RARγ is critical for maintaining a balance between hematopoietic stem cell self-renewal and differentiation

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Hematopoietic stem cells (HSCs) sustain lifelong production of all blood cell types through finely balanced divisions leading to self-renewal and differentiation. Although several genes influencing HSC self-renewal have been identified, to date no gene has been described that, when activated, enhances HSC self-renewal and, when activated, promotes HSC differentiation. We observe that the retinoic acid receptor (RAR)γ is selectively expressed in primitive hematopoietic precursors and that the bone marrow of RARγ knockout mice exhibit markedly reduced numbers of HSCs associated with increased numbers of more mature progenitor cells compared with wild-type mice. In contrast, RARα is widely expressed in hematopoietic cells, but RARα knockout mice do not exhibit any HSC or progenitor abnormalities. Primitive hematopoietic precursors overexpressing RARα differentiate predominantly to granulocytes in short-term culture, whereas those overexpressing RARγ exhibit a much more undifferentiated phenotype. Furthermore, loss of RARγ abrogated the potentiating effects of all-trans retinoic acid on the maintenance of HSCs in ex vivo culture. Finally, pharmacological activation of RARγ ex vivo promotes HSC self-renewal, as demonstrated by serial transplant studies. We conclude that the RARs have distinct roles in hematopoiesis and that RARγ is a critical physiological and pharmacological regulator of the balance between HSC self-renewal and differentiation.

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Abbreviations used: ATRA, all-trans retinoic acid; BMT, BM transplant; CFU-GEMM, CFU-granulocyte, erythrocyte, macrophage, megakaryocyte; CFU-S, CFU-spleen; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; Hes1, hairy and enhancer of split 1; HSC, hematopoietic stem cell; LKS$^+$, lineage-negative, c-*kit*–positive, Sca-1–positive cells; LKS$^-$, lineage-negative, c-*kit*–positive, Sca-1–negative cells; RAR, retinoic acid receptor; RU, repopulating unit.

Organogenesis requires a balance between self-renewal and differentiation of the organ's stem cells. This is important not only for producing the mature cells essential for its normal function but also to maintain a pool of multipotent stem cells in organs, especially those that undergo continual developmental processes during an organism's lifespan.

Hematopoiesis requires a continuous production of progenitors and mature blood cells from hematopoietic stem cells (HSCs) through differentiation processes. Simultaneously, maintenance of the HSC pool occurs via cell fate decisions of self-renewal, apoptosis/senescence, or differentiation. Studies involving genetically modified HSCs such as overexpression of HOXB4 (1, 2), Notch1 (3), and β-catenin (4) in HSCs have provided evidence of genes that increase HSC self-renewal. Although each of these genes increases HSC potential when artificially induced, their physiological roles in HSC homeostasis are unclear, as mice deficient for Hoxb4, β-catenin, and Notch1 have been reported to have normal HSC content and function (5–7). Furthermore, to date, there has been a lack of studies describing genes that are physiologically required for maintaining the HSC pool in vivo and that reciprocally regulate HSC fate in an activation state–dependent manner.

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We have previously described a novel role for the vitamin A derivative, all-trans retinoic acid (ATRA), in enhancing the maintenance of HSCs in ex vivo liquid suspension culture (8). ATRA activates the retinoic acid receptors (RARs), of which there are three subtypes: RARα, RARβ, and RARγ. In these studies, we sought to determine whether any of the RARs had a physiological role in the regulation of HSCs.

