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Definitive Hematopoiesis Requires the *Mixed-Lineage Leukemia* Gene

Short Article

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Summary

The *Mixed-Lineage Leukemia* (*MLL*) gene encodes a Trithorax-related chromatin-modifying protooncogene that positively regulates *Hox* genes. In addition to their well-characterized roles in axial patterning, Trithorax and Polycomb family proteins perform less-understood functions in vertebrate hematopoiesis. To define the role of MLL in the development of the hematopoietic system, we examined the potential of cells lacking MLL. *Mll*-deficient cells could not develop into lymphocytes in adult *RAG-2* chimeric animals. Similarly, in vitro differentiation of B cells required MLL. In chimeric embryos, *Mll*-deficient cells failed to contribute to fetal liver hematopoietic stem cell/progenitor populations. Moreover, we show that aorta-gonad-mesonephros (AGM) cells from *Mll*-deficient embryos lacked hematopoietic stem cell (HSC) activity despite their ability to generate hematopoietic progeny in vitro. These results demonstrate an intrinsic requirement for MLL in definitive hematopoiesis, where it is essential for the generation of HSCs in the embryo.

Introduction

The development of the hematopoietic system in vertebrates occurs at several distinct anatomic sites during embryogenesis, providing populations that meet the needs of the rapidly developing embryo. During this dynamic developmental process, HSCs and progenitors with distinct properties are generated transiently (Godin and Cumano, 2002; Palis and Yoder, 2001). The potential utility of manipulating such pathways in normal and malignant hematopoiesis warrants a complete understanding of the genetic pathways involved in the generation and maintenance of HSCs. Genes found at sites of chromosomal translocations in human leukemias have coincided with genes critical for early steps of hematopoietic commitment (Elefanty et al., 1997; Orkin et al., 1999). Genes identified in this manner include *AML-1 (Runx-1/CBFα)*, *LMO-2 (rbtn-2, Ttg-2)*, *SCL/TAL-1*, and *TAN-1/Notch-1*, all of which have subsequently been shown to be critical for embryonic hematopoiesis in murine knock-out studies (Godin and Cumano, 2002; Kumano et al., 2003). Such analyses demonstrate that loss of these individual genes can block hematopoiesis, although the number of independent pathways that promote the development of blood lineages remains uncertain.

This study focuses on the role of MLL, a chromatin-modifying transcriptional regulator, in the development of definitive hemato-lymphoid system. The *MLL* gene (*Mixed-Lineage Leukemia* gene, also *ALL-1*, *HRX*) was identified at human chromosome segment 11q23 where it is frequently disrupted by chromosomal translocations in acute lymphoblastic leukemia of infants and therapy-induced myeloid leukemia (reviewed in Biondi et al., 2000). Gene expression profiling demonstrated that leukemias bearing an *MLL* translocation comprise a distinct disease characterized by the unique coexpression of early lymphoid progenitor-associated genes as well as myeloid-specific genes (Armstrong et al., 2002), suggesting immortalization of an early multipotent progenitor. The precise mechanism by which Trithorax group proteins act to maintain different target genes is not yet clear, nor is the range of tissue-specific *Mll*-dependent gene programs. *Mll* is expressed in most fetal and adult hematopoietic cell types throughout development, including stem cell and progenitor populations (Kawagoe et al., 1999; Phillips et al., 2000). When hematopoiesis was examined, reduced clonogenic capacity was demonstrated in *Mll*-deficient progenitors in the yolk sac (Hess et al., 1997) and fetal liver (Yagi et al., 1998). Using a different allele of *Mll*, enhanced clonogenic capacity in homozygous mutant embryoid bodies was reported (Fidanza et al., 1996). Although the exact cause of the early embryonic death of homozygous *Mll* mutants is uncertain, they exhibit pleiotropic defects in multiple tissues (Yagi et al., 1998; Yu et al., 1998, 1995). Consequently, the defects in hematopoeitic populations in such embryos may have been influenced by cell-extrinsic factors.

