HEMATOPOIESIS

Runx1 is essential for hematopoietic commitment at the hemangioblast stage of development in vitro

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In this report we demonstrate a role for Runx1 (AML1) at the hemangioblast stage of hematopoietic and endothelial development in embryonic stem (ES) cell-derived embryoid bodies (EBs). Runx1 is expressed in EBs during the appearance of precursors with hemangioblast properties, the blast colony-forming cells (BL-CFCs). Cell sorting studies revealed that all BL-CFCs within EBs express Runx1. Runx1-deficient EBs consistently generate 10- to 20-fold fewer blast colonies than wild-type controls and display a complete block in definitive hematopoiesis. Despite this defect, Runx1-/- EBs and yolk sacs from mutant embryos generate normal numbers of primitive erythroid precursors. These observations clearly demonstrate that Runx1 functions early in hematopoietic development, and they support the interpretation that the primitive erythroid lineage is established early by a subset of BL-CFCs that develop in a Runx1-independent fashion. (Blood. 2002; 100:458-466)

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Introduction

Hematopoiesis in the mouse embryo begins in the yolk sac, where blood islands of mesodermal origin develop at approximately day 8 of gestation (EB).1,2 These blood islands consist of 2 distinct lineages, a population of erythroblasts and a surrounding layer of angioblasts that will form the first vascular structures.3 The parallel temporal development of these lineages in physical proximity provided the basis for the hypothesis that they arise from a common precursor, a cell called the hemangioblast.4,5 Erythroid cells within the blood islands, known as embryonic or primitive erythrocytes, are large and nucleated, and they produce the embryonic forms of globin.1,6,7 Generation of the primitive embryonic or primitive erythrocytes, are large and nucleated, and they be detected as early as the primitive streak stage of development.10,11 Definitive hematopoiesis encompasses the development of all lineages other than primitive erythroid and includes definitive erythroid, myeloid, and lymphoid. As with primitive hematopoiesis, the first definitive hematopoietic precursors also develop in the yolk sac and can be detected as early as the primitive streak stage of development.10 Although initiated in this extra-embryonic region, definitive hematopoiesis is most often associated with intra-embryonic sites such as the para-aortic splanchnopleura (P-Sp), the aorta–gonad–mesonephros (AGM), and the fetal liver, where long-term repopulating stem cells and precursor populations from different lineages undergo significant expansion and maturation.11-15

Although yolk sac blood islands were identified as the earliest site of hematopoietic and endothelial development almost 100 years ago, attempts to identify, isolate, and characterize the precursors representing these initial stages of lineage commitment, including the elusive hemangioblast, have been largely hampered by the inaccessibility of the early mammalian embryo. One promising alternative approach to study early hematopoietic development is the model system based on the differentiation potential of embryonic stem (ES) cells in culture.16-21 Most evidence suggests that the events leading to the establishment of the hematopoietic and endothelial lineages in embryoid bodies (EBs) generated from ES cells in culture are similar, if not identical, to those in the early yolk sac.19,22-29 Using the ES/EB differentiation model, cells with hemangioblast potential have been identified.30,31 In the presence of vascular endothelial growth factor (VEGF) in methylcellulose cultures, these EB-derived precursors generate blast cell colonies that display hematopoietic and endothelial potential.30,32 The cells that give rise to these blast colonies, the blast colony-forming cells (BL-CFCs), represent a transient population that appears in EBs before the establishment of any other hematopoietic lineages. The developmental potential of the BL-CFC strongly suggests that it represents the in vitro equivalent of the hemangioblast and, as such, the earliest stage of hematopoietic and endothelial commitment.