Here, we show that loss of RARγ results in reduced numbers of HSCs and increased numbers of more differentiated progenitor cells in the mutant mice. Furthermore, unlike wild-type HSCs, RARγ null HSCs did not repopulate recipients after 14 d of ex vivo culture with ATRA. In contrast, loss of the other RAR predominantly expressed in hematopoietic cells, RARα, did not result in any hematopoietic defects in the mice and ATRA-treated RARα null HSCs had comparable repopulating activity to wild-type HSCs cultured with ATRA. Finally, treatment of cultures of highly enriched populations of hematopoietic precursors/stem cells with ATRA, the natural ligand for RARγ, resulted in increased Notch1 and Hoxb4 expression in these cells accompanied by enhanced HSC self-renewal, as shown by increased potential of ATRA-treated HSCs to repopulate mice after serial transplantation. These results, therefore, demonstrate that RARγ is a critical regulator of the balance between HSC self-renewal and differentiation; loss of RARγ results in reduced numbers of HSCs as the result of enhanced HSC differentiation, whereas RARγ activation results in HSC self-renewal.

RESULTS

RARγ1 and RARα have different effects on hematopoietic progenitor cells

RARα and RARγ subtypes are most widely expressed in hematopoiesis and loss of both of these RARs results in early postnatal lethality accompanied by impaired granulopoiesis in vivo (9). To determine which of the RARs may have a role in regulating HSCs, expression analysis of the major isoforms of the three RAR subtypes was performed in the HSC-containing lineage-negative, c-kit–positive, Sca-1–positive (LKS^+) cells and the lineage-negative, c-kit–positive, Sca-1–negative (LKS^-) cell population, which does not contain HSCs (10) and differentiates in response to ATRA treatment (11). RARα was expressed in both LKS^+ and LKS^- cells. In contrast, RARβ2 and RARγ1 were expressed preferentially by LKS^+ (Table S1, available at http://www.jem.org/cgi/content/full/jem.20052105/DC1). RARβ null mice are essentially normal (12) in contrast with the RARα and RARγ null mice, which have profound defects in multiple organs (13, 14). Therefore, we focused our attention on a role for RARα or RARγ in the regulation of HSCs.

To further define the roles of RARα and RARγ1 in HSCs, we used retroviral vector-mediated gene transduction to individually overexpress RARα and RARγ1 (15) in 5-fluorouracil pretreated BM and monitored the proliferation and differentiation of the transduced cells during 4 wk of culture in cytokine-containing media (see supplemental Materials and methods, available at http://www.jem.org/cgi/content/full/jem.20052105/DC1). 48 h after the infection, the transduction efficiencies (GFP^+ cells) were 8.8% (control), 4.3% (RARα), and 4.5% (RARγ1). During the 4 wk of culture, cell viabilities of the GFP^+ cells within each culture were similar. Both the control and RARα-overexpressing cells proliferated well in culture, comprising >50% of the cells in the cultures at 4 wk (Fig. S1, A–C, available at http://www.jem.org/cgi/content/full/jem.20052105/DC1). At this time point, the RARα-overexpressing cells predominantly expressed the granulocyte marker, Gr-1 (Fig. S1 D). In contrast, cells overexpressing RARγ1 proliferated slowly in culture (comprising <2% of the culture), did not express Gr-1, were negative for all other lineage markers assessed, and expressed high levels of Sca-1 (Fig. S1). Thus, there was a marked difference in the proliferation and differentiation of the RARα- vs. RARγ1-transduced cells, with the RARα-transduced cells exhibiting rapid proliferation and differentiation to granulocytes, whereas the RARγ transduced cells exhibited much slower proliferation associated with a much more immature phenotype.

RARγ null BM cells have increased numbers of progenitors

To confirm that RARα and RARγ have distinct roles in hematopoiesis, especially with respect to HSCs, we investigated the multipotential progenitor cell compartment of RARα and RARγ+/+, +/−, and −/− single mutant mice. BM obtained from the $RAR\alpha^{-/-}$ and $RAR\alpha^{+/-}$ mice had equivalent numbers of immature mixed colonies (CFU-granulocyte, erythrocyte, macrophage, megakaryocyte [GEMM]) and CFU-spleen (CFU-S) compared with $RAR\alpha^{+/+}$ BM (Fig. 1, A and B). In contrast, BM from $RAR\gamma^{-/-}$ mice had twofold increased CFU-GEMM and threefold increased CFU-S compared with $RAR\gamma^{+/+}$ BM (Fig. 1, C and D; $P < 0.05$). Further investigation of $RAR\gamma^{-/-}$ BM revealed that this was also accompanied by significantly increased numbers of common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) compared with $RAR\gamma^{+/+}$ BM (Fig. 1, E and F; $P < 0.05$).

