ}$] E13.5 livers were analyzed for donor (Ly5.2/2) myeloid and lymphoid contribution into peripheral blood and bone marrow. Embryo genotypes are designated as indicated in legend for Figure 6. Abbreviations: APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; Re/Re, [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryos; wt/LacZ, Runx1$^{LacZ/wt}$ embryos; wt/Re, [Tie2-Cre: Runx1$^{LacZ/wt}$] embryos; wt/wt, Runx1$^{wt/wt}$ embryos.

isolated from E13.5 embryos and transplanted into irradiated mice (Fig. 7A). Livers from [Tie2-Cre: Runx1$^{LacZ/LacZ}$] embryos successfully repopulated irradiated adult recipients for a period of more than 200 days. Further comparison with recipients of wild-type E13.5 fetal livers showed an unbiased multilineage contribution of rescued [Tie2-Cre: Runx1$^{LacZ/LacZ}$] HSCs into the hematopoietic system (Fig. 7B). Secondary transplantations of bone marrow cells from mice reconstituted with rescued HSCs have also been successful (supporting information Fig. 4).

## Discussion

The structural organization of the developing hematopoietic hierarchy is complex. It remains unclear whether the first

generation of clonogenic myeloid progenitors emerges independently of the generation of definitive HSCs that appear later [55, 56]. Given the hierarchical complexity of the developing hematopoietic system, it is important to establish in which cell population the Runx1 transcription factor plays a critical role in the development of the hematopoietic system.

Here we investigated the effects of selective Runx1 rescue in the Tie2 embryonic compartment. Previously, selective genetic ablation of Runx1 in the embryonic $Tie2^+$ cell compartment resulted in hematopoietic deficiency similar to that observed in E12.5 Runx1 knockout embryos [57]. Due to early embryonic death and unknown proliferative response of early embryonic HSCs in a standard methylcellulose assay, it remains unclear whether development of the first definitive HSCs was abolished. Here, we functionally tested if Runx1 expression in the $Tie2^+$ cell compartment is sufficient to rescue clonogenic hematopoietic progenitors and/or definitive HSCs. To this end, [Tie2-Cre: $Runx1^{LacZ/LacZ}$] transgenic embryos were generated in which Cre-recombinase caused restoration of the Runx1 locus selectively in the $Tie2^+$ cell compartment.

We have found that yolk sac myeloid clonogenic progenitors in E10.5 [Tie2-Cre: $Runx1^{LacZ/LacZ}$] embryos are fully rescued, as they are generated in normal numbers and produce a normal morphological variety of methylcellulose colonies. The hemorrhage phenotype of Runx1-null embryos almost fully disappears, suggesting that it is caused by hematopoietic/vascular abnormalities rather than neurological defects. Normal CFU-C development was rescued in early embryonic life, and lymphoid and myeloid populations were proportionally represented in the late fetus, although the cellularity of fetal hematopoietic organs (liver and the spleen) was decreased. These animals survive until birth (whereas $Runx1^{LacZ/LacZ}$ embryos die by E12.5), and we tested whether this early death is caused by lack of HSCs. E13.5 fetal livers from rescued animals were transplanted into adult irradiated recipients. The recipients were successfully engrafted over a 200-day period, and the pattern of donor-derived myeloid and lymphoid repopulation was phenotypically indistinguishable from wild-type transplants.

Runx1 plays an important role in development of selected neural cell types [25, 58, 59]. As Tie2-Cre-mediated recombination is selective and does not restore expression of Runx1 in the neural system, neural development failure could be the main cause of lethality of [Tie2-Cre: $Runx1^{LacZ/LacZ}$] embryos at birth [59]. In addition, Runx1 deficiency causes abnormal development of the sternum that may affect the rigidity of the rib cage and contribute to the lethal phenotype (A. Liakhovitskaia et al., manuscript submitted for publication). Interestingly, previous rescue of the Cbf$\beta$ (common subunit for Runx1, 2, and 3 transcription complexes)-null embryos, which normally die between E12.5 and E13.5, using a Tie2-Cbf$\beta$ transgene also resulted in death at birth despite rescue of the fetal liver hematopoiesis [60].