Although a precursor with hemangioblast properties remains to be identified in the early embryo, insights into the molecular events involved in the establishment of the earliest hematopoietic and endothelial lineages have been provided by gene-targeting experiments. Such studies have uncovered the role of specific genes at distinct stages in this process and, in doing so, have been instrumental in defining key developmental steps in the commitment, growth, and maturation of these lineages. Flk-1, a gene that

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encodes a receptor tyrosine kinase, is required early in ontogeny and is essential for the development of the hematopoietic and the endothelial components of the blood island.33 Cell tracking studies have indicated that the Flk-1 receptor is initially involved in migration of the mesodermal precursor cells to the extraembryonic region of the embryo, the site of yolk sac development.34 These migrating precursors may be similar to the EB-derived BL-CFCs that have been shown to express Flk-135 and to grow in response to its ligand, VEGF.32

Scl, a member of the helix-loop-helix family of transcription factors,36 appears to function at a slightly later developmental stage than Flk-1. The yolk sacs of Scl−/− embryos develop a primary vascular network but contain no primitive or definitive hematopoietic cells, indicating a pivotal role for Scl in hematopoietic commitment but not in the early stages of vasculogenesis.36-38 Although not essential for establishment of the endothelial lineage, Scl does appear to be required for remodeling and maturation of the vascular system.39 In vitro differentiation studies with Scl−/− ES cells showed a complete block in primitive and definitive hematopoietic potential, confirming the developmental defect observed in vivo. Further analysis of the Scl−/− EBs revealed a defect as early as the BL-CFC because the mutant ES cells were unable to generate blast cell colonies.29,35 Scl−/− ES cells were, however, able to generate precursors that gave rise to transitional colonies that represent a stage of development slightly earlier than the blast cell colony.29

The AML1 gene (recently renamed Runx1), which encodes the DNA-binding subunit of a transcription factor of the core binding factor (CBF) family,30,42 is required for the establishment of definitive but not primitive hematopoiesis. Runx1−/− embryos develop normal blood islands and progress through the yolk sac phase of hematopoiesis but die between E11 and E12.5,26,27 Before death, the liver rudiment contains primitive nucleated erythrocytes but lacks all definitive erythroid, myeloid, and megakaryocytic cells, indicating a complete block in the development of the definitive hematopoietic program. Analysis of Runx1−/− EBs revealed a similar block in definitive hematopoietic potential.26,43 In addition to the hematopoietic abnormalities, Runx1−/− deficient animals show extensive central nervous system hemorrhage and necrosis, suggesting an additional vascular defect.

Although these targeting studies position Runx1 at the establishment and expansion of the definitive hematopoietic program, expression analysis suggest that it may function at earlier stages of development. Using mice with the LacZ gene targeted to the Runx1 locus (Runx1LacZ mice), North et al44 demonstrated expression of Runx1 (LacZ) in subpopulations of endothelial cells in the yolk sac, in the vitelline and umbilical arteries, and in the ventral wall of the dorsal aorta. Expression was also detected in emerging primitive erythrocytes early in the yolk sac. Expression in these cells was transient, declined significantly by E8.5, and was undetectable by E10.5. The presence of Runx1 transcripts in primitive erythrocytes and in subpopulations of endothelial cells suggests that this gene may be expressed and may function at the level of a cell with hemangioblast properties.

To investigate the role of Runx1 at the earliest stage of hematopoietic commitment, we analyzed its expression pattern and function during ES/EB differentiation and in early yolk sac development. Our results indicate that Runx1 is expressed in yolk sac mesodermal cells before the establishment of the blood islands and within the BL-CFCs in EBs. Analysis of Runx1−/− deficient ES cells demonstrated that this gene is essential for the generation of normal numbers of blast colonies and, as such, provides evidence that it does function at the equivalent of the hemangioblast stage of development. BL-CFCs that develop in the deficient EBs appear to be primitive erythroid restricted, suggesting that the functional requirement for Runx1 may define subpopulations of these precursors.

### Materials and methods

#### In situ hybridization

In situ hybridization of outbred ICR (Taconic, Germantown, NY) murine embryos was performed as previously described32 with the following modifications. Single-stranded 32P-labeled antisense Runx1 and sense control probes (accession number W29200) were prepared at specific activities of 4 × 106 dpm/μg. After hybridization and high-stringency washes, tissues were counterstained with hematoxylin. Bright-field and dark-field images were captured with a Polaroid digital camera and were processed, including pseudocolorizing grains, using Adobe Photoshop (Adobe Systems). No signal above background was observed in the negative controls.

