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An Unexpected Role for IL-3 in the Embryonic Development of Hematopoietic Stem Cells

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Summary

Cytokines are important in adult hematopoiesis, yet their function in embryonic hematopoiesis has been largely unexplored. During development, hematopoietic stem cells (HSCs) are found in the aorta-gonad-mesonephros (AGM) region, yolk sac (YS), and placenta and require the Runx1 transcription factor for their normal generation. Since *IL-3* is a Runx1 target and this cytokine acts on adult hematopoietic cells, we examined whether IL-3 affects HSCs in the mouse embryo. Using *Runx1* haploinsufficient mice, we show that IL-3 amplifies HSCs from E11 AGM, YS, and placenta. Moreover, we show that IL-3 mutant embryos are deficient in HSCs and that *IL-3* reveals the presence of HSCs in the AGM and YS prior to the stage at which HSCs are normally detected. Thus, our studies support an unexpected role for IL-3 during development and strongly suggest that IL-3 functions as a proliferation and/or survival factor for the earliest HSCs in the embryo.

Introduction

Hematopoietic stem cells (HSCs) are rare self-renewing, multipotential cells localized in the bone marrow (BM) of the adult. HSCs extensively proliferate and differentiate into myeloid and lymphoid lineages to constantly replenish mature blood cells. In the adult, many growth factors regulate hematopoietic processes during homeostasis and trauma. Among these is IL-3, one of the earliest identified cytokines (Ihle et al., 1981). Extensive in vivo and in vitro studies have implicated IL-3 in the survival, proliferation, and differentiation of hematopoietic progenitors/stem cells and also mature hematopoietic cells (Ihle et al., 1983; Metcalf et al., 1986). IL-3 is produced by activated T and NK cells, keratinocytes, some myeloid, stromal, neuronal, and microglia cells. IL-3 receptors are expressed mainly by hematopoietic cells (e.g., HSCs, myeloid cells, B cells), although their expression is also found in testis, placenta, and brain (Morikawa et al., 1996). Most species express one receptor for IL-3, formed by the IL-3Rα chain (Gorman et al., 1990) and the common β chain (βc, shared with IL-5 and GM-CSF receptors). A second IL-3 receptor is expressed only in the mouse and is formed by the $\beta_{IL\text{-}3}$ chain in association with the IL-3Rα chain (Gorman et al., 1992).

The effect of IL-3 on adult HSCs remains controversial. IL-3 has been shown both to significantly increase (Bryder and Jacobsen, 2000) and decrease (Peters et al., 1996; van der Loo and Ploemacher, 1995; Yonemura et al., 1996) the expansion and/or self-renewal capacity of adult HSCs. The seemingly contradictory effects of IL-3 are most likely influenced by its concentration, the presence/absence of other cytokines, serum, culture conditions, tested cell population, etc. Surprisingly, the targeted disruption of the *IL-3* (Lantz et al., 1998) or *IL-3 receptor* genes (Nishinakamura et al., 1995, 1996; Robb et al., 1995) in mice revealed only very minor alterations of adult hematopoiesis. Hence, the role of IL-3 was not further explored during embryonic HSC development.

HSC development in the mouse embryo is of intense current interest. The first adult-type HSCs are autonomously produced at mouse embryonic day (E) 10.5 (>35 somite pairs, sp) in the intraembryonic AGM (aorta-gonad-mesonephros) region (Medvinsky and Dzierzak, 1996; Muller et al., 1994). During midgestation, HSCs are also found in the vitelline and umbilical arteries (V/U) (de Bruijn et al., 2000), vascular labyrinth of the placenta (Gekas et al., 2005; Ottersbach and Dzierzak, 2005), yolk sac (YS), and fetal liver (FL). The Runx1 transcription factor is expressed in all these embryonic sites (North et al., 1999) and is essential for the embryonic development of HSCs (Okuda et al., 1996; Wang et al., 1996). *Runx1*$^{-/-}$ embryos die at E12/13 with a complete lack of AGM and FL definitive hematopoiesis. Haploinsufficiency of *Runx1* results in reduced adult-repopulating ability and alters the temporal and spatial distribution of HSCs (Cai et al., 2000). Although several target genes of Runx1 have been identified in hematopoietic cells (for review, see Otto et al., 2003) and include the hematopoietic cytokine genes *IL-3* (Mao et al., 1999; Uchida et al., 1997) and *GM-CSF* (Takahashi et al., 1995), these cytokines have not been tested for their effects on embryonic HSCs. Hence, we examined whether IL-3 and other cytokines play a role in embryonic HSC development.

We show here, by using the *Runx1* haploinsufficient mouse model, that among the tested cytokines, only IL-3 rescues *Runx1*$^{+/-}$ AGM HSC numbers. IL-3, which is expressed in the major embryonic vessels, acts as a survival and proliferation factor for E11 AGM HSCs. It also amplifies HSCs from E11 YS and placenta. Blocking experiments and transplantation studies with *IL-3* mutant embryos reveal a role for IL-3 in the development of HSCs and demonstrate that IL-3 acts on HSCs at the earliest stages of their emergence in the mouse embryo.

Results

HSC Numbers Are Reduced in *Runx1*$^{+/-}$ AGMs

We have shown previously that *Runx1*$^{+/-}$ embryos suffer from defects in the temporal and spatial emergence and/or maintenance of HSCs (Cai et al., 2000). To more

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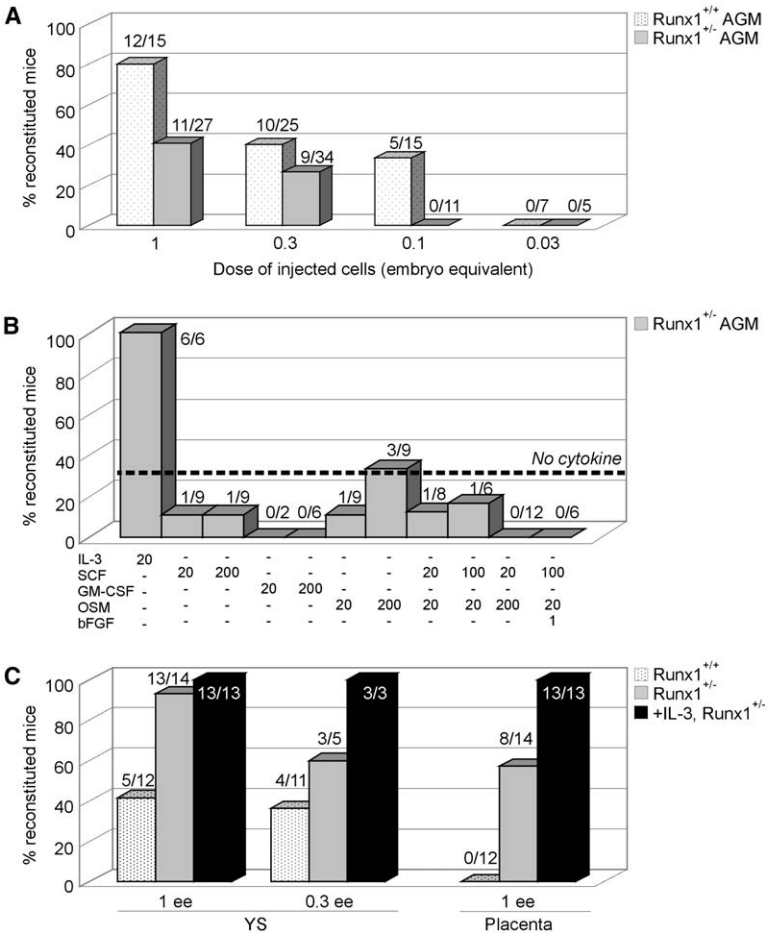

Figure 1. IL-3 Increases HSC Activity in E11 *Runx1*$^{+/-}$ Tissue Explants

(A) Percentage of mice reconstituted with injected donor cells (1, 0.3, 0.1, or 0.03 embryo equivalent [ee]/mouse) from E11 *Runx1*$^{+/+}$ or *Runx1*$^{+/-}$ AGM explants (n = 7). Donor chimerism was determined 4 months after transplantation by semiquantitative PCR. Numbers above each bar represent "number of reconstituted mice/total number transplanted."