To assess *Mll*-dependent functions intrinsic to hematopoietic lineages, we analyzed chimeric fetal and adult animals reconstituted with *Mll*-deficient or heterozygous embryonic stem (ES) cells. In this context, we demonstrated a cell-intrinsic requirement for MLL in the generation of lymphoid and myeloid populations in adult animals, and found a surprisingly stringent requirement for MLL in generating fetal HSC/progenitor populations. The lack of *Mll*-deficient HSC/progenitor populations in the fetal liver is preceded by a deficiency in HSCs in the AGM region, an intraembryonic site in which definitive HSCs are localized (reviewed in Godin and Cumano, 2002). These results demonstrate that MLL is required during embryogenesis for the specification or expansion of hematopoietic stem cells.

Results

Lymphopoiesis in the Absence of MLL

To examine lymphopoiesis in the absence of MLL in vivo, independent ES clones ($Mll^{+/+}$, $Mll^{+/-}$, and

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Mll$^{-/-}$, Supplemental Figures S1 and S2 [http://www.developmentalcell.com /cgi /content /full /6/3/437/DC1]) were injected into *RAG-2*-deficient blastocysts (Chen et al., 1993). *Mll*$^{+/-}$ ES cells developed into B220^{+}IgM^{+} B lymphocytes in the spleen (Figure 1A) as well as in the lymph nodes, peripheral blood, and peritoneum (data not shown). In contrast, no B220^{+} cells were present in spleens of *Mll*$^{-/-}$ chimeras (Figure 1A). Using a stromal coculture system, *Mll*$^{-/-}$ ES clones yielded a brief wave of erythropoiesis, but no B lymphopoiesis (Figure 2 and Supplemental Figure S4). An analysis of developing T cells demonstrated that *Mll*$^{+/-}$ ES cells give rise to CD4^{+}CD8^{+} double positive (DP) thymocytes, as well as CD4^{+} and CD8^{+} single positive (SP) thymocytes. In contrast, *Mll*$^{-/-}$ ES clones produced neither SP nor DP thymocytes (Figure 1B). These results demonstrate a cell-intrinsic block in lymphopoiesis when competition with host lymphocytes is eliminated (as in the *RAG-2* chimeras) or even when competition with progenitors is eliminated (as in the OP9 cocultures).

The complete absence of B and T cells in *Mll*$^{-/-}$ chimeras prompted us to examine other hematopoietic populations in MLL-deficient chimeras using the pan-leukocyte, strain-specific marker Ly9.1 (Miller et al., 1985). Chimeras generated with *Mll*$^{+/+}$ ES cells exhibited a mixture of ES-derived (Ly9.1^{+}DX5^{+}, boxed) and host-derived (Ly9.1^{neg}DX5^{+}) NK cells, whereas NK cells were not produced from *Mll*$^{-/-}$ ES cells (Figure 1C). Similarly, *Mll*$^{-/-}$ ES clones did not contribute to peritoneal macrophage populations (data not shown). Collectively, these results indicate that the multilineage block observed in adult MLL-deficient chimeras is due to a requirement for MLL at a multipotent progenitor or stem cell stage.

Analysis of Fetal Liver Chimeras Identifies an Early *Mll*-Dependent Step in Hematopoiesis

To more easily assess progenitor and HSC populations in vivo, we analyzed fetal hematopoiesis in chimeras generated from *lacZ* marked *Mll*$^{+/-}$ and *Mll*$^{-/-}$ ES clones (Supplemental Figure S2) injected into wild-type blastocysts. Using the ES strain-specific Ly9.1 cell surface marker, we found that *Mll*-deficient ES clones did not contribute to any lineage-positive (Gr-1^{+}, CD11b^{+}, B220^{+}, CD19^{+}, DX5^{+}, or CD3^{+}, data not shown) cells in embryonic day (E) 14–15 chimeras. Therefore, we analyzed individual fetal livers from chimeric embryos for contribution to the lineage-negative, c-Kit-positive pool that includes both HSC and progenitor populations (Morrison et al., 1995; Traver et al., 2001). To determine overall chimerism, we incubated embryos with X-Gal to reveal cells of ES origin (Figures 2E and 2F). Embryos derived from *Mll*$^{+/-}$ ES cell injections varied in their degree of chimerism, but always gave rise to lineage-negative, c-Kit^{+}/Ly9.1^{+} cells in proportion to the degree of chimerism (Figure 2E). In sharp contrast, *Mll*-deficient chimeras exhibited background levels of lineage-negative, c-Kit^{+} ES-derived cells, despite high levels of overall chimerism (Figure 2F).