#### Embryonic stem cell growth and differentiation

The generation of Runx1+/−, Runx1−/−, and Runx1−/− ES cells has been previously described.27,44 ES cells were maintained on irradiated embryonic feeder cells in Dulbecco modified Eagle medium supplemented with 15% fetal calf serum (FCS), penicillin, streptomycin, leukemia inhibitory factor (LIF) (1% conditioned medium), and 1.5 × 10−8 M monothioglycerol (MTG; Sigma, St Louis, MO). Two days before the onset of differentiation, cells were transferred to gelatinized plates in the same media. For the generation of ES cells, ES cells were tyrosinized and plated at various densities and grown in differentiation cultures. Differentiation of EBs was carried out in 60-mm Petri-grade dishes in Iscoves modified Dulbecco medium (IMDM) supplemented with 15% FCS, 2 mM L-glutamine (Gibco/BRL, Grand Island, NY), transferrin (200 μg/mL), 0.5 mM ascorbic acid (Sigma), and 4.5 × 10−4 M MTG. Cultures were maintained in a humidified chamber in a 5% CO2-air mixture at 37°C.

#### Colony assays

For the generation of blast cell colonies (BL-CFC assay), EB cells were plated in 1% methylcellulose supplemented with 10% FCS (Summit, Fort Collins, CO), VEGF (5 ng/mL), c-kit ligand (KL; 1% conditioned medium), interleukin-6 (IL-6; 5 ng/mL), and 25% D4T endothelial cell-conditioned medium.32 Transitional colonies were generated in the same basic conditions in the absence of VEGF. Colonies were scored after 4 days of culture. For the growth of hematopoietic precursors, cells were plated in 1% methylcellulose containing 10% plasma-derived serum (Antech, Tyler, TX), 5% protein-free hybridoma medium (PFHM-II; Gibco-BRL), and cytokines KL (1% conditioned medium), thrombopoietin (5 ng/mL), Erythropoietin (2 U/mL), IL-11 (25 ng/mL), IL-3 (1% conditioned medium), granulocyte-macrophage colony-stimulating factor (GM-CSF; 3 ng/mL), G-CSF (30 ng/mL), M-CSF (5 ng/mL), and IL-6 (5 ng/mL). Cultures were maintained at 37°C, 5% CO2. Primary erythroid colonies were scored at day 5 to 6 of culture, whereas definitive erythroid, macrophage, and multilineage colonies were counted after 7 to 10 days of culture.

For expansion of blast cell colonies, individual colonies were transferred to Matrigel-coated (Collaborative Research, San Jose, CA) microtitre wells containing IMDM with 10% FCS (Hyclone, Logan, UT), 10% horse serum (Biocell, Rancho Dominguez, CA), VEGF (5 ng/mL), insulinlike growth factor 1 (IGF-1) (10 ng/mL), etoposide (2 μM), basic fibroblast growth factor (bFGF) (10 ng/mL), IL-11 (50 ng/mL), KL (1% conditioned medium), IL-3 (1% conditioned medium), L-glutamine (1%), and 4.5 × 10−5 M MTG. After 4 days of growth, the nonadherent cells of each well were harvested and cultured in 1 mL methylcellulose containing the above mixture of cytokines used for the growth of hematopoietic precursors. LIF and c-kit ligand were derived from media conditioned by Chinese hamster ovary cells transfected with LIF and KL expression vectors, respectively (kindly provided by Genetics Institute, Cambridge, MA). IL-3 was obtained from medium conditioned by X63 AG8-653 myeloma cells transfected...
with a vector expressing IL-3, VEGF, GM-CSF, M-CSF, G-CSF, thrombopoietin, IL-6, and IL-11 were purchased from R&D Systems (Minneapolis, MN).

X-gal staining

Undifferentiated ES cells, differentiated EBs, and hematopoietic colonies were fixed in 1× phosphate-buffered saline (PBS) containing 0.5% glutaraldehyde (Sigma) and 1 mM MgCl₂ for 10 to 15 minutes. After fixation, the cells were rinsed in 1× PBS and stained with 1 mM MgCl₂, 3.3 mM K₄Fe(CN)₆, 3.3 mM K₃Fe(CN)₆, 0.02% NP-40, and 0.1 vol 2% X-gal (Sigma) overnight at 37°C. Positive cells were detected by the presence of blue staining visualized under light microscopy.