(B) Percentage of mice reconstituted 4 months posttransplantation with donor cells (0.3 ee) from *Runx1*$^{+/-}$ AGM explants cultured with cytokines (ng/ml): interleukin (IL)-3 (n = 3), stem cell factor (SCF) (n = 7), granulocyte macrophage-colony stimulating factor (GM-CSF) (n = 4), oncostatin M (OSM) alone or in combination with SCF and/or basic fibroblast growth factor (bFGF) (n = 3). The hatched line indicates the percentage of mice reconstititued with *Runx1*$^{+/-}$ AGM explants cultured in the absence of added cytokine (0.3 ee).

(C) Percentage of mice reconstituted 4 months posttransplantation with donor cells (0.3 or 1 ee) from E11 *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ yolk sac (YS) (n = 7) and placenta explants (n = 5) cultured in the absence or presence of IL-3 (200 ng/ml).

precisely evaluate the HSC defects observed in *Runx1*$^{+/-}$ AGMs, we performed limiting dilution in vivo transplantations of cells from cultured E11 *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ AGM explants (described in Medvinsky and Dzierzak, 1996). At greater than 4 months posttransplantation, the percentage of reconstituted recipients is reduced in the groups receiving *Runx1*$^{+/-}$ AGM explant cells as compared to *Runx1*$^{+/+}$ cells (Figure 1A). The frequency of HSCs per *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ AGM was determined by Poisson statistics (Figure S1A and S1B, no cytokine), and the absolute number of HSCs was calculated to be 1.7 HSCs per *Runx1*$^{+/+}$ AGM and 0.5 HSC per *Runx1*$^{+/-}$ AGM (Table 1). Thus, *Runx1*$^{+/-}$ E11 AGM explants contain 3.4-fold fewer HSCs.

IL-3 Addition to *Runx1*$^{+/-}$ AGM Explants Rescues the HSC Deficiency

Since two known downstream targets of Runx1 are the cytokine genes, *IL-3* and *GM-CSF*, our hypothesis concerning the *Runx1*$^{+/-}$ AGM hematopoietic deficiency was that these factors are important for the emergence, survival, and/or expansion of HSCs and are not optimally expressed in *Runx1*$^{+/-}$ embryos. To test this, *Runx1*$^{+/-}$ AGM explants were cultured in the presence of IL-3 or GM-CSF (20–200 ng/ml) and HSC activity was assayed by in vivo transplantation. We also examined the effects of other cytokines that are known either to act synergistically or to induce the in vitro endothelial/hematopoietic differentiation of AGM cells (Mukouyama et al., 1998). As shown in Figure 1B, the addition of GM-CSF, SCF, OSM, and/or bFGF did not increase *Runx1*$^{+/-}$ AGM HSC activity when compared to the control (no cytokine, 26%). Some conditions induced a negative effect

Table 1. Frequencies and Numbers of HSCs per *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ AGM Explants Cultured in the Absence or Presence of Different Doses of IL-3

IL-3	*Runx1*$^{+/+}$			*Runx1*$^{+/-}$		
(ng/ml)	f_{HSCs}[a]	HSCs/AGM[b]	AF[c]	f_{HSCs}	HSCs/AGM	AF
0	1/165709	1.7	—	1/497126	0.5	—
2	1/49713	5.9	3.5	1/49713	5.0	10
20	1/19885	14.7	8.6	1/16571	14.9	29.8
200	1/4971	58.6	34.5	1/14204	17.4	34.8

Runx1$^{+/+}$ and *Runx1*$^{+/-}$ AGM explants were cultured for 3 days prior to transplantation in the absence (0) or in the presence of 2, 20, or 200 ng/ml of IL-3.

[a] Limiting dilution transplantations were performed (see Figure 1A and Table S1), and Poisson statistics (Figure S1) was used to calculate the frequencies (f) of HSCs.

[b] Number of HSCs per AGM was calculated by multiplying the f_{HSCs} with the average number of total cells per AGM. The number of cells/AGM is $2.9 \times 10^5 \pm 0.8$ (n = 13) and $2.5 \times 10^5 \pm 0.8$ (n = 10) for *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ embryos, respectively. When cultured in the presence of IL-3, the number of cells/AGM is $2.9 \times 10^5 \pm 1.0$ (n = 10) and $2.5 \times 10^5 \pm 0.7$ (n = 10) for *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ embryos, respectively.

[c] AF is the amplification factor of HSCs in the explant cultures performed in the presence of IL-3 compared to no IL-3.