These data demonstrate a cell-autonomous requirement for the *Mll* gene in fetal liver hematopoiesis. Given the stringency of this requirement, it is possible that *Mll*-deficient HSCs either fail to populate the fetal liver rudiment or may arrive but fail to expand competitively. We next analyzed *Mll*-deficient embryos to determine whether definitive HSCs were produced prior to the fetal liver stage of hematopoiesis.

Pre-Liver, Intraembryonic Hematopoiesis in the Absence of MLL

The precise origin of definitive HSCs that seed the fetal liver is not completely resolved (Godin and Cumano, 2002; Palis and Yoder, 2001). Using appropriate recipients, several hematopoietic populations including the AGM region have been shown to harbor HSCs (Cumano et al., 1996; Muller et al., 1994; Rodewald et al., 1994; Yoder et al., 1997). Transplantation of AGM-derived cells can serve as an important functional test for HSC activity in embryos exhibiting early lethality (Cai et al., 2000; Kumano et al., 2003). Therefore, to assess HSCs prior to the fetal liver stage of hematopoiesis, we performed a phenotypic and functional analysis of AGM cells from *Mll*$^{-/-}$ embryos.

The peak of HSC activity in the AGM occurs at E11–11.5, and can be enriched by sorting for cells that express c-Kit, CD34, CD45, PECAM/CD31, or *Runx-1* (Delassus et al., 1999; Kumaravelu et al., 2002; North et al., 2002; Sanchez et al., 1996). AGM cells from *Mll*-deficient embryos exhibited a reduction in many of these markers relative to *Mll*$^{+/+}$ littermate controls, although c-Kit^{+} cells were not reduced overall (Figure 3A). *Runx-1* expression in the dorsal aorta, as detected by in situ hybridization, was reduced, yet the endothelium appeared otherwise intact (Figures 2B–2G).

In short-term explant cultures, we found that *Mll*-deficient E11.5 AGM cells consistently produced some CD45^{+} hematopoietic cells that differentiated into macrophages, whereas wild-type cultures produced a range of myeloid cell types in these culture conditions (Figure 4A). To determine whether HSCs were produced in MLL-deficient embryos, we transplanted E11.5 AGM cells into sublethally irradiated *RAG*γC$^{-/-}$ recipients. AGM cells derived from *Mll*$^{+/+}$ littermates efficiently reconstituted most recipients (75%) and persisted for greater than 6 months. In contrast, none of the recipients of *Mll*$^{-/-}$ AGM cells were significantly reconstituted (Figure 4B). Donor-derived cells in *Mll*$^{-/-}$ AGM-engrafted recipients could not be detected within erythroid, myeloid, or lymphoid populations of peripheral blood (Supplemental Figure S5 and data not shown). Unexpectedly, only two of nine recipients engrafted with *Mll*$^{+/-}$ AGM cells yielded any detectable donor contribution (Figure 4B). One of these two cases exhibited transient reconstitution and the other exhibited low-level reconstitution (Supplemental Figure S5). Thus, *Mll* haploinsufficiency is incompatible with the generation of HSCs at normal frequencies in the E11.5 AGM. Together, these data indicate that MLL deficiency blocks the generation of functional HSCs in the AGM.

Discussion

In the present study, we demonstrate a cell-intrinsic requirement for MLL in the development of definitive hematopoietic cells. Using chimeric animals, we show that *Mll*-deficient cells fail to generate adult lymphoid and myeloid cells, and that even fetal HSC/progenitor