FACS-gal analysis

Fluorescein di-β-D-galactopyranoside (FDG; Sigma), hydrolyzed to fluorescein by intracellular β-galactosidase, was used to detect LacZ activity. EB-derived cells were washed and resuspended in 1× PBS–20% FCS to a final maximum concentration of 10⁶ cells/mL. Hypotonic loading was achieved by a 2-minute incubation at 37°C with an equal volume of prewarmed 2 mM FDG (in water). After the loading procedure, 10 vol cold IMDM–15% FCS were added, and the mixture was incubated 20 minutes on ice. Stained suspensions were analyzed on a FACSscan (Becton Dickinson, San Jose, CA) or sorted on a MoFlo (Cytomation Systems, Fort Collins, CO) cell sorter.

Gene expression analysis

For polyA⁺ global amplification polymerase chain reaction (PCR), reverse transcription (RT), poly-A tailing, and PCR procedures were performed as described by Brady et al. with the exception that the X-dT oligonucleotide was shortened to 5'-GGTAACTCGAGAATTCTTCC-3'. Amplified products from PCR were separated on agarose gels and transferred to a Zeta-probe (Qiagen). PCR was carried out using the following oligonucleotides:

- For Runx1, 5'-AGAAGATCTGCGACGCG3' (sense), 5'-TTTGGCGCTCAGGAGGAGCG3' (antisense); and 5'-GGTTCGGGAAAGCCATGG3' (antisense; Runx1) and 5'-GGTGAAGAGGAGCGGCG3' (sense), 5'-GGTGAAGAGGAGCGGCG3' (antisense; Runx1).

- For Flk-1, 5'-CACCTGACCTCCTACACTGC3' (sense), 5'-GATTTCACCTAATACACCG3' (antisense; Flk-1).

PCR was performed with 2.5 U Taq polymerase (Promega, Madison, WI), PCR buffer, 2.5 mM MgCl₂, 0.2 μM each primer, and 0.2 mM dNTP. Cycling conditions were as follows: 94°C for 5 minutes followed by 35 cycles of amplification (94°C denaturation for 1 minute, 60°C annealing for 1 minute, 72°C elongation for 1 minute), with a final incubation at 72°C for 7 minutes.

Results

Runx1 is expressed during gastrulation in extra-embryonic mesoderm before formation of blood islands

To further assess the role of Runx1 in the establishment of the hematopoietic system, we mapped its expression before and during the development of blood islands in E7.25 to E8.25 embryos. Runx1 transcripts were detected at the mid-to-late primitive streak stage (E7.25), specifically in extraembryonic mesoderm cells adjacent to visceral endoderm (Figure 1A). This pattern of expression in extraembryonic mesoderm persisted in early neural plate embryos (E7.5, Figure 1B). At mid-to-late neural plate stages, Runx1 mRNA was present predominantly in nascent yolk sac blood islands (Figure 1C and data not shown). In addition, there was a low level of expression in the developing chorion that increased by early somite pair stages (E8.25, Figure 1D). At E8.25, the predominant accumulation of Runx1 mRNA was in the developing yolk sac blood islands (Figure 1D). The early expression of Runx1 described here is similar to that observed for Scl and is consistent with a role in the commitment of mesoderm to hematopoietic–endothelial fates.