Tie2 expression in the early embryo labels the endothelial, mesodermal, and $CD41^+$ populations. Tie2 is also expressed in developing HSCs [30–32]. However, in Runx1-null embryos, $CD41^+$ and $CD45^+$ cells are not detectable, either phenotypically by flow cytometry or functionally. Therefore, the rescue of the definitive hematopoiesis through reconstitution of the Runx1 locus occurs either in the hematogenic endothelium [44, 46, 61–64] or in a common progenitor for hematopoietic and endothelial cells [43]. Further experiments are required to establish precise cell targets in which Runx1 deficiency blocks development of HSCs.

## Conclusion

In conclusion, we have shown here that expression of Runx1 in the embryonic Tie2 compartment (and/or downstream rescued progeny) is necessary and sufficient for normal embryonic development of both early CFU-C and definitive HSC populations.

## Acknowledgments

We thank D. Hills for useful comments on the manuscript; Y. Gibson, C. Manson, and J. Verth for animal management. We thank M. Yanagisawa for providing us with Tie2-Cre deletor mice. This work was supported by the Medical Research Council, the Leukemia Research Fund, the Stem Cell Research Foundation, and the European Union Sixth Framework Programme (FPVI) integrated project EuroStemCell.

## Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

## References

1 Michaud J, Scott HS, Escher R. AML1 interconnected pathways of leukemogenesis. Cancer Invest 2003;21:105–136.
2 Perry C, Eldor A, Soreq H. Runx1/AML1 in leukemia: disrupted association with diverse protein partners. Leuk Res 2002;26:221–228.
3 Downing JR, Higuchi M, Lenny N et al. Alterations of the AML1 transcription factor in human leukemia. Semin Cell Dev Biol 2000;11:347–360.
4 de Bruijn MF, Speck NA. Core-binding factors in hematopoiesis and immune function. Oncogene 2004;23:4238–4248.
5 Okuda T, van Deursen J, Hiebert SW et al. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell 1996;84:321–330.
6 Wang Q, Stacy T, Binder M et al. Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. Proc Natl Acad Sci U S A 1996;93:3444–3449.
7 North T, Gu TL, Stacy T et al. Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. Development 1999;126:2563–2575.
8 Yokomizo T, Hasegawa K, Ishitobi H et al. Runx1 is involved in primitive erythropoiesis in the mouse. Blood 2008;111:4075–4080.
9 Ichikawa M, Asai T, Saito T et al. AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis. Nat Med 2004;10:299–304.
10 Putz G, Rosner A, Nuesslein I et al. AML1 deletion in adult mice causes splenomegaly and lymphomas. Oncogene 2006;25:929–939.
11 Growney JD, Shigematsu H, Li Z et al. Loss of Runx1 perturbs adult hematopoiesis and is associated with a myeloproliferative phenotype. Blood 2005;106:494–504.
12 Kundu M, Compton S, Garrett-Beal L et al. Runx1 deficiency predisposes mice to T-lymphoblastic lymphoma. Blood 2005;106:3621–3624.
13 Palis J, Robertson S, Kennedy M et al. Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. Development 1999;126:5073–5084.
14 Medvinsky A. Ontogeny of the mouse hematopoietic system. Semin Dev Biol 1993;4:333–340.
15 Muller AM, Medvinsky A, Strouboulis J et al. Development of hematopoietic stem cell activity in the mouse embryo. Immunity 1994;1:291–301.