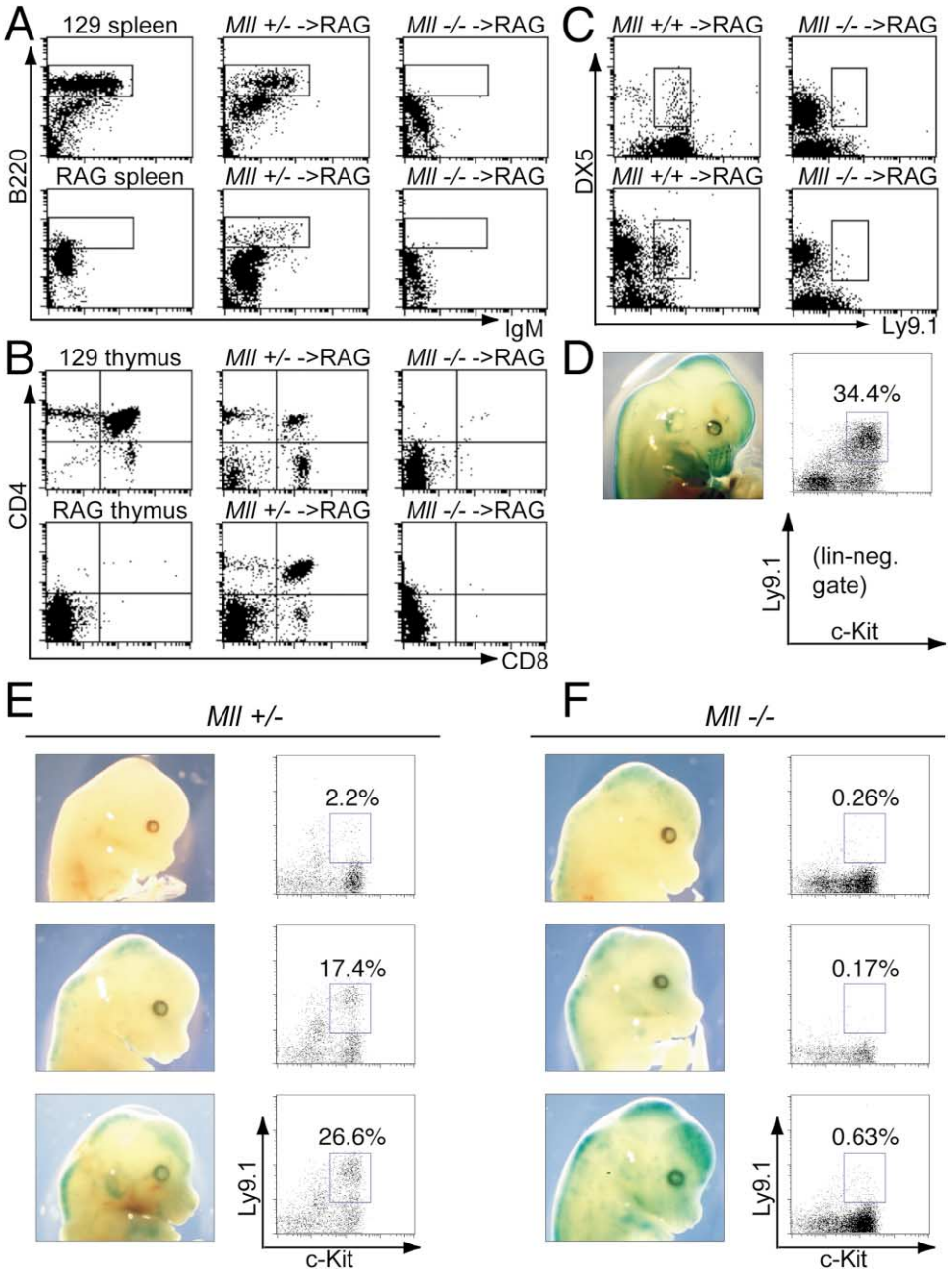


Figure 1. MLL-Deficient ES Cells Fail to Reconstitute Hematopoietic Organs in Chimeric Animals

(A) Spleen cells of two representative mice for control (*Mll*$^{+/-}$→RAG) or *Mll*-deficient (*Mll*$^{-/-}$→RAG) chimeras were analyzed by flow cytometry. Control 129SvJ strain (wild-type) and *RAG-2* animals were analyzed in parallel (left panels).

(B) Cells from the thymus of two representative chimeras were analyzed as in (A) using the indicated antibodies.

(C) Spleen cells of two representative control (*Mll*$^{+/+}$→RAG) or *Mll*-deficient (*Mll*$^{-/-}$→RAG) chimeras were analyzed by flow cytometry to reveal ES-derived NK cells (boxed). All experiments are representative of more than 40 chimeric animals (based on coat color and PCR-positive tail biopsies) analyzed per genotype.