RARγ null BM cells contain markedly reduced numbers of HSCs

The increased numbers of CFU-GEMM, CFU-S, CMPs, and CLPs in the $RAR\gamma^{-/-}$ BM may have resulted from increased numbers of HSCs, or alternatively from enhanced proliferation or differentiation of the HSCs into the more mature progenitor pool. Therefore, we performed limiting dilution assays (16) using BM from wild-type, $RAR\gamma^{+/-}$, $RAR\gamma^{-/-}$, and $RAR\alpha^{-/-}$ mice to compare the numbers of transplantable HSCs in these mice. Lethally irradiated (10 Gy) recipient mice (8–10 mice per cell dose) were injected with limiting numbers of donor cells together with 2×10^5 competing cells, and the frequency of HSCs estimated at 6 mo posttransplant using Poisson statistics (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20052105/DC1).

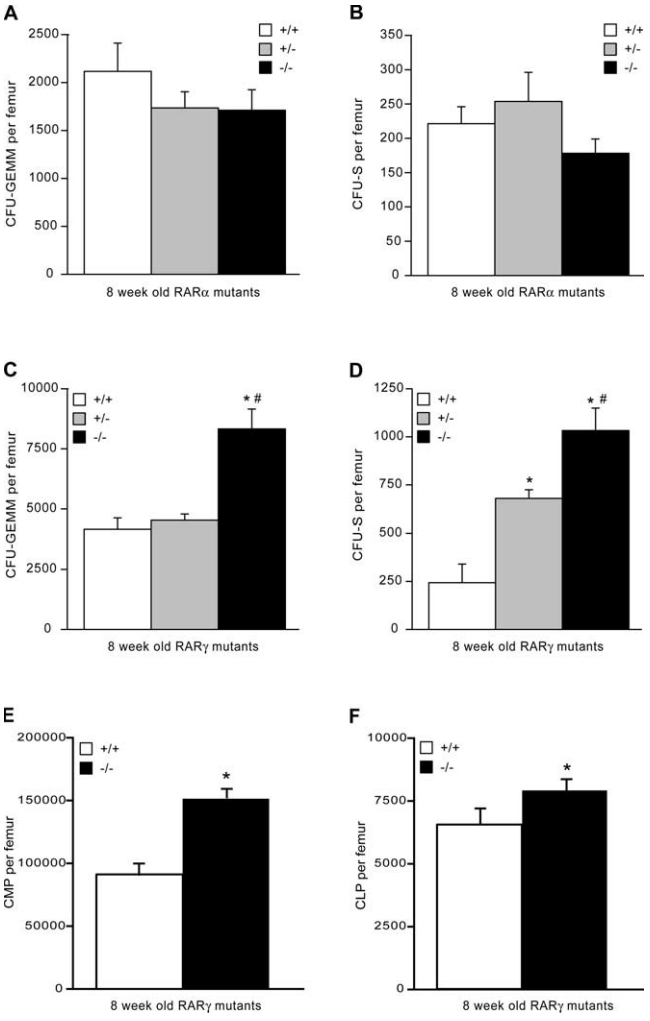


Figure 1. BM cells derived from 8-wk-old mice deficient for RARγ have increased numbers of progenitors. (A) Numbers of CFU-GEMM produced per femur from 8-wk-old RARα$^{+/+}$, RARα$^{+/-}$, and RARα$^{-/-}$ mice ($n = 4$). (B) CFU-S content per femur from 8-wk-old RARα$^{+/+}$, RARα$^{+/-}$, and RARα$^{-/-}$ mice ($n = 4$). (C) Numbers of CFU-GEMM produced per femur from 8-wk-old RARγ$^{+/+}$, RARγ$^{+/-}$, and RARγ$^{-/-}$ mice ($n = 4$). *, $P < 0.05$ compared with RARγ$^{+/+}$ BM (paired Student's *t* test). (D) CFU-S content per femur from 8-wk-old RARγ$^{+/+}$, RARγ$^{+/-}$, and RARγ$^{-/-}$ mice ($n = 4$). *, $P < 0.05$ compared with RARγ$^{+/+}$ BM; #, $P < 0.05$ compared with RARγ$^{+/-}$ BM (paired Student's *t* test). (E) Numbers of CMP