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

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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

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 megakaryocytopenia and perturbs T- and B-cell differentiation, but does not deplete hematopoietic stem cells (HSC's) [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.

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

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

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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 deleter mice were kindly provided by M. Yanagisawa [23]. To obtain Tie2-Cre: Runx1LacZ/LacZ triple-transgenic animals [Tie2-Cre: Runx1LacZ/LacZ] males were crossed with Runx1LacZ/LacZ 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 amplification 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 Runx1lacZ and Runx1Cre; reaction b, for detection of Runx1lacZ. 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’-GGCTTCTAAGGTTTGATGTGTT-3’ and 5’-TTCCACACGCGTGAGGAGCTT-3’ (gives 455-bp product for wild type and 515-bp product for reactivated Runx1 allele); for reaction b, 5’-GTTTTCTATGGGGGTGAGG-3’ and 5’-TTCCACACGCGTGAGGAGCTT-3’ (gives 1000-bp product for the targeted Runx1/LacZ allele).

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. Runx1lacZ/LacZ phenotype is embryonic lethal by E12.5. If Cre-recombinase is expressed in the Runx1lacZ/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 deleter mice with reversible knockout Runx1 mice enables lineage-specific rescue of Runx1 expression. Here we used Tie2-Cre deleter mice to test if rescue of the Runx1 locus in the Tie2 expressing cell compartment is sufficient to...
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. 2D–2G). These observations underscore the importance of Tie2 in the development of the vascular system.
Figure 3. Recombination in E11.5 [Tie2-Cre: Runx1\textsuperscript{LacZ/LacZ}] embryos. (A, C, D, E): Heterozygous Runx1\textsuperscript{LacZ/wt} embryo; (B, C’, D’, E’) [Tie2-Cre: Runx1\textsuperscript{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\textsuperscript{LacZ/LacZ}] embryos is either fully absent (placenta) or significantly attenuated (umbilical cord). Clusters inside the cord of the Runx1\textsuperscript{LacZ/wt} embryo (E, arrowhead) are readily observed; compare with weak LacZ staining in [Tie2-Cre: Runx1\textsuperscript{LacZ/LacZ}] embryos (E’, arrowhead). (This observation is supported by histological analysis shown in Fig. 4.) Note that LacZ\textsuperscript{+} stained hematopoietic cells, typical for heterozygous Runx1\textsuperscript{LacZ/wt} embryos, are not seen in the head or the yolk sac of the [Tie2-Cre: Runx1\textsuperscript{LacZ/LacZ}] embryo (compare D with D’ and C with C’, respectively). Note slight hemorrhage in the head of the [Tie2-Cre: Runx1\textsuperscript{LacZ/LacZ}] embryo.

Figure 4. Rescue of intravascular hematopoietic cell clusters in the umbilical cord of E11.5 [Tie2-Cre: Runx1\textsuperscript{LacZ/LacZ}] embryos. (A, A’): Runx1\textsuperscript{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\textsuperscript{+} (A’, arrow). In addition, many cells in the surrounding mesenchyme are LacZ\textsuperscript{+}. (B, B’): [Tie2-Cre: Runx1\textsuperscript{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\textsuperscript{+} 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 matenal 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 Compart-ment of [Tie2-Cre: Runx1LacZ/LacZ] Embryos: Phenotypic Analysis

As described previously, LacZ expression is observed in various tissues of Runx1LacZ/LacZ heterozygous embryos, such as motoneurons, 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 clonal progenitors and definitive HSCs [7, 51–53]. Similar cell 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 Runx1LacZ/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: Runx1LacZ/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 Run-\textit{x}LacZ/LacZ embryos, [Tie2-Cre: Runx1LacZ/LacZ] embryos contained rescued clusters that were LacZ-negative due to excision of the LacZ reporter (Figs. 3E, 3E₀ 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: Run-x1LacZ/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: Runx1LacZ/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 Runx1LacZ/LacZ embryos, which die by E12.5 of severe hematopoietic deficiency, [Tie2-Cre: Runx1LacZ/LacZ] embryos survive, but only until birth. E16.5–E18.5 litters cumulatively contained 11.6% of rescued [Tie2-Cre: Runx1LacZ/LacZ] embryos (supporting information Table 1). Extensive hemorrhaging was observed in the neural system of Runx1LacZ/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: Runx1LacZ/LacZ] embryos (Fig. 3C'). The number of CFU-C and morphological types of hematopoietic colonies formed by E12.5 yolk sacs of [Tie2-Cre: Runx1LacZ/LacZ] embryos was similar to those in Runx1LacZ/LacZ embryos (Fig. 5A). The restoration of the Runx1 locus in hematopoietic colonies was confirmed by PCR analysis (Fig. 5B).

By E18.5, the cellularity of the fetal liver in [Tie2-Cre: Runx1LacZ/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
spleen of E18.5 [Tie2-Cre: Runx1lacZ/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 Runx1lacZ/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: Runx1lacZ/lacZ] Embryo**

To determine whether the development of HSCs was rescued in [Tie2-Cre: Runx1lacZ/lacZ] embryos, fetal livers were
isolated from E13.5 embryos and transplanted into irradiated mice (Fig. 7A). Livers from [Tie2-Cre: Runx^{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: Runx^{1^{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: Runx1LacZ/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: Runx1LacZ/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 Runx1LacZ/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: Runx1LacZ/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 Cbfβ (common subunit for Runx1, 2, and 3 transcription complexes)-null embryos, which normally die between E12.5 and E13.5, using a Tie2-Cbfβ 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.

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**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.

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