(D) Control *Mll*$^{+/-}$ embryo at E14.5 stained with X-Gal to demonstrate maximum *lacZ* expression, and control flow cytometry analysis of wild-type 129SvJ E14.5 fetal liver. Fetal liver cells were analyzed by excluding lineage-positive cells using a cocktail of lineage-specific antibodies (Supplemental Data) and the remaining cells are shown stained with Ly9.1 (*y* axis) and c-Kit (*x* axis). The percentage of lineage-negative cells that were c-Kit^{+}/Ly9.1^{+} (boxed) is shown.

(E) Three individual chimeric embryos generated with *Mll*$^{+/-}$ ES cells are shown next to results of flow cytometry performed as described above.

(F) Three individual chimeric embryos generated with *Mll*$^{-/-}$ ES cells, and flow cytometry results as in (D). The embryos shown are representative of the range of chimerism observed in all experiments.

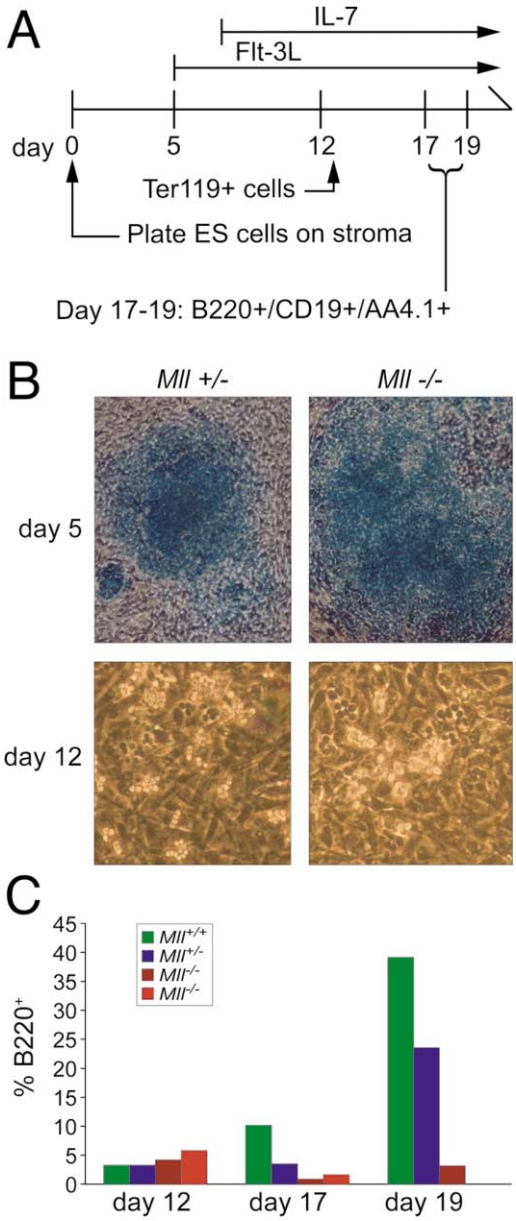


Figure 2. OP9 Coculture Demonstrates Cell-Autonomous Block in B-Lymphopoiesis from *Mll*-Deficient ES Cells

(A) Scheme and factors used for differentiating ES cells to B cells. (B) Phase-contrast image of mesodermal (top panels, 40× magnification) and hematopoietic colonies (lower panels, 100× magnification). Cultures were fixed and stained with X-Gal to reveal mesodermal colonies at day 5, or photographed live at day 12. Most suspension cells were Ter119$^+$ at day 12 (Supplemental Figure S4). (C) Time course analysis of B220-positive cells produced in coculture experiments. Cultures from two independent *Mll*$^{-/-}$ ES clones are shown (dark and light red bars), one *Mll*$^{+/-}$ ES clone (blue), and wild-type ES cells (green). Data from one representative experiment of three are shown. Control (*Mll*$^{+/+}$) cells were ~60% B220$^+$AA4.1$^+$CD19$^+$ (Supplemental Figure S4).

pools cannot develop in the absence of MLL. Moreover, the deficit of HSC activity within both the *Mll*$^{+/-}$ and *Mll*$^{-/-}$ AGM indicates that a critical threshold level of MLL is required for the development of normal numbers of HSCs in the embryo.