Runx1 expression is up-regulated at the hemangioblast stage of EB differentiation

The above analyses indicate that Runx1 is expressed at the earliest stage of blood island development, suggesting a potential role at the level of the putative hemangioblast. To further investigate the function of Runx1 at the onset of hematopoietic development, we analyzed its expression pattern in EBs over a 10-day differentiation period. A low level of Runx1 expression was detected in undifferentiated ES cells and in EBs after the first few days of differentiation (Figure 2A). Runx1 expression increased significantly between days 3 and 4 of differentiation and remained elevated thereafter. Based on real-time PCR analysis, the magnitude of the increase in Runx1 expression over this 24-hour period was found to be approximately 10-fold (not shown). No Runx1 cDNA was detected in EBs generated from Runx1−/− ES cells. Comparative analysis of Runx1 expression with other genes demonstrated a striking similarity to the temporal expression pattern of Flk-1. The up-regulation of both Runx1 and Flk-1 was preceded by the expression of Brachyury, a marker of early mesoderm development. The zinc finger transcription factor expressed in ES cells but not in their differentiated progeny was readily detected in undifferentiated ES cells but not significantly in day 3 EBs. Scl was expressed at low levels as early as day 3.5 of differentiation. The levels increased over the next few days and then remained relatively constant. Gata-1, a transcription factor expressed in hematopoietic but not endothelial cells and in the embryonic β H1 and adult β major globins, followed the onset of Scl expression. These findings suggest that Runx1 expression is up-regulated at the hemangioblast stage of development (as defined by Flk-1 and Scl), following the establishment of the earliest mesodermal population (Brachyury) but...
preceeding the commitment to the hematopoietic lineage (Gata-1, β H1, β major globin).

The up-regulation of Runx1 expression detected during EB differentiation could reflect an increase in the level of expression within a subset of cells or an increase in the number of cells expressing this gene. To distinguish between these 2 possibilities, we analyzed EBs generated from Runx1+/− ES cells that contain the LacZ gene targeted to the Runx1 gene. LacZ expression was evaluated either by direct X-gal staining or by FDG staining followed by flow cytometry analyses. Using both methods of detection, no significant staining was observed in undifferentiated ES cells (day 0) or in early EBs (up to 2.5 days) (Figure 2B-C). LacZ+ cells were first detected within the EBs by day 3 of differentiation, at which time approximately 5% of cells expressed this marker as determined by fluorescence-activated cell sorter (FACS) analysis. The number of positive cells increased dramatically over the next 24 hours, reaching levels greater than 30% of the total EB population by day 4 of differentiation. The frequency of LacZ+ cells remained elevated (from 30% to 50%) in EBs between days 5 and 10 of differentiation (Figure 2B and not shown). X-gal staining revealed that a large portion of individual EBs at day 4 to 6 of differentiation expressed LacZ. No significant level of β-galactosidase activity was detected in control wild-type (Runx1+/+) EBs. These findings demonstrate an early and rapid expansion of cells expressing Runx1, with kinetics almost identical to the expansion of the Flk-1+ population and the development of the BL-CFC. To determine whether the Runx1+ and Flk-1+ cells represent distinct or overlapping populations, we analyzed developing EBs for the presence of both markers. At days 3.0 and 3.5 of differentiation, most LacZ+ cells also expressed Flk-1 (Figure 2D). By day 4 of differentiation, however, a significant population of Flk1+/− LacZ+ cells was detected. These patterns suggest that the expression of Runx1 within a subpopulation of Flk-1+ cells at these early stages of differentiation could define the emergence of the BL-CFC.

**Runx1 is expressed in blast colonies and BL-CFCs**

Given the early expression pattern observed in EBs, we next investigated Runx1 expression in blast colonies and populations derived from them. As shown in Figure 3A, blast colonies generated from Runx1+/− EBs uniformly stained positive for β-galactosidase activity. In contrast, no staining was observed in control Runx1+/+ blast colonies. This indicates that the cells within these colonies, which have previously been shown to represent hematopoietic and endothelial precursors, express Runx1. When individual blast colonies are transferred to microricter wells under appropriate growth conditions, these precursors grow and mature into adherent endothelial cells and a nonadherent population of hematopoietic cells after 4 days of culture. Analysis of these subpopulations demonstrated extensive LacZ expression in the
hematopoietic cells (Figure 3B). Expression was also detected in the adherent cells, though the levels were significantly lower than in the hematopoietic population (Figure 3B, arrowheads). Expression in the adherent population is consistent with the observation that Runx1 is expressed in subsets of endothelial cells in the yolk sac and embryo proper.44