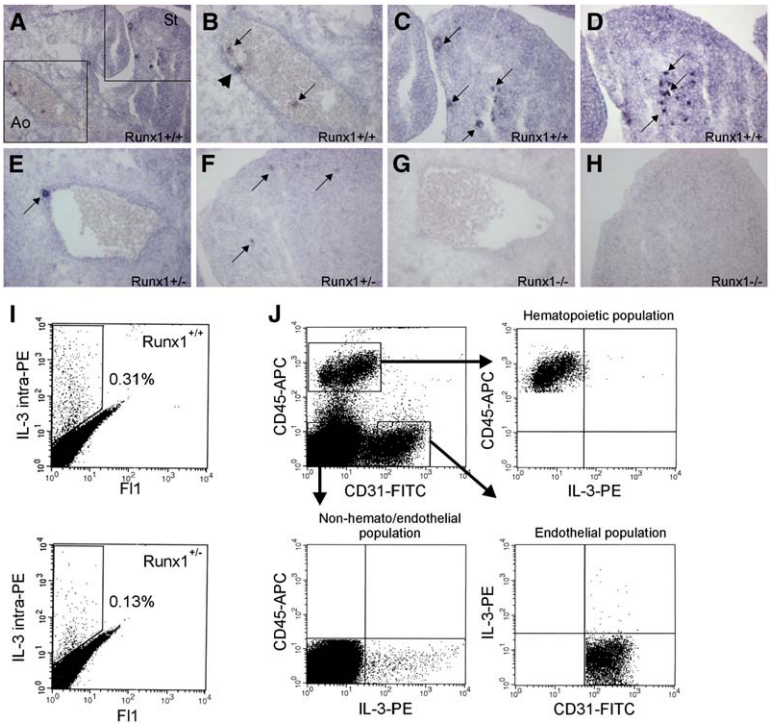

Figure 2. IL-3 Expressing Cells in E11 AGMs

IL-3 specific in situ hybridization on cryosections from E11 *Runx1*$^{+/+}$ (A–D), *Runx1*$^{+/-}$ (E and F), and *Runx1*$^{-/-}$ (G and H) embryos. Sections are at the level of the intersection of the aorta with the vitelline artery. (B), (E), and (G) show the dorsal aorta (Ao), and (C), (D), (F), and (H) show the developing stomach (St). (A) Dorsal aorta boxed-in bottom area is shown in (B), and developing stomach boxed-in top area is shown in (C). Arrows highlight IL-3 expressing cells in the lumen (arrow) and attached to the Ao endothelium (arrow head) and also disseminated in the St (arrows). As a positive control, cytospins of WEHI3 cells were used (not shown). IL-3 sense mRNA probe showed no signal (not shown). Magnification 20× (A) and 40× (B–H). (I) Flow cytometric plots of cells from *Runx1*$^{+/+}$ (top dot plot) and *Runx1*$^{+/-}$ (bottom dot plot) AGM explants intracytoplasmically stained with anti-IL-3 antibodies. Percentage of positive cells is indicated. Representative plots are shown (n = 3). (J) Flow cytometric plots of *Runx1*$^{+/+}$ AGM explant cells showing IL-3 expression in hematopoietic (CD45^{+}CD31^{-}), endothelial (CD45^{-}CD31^{+}), and nonhemato/nonendothelial (CD45^{-}CD31^{-}) populations. Representative data are shown (n = 2).

(SCF and GM-CSF at 20 or 200 ng/ml, OSM at 20 ng/ml alone or with SCF and/or bFGF). While OSM at high concentration (200 ng/ml) had a slight positive effect (33%), the only cytokine able to significantly increase the HSC activity was IL-3. At a low concentration of IL-3 (20 ng/ml), repopulation by *Runx1*$^{+/-}$ cells was found in six out of six mice transplanted. Limiting dilution transplantation of *Runx1*$^{+/-}$ E11 AGM explants (0.03, 0.1, 0.3, and 1 ee) cultured with 2, 20, or 200 ng/ml of IL-3 revealed that HSC rescue was IL-3 dose dependent (Table S1). These HSCs provide high-level multilineage engraftment (T and B lymphoid and myeloid lineages) in primary recipients and self-renew, as demonstrated by secondary transplantation (Figure S2A). HSC numbers in *Runx1*$^{+/-}$ explants cultured in 2 ng/ml IL-3 surpassed the number of HSCs in *Runx1*$^{+/+}$ explants cultured without IL-3 (Table 1 and Figures S1A and S1B). In the presence of increasing concentrations of IL-3, both *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ explants amplify HSC numbers, reaching a 35-fold increase as compared to starting numbers. However, since the starting numbers of *Runx1*$^{+/-}$ HSCs are at least 3-fold lower than in *Runx1*$^{+/+}$ AGMs, the total number of *Runx1*$^{+/-}$ HSCs after IL-3 amplification remains 3-fold lower than in *Runx1*$^{+/+}$ AGMs (Table 1). Thus, IL-3 acts in a dose-dependent manner to amplify HSC activity in *Runx1*$^{+/-}$ AGM explants, rescuing this activity to levels achieved by *Runx1*$^{+/+}$ AGM explants in the absence of exogenous IL-3.

Effects of Runx1 Deficiency and Exogenous IL-3 on Yolk Sac and Placenta HSCs

To evaluate if *Runx1* haploinsufficiency affects HSCs in the other major hematopoietic tissues, we performed explant culture and transplantation experiments with E11 *Runx1*$^{+/-}$ YS and placenta. In contrast to the results of AGM transplantations, *Runx1*$^{+/-}$ YS explants resulted in a higher percentage of reconstituted recipients than *Runx1*$^{+/+}$ YS explants (Figure 1C) (Cai et al., 2000). The absolute number of HSCs was at least 4-fold increased in *Runx1*$^{+/-}$ YS as compared to *Runx1*$^{+/+}$ YS (Figure S1C). Also, HSCs were increased in *Runx1*$^{+/-}$ as compared to *Runx1*$^{+/+}$ placenta explants (Figure 1C). Thus, while *Runx1*$^{+/-}$ embryos are deficient in AGM HSCs, the YS and placenta contain more HSCs than normal. These Runx1 related changes suggest that a full dose of Runx1 suppresses HSC activity in the YS and placenta (but not in the AGM) and downstream targets may differ between tissues.

To test if the growth effect of IL-3 was restricted to the HSCs within the AGM, we performed transplantations of cells from E11 *Runx1*$^{+/-}$ YS and placenta explants cultured in the presence of IL-3. The percentage of reconstituted recipients was increased (to 100%) in the groups receiving YS and placental cells cultured with IL-3 (Figure 1C) as compared to those cultured without IL-3. A similar increase was observed for *Runx1*$^{+/+}$ YS and placental explant cells cultured with IL-3 (data not shown). Thus, similar to AGM HSCs, IL-3 also stimulates the growth of YS and placental HSCs.

IL-3 Is Expressed in Wild-Type but Not Runx1-Deficient Embryos

Since gene targeting studies showed no embryonic lethality or gross hematological defects in adult IL-3-deficient mice, the expression pattern of this cytokine during midgestation had not been previously explored. We performed *IL-3*-specific in situ hybridization on transverse sections of E11 *Runx1*$^{+/+}$, *Runx1*$^{+/-}$, and *Runx1*$^{-/-}$ embryos (Figure 2). *Runx1*$^{+/+}$ sections contain strongly *IL-3*-expressing cells within the lumen of the aorta (Figure 2B, arrows), and some of these cells

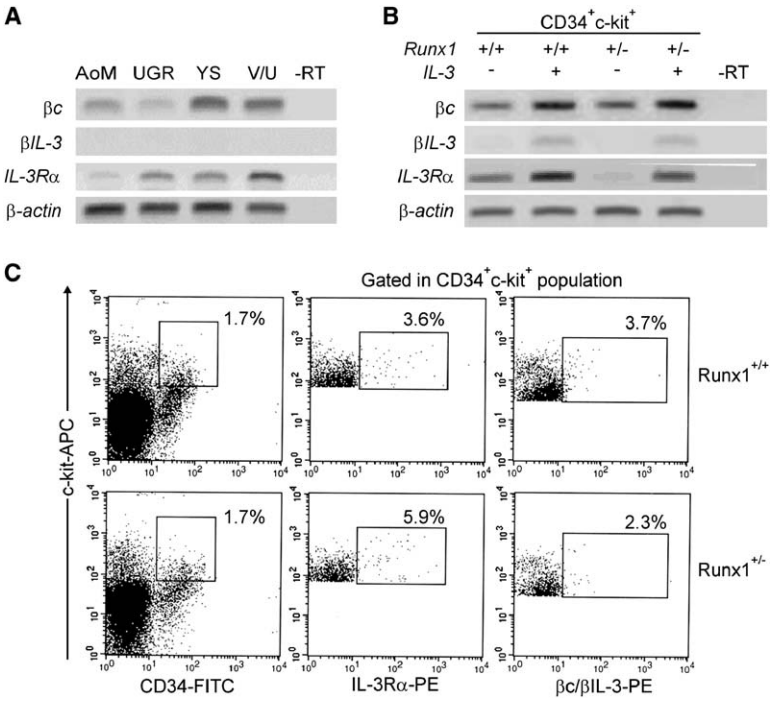

Figure 3. IL-3 Receptor Chain Expression in Embryonic Tissues and Sorted CD34$^+$c-kit$^+$ Cells from E11 *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ AGM Explants