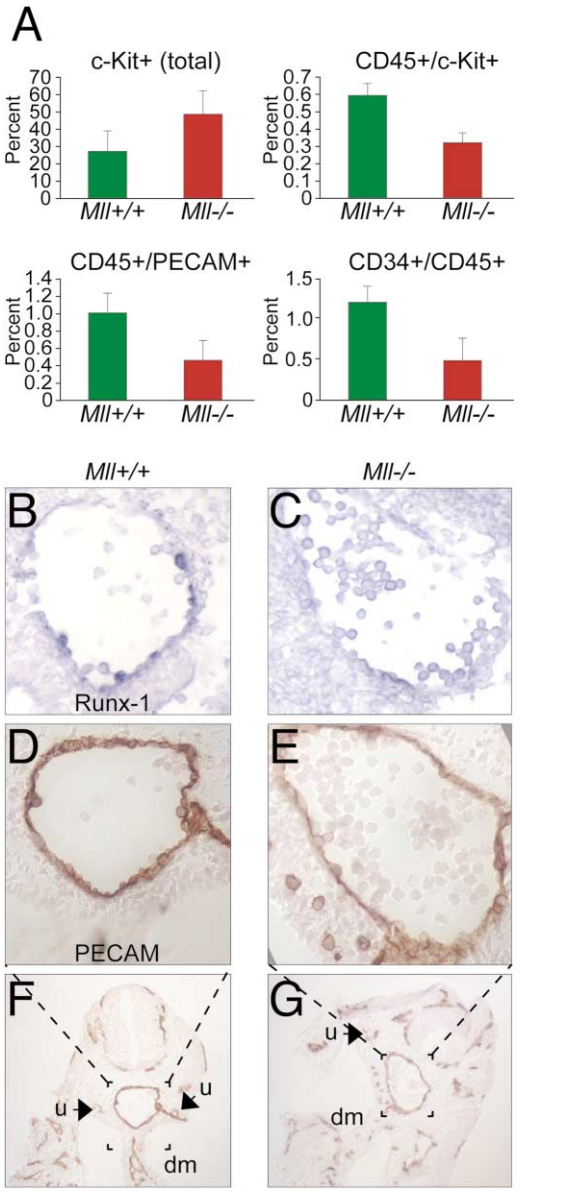
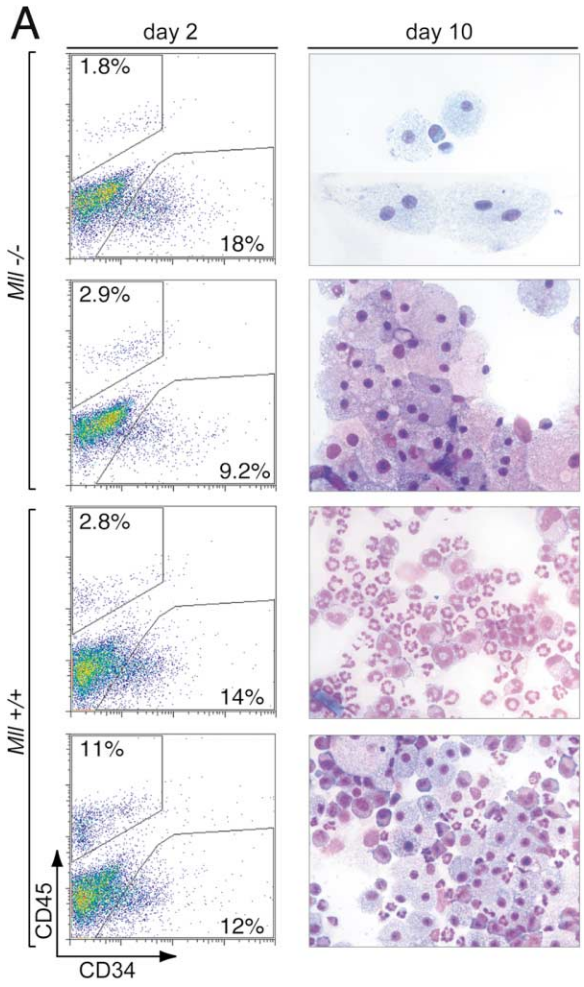


Figure 3. Phenotypic and Functional Consequences of MLL Deficiency in Cells of the AGM

(A) Cell populations in dissociated E11.5 AGM cells were quantified by flow cytometry. Individual AGM regions were dissected, dissociated, and analyzed by flow cytometry (see Experimental Procedures). Values shown are averages of two to five individual embryos within a single litter. Results were reproduced using at least three different litters.