per femur from 8-wk-old RARγ$^{+/+}$ and RARγ$^{-/-}$ mice ($n = 3$). *, $P < 0.05$ compared with RARγ$^{+/+}$ BM (paired Student's *t* test). (F) Numbers of CLP per femur from 8-wk-old RARγ$^{+/+}$ and RARγ$^{-/-}$ mice ($n = 3$). *, $P < 0.05$ compared with RARγ$^{+/+}$ BM (paired Student's *t* test). Data are presented as means ± SEM.

Mice that had ≥1% multilineage repopulating donor cells were considered to be positive for donor cell reconstitution and those that had <1% donor cells were considered to be negative.

The number of long-term repopulating HSCs per femur of each genotype is shown in Fig. 2, derived from data given in Fig. S2. There was a 3.3-fold reduction in the number of

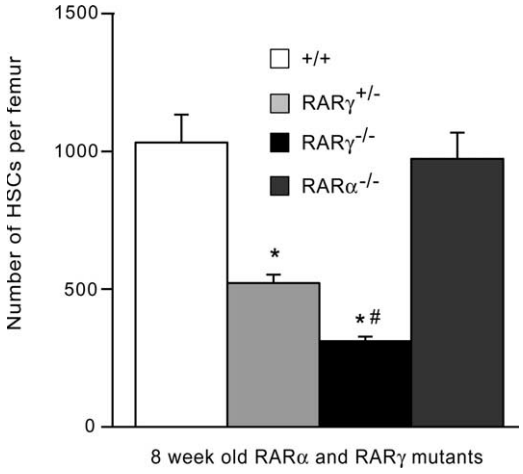


Figure 2. BM cells derived from 8-wk-old mice deficient for RARγ have reduced numbers of HSCs. Number of HSCs per femur of 8-wk-old wild-type (+/+), RARγ$^{+/-}$, RARγ$^{-/-}$, and RARα$^{-/-}$ mice ($n = 8$–10). *, $P < 0.05$ compared with wild-type and RARα$^{-/-}$ BM; #, $P < 0.05$ compared with RARγ$^{+/-}$ BM (paired Student's *t* test). Data are presented as means ± SEM.

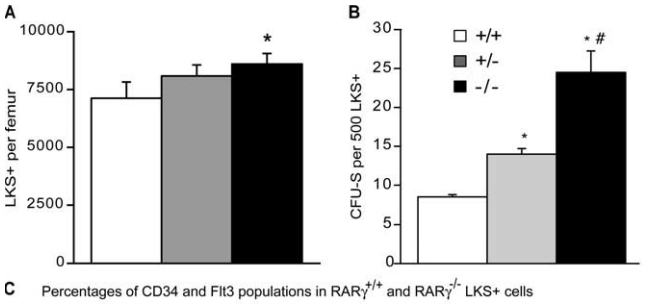
HSCs per femur in BM from RARγ$^{-/-}$ mice compared with wild-type BM (Fig. 2; $P < 0.05$). Furthermore, this profound decrease occurred in the very primitive, multilineage repopulating HSCs, as repopulation of the myeloid and lymphoid lineages was equally affected by loss of RARγ (unpublished data). In marked contrast, there were similar numbers of long-term repopulating HSCs per femur in BM from RARα$^{-/-}$ mice compared with wild-type BM (Fig. 2).