16 de Bruijn MF, Speck NA, Peeters MC et al. Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. EMBO J. 2000;19:2465–2474.
17 Ottersbach K, Dzierzak E. The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. Dev Cell 2005;8:377–387.
18 Gekas C, Dieterlen-Lievre F, Orkin SH et al. The placenta is a niche for hematopoietic stem cells. Dev Cell 2005;8:365–375.
19 Medvinsky A, Dzierzak E. Definitive hematopoiesis is autonomously initiated by the AGM region. Cell 1996;86:897–906.
20 Kumaravelu P, Hook L, Morrison AM et al. Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. Development 2002;129:4891–4899.
21 Samokhvalov IM, Thomson AM, Lalancette C et al. Multifunctional reversible knockout/reporter system enabling fully functional reconstitution of the AML1/Runx1 locus and rescue of hematopoiesis. Genesis 2006;44:115–121.
22 Gilchrist DS, Ure J, Hook L et al. Labeling of hematopoietic stem and progenitor cells in novel activatable EGFP reporter mice. Genesis 2003;36:168–176.
23 Kisanuki YY, Hammer RE, Miyazaki J et al. Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. Dev Biol 2001;230:230–242.
24 Beddington RS, Morgernstern J, Land H et al. An in situ transgenic enzyme marker for the midgestation mouse embryo and the visualization of inner cell mass clones during early organogenesis. Development 1989;106:37–46.
25 Gribi R, Hook L, Ure J et al. The differentiation program of embryonic definitive hematopoietic stem cells is largely alpha4 integrin independent. Blood 2006;108:501–509.
26 North TE, de Bruijn MF, Stacy T et al. Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. Immunity 2002;16:661–672.
27 Lian JB, Balint E, Javed A et al. Runx1/AML1 hematopoietic transcription factor contributes to skeletal development in vivo. J Cell Physiol 2003;196:301–311.
28 Theriault FM, Roy P, Stifani S. AML1/Runx1 is important for the development of hindbrain cholinergic branchiovisceral motor neurons and selected cranial sensory neurons. Proc Natl Acad Sci U S A 2004; 101:10343–10348.
29 Theriault FM, Nuthall HN, Dong Z et al. Role for Runx1 in the proliferation and neuronal differentiation of selected progenitor cells in the mammalian nervous system. J Neurosci 2005;25:2050–2061.
30 Sato TN, Qin Y, Kozak CA et al. Tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system [published erratum appears in Proc Natl Acad Sci U S A 1993;90:12056]. Proc Natl Acad Sci U S A 1993;90:9355–9358.
31 Takakura N, Huang XL, Naruse T et al. Critical role of the TIE2 endothelial cell receptor in the development of definitive hematopoiesis. Immunity 1998;9:677–686.
32 Hsu HC, Ema H, Osawa M et al. Hematopoietic stem cells express Tie-2 receptor in the murine fetal liver. Blood 2000;96:3757–3762.
33 Holtz ML, Misra RP. Endothelial-specific ablation of serum response factor causes hemorrhaging, yolk sac vascular failure, and embryonic lethality. BMC Dev Biol 2008;8:65.
34 Zhu BM, McLaughlin SK, Na R et al. Hematopoietic-specific Stat5-null mice display microcytic hypochromic anemia associated with reduced transferrin receptor gene expression. Blood 2008;112:2071–2080.
35 Yoon MJ, Koo BK, Song R et al. Mind bomb-1 is essential for intraembryonic hematopoiesis in the aortic endothelium and the subaortic patches. Mol Cell Biol Aug 2008;28:4794–4804.
36 Gitler AD, Kong Y, Choi JK et al. Tie2-Cre-induced inactivation of a conditional mutant Nf1 allele in mouse results in a myeloproliferative disorder that models juvenile myelomonocytic leukemia. Pediatr Res 2004;55:581–584.
37 Schlaeger TM, Mikkola HK, Gekas C et al. Tie2Cre-mediated gene ablation defines the stem-cell leukemia gene (SCL/tal1)-dependent window during hematopoietic stem-cell development. Blood 2005; 105:3871–3874.
38 Ulyanova T, Scott LM, Priestley GV et al. VCAM-1 expression in adult hematopoietic and nonhematopoietic cells is controlled by tissue-inductive signals and reflects their developmental origin. Blood 2005;106:86–94.
39 Rivera-Feliciano J, Lee KH, Kong SW et al. Development of heart valves requires Gata4 expression in endothelial-derived cells. Development 2006;133:3607–3618.