(B and C) In situ hybridization revealing *Runx-1* transcripts in the dorsal aorta of the AGM region of *Mll*$^{+/+}$ (B) and *Mll*$^{-/-}$ (C) E10.5 embryos photographed at 600× magnification.

(D–G) Immunohistochemistry using anti-PECAM/CD31 antibodies on adjacent sections of *Mll*$^{+/+}$ (D and F) or *Mll*$^{-/-}$ (E and G) embryos. The low-power view of the embryo section is shown in (F) and (G) for orientation. Dorsal mesentery of the hindgut (dm), urogenital ridges (u), and mesonephric tubules (arrows) are labeled. All results were reproduced using multiple sections from at least two embryos per genotype.



B Reconstitution of *RAG*γC-/- Recipients

embryo genotype	reconstituted/transplanted
Mll -/-	0/8 (0%)
Mll +/-	2/9 (22%)
Mll +/+	6/8 (75%)

Figure 4. *Mll*-Deficient AGM Cells Can Generate Hematopoietic Progeny but Lack HSC Activity

(A) Results of short-term explant cultures. Intact AGM from E11.5 embryos were plated on OP9 monolayers. After 2 days of culture, cells were trypsinized and allowed to differentiate 2 additional days with half analyzed by flow cytometry (left column) using CD45 (*y* axis) and CD34 (*x* axis) antibodies. After 8 additional days, modified Wright-Giemsa staining of suspension cells was performed (right column). Data shown are representative of embryos from four different litters.

(B) Reconstitution of *RAG*γ$C^{-/-}$ recipients with wild-type and *Mll*-deficient AGM cells. AGM cell suspensions were prepared from E11.5 embryos and were injected intravenously into sublethally irradiated hosts. All wild-type AGM-reconstituted animals exhibited long-term (>20 week) persistence of donor-type cells in at least three lineages.

While short-term in vitro assays reveal that some myeloid and erythroid differentiation can occur in the absence of MLL, our in vivo approaches found no contribution of *Mll*-deficient cells to myeloid or erythroid populations in either chimeric animals or recipients of AGM cells. The striking defect in HSC activity in vivo, in concert with these observations, place the block in definitive hematopoiesis at the development of the HSC itself and/or in the transition from HSC to multipotent progenitor.

Comparison of the phenotype described here to other loss-of-function models of genes critical for embryonic hematopoiesis positions *Mll* within a select set of genes required for all definitive blood lineages in the embryo. Disruption of the *c-Myb* (Sumner et al., 2000), *Lmo-2* (Yamada et al., 1998), *Gata-2* (Tsai et al., 1994), *Runx-1* (Okuda et al., 1996), or *SCL* gene (Porcher et al., 1996) results in a cell-intrinsic block in all definitive blood lineages when tested in blastocyst chimeras, as shown here for *Mll*. The *Lmo-2*-, *SCL*-, *Notch-1*-, and *GATA-2*-deficient embryos exhibit earlier lethality than the *Mll* mutants reported here, possibly due to more severe effects on vasculogenesis or embryonic erythropoiesis. *Mll*- and *Runx-1*-deficient embryos both lack fetal liver hematopoiesis with primitive erythropoiesis remaining relatively unaffected (Hess et al., 1997; Okuda et al., 1996; Wang et al., 1996; Yagi et al., 1998; this study). Furthermore, *Mll*- and *Runx-1*-heterozygous embryos both exhibit defects in the generation of HSC in the E11.5 AGM although the temporally disordered development of HSCs in the *Runx-1*$^{+/-}$ embryo contrasts with the consistent reduction in HSC or progenitor numbers in the *Mll*$^{+/-}$ embryo (Cai et al., 2000; Hess et al., 1997; Yagi et al., 1998; this study). Further experiments will clarify the relationships between MLL-dependent gene programs and those of other genes in this group.