The reduction in HSCs together with the increased numbers of CFU-GEMM, CFU-S, CMPs, and CLPs in RARγ$^{-/-}$ BM (Fig. 1, C–F) suggests that the loss of RARγ in vivo likely results in enhanced differentiation of HSCs into more mature progenitor compartments. Interestingly, the RARγ$^{+/-}$ mice also showed signs of haplo-insufficiency with respect to their HSC content, having twofold fewer HSCs per femur than wild-type mice (Fig. 2; $P < 0.05$) and twofold increased numbers of CFU-S (Fig. 1 D). In marked contrast, we did not detect an altered HSC content in RARα$^{-/-}$ mice compared with their wild-type littermates (Fig. 2). Collectively, these data underscore the importance of RARγ in the maintenance of the HSC pool.

Enriched populations of RARγ null HSCs have reduced long-term repopulating potential accompanied by increased production of progenitor cells

To further explore a potential role of RARγ in regulating HSCs, we assessed the repopulating potential of a more enriched population of RARγ-deficient HSCs, the LKS$^+$ cell population, which are the HSC-containing population used in our previous studies (8, 11).

The numbers of LKS$^+$ cells in RARγ$^{-/-}$ BM were significantly elevated compared with that of RARγ$^{+/+}$ BM (Fig. 3 A, $P < 0.05$). Interestingly and similar to the results



C Percentages of CD34 and Flt3 populations in RARγ$^{+/+}$ and RARγ$^{-/-}$ LKS+ cells

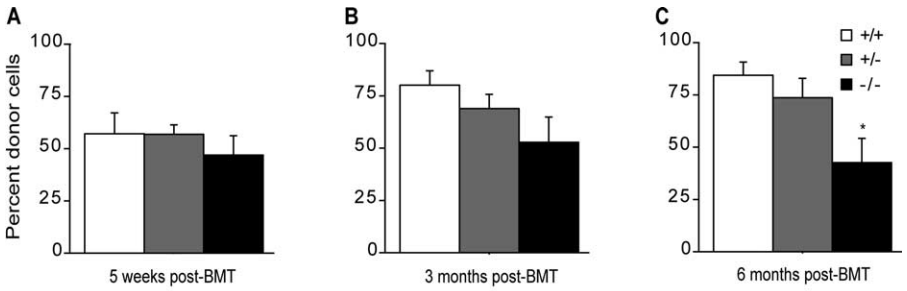
CD34- Flt3-		CD34+ Flt3-		CD34+ Flt3+	
RARγ+/+	7.55 ± 1.04	RARγ+/+	32.82 ± 1.97	RARγ+/+	53.28 ± 2.35
RARγ-/-	8.57 ± 1.21	RARγ-/-	39.76 ± 1.87*	RARγ-/-	44.24 ± 1.35*

Figure 3. Properties of RARγ mutant LKS$^+$ cells. (A) Number of LKS$^+$ cells per femur from 8-wk-old RARγ$^{+/+}$, RARγ$^{+/-}$, and RARγ$^{-/-}$ mice (n = 7–12). *, P < 0.05 compared with RARγ$^{+/+}$ and RARγ$^{+/-}$ LKS$^+$ cells (paired Student's *t* test). (B) Number of CFU-S produced per 500 LKS$^+$ cells ($n = 4$). *, P < 0.05 compared with RARγ$^{+/+}$ LKS$^+$ cells; #, P < 0.05 compared with RARγ$^{+/-}$ LKS$^+$ cells (paired Student's *t* test). Data are presented as means ± SEM. (C) Percentages of CD34 and Flt3 populations in LKS$^+$ cells obtained from 8-wk-old RARγ$^{+/+}$ and RARγ$^{-/-}$ mice ($n = 5$). *, P < 0.05 compared with RARγ$^{+/+}$ LKS$^+$ cells.