To determine whether the BL-CFC also expresses Runx1, day 3.75 Runx1+/Z EB cells were fractionated for β-galactosidase activity by cell sorting (Figure 3C). Higher levels of Runx1 expression were detected in cells from the LacZ-positive fraction than in those from the negative fraction, confirming that separation of cells based on β-galactosidase activity resulted in an enrichment of Runx1-expressing cells. Analysis of BL-CFC content revealed that most precursors were present in the LacZ+ population, demonstrating that most BL-CFCs isolated from day 3.75 EBs express Runx1. To further define the developmental status of the day 3.75 LacZ+ (Runx1+) cells, we analyzed this fraction for the expression of other genes known to play a role in early hematopoietic commitment and maturation. Runx1+ cells were found to contain lower levels of Scl and Gata-1 than those in the negative fraction, indicating that this population is exiting the mesodermal stage of development. Flk-1 was expressed in both the positive and the negative fractions, a finding consistent with the previous FACS analyses that demonstrated the presence of Flk-1+/Runx1+ and Flk-1+/Runx1− populations in comparably staged EBs (Figure 2D). The higher levels of Scl and Gata-1 in the positive fraction are consistent with its elevated BL-CFC content and further support the interpretation that Runx1 expression defines one of the earliest stages of hematopoietic commitment. Low levels of βH1 globin were present in the LacZ− (Runx1−) fraction, indicating the onset of differentiation to the primitive erythroid lineage.

To evaluate whether earlier developing BL-CFCs also express Runx1, day 3 Runx1+/Z EBs were fractionated for β-galactosidase activity. In addition to BL-CFCs, EBs at this stage of development contain some residual ES cells and precursors (Trans-CFC) that generate transitional colonies. Transitional colonies represent an earlier stage of development than blast colonies and contain cell populations undergoing commitment of mesoderm to the hematopoietic and endothelial lineages.29 As observed for day 3.75 BL-CFCs, almost all day 3.0 BL-CFCs were present in the LacZ+ fraction (Figure 3D). In contrast, the Trans-CFC and the ES cells that generate secondary EBs segregated to the LacZ− fraction, indicating that they do not express Runx1 (Figure 3D). Taken together, the findings from the cell-sorting studies clearly demonstrate that the BL-CFC, a precursor with hemangioblast characteristics, expresses Runx1.

Runx1+/− ES cells display a defect in BL-CFC developmental potential

To examine the requirement for the Runx1 gene in BL-CFC development, day 3.5 and 4 EBs generated from Runx1+/−, Runx1+/−, and Runx1+/− ES cells were analyzed for BL-CFC content. At both time points, the Runx1+/− EB cells displayed a profound defect in BL-CFC potential. Runx1-deficient EBs consistently generated 10- to 20-fold fewer blast colonies than wild-type controls (Figure 4A). The blast colonies that did develop from the Runx1+/− EB cells were similar in morphology to those generated from wild-type cells (Figure 5A, top). To confirm that Runx1 was indeed critical for blast colony development, we attempted to rescue the defect by retroviral-mediated expression of this gene in deficient cells. As shown in Figure 4B, day 3 and 3.75 EBs generated from Runx1−/− ES cells infected with a retroviral vector encoding Runx1, and selected for puromycin resistance, produced higher numbers of blast colonies than EBs from ES cells infected with the empty retrovirus. These findings strongly suggest that Runx1 plays a pivotal role at the stage of BL-CFC development.

In the next set of experiments, we assessed the developmental potential of the blast colonies generated from the Runx1+/− and the rescued Runx1+/− ES cells and compared it to that of colonies from heterozygous and wild-type cells. Two different assays were used in this analysis. As a first approach, colonies were picked, transferred to microtiter wells, and cultured for 4 days to determine their potential to generate adherent (endothelial) and nonadherent (hematopoietic) cells. Colonies from each of the groups generated both types of cells (Runx1+/− and Runx1+/−, shown in Figure 5A, bottom). After expansion, a fraction of the nonadherent cells of each well was replated in methylcellulose to assay hematopoietic potential. The remainder of the
nonadherent population was analyzed for \( \beta H1 \) globin gene expression as a measure of primitive erythroid potential. Adherent cells from the wild-type and Runx1 \(^{-/-} \) colonies were cultured for an additional week and then harvested and analyzed for the expression of genes associated with the endothelial lineage. This expression analysis demonstrated the presence of Flk-1 and Tie-2 transcripts in the adherent populations derived from Runx1 \(^{-/-} \) and the Runx1 \(^{+/+} \) individual blast colonies, indicating that these cells are of the endothelial lineage (Figure 5B).