Mll regulates the expression of several *Hox* genes including *Hoxa7*, *a9, a10, c6, c8*, and *c9* (Hanson et al., 1999; Nakamura et al., 2002; Yagi et al., 1998; Yu et al., 1998, 1995). Both loss- and gain-of-function studies have indicated that *Hox* genes play important roles in regulating HSC numbers, progenitor expansion, and lineage choice (Buske and Humphries, 2000). Thus, one explanation for the severe block in definitive hematopoiesis in *Mll*-deficient animals is that a particular subset of critical *Hox* genes is under- or mis-expressed at an early stage of blood development. Expression of select *Hox A*, *B*, and *C* genes has been reported in early bone marrow and fetal hematopoietic populations (Kawagoe et al., 1999; McGrath and Palis, 1997; Pineault et al., 2002). To date, no individual or compound *Hox* gene knockout recapitulates the block in definitive hematopoiesis observed in *Mll* mutants, suggesting that the *Mll*-dependent hematopoietic program will prove to be a broader set of target genes, or a specific subset of *Hox* genes.

Experimental Procedures

Embryonic Stem Cell Culture and Differentiation

ES cells were maintained on mitomycin-C inactivated feeder layers in DMEM containing 200 mM nonessential amino acids, 200 mM L-glutamine, 10 U/ml penicillin G, 10 μg/ml streptomycin (Invitrogen), 100 μM 2-ME (Sigma), 15% fetal bovine serum (Hyclone), and

400 U/ml ESGRO (Chemicon). Targeting details are described in the Supplemental Data). OP9 monolayers were maintained and used to initiate differentiation of ES cells as described (Cho et al., 1999). Murine recombinant Flt-3 ligand and IL-7 were used at 20 ng/ml and 5 ng/ml, respectively; both were purchased from R&D Systems.

Mouse and Embryo Analyses

Flow cytometry was performed using standard methods with a Becton Dickinson FACSCalibur and FlowJo software. *RAG-2* chimeras were analyzed between 4 and 8 weeks of age. Embryo age was defined as 0.5 dpc at 8 a.m. the day of vaginal plug observation. *LacZ* expression in chimeric embryos was revealed by fixing the embryos in 2% paraformaldehyde/0.2% glutaraldehyde/PBS for 1 hr at 4°C, washing the embryo in several changes of X-Gal wash solution (0.1 M phosphate buffer [pH 7.3], 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% NP-40), and then incubating the embryo in X-Gal substrate solution (X-Gal wash solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml X-Gal) at 37°C for 2–6 hr. Embryos were post-fixed in 4% paraformaldehyde/PBS for 1 hr at 4°C and photographed at 7× magnification. AGM regions of E11.5 embryos were dissected as diagrammed (Medvinsky et al., 1996) and incubated in culture for 1–4 hr before transplantation. Single-cell suspensions were prepared by digestion with 0.2% collagenase (Boehringer) for 30 min at 37°C, or by mechanical dissociation with a 26G syringe in Hank's Balanced Salt Solution (Invitrogen) containing 2% fetal bovine serum. Cell suspensions were injected intravenously into 5- to 8-week-old *RAG*$\gamma C^{-/-}$ animals (Taconic) irradiated with a single dose of 450 cGy from a ^{137}Cesium source. For short-term culture experiments, the intact AGM was placed on an OP9 stromal layer in IMDM (Invitrogen) containing 50 ng/ml SCF, 10 ng/ml IL-6, and 10 ng/ml IL-3 (R&D Systems). After 2 days of explant culture, all cells were trypsinized and replated in six-well dishes. Cells were then passaged as necessary. Cytospin preparations were performed with a Cytospin 3 cytocentrifuge (Shandon) for 4 min at 700 rpm and cells were fixed and stained with Dip-Quick (Jorgensen Laboratories).

In Situ Hybridization and Immunohistochemistry

E10–11.5 embryos were fixed in 4% paraformaldehyde in phosphate buffer (pH 7.2) 4–16 hr, washed in PBS, equilibrated in 15% sucrose/phosphate buffer, and then frozen in OCT medium (Tissue-Tek). Eight to twelve micron sections were cut using a Reichert-Jung Cryocut 1800 and air-dried for >1 hr on Superfrost Plus slides (Fisher Scientific). Nonradioactive in situ hybridization with *Runx-1* probe was performed as described (Birren et al., 1993). Immunohistochemistry was performed with rat anti-CD31 and developed using an anti-rat Vectastain kit (Vectastain) and Nova Red substrate (Vector Labs). Sections were photographed at 600× total magnification (Figures 3B–3E) or 40× magnification (Figures 3F and 3G) with a Nikon Eclipse 6000 microscope using a Spot digital camera.

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