RT-PCR analysis of *IL-3R*β_c, $\beta_{IL\text{-}3}$, and *IL-3R*α mRNA in (A) subdissected aorta/mesenchyme (AoM) and urogenital regions (UGR), yolk sac (YS), and vitelline/umbilical vessels (V/U) from E11 *Runx1*$^{+/+}$ embryos and (B) sorted CD34$^+$c-kit$^+$ cells from *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ E11 AGM explants cultured in the presence (+) or absence (−) of IL-3. −RT, negative control; *β-actin*, RNA loading control. (C) Flow cytometric plots showing IL-3Rα and β_c/$\beta_{IL\text{-}3}$ chain expression on CD34$^+$c-kit$^+$ cells from *Runx1*$^{+/+}$ (top row) and *Runx1*$^{+/-}$ (bottom row) E11 AGM explants. Dot plots (left panel) show the gate including CD34$^+$c-kit$^+$ cells. The percentage of IL-3R chain expression is shown for the CD34$^+$c-kit$^+$-gated population.

adhere closely to the endothelial wall (Figure 2B, arrow head). *IL-3*-expressing cells localize also within the developing stomach region (Figures 2C and 2D), skin, cardinal veins, and vitelline artery (Figures S3A–S3C). Sections through *Runx1*$^{+/-}$ embryos show fewer *IL-3*-expressing cells in these regions (Figures 2E and 2F and Figures S3D and S3E), and no *IL-3*-expressing cells are present in *Runx1*$^{-/-}$ embryo sections (Figures 2G and 2H and Figures S3F and S3G). Thus, the presence of *IL-3*-expressing cells in the normal midgestation embryo and the diminished/absent expression of *IL-3* in *Runx1* mutant embryos demonstrate that *IL-3* is downstream of Runx1 in the mouse.

To determine the number and identify the lineage of *IL-3*-expressing cells, we performed flow cytometry on E11 *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ AGM cells. Intracellular staining with an IL-3-specific antibody revealed 0.31% and 0.13% of AGM cells positive for IL-3 (Figure 2I), representing 688 and 298 IL-3$^+$ cells per *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ AGM, respectively. Multicolor staining with antibodies specific for hematopoietic (CD45), endothelial (CD31), and lymphoid progenitors (IL-7R) revealed that all IL-3-expressing cells are within the nonhemato/nonendothelial CD45$^-$CD31$^-$ and IL-7R$^-$ fractions of *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ AGM cells (Figure 2J and data not shown). Thus, although limited to a few cells of unknown lineage, IL-3 is produced in the midgestation embryo and may play a physiological role in HSC regulation.

CD34$^+$c-kit$^+$ AGM Cells Express IL-3 Receptors

IL-3 signaling is known to occur through IL-3Rα:βc and IL-3Rα:$\beta_{IL\text{-}3}$ surface receptors. We tested whether embryonic hematopoietic tissues and enriched AGM HSCs express the IL-3R subunits. Semiquantitative RT-PCR shows *βc* and *IL-3Rα* but not $\beta_{IL\text{-}3}$ expression by all E11 *Runx1*$^{+/+}$ tissues tested, aorta-mesenchyme and urogenital ridge subregions of the AGM, YS, and the V/U vessels (Figure 3A). CD34$^+$c-kit$^+$ cells sorted from *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ AGM explants cultured in the presence or absence of IL-3 also express *βc* and *IL-3Rα* (Figure 3B). Expression levels do not appear to differ between the two genotypes (except for a lower *IL-3Rα* expression in the *Runx1*$^{+/-}$ AGM cells). Interestingly, in the presence of IL-3, $\beta_{IL\text{-}3}$ begins to be expressed and the levels of *IL-3Rα* and *βc* expression are increased in CD34$^+$c-kit$^+$ cells, indicating some autoregulation of these receptors.

Flow cytometric analyses with antibodies specific for IL-3Rα and for both $\beta_{IL\text{-}3}$ and βc chains confirm that indeed the IL-3 receptors are expressed on the surface of AGM cells (Figure 3C). In both *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ AGM explants, a small fraction of CD34$^+$c-kit$^+$ cells showed expression of the IL-3Rα and $\beta_{IL\text{-}3}$/βc chains. IL-3R chains are not expressed by endothelial (CD45$^-$CD31$^+$) cells but are expressed by a very small number of nonhemato/nonendothelial cells (CD45$^-$CD31$^-$) (data not shown). Thus, phenotypically defined AGM HSCs express all three IL-3 receptor chains, indicating that IL-3 may mediate its effect directly on HSCs.

IL-3 Regulates AGM HSCs In Vivo

We next examined whether an absence of IL-3 interferes with AGM HSC function. Two approaches were used: blocking with an IL-3 specific antibody and a genetic approach with *IL-3* mutant mice. E11 *Runx1*$^{+/-}$ AGM explants were cultured prior to transplantation in the presence of an IL-3-blocking antibody to neutralize endogenous IL-3 or an IgG1 control antibody. As shown in Table 2, the percentage of repopulated mice decreased from 30% to 5% in the presence of the IL-3-blocking antibody. A similar decrease in the HSC activity in E11 *Runx1*$^{+/+}$ AGMs cultured in the presence of IL-3-specific blocking antibody was also observed (data not shown). Explants cultured in the presence of IgG1

Table 2. Effect of a Decrease or Total Absence of IL-3 on the HSC Activity in E11 AGM Explants

	Transplantation	Number of Mice Repopulated/ Total Transplanted (% Mice Reconstituted)		Percent Chimerism[d]
Antibody[a]				
None	after explant	6/20	(30)	13–37
IgG1	after explant	9/30	(30)	12–49
Anti-IL-3	after explant	1/20	(5)	13
Gene targeted[b]				
IL-3$^{+/+}$	after explant	5/6	(83.3)	36–100
IL-3$^{+/-}$	after explant	5/21	(23.8)	21–72
IL-3$^{-/-}$	after explant	1/6	(16.7)	26
Gene targeted[c]				
IL-3$^{+/+}$	direct	4/9	(44.4)	100
IL-3$^{+/-}$	direct	4/12	(33.3)	17–100
IL-3$^{-/-}$	direct	0/5	(0)	0

[a] AGMs from E11 *Runx1*$^{+/-}$ embryos were cultured as explants in the absence (none) or presence of 450 ng/ml of IgG_1 (control) or blocking IL-3 antibody (Anti-IL-3) for 3 days prior to transplantation. One embryo equivalent of cells were injected per mouse. Data from explant cultures in the absence or presence of IgG1 and anti-IL-3 are from a total of five, six, and four independent experiments, respectively.

[b] AGMs from E11 *IL-3*$^{+/+}$, *IL-3*$^{+/-}$, and *IL-3*$^{-/-}$ embryos were cultured as explants for 3 days prior to transplantation. 0.3–1 ee of cells were injected per mouse. Data from *IL-3*$^{+/+}$, *IL-3*$^{+/-}$, and IL-3$^{-/-}$ explant cultures are from a total of two, three, and two independent experiments, respectively.