40 Lincoln J, Kist R, Scherer G et al. Sox9 is required for precursor cell expansion and extracellular matrix organization during mouse heart valve development. Dev Biol. 2007;305:120–132.
41 Srinivasan RS, Dillard ME, Lagutin OV et al. Lineage tracing demonstrates the venous origin of the mammalian lymphatic vasculature. Genes Dev 2007;21:2422–2432.
42 Yokomizo T, Takahashi S, Mochizuki N et al. Characterization of GATA-1(+) hemangioblastic cells in the mouse embryo. EMBO J 2007;26:184–196.
43 Huber TL, Kouskoff V, Fehling HJ et al. Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. Nature 2004; 432:625–630.
44 Pearson S, Sroczynska P, Lacaud G et al. The stepwise specification of embryonic stem cells to hematopoietic fate is driven by sequential exposure to Bmp4, activin A, bFGF and VEGF. Development 2008; 135:1525–1535.
45 Nishikawa SI, Nishikawa S, Hirashima M et al. Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. Development 1998;125:1747–1757.
46 Nishikawa SI, Nishikawa S, Kawamoto H et al. In vitro generation of lymphohematopoietic cells from endothelial cells purified from murine embryos. Immunity 1998;8:761–769.
47 Era T, Izumi N, Hayashi M et al. Multiple mesoderm subsets give rise to endothelial cells, whereas hematopoietic cells are differentiated only from a restricted subset in embryonic stem cell differentiation culture. Stem Cells 2008;26:401–411.
48 Bertrand JY, Jalil A, Klaine M et al. Three pathways to mature macrophages in the early mouse yolk sac. Blood 2005;106:3004–3011.
49 Mikkola HK, Fujiwara Y, Schlaeger TM et al. Expression of CD41 marks the initiation of definitive hematopoiesis in the mouse embryo. Blood 2003;101:508–516.
50 Ferkowicz MJ, Starr M, Xie X et al. CD41 expression defines the onset of primitive and definitive hematopoiesis in the murine embryo. Development 2003;130:4393–4403.
51 Taoudi S, Medvinsky A. Functional identification of the hematopoietic stem cell niche in the ventral domain of the embryonic dorsal aorta. Proc Natl Acad Sci U S A. 2007;104:9399–9403.
52 Taoudi S, Gonneau C, Moore K et al. Extensive hematopoietic stem cell generation in the AGM region via maturation of VE-cadherin(+)CD45(+) pre-definitive HSCs. Cell Stem Cell. 2008;3: 99–108.
53 Zeigler BM, Sugiyama D, Chen M et al. The allantois and chorion, when isolated before circulation or chorio-allantoic fusion, have hematopoietic potential. Development 2006;133:4183–4192.
54 Hayashi K, Natsume W, Watanabe T et al. Diminution of the AML1 transcription factor function causes differential effects on the fates of CD4 and CD8 single-positive T cells. J Immunol 2000;165:6816–6824.
55 Dzierzak E, Medvinsky A. The discovery of a source of adult hematopoietic cells in the embryo. Development 2008;135:2343–2346.
56 Dzierzak E, Medvinsky A. Mouse embryonic hematopoiesis. Trends Genetics 1995;11:359–366.
57 Li Z, Chen MJ, Stacy T et al. Runx1 function in hematopoiesis is required in cells that express Tek. Blood 2006;107:106–110.
58 Marmigère F, Montelius A, Wegner M et al. The Runx1/AML1 transcription factor selectively regulates development and survival of TrkA nociceptive sensory neurons. Nat Neurosci. 2006;9:180–187.
59 Stifani N, Freitas AR, Liakhovitskaia A et al. Suppression of interneuron programs and maintenance of selected spinal motor neuron fates by the transcription factor AML1/Runx1. Proc Natl Acad Sci U S A 2008;105:6451–6456.
60 Miller J, Horner A, Stacy T et al. The core-binding factor beta subunit is required for bone formation and hematopoietic maturation. Nat Genet 2002;32:645–649.
61 Oberlin E, Tavian M, Blazsek I et al. Blood-forming potential of vascular endothelium in the human embryo. Development 2002;129: 4147–4157.
62 Jaffredo T, Gautier R, Eichmann A et al. Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. Development 1998;125:4575–4583.
63 Zovein AC, Hofmann JJ, Lynch M et al. Fate tracing reveals the endothelial origin of hematopoietic stem cells. Cell Stem Cell 2008;3: 625–636.
64 Chen MJ, Yokomizo T, Zeigler BM et al. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. Nature 2009.


See www.StemCells.com for supporting information available online.