obtained from the whole BM cell studies, the numbers of CFU-S formed per 500 LKS$^+$ cells were significantly increased from both RARγ$^{+/-}$ and RARγ$^{-/-}$ cells compared with that of RARγ$^{+/+}$ cells (Fig. 3 B, P < 0.05). It is known that LKS$^+$ cells are heterogeneous; therefore, we further investigated the LKS$^+$ cell compartment of RARγ wild-type and null mice by phenotypic analysis of the CD34 and Flt3 subsets within LKS$^+$ cells, which discriminate between long-term HSCs (CD34$^-$Flt3$^-$), short-term HSCs (CD34$^+$Flt3$^-$), and multipotent progenitors (CD34$^+$Flt3$^+$) (17). RARγ$^{-/-}$ LKS$^+$ cells had significantly reduced percentages of CD34$^+$Flt3$^+$ cells, significantly increased content of CD34$^+$Flt3$^-$, and similar percentages of CD34$^-$Flt3$^-$ compared with RARγ$^{+/+}$ LKS$^+$ cells (Fig. 3 C).

We further examined the functional capacity of LKS$^+$ cells by performing competitive repopulating assays with LKS$^+$ cells obtained from RARγ$^{+/+}$, RARγ$^{+/-}$, and RARγ$^{-/-}$ mutants (8). The numbers of repopulating units (RU) were estimated as previously described for comparison of HSC potential across the genotypes (18).

All populations had similar RU at 5 wk after BM transplant (BMT) (Fig. 4 A). Thereafter, there was a progressive decline in RU from RARγ$^{-/-}$ LKS$^+$ cells compared with RARγ$^{+/+}$ LKS$^+$ cells, resulting in a 2.5-fold reduction by 3 mo and a significant 6.6-fold reduction by 6 mo (Fig. 4, B and C; P < 0.05). Hence, RARγ$^{-/-}$ LKS$^+$ cells had reduced long-term repopulating potential compared with RARγ$^{+/+}$ LKS$^+$ cells, results that are consistent with those obtained using unfractionated BM. The functional potential of the RARγ$^{-/-}$ LKS$^+$ cells therefore did not correlate



Genotype	RU
RARγ+/+	1.93 ± 0.48
RARγ+/-	1.55 ± 0.32
RARγ-/-	1.22 ± 0.28

Genotype	RU
RARγ+/+	6.17 ± 1.15
RARγ+/-	3.34 ± 0.84
RARγ-/-	2.42 ± 0.80*

Genotype	RU
RARγ+/+	13.14 ± 3.93
RARγ+/-	6.06 ± 1.82
RARγ-/-	1.99 ± 1.11*

D Survival and Cell Cycle Properties of RARγ$^{+/+}$ and RARγ$^{-/-}$ LKS+ populations

% apoptotic cells in LKS+		% G0/G1 in LKS+		% G0 in G0/G1 of LKS+	
RARγ+/+	3.42 ± 1.12	RARγ+/+	98.1 ± 0.10	RARγ+/+	8.70 ± 0.49
RARγ-/-	2.86 ± 0.79	RARγ-/-	97.1 ± 1.05	RARγ-/-	9.38 ± 2.29

Figure 4. RARγ$^{-/-}$ LKS$^+$ cells from 8-wk-old mice have reduced long-term competitive repopulating potential accompanied by increased production of progenitor cells. (A–C) 1,000 LKS$^+$ cells isolated from RARγ$^{+/+}$, RARγ$^{+/-}$, or RARγ$^{-/-}$ BM were mixed with 10^5 congenic BM cells and transplanted into each lethally irradiated congenic recipient ($n = 8$). Peripheral blood was analyzed at (A) 5 wk, (B) 3 mo, and (C) 6 mo after BMT for donor-derived multilineage repopulating cells. The numbers of RU in each population are depicted below their respective graphs. *, P < 0.05 compared with RARγ$^{+/+}$ LKS$^+$ cells (paired Student's *t* test). Data are presented as means ± SEM. (D) Apoptosis and cell cycle properties of RARγ mutant LKS$^+$ cells ($n = 3$–5).

with their CD34 phenotype, which is consistent with reports by other investigators that CD34 subsets do not necessarily provide an accurate measurement of HSC numbers in mutant mice or mice that are not in homeostatic conditions (19–22). In contrast, RAR$\alpha^{-/-}$ LKS$^+$ cells did not have altered repopulating potential compared with wild-type LKS$^+$ cells (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20052105/DC1).