Replating studies revealed that the nonadherent populations from most Runx1 \(^{+/+} \) and Runx1 \(^{-/-} \) blast colonies contained precursors with colony-forming potential (Figure 5C). All expanded populations that replated were found to contain definitive hematopoietic precursors. Approximately 30% of the cultures with definitive precursors also contained primitive erythroid precursors. Cultures with only primitive erythroid precursors were not detected in this set of experiments. In contrast to the wild-type cells, nonadherent populations from Runx1 \(^{-/-} \) blast colonies contained few, if any, precursors. No precursors were detected in the experiment shown in Figure 5C; however, primitive erythroid precursors were occasionally found in other studies. The nonadherent fraction of the rescued Runx1 \(^{-/-} \) blast colonies did contain precursors of the definitive hematopoietic lineages (macrophage and mix), demonstrating that this potential was rescued in the BL-CFCs. Although the precursor content was low in the nonrescued Runx1 \(^{-/-} \) cultures, \( \beta H1 \) globin expression was readily detectable in the nonadherent cells, indicating the presence of primitive erythroid precursors (Figure 5D). The presence of \( \beta H1 \), together with the lack of clonal precursors in the Runx1-deficient cultures, suggests that the primitive erythroid cells had matured beyond the precursor stage during the expansion phase. In the second analysis, we assayed the hematopoietic potential of the Runx1 \(^{-/-} \) blast colonies before expansion in culture to determine if they did contain primitive erythroid precursors. For this assay, individual Runx1 \(^{-/-} \) blast colonies were picked, and the cells were dispersed and replated directly in secondary methylcellulose cultures. Under these conditions, approximately 40% of the replated colonies gave rise to secondary primitive erythroid colonies. Colonies of definitive hematopoietic cells were never observed in these secondary cultures. Collectively, these findings demonstrate that Runx1 is essential for the generation of normal numbers of blast colonies. Blast colonies that develop from Runx1 \(^{-/-} \) EBs appear to be restricted to the primitive erythroid and endothelial lineages, a finding consistent with the interpretation that Runx1 is essential for definitive hematopoietic development.

**Runx1 deficiency does not affect primitive hematopoietic development**

The defect in blast colony development in Runx1 \(^{-/-} \) EBs prompted us to investigate the primitive hematopoietic potential of these cells.
and those of the yolk sac of Runx1−/− embryos. Previous analysis of the primitive erythroid lineage, by estimation of circulating red cell number in Runx1−/− embryos, suggested that this population was relatively unaffected by the deletion of this gene.26,27 However, because quantitative analysis of primitive erythroid precursors was not performed, it is possible that the Runx1 mutation did cause subtle defects in the development of this lineage.

Analyses of the yolk sac of Runx1−/− embryos, ranging in stage from 4 to 6 sp (E8.25) to 13 to 15sp (E8.5), revealed the presence of normal numbers of primitive erythroid precursors (Figure 6A). This tissue was devoid of definitive hematopoietic precursors, including those of the macrophage and definitive erythroid lineages. In contrast, definitive precursors could be detected in the yolk sac of Runx1+/− and Runx1+/+ embryos as early as the 7 to 9sp stage of development. Yolk sacs from Runx1+/− embryos contained fewer definitive precursors than those from wild-type embryos, indicating a hemizygous affect of Runx1 at these early stages of definitive hematopoiesis. These findings clearly demonstrate that the primitive lineage, at the level of the precursor stage, is intact in the Runx1−/− embryos and that the definitive hematopoietic program is affected at the earliest stages of its development.