[c] Cells from *IL-3*$^{+/+}$, *IL-3*$^{+/-}$, and *IL-3*$^{-/-}$ E11 AGMs (1 ee) were directly injected into irradiated adult recipients. Data from *IL-3*$^{+/+}$ and *IL-3*$^{+/-}$ transplantations are from a total of three and four independent experiments. Data from *IL-3*$^{-/-}$ transplantations are from one experiment. Only mice with $\geq$10% donor chimerism in the peripheral blood at >4 months after transplantation were included as positive.

[d] Donor cell contribution to peripheral blood of individual recipient mice was determined by semiquantitative PCR.

control antibody were not reduced in their HSC activity. These results show that the IL-3-blocking antibody specifically interferes with HSC activity and that IL-3 present, and/or produced, in the AGM is important for the survival and/or amplification of HSCs.

IL-3 mutant embryos were examined for AGM HSC activity. We performed transplantations of E11 *IL-3*$^{+/+}$, *IL-3*$^{+/-}$, and *IL-3*$^{-/-}$ AGM explant cells into adult irradiated recipients. Transplantation of *IL-3*$^{+/+}$ cells resulted in 83.3% of recipients repopulated, while only 23.8% of recipients were repopulated with *IL-3*$^{+/-}$ cells (Table 2). Of the 6 *IL-3*$^{-/-}$ embryos available for explant culture, only 1 AGM resulted in hematopoietic repopulation (16.7%). When the primary recipients were tested for multilineage engraftment, high-level donor repopulation was found in all hematopoietic tissues and in sorted myeloid, T lymphoid, and B lymphoid cells (Figure S2B, top panel). Moreover, the transplantation of the BM from these primary recipients into secondary adult irradiated recipients resulted in similar high-level repopulation (Figure S2B, bottom panel). Thus, HSCs from IL-3 deficient embryos are functional (multipotential and self-renewing) but are greatly reduced in E11 AGM explants.

To verify the in vivo requirement for IL-3 (in the absence of the explant culture step), we transplanted E11 *IL-3* mutant AGMs directly into irradiated adult recipients. Again, HSCs were decreased in *IL-3*$^{+/-}$ AGMs and were undetectable in *IL-3*$^{-/-}$ AGMs (Table 2). Similar results were found when *IL-3* mutant E11 YS and placenta were transplanted directly (data not shown). Thus, we conclude that wild-type levels of IL-3 are required for the growth of normal numbers of HSCs as they emerge in the mouse embryo.

Proliferation and Survival Effects of IL-3 on *Runx1*$^{+/-}$ HSCs

To determine if IL-3 promotes an increase in HSC numbers by proliferation or survival, we first analyzed the absolute number of two phenotypically enriched HSC populations (CD34$^+$c-kit$^+$ and Ly6A-GFP$^+$c-kit$^+$) (de Bruijn et al., 2002; Sanchez et al., 1996) in E11 *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ AGM explants after culture with and without IL-3. In the absence of exogenously added IL-3, the number of CD34$^+$c-kit$^+$ cells in *Runx1*$^{+/-}$ AGMs is slightly lower than in *Runx1*$^{+/+}$ AGMs (Figure 4A). In the presence of IL-3, CD34$^+$c-kit$^+$ cells increase in both *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ AGMs. We also observed similarly increased numbers of Ly6A-GFP$^+$c-kit$^+$ cells in AGM explants cultured with IL-3 (data not shown). Hence, IL-3 induces an increase in the absolute number of phenotypically defined HSC populations in both *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ AGMs. The cell-cycle status of c-kit$^+$ cells (a broader population of progenitors including HSCs) from E11 *Runx1*$^{+/-}$ AGMs cultured in the absence or presence of IL-3 was also analyzed. BrdU staining revealed that IL-3 induces a small percentage of c-kit$^+$ cells to exit G_0/ G_1 (Figure 4B). Moreover, Ki67 staining shows that in the presence of IL-3, some G_0 cells enter into G_1. Thus, IL-3 appears to act as an HSC proliferation factor.

We further tested if IL-3 affects the proliferation and/or survival of AGM HSCs in a two-step culture system (Figure 4C). The first step is the AGM explant culture in the presence/absence of IL-3. The second step is an amplification culture (with equivalent numbers of harvested CD34$^+$c-kit$^+$ cells) that allows for a more direct comparison of the influence of IL-3 on the HSC population during step one. Following the step-one culture of E11 *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ AGM explants, hematopoietic (CD45$^+$) cells were analyzed by flow cytometry for apoptotic markers. As shown in Figure 4Ca, in the absence of IL-3, the percentage of healthy cells (AnnexinV$^-$7AAD$^-$) is lower, and the percentage of preapoptotic cells (AnnexinV$^+$7AAD$^-$) is higher in *Runx1*$^{+/-}$ explants as compared to *Runx1*$^{+/+}$ explants. When IL-3 was present in the culture, the percentages of healthy and preapoptotic CD45$^+$ cells in *Runx1*$^{+/-}$ explants are restored to percentages observed in the *Runx1*$^{+/+}$ explants (no IL-3). The same results were obtained from *Runx1*$^{+/-}$ AGMs characterized by the expression of hematopoietic stem/progenitor markers c-kit, Sca-1, or CD34 (data not shown). Thus, hematopoietic cells in *Runx1*$^{+/-}$ AGM explants are more sensitive to apoptosis than *Runx1*$^{+/+}$ cells, and addition of IL-3 to the cultures promotes their survival.

Following step one, *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ AGM explants were harvested and CD34$^+$c-kit$^+$ cells were sorted, seeded at a density of 3.5×10^3 cells per well, and cultured in suspension for 10 days in the presence

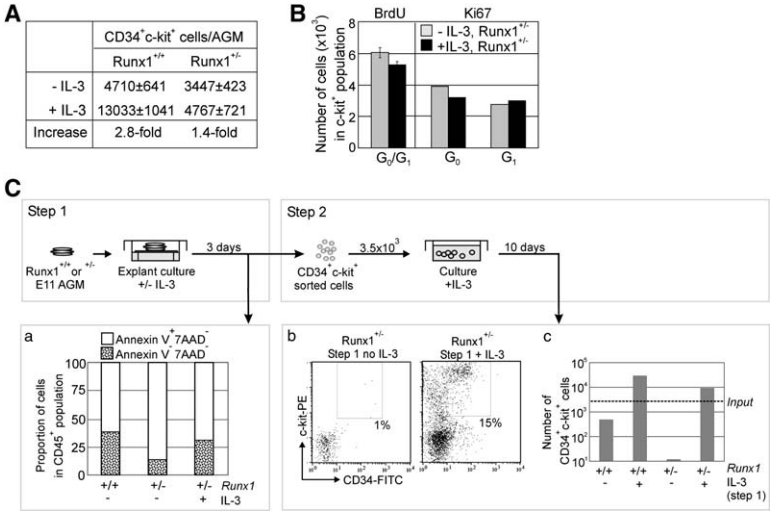

Figure 4. Proliferation and Survival Effects of IL-3 on AGM HSCs In Vitro

(A) Absolute number of CD34$^+$c-kit$^+$ cells per *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ AGM explants cultured in the absence/presence of IL-3 (200 ng/ml) was calculated from the average number of cells per AGM and the percentage of CD34$^+$c-kit$^+$ cells (see legend of Table 1) (n = 3–5). Fold increase of CD34$^+$c-kit$^+$ cells in the presence of IL-3 is indicated.