We further investigated properties of RAR$\gamma^{+/+}$ and RAR$\gamma^{-/-}$ LKS$^+$ cell populations to determine if altered survival or cell cycle properties could account for the reduced repopulating activity of the RAR$\gamma^{-/-}$ LKS$^+$ cells. There were no differences in apoptosis, the percentages of cells in G_0/G_1 or the proportion of G_0/G_1 LKS$^+$ cells that were in G_0 in RAR$\gamma^{-/-}$ LKS$^+$ cells compared with RAR$\gamma^{+/+}$ LKS$^+$ cells (Fig. 4 D). Hence, it did not appear that altered survival or cell cycle properties were contributing to the reduced long-term repopulating potential of RAR$\gamma^{-/-}$ LKS$^+$ cells.

BM obtained from 12-mo-old RARγ null mice have reduced CFU-GEMM colony-forming cell potential

RAR$\gamma^{-/-}$ mice have been reported to exhibit early lethality from unknown causes (14). In our care, a small number of RAR$\gamma^{-/-}$ mice have survived to 12 mo of age, which appears to be the maximum lifespan of these mutants. In these older mice, the PB and BM cellularities were slightly elevated compared with their wild-type age-matched littermates (Fig. 5, A and B). The total CFC content was similar between all genotypes (Fig. 5 C). In contrast and opposite to that of 8-wk-old RAR$\gamma^{-/-}$ mice, the numbers of the immature CFU-GEMM were markedly reduced in RAR$\gamma^{-/-}$ BM compared with their wild-type littermates (Fig. 5 D; $P < 0.05$). The CFU-GEMM content, the opposite of that of BM from 8-wk-old RARγ null mice (Fig. 2 D), is also consistent with enhanced differentiation and reduced self-renewal capacity of RARγ HSCs, which, as the HSCs exhaust over time, would likely result in reduced immature colony-forming cell potential in the BM of ageing mice.

ATRA-induced enhancement/maintenance of HSCs in ex vivo cultures requires RARγ

We have previously shown that ATRA-treated LKS$^+$ cells have enhanced long-term repopulating activity after 14 d of culture compared with LKS$^+$ cells cultured without ATRA (8). We wished to determine if RARγ was the major RAR responsible for this maintenance of HSCs in ATRA-treated

A Peripheral Blood Cellularity

RARγ Genotype	WBC (x10^6/ml)	RBC (x10^9/ml)	PLT (x10^6/ml)	HGB (g/dl)	HCT (%)
+/+	10.75 ± 2.15	9.51 ± 0.14	1673 ± 109	13.65 ± 0.85	41.05 ± 3.05
+/-	11.73 ± 1.67	9.46 ± 0.10	1491 ± 114	13.33 ± 0.12	39.63 ± 0.12
-/-	19.85 ± 5.55	9.16 ± 0.15	1950 ± 50	12.15 ± 0.05	36.45 ± 0.45

B Bone Marrow Cellularity (x10^6/femur)

RARγ Genotype	WBC	RBC	PLT
+/+	40.40 ± 2.70	55 ± 5	103 ± 5
+/-	42.80 ± 5.37	53 ± 12	112 ± 14
-/-	51.85 ± 0.65	35 ± 15	124 ± 16