As observed in the yolk sac, EBs generated from Runx1−/− ES cells showed little or no defect in primitive erythroid potential (Figure 6B). The only discernable difference was that primitive erythroid development in the Runx1−/− EBs appeared to be restricted to a narrower window of differentiation than found in wild-type and heterozygous EBs. The reason for this restriction is unknown. No definitive precursors were found in the Runx1+/− EBs, confirming the findings in the mouse embryo that this gene is absolutely essential for the development of these lineages (Figure 6C). As with the yolk sacs, Runx1+/− ES cells generated fewer definitive precursors than those differentiated from wild-type ES cells (Figure 6C).

The previous observation of Runx1 expression in yolk sac primitive erythrocytes24 and our sorting studies indicating that all BL-CFCs are Runx1+ suggested that ES-derived committed primitive erythroid precursors are also likely to express this gene. To address this issue, we sorted day 4.75 Runx1+/−/− EBs for LacZ expression and analyzed the fractions for primitive erythroid potential (Figure 6D). Replating studies indicated that almost all primitive erythroid precursors were found in the Runx1+ fraction (Figure 6D). As expected, the earliest macrophage precursors developing in these EBs were also Runx1+. Additional studies demonstrated that all definitive precursors found in day 6 EBs were Runx1+ (data not shown). These findings extend our BL-CFC analysis and demonstrate that Runx1 expression defines the earliest stages of primitive and definitive hematopoiesis.

Although primitive erythroid precursors express Runx1, mature cells within colonies generated from them appear to have downregulated expression as indicated by the low levels of β-galactosidase staining (Figure 6E). In contrast, colonies of definitive hematopoietic cells maintain high levels of expression (Figure 6E and data not shown). These results indicate that all hematopoietic precursors express Runx1. As the primitive erythroid lineage matures, the cells appear to rapidly lose Runx1 expression, a pattern similar to that observed in vivo in the yolk sac blood islands.

Discussion

Previous studies have established Runx1 as a pivotal player in the development of the definitive hematopoietic system in the mouse embryo.26,27 Given this association with definitive hematopoiesis, Runx1 is generally considered to exert its primary function within the embryo proper, following the primitive erythroid stage of development in the yolk sac. In this report, we investigated the role of Runx1 in hematopoietic development in ES cell–derived EBs and demonstrated that it is essential for the establishment or differentiation of the BL-CFC, a precursor that represents the earliest stage of hematopoietic and endothelial commitment in this model system. Our data clearly show that Runx1 is expressed within BL-CFCs and is required for the development of normal numbers of blast colonies from these precursors. Several lines of evidence indicate that the observed effect on the number of blast colonies truly reflects a critical role for Runx1 at this stage of development and is not simply due to reduced potential resulting from ES cell clonal variation. First, the defect in BL-CFC...
was found to be up-regulated between days 3 and 4 of differentiation. Flk-1, Scl provide a unique opportunity to discriminate restricted hema-

globlasts. Evidence for a role for Runx1 in vivo is most likely to be found in the P-Sp/AGM region of the embryo. These in vivo studies do support a role for Runx1 at this stage of development has been provided by North et al., who demonstrated that a functional gene is essential for the development of these hematopoietic cell clusters but not for the underlying endothelial cells. Runx1-dependent hematopoietic clusters were also found associated with the endothelial lining of the vitelline and umbilical arteries and with vessels in the yolk sac capillaries.

The function of the definitive hemangioblast in vivo would be first to generate the definitive precursors found early in the yolk sac and subsequently to establish intra-embryonic hematopoiesis in the P-Sp/AGM region of the embryo. These in vivo studies do support this model, but formal proof for the existence of a hemangioblast in the yolk sac or AGM will require clonal analysis.

In summary, the data presented in this report position Runx1 functionally at the earliest stages of hematopoietic and endothelial commitment in developing EBs. Expression of Runx1 in early EBs defines a subset of the Flk-1+/− population that contains most, if not all, BL-CFCs, the in vitro equivalent of the putative hemangioblast. Access to these early populations through Runx1 expression provides a unique opportunity to define the molecular events involved in the establishment of the primitive and definitive hematopoietic programs.

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