(B) Cell cycle status of c-kit$^+$ cells from *Runx1*$^{+/-}$ AGM explants cultured in the presence/absence of IL-3 (200 ng/ml). BrdU (20 μM) incorporation was analyzed by flow cytometry and the absolute number of c-kit$^+$ cells in G_0/G_1 was calculated (n = 3). Ki67 and 7AAD stainings discriminate cells in G_0 from cells in G_1 phase (representative experiment shown; n = 2). Data shown are the mean ± SD of three experiments.

(C) A two-step culture of *Runx1*$^{+/+}$ and *Runx1*$^{+/-}$ AGMs was performed in the absence/presence of IL-3 (200 ng/ml). Step one, explant culture with/without IL-3. Step two, CD34$^+$c-kit$^+$ cells were sorted from AGM explants, seeded at 3.5 × 10^3 cells/well, and cultured in suspension for 10 days with IL-3 (200 ng/ml). (Ca) Apoptotic status of CD45$^+$ cells from AGM explants after step one culture. Flow cytometry determined the proportions of healthy (AnnexinV$^-$7AAD$^-$) and preapoptotic (AnnexinV$^+$7AAD$^-$) CD45$^+$ cells (n = 3). (Cb) Percentage of CD34$^+$c-kit$^+$ cells remaining after two-step culture as determined by flow cytometry (n = 3). Dot plots are shown for *Runx1*$^{+/-}$ AGM cells. (Cc) Absolute numbers of CD34$^+$c-kit$^+$ cells obtained after the two-step culture (n = 4). Hatched line indicates input number (3.5 × 10^3 cells/well).

of IL-3. When no IL-3 was present during the step one explant culture, we observed a complete disappearance of *Runx1*$^{+/-}$ CD34$^+$ckit$^+$ cells in the step two suspension cultures (Figures 4Cb and 4Cc). This is consistent with increased apoptotic activity observed by Annexin staining. In contrast, when IL-3 was included during step one, *Runx1*$^{+/-}$ CD34$^+$ckit$^+$ cells survived and were increased 2.6-fold in the suspension culture as compared to the input cell number. *Runx1*$^{+/+}$ CD34$^+$ckit$^+$ cells were also increased (8.3-fold) in the step two culture when IL-3 was present during step one. Thus, IL-3 promotes the survival and proliferation of phenotypically defined HSCs in E11 AGM explants.

IL-3 Promotes the Early Detection of AGM and YS HSCs

Since IL-3 has such potent effects on E11 HSC survival and proliferation, we tested whether IL-3 could also affect hematopoiesis at earlier stages. Thus far, adult-repopulating HSCs have been detected only in embryos from the 35 somite pair (sp) stage (E10.5) onward, suggesting that this is a critical time point for their emergence in the AGM region. To test for effects of IL-3 prior to this time point, we performed explant cultures of early E10 AGMs (30–34 somites pairs) in the absence or presence of IL-3 and measured HSC activity by in vivo long-term repopulation of irradiated adult recipients. Transplantation of cells (2–2.5 ee) from E10 AGM explants cultured with IL-3 resulted in 64% of recipients repopulated, while none of the recipients (0/17) were repopulated with cells from AGMs cultured in the absence of IL-3 (Table 3). Successful repopulation was also obtained with the injection of as few as 1 ee of E10 AGM cells (Table 3). Indeed, 20% of the mice transplanted were reconstituted (with one recipient showing 64% donor chimerism). E10 HSCs result in high-level multilineage engraftment (normal levels of T and B lymphoid and myeloid lineage cells) in primary recipients (Figure S2C, top panel) and successful high-level repopulation in secondary recipients (Figure S2C, bottom panel). In parallel, we also transplanted the cells from E10 YS and placenta (Table 3). Similar to the AGM transplantation results, 20% of the mice injected with 1 ee of E10 YS were reconstituted (including one recipient with 45% donor chimerism). No mice were reconstituted after the injection of E10 placenta. Thus, IL-3 promotes HSC detection in early E10 AGM and YS either through the survival and/or proliferation of a limiting number of previously undetectable HSCs or the emergence of new HSCs.

Discussion

We have demonstrated here that the well-known adult hematopoietic cytokine IL-3 plays an in vivo role in the amplification of the HSC pool in the midgestation mouse embryo. We have also shown that exogenously provided IL-3 promotes HSC activity in the early E10 AGM and YS (30–34 somites pairs), at a time when typically no HSCs are detected (as stringently defined by in vivo long-term, high-level multilineage repopulation in irradiated adult wild-type recipients). Considering that this cytokine does not produce dramatic effects in the hematopoietic system of the adult, our finding that IL-3 is an important embryonic HSC regulator offers new possibilities for the examination and manipulation of the earliest events in HSC fate determination and growth.

Hematopoietic Growth Factors in the Embryo

The emergence and growth of HSCs in the midgestation mouse embryo is complex and the processes leading to the establishment of these cells are influenced by the specific microenvironments of the hematopoietic sites. Careful dissection of the AGM, YS, and placental microenvironments and study of the HSCs that reside within them can reveal important regulatory molecules. Several factors already shown to affect early hematopoietic and endothelial cell development include the vascular

Table 3. Effect of IL-3 on the HSC Activity in E10 AGM, YS, and Placenta Explants

Tissue	ee	IL-3	Number of Mice Repopulated/ Total Transplanted (% Mice Reconstituted)	Percent Chimerism
AGM	2–2.5	–	0/17 (0)	0
		+	9/14 (64.3)	1; 27; 83; 88; 95; 98; 99; 100; 100
	1	–	0/8 (0)	0
		+	3/15 (20)	4.8[a]; 8.9[a]; 64[b]
YS	1	–	0/9 (0)	0
		+	3/15 (20)	3.6[b]; 9.1[a]; 45[a]
Placenta	1	–	0/15 (0)	0
		+	0/10 (0)	0

Early E10 (30 to 34 somite pairs [sp]) AGM, YS, and placenta explants were cultured in the absence (–) or the presence (+) of 200 ng/ml of IL-3 for 3 days prior to transplantation. Only mice with $\geq$1% donor chimerism in the peripheral blood at 2 and 4 months after transplantation were included in the number of repopulated mice. Donor cell chimerism in the peripheral blood of individual recipient mice was determined by semiquantitative PCR. Data are from a total of three to six independent experiments. ee corresponds to the number of embryo equivalent injected per mouse. AGMs from embryos ranging from 30 to 34 sp were pooled for the transplantations of 2–2.5 ee.
[a] Mice reconstituted with cells from 33 sp embryos.
[b] Mice reconstituted with cells from 34 sp embryos.

endothelial growth factor (VEGF) (Shalaby et al., 1995), TGFβ1 (Dickson et al., 1995), M-CSF (Minehata et al., 2002), and the IL-6 family member, oncostatin M (Mukouyama et al., 1998). Many other growth factors (TPO, Flt3L, G-CSF, IL-1β, IL-6, Weche, IL-11, LIF) have been found to be expressed in cell lines from the AGM microenvironment (Ohneda et al., 1998, 2000; Oostendorp et al., 2002) and await further study.

Until our studies, the expression of IL-3 in the embryo was undetermined, and its function during development was thought to be redundant due to the viability of *IL-3*$^{-/-}$ mice and the lack of major hematopoietic defects in the adult. Sensitive in situ hybridization analysis revealed the expression of IL-3 in appropriate sites and tissues at E11, coincident with HSC activity. Similarly, expression of IL-3 receptors was found on E11 cell populations enriched for HSC activity. We also confirmed the presence of IL-3- and IL-3R-expressing cells in the early E10 AGM region and found upregulated IL-3R expression when AGMs were cultured in presence of IL-3 (data not shown). In the two-step in vitro culture, only AGMs cultured with IL-3 in step one were able to yield CD34$^+$ckit$^+$ cells that survived and proliferated in step two. Furthermore, HSCs in E11 *IL-3*$^{+/-}$ AGMs do not appear to amplify (as do *IL-3*$^{+/+}$ AGM HSCs) during explant culture. Thus, IL-3 is an important early regulator of HSCs, which most likely becomes redundant as the expression of other growth factors is initiated at later developmental stages.

The Relationship of Runx1 and IL-3

Initially we used Runx1 haploinsufficient mice as a model to test if adult cytokines play a role in embryonic hematopoiesis since cytokine genes *IL-3* (Mao et al., 1999; Uchida et al., 1997) and *GM-CSF* (Takahashi et al., 1995) are known Runx1 targets. Out of the panel of cytokines tested, only IL-3 was able to rescue the *Runx1*$^{+/-}$ AGM HSC defect. If IL-3 is indeed one of the Runx1 downstream effectors, at least some aspects of the HSC defects in *IL-3*$^{+/-}$ AGMs should be similar to *Runx1*$^{+/-}$ AGMs. We found an approximately 2-fold decrease in repopulation potential in both *Runx1*$^{+/-}$ AGM cells and *IL-3*$^{+/-}$ AGM cells, as compared to wild-type AGMs. Moreover, we found that IL-3 expression, normally present in circulating cells and developing stomach cells of wild-type E11 embryos, was absent and much decreased in *Runx1*$^{-/-}$ and *Runx1*$^{+/-}$ embryos, respectively. Thus, as one of many putative targets of Runx1, IL-3 is responsible for some part of the AGM defects (survival and/or proliferation of HSCs) observed in the *Runx1* mutant embryos.

A direct role for Runx1 in activating the transcription of *IL-3* would require Runx1 expression in the AGM microenvironment. Runx1 expression has been found in some circulating cells within the lumen of the E11.5 aorta (North et al., 2002). Since some IL-3 expressing cells circulate, and it has been reported that circulating cells can attach to the aortic endothelium (Takakura et al., 1998), it is possible that localized release of IL-3 at these sites where hematopoietic clusters emerge from the endothelium affects the survival/expansion of newly formed HSCs. Flow cytometry and RT-PCR analysis suggest that the IL-3-producing cells are not hematopoietic or endothelial (CD45$^-$CD31$^-$ cells) nor early T cell progenitors (IL-7R$^-$) or hematopoietic "lymphoid tissue inducer cells" (CD4$^+$CD3$^-$IL-7Rα^+) (D. Finke, personal communication). However, due to ontogenic expression changes in hematopoietic markers and the finding that some AGM hematopoietic cells are CD45$^-$ (Bertrand et al., 2005; Matsubara et al., 2005), we cannot rule out the possibility of their hematopoietic nature. Positive identification of these cells is ongoing.

The effects of Runx1 haploinsufficiency are more wide spread than those of IL-3 haploinsufficiency. As previously mentioned by Cai et al. (2000), HSC activity in *Runx1*$^{+/-}$ AGMs is decreased, while it is increased in *Runx1*$^{+/-}$ YS. We confirmed this and found that *Runx1*$^{+/-}$ YS contains at least 4-fold more HSCs than *Runx1*$^{+/+}$ YS. We have additionally shown that the placentas of *Runx1*$^{+/-}$ embryos are increased in HSCs. The discordant effect of half a dose of Runx1 in the AGM and the extraembryonic tissues is puzzling. *Runx1*$^{+/-}$ BM has been reported to contain a decreased number of HSCs but has increased numbers of progenitor cells (Sun and Downing, 2004). Also, an acceleration of the mesodermal commitment and specification to Blast-CFC and hematopoietic lineages was reported in the *Runx1*$^{+/-}$ embryoid bodies (Lacaud et al., 2004). As we have preliminary data showing an early appearance (E10) of HSCs in *Runx1*$^{+/-}$ YS and placenta, it is possible that the higher HSC numbers found at E11 in these tissues are a consequence of HSC accumulation (and is unrelated to IL-3). This would suggest that normal levels of Runx1 suppress the appearance of HSCs in the YS and placenta while promoting HSC appearance in the AGM. This could occur at the level of Runx1-interacting proteins in the specific microenvironments. Such interacting

partners could positively/negatively regulate transcription in a spectrum of downstream targets. Nonetheless, we have shown that IL-3 acts similarly in E11 AGM, YS, and placenta to increase HSC numbers, supporting the notion that *IL-3* is a Runx1 target.

How Does IL-3 Regulate AGM HSCs?

Our data showing expression of IL-3R on $CD34^+$c-kit^+ AGM enriched HSC populations are consistent with the expression of IL-3Rs on adult mouse BM HSCs ($Rho^{lo}Lin^-Ly6A/E^+$c-kit^+) (McKinstry et al., 1997). Interestingly, the levels of IL-3R expression on adult HSCs appear to be directly related to the proliferative response (Murthy et al., 1989), and thus, it is thought that the low level expression of IL-3R on adult BM HSCs protects them from inappropriate proliferation. The low expression of IL-3R on AGM HSCs and the lack of receptor expression on most other AGM cells suggest that IL-3 acts directly on AGM HSCs.

IL-3 is known to act as a survival factor. In vitro analyses of AGM hematopoietic populations (including $CD34^+$c-kit^+) demonstrate that IL-3 promotes their survival. This is in agreement with the survival effect of IL-3 on adult hematopoietic cells (Kinoshita et al., 1995; Lotem and Sachs, 2002) and the fact that survival pathways function in AGM HSCs (Orelio et al., 2004). IL-3 also acts as a proliferation factor. We have shown by in vivo transplantation assays that IL-3 acts to increase the absolute number of AGM HSCs. Our in vitro culture and phenotypic studies demonstrate that IL-3 increases the number of AGM HSC-enriched populations ($CD34^+$c-kit^+ and Ly6A-GFP^+c-kit^+) by promoting proliferation. Thus, IL-3 acts to increase E11 AGM (and most likely YS and placenta) HSC numbers by both processes.

In contrast to the apparent hematopoietic normality of adult *IL-3*$^{-/-}$ and *IL-3R*$^{-/-}$ (*βc*, $\beta_{IL\text{-}3}$, and *IL-3:βc*) mice (Lantz et al., 1998; Nicola et al., 1996; Nishinakamura et al., 1995, 1996; Robb et al., 1995), we found a decrease in AGM HSCs in *IL-3*$^{+/-}$ and *IL-3*$^{-/-}$ embryos by both direct transplantation and transplantation after explant culture. HSCs are also decreased in *IL-3* mutant E11 YS and E11/E12 placenta (by direct transplantation, not shown). This suggests that while IL-3 shares functional redundancy with other cytokines for HSC growth in the adult, this is not the case in the embryo. In early/midgestation embryos the functionally overlapping cytokines or their receptors may not yet be expressed, thus revealing an early role for IL-3 in the expansion of the first HSCs. We propose that the limited numbers of HSCs surviving in *IL-3*$^{+/-}$ and *IL-3*$^{-/-}$ AGMs may be sufficiently expanded at later developmental times by redundant cytokines to provide a normal adult hematopoietic system. Although less likely (since HSCs are not completely absent in *IL-3*$^{-/-}$ mice), IL-3 may influence the de novo generation of HSCs in the AGM, as well as in the placenta and YS (Cai et al., 2000; Gekas et al., 2005; Kumaravelu et al., 2002; Ottersbach and Dzierzak, 2005). We found that the addition of IL-3 to explant culture of early E10 AGM and YS (30–34 sp) promotes a high level of HSC activity, prior to the time when they are normally detected. As others have detected multipotential in vivo repopulating cells in the AGM and YS at pre-E10 stages when transplanted under less stringent conditions (neonatal [Yoder et al., 1997] or immunodeficient adult mice [Cumano et al., 2001]), IL-3 may promote the induction of HSCs from these earlier hematopoietic progenitors or alternatively, from other precursors. To test the HSC induction properties of IL-3, we attempted to rescue the complete HSC deficiency of *Runx1*$^{-/-}$ embryos. We explant cultured E11 *Runx1*$^{-/-}$ AGM or V/U arteries in presence of IL-3 (200 ng/ml) for 3 days and transplanted these cells into irradiated adult recipient mice. No repopulation was obtained even at 9 months posttransplantation of 1 ee of *Runx1*$^{-/-}$ AGM (0/5 recipients repopulated, n = 3) or 3 ee of *Runx1*$^{-/-}$ AGM (0/3, n = 1) or V/U cells (0/3, n = 1). These data suggest that IL-3 does not promote the de novo generation of HSCs but most likely acts as a survival and proliferation factor for preexisting HSCs that were undetectable due to limiting numbers. Thus, despite its long history of study, IL-3 is one of the earliest acting HSC cytokines and is an important factor in the future studies of HSC fate determination and expansion in the embryo.

Experimental Procedures

Mice and Embryo Generation

Embryos were generated as indicated in Table S2. The date of vaginal plug discovery was considered E0. Animals were housed according to institutional guidelines with free access to water and food. Animal procedures were carried out in compliance with the Standards for Humane Care and Use of Laboratory Animals.

Dissections, Explant Cultures, and Cell Preparation

Tissues were dissected from E10, E11, and E12 embryos (Robin and Dzierzak, 2005) and dissociated by collagenase treatment (0.12% w/v, type I, Sigma) for 1 hr. In some cases, whole tissues explants were cultured for 3 days at 37°C on permeable membrane filters in myeloid long-term medium (StemCell Technologies) with hydrocortisone (10^{-6} M, Sigma). Placenta explants were cut into small pieces before culture on the filter. Medium was supplemented with 0, 1, 2, 20, or 200 ng/ml of cytokines (recombinant murine IL-3 [BD Pharmingen], SCF [AbCys], GM-CSF [R&D Systems], Oncostatin M [R&D Systems], bFGF [R&D Systems]) or with 450 ng/ml of IL-3-blocking antibody or IgG1 as a control (BD Pharmingen).

In Vivo Transplantation Assay

Intravenous injection of genetically marked cells into irradiated adult female recipients was as previously described (Robin and Dzierzak, 2005). 129/SV × C57BL/6, C57BL/6, (CBA × C57BL/10), or BALB/c mice were exposed to a split dose of 9 Gy of γ-irradiation (^{137}Cs source) and injected with several dilutions of cells (0.03, 0.1, 0.3, or 1 embryo equivalent [ee]). 2×10^5 spleen cells (recipient background) were coinjected to promote short-term survival. Mice were maintained on 0.16% Neomycin (Sigma) water. Blood DNA was obtained at 4 months after transplantation and analyzed for donor cell genetic markers by semiquantitative PCR (*Runx1*, *Neo*, *hβ-globin*, and/or *Ymt*) (Cai et al., 2000; Muller et al., 1994; Strouboulis et al., 1992). The percentage of donor chimerism was determined from phosphoimaging of ethidium bromide stained gels (Imagequant) and calculated from a standard curve of DNA control dilutions (0%, 1%, 3%, 6%, 10%, 30%, 60%, and 100% donor marker). Recipients of E11 and E10 cells were considered repopulated only when donor chimerism was respectively >10% and >1%. HSC frequencies were determined with Poisson statistics based on the logarithmic proportion of nonrepopulated mice and the doses of injected cells. HSC number/tissue was estimated according to the average total cell number/explant tissue (see legend Table 1).

Flow Cytometric Analysis after Surface/Intracytoplasmic Antibody Staining

Monoclonal antibodies (BD Pharmingen) include: APC or PE-anti-c-kit (clone 2B8), FITC-anti-CD34 (RAM 34), FITC-anti-CD31, APC or PE-anti-CD45, PE-anti-IL3Rα chain (kindly provided by Dr. Rodewald), PE-anti-$\beta_{IL\text{-}3}$/βc, and APC-anti-CD127 (IL-7 Receptor α chain).

Cells were incubated with antibodies for 20 min on ice, washed twice, and resuspended in PBS supplemented with 10% FCS and 7AAD (Molecular Probes, Leiden, The Netherlands) or Hoechst 33258 (1 μg/ml, Molecular Probes) for dead cell exclusion. Cells were analyzed on a FACScan or FACS Aria (Becton Dickinson). For intracytoplasmic PE-anti-IL3 antibody staining, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen). For apoptotic status, cells were stained with FITC-anti-AnnexinV (BD Pharmingen) and 7AAD. For cell-cycle analysis, BrdU (20 μM, Sigma) was added to explant cultures during the final 16 hr. After collagenase treatment, cells were stained with PE-anti-c-kit and FITC-BrdU (BrdU Flow kit; BD Pharmingen). FITC-anti-Ki67 (BD Pharmingen) and 7AAD stainings were also performed.

RT-PCR Analysis

Total RNA was extracted from 10–20 × 10^3 sorted cells or embryonic tissues with Trizol (Invitrogen). RNA was treated with RNase-free DNaseI (Promega). After phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation, cDNA synthesis was performed by using oligodT primers (Promega) and reverse transcriptase (Superscript II, Stratagene). Table S3 lists the primer sequences and PCR fragment sizes.

In Situ Hybridization

A 606 bp product was obtained by RT-PCR with cDNA prepared from WEHI-3 cells and the IL-3-specific primers (5′-TTGGAGGACC AGAACGAGAC-3′ and 5′-CATTTGATGGCATAAAGGAATG-3′) and cloned into the p-GEM-T Easy (Promega). Ten micron cryosections were prepared from quick frozen embryos and hybridized with digoxigenin-labeled riboprobes as described previously (Ottersbach and Dzierzak, 2005).

Supplemental Data

Supplemental Data include graphs of Poisson statistics used to calculate the HSC frequencies (Figure S1), hematopoietic multilineage engraftment data (Figure S2), IL-3 mRNA expression analysis in Runx1 mutant skin and embryonic vessels (Figure S3), transplantation data on the dosage effect of IL-3 (Table S1), details of embryos used in the studies (Table S2), and RT-PCR primer sequences (Table S3) and are available at http://www.developmentalcell.com/cgi/content/full/11/2/171/DC1/.

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