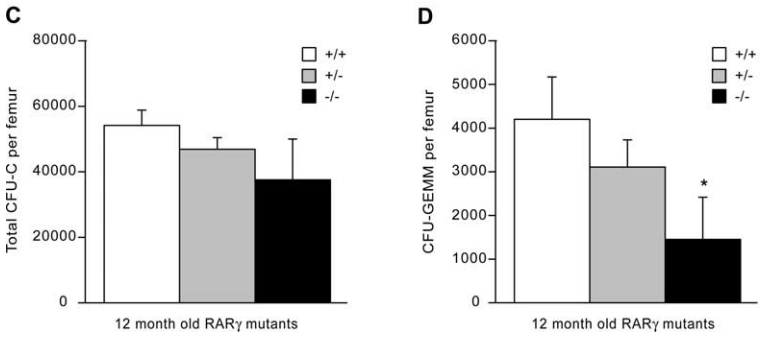


Figure 5. Altered hematopoiesis in 12-mo-old mice deficient for RARγ. (A) Peripheral blood cellularity of 12-mo-old mice ($n = 3$). (B) BM cellularity of 12-mo-old mice ($n = 3$). (A and B) WBC, white blood cells; RBC, red blood cells; PLT, platelets; HGB, hemoglobin; HCT, hematocrit. (C) Numbers of CFU-GEMM produced per femur from 12-mo-old RAR$\gamma^{+/+}$, RAR$\gamma^{+/-}$, and RAR$\gamma^{-/-}$ mice ($n = 3$). *, $P < 0.05$ compared with RAR$\gamma^{+/+}$ BM (paired Student's *t* test). (D) Numbers of total colonies produced per femur from 12-mo-old RAR$\gamma^{+/+}$, RAR$\gamma^{+/-}$, and RAR$\gamma^{-/-}$ mice ($n = 3$). Data are presented as means ± SEM.

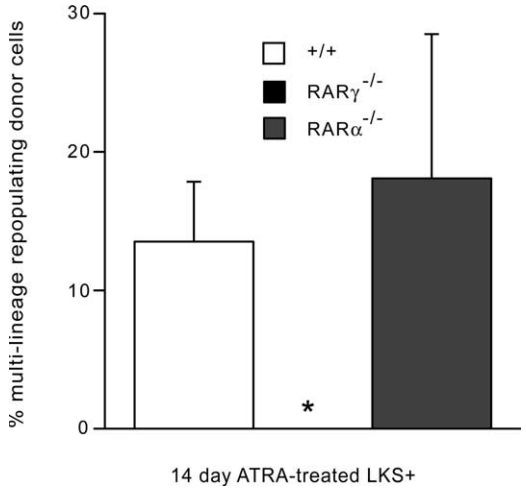


Figure 6. ATRA does not enhance the repopulating ability of RARγ$^{-/-}$ LKS^{+} cells. Shown are the competitive repopulation ability of wild-type (+/+), RARα$^{-/-}$, and RARγ$^{-/-}$ LKS^{+} cells after 14 d of culture with ATRA. Data are expressed as the mean ± SEM donor cell multilineage reconstitution in the peripheral blood of transplanted recipients ($n = 8$) analyzed at 6 mo after BMT. *, $P < 0.05$ compared with wild-type and RARα$^{-/-}$ LKS^{+} cells (paired Student's *t* test).

ex vivo cultures and thus compared ATRA-treated cultures of RARγ$^{+/+}$ and RARγ$^{-/-}$ LKS^{+} cells.

Consistent with our previous studies, neither RARγ$^{+/+}$ nor RARγ$^{-/-}$ LKS^{+} cells cultured without ATRA for 14 d displayed any competitive repopulating potential (unpublished data). After 14 d of culture, ATRA-treated RARγ$^{+/+}$ LKS^{+} cells comprised an average of 14% of peripheral blood cells in the transplanted mice, and these were multilineage repopulating HSCs (Fig. 6). In contrast, ATRA-treated RARγ$^{-/-}$ LKS^{+} cells did not show any multilineage repopulating potential after 14 d of culture (Fig. 6). Furthermore, RARα$^{-/-}$ LKS^{+} cells cultured for 14 d with ATRA had similar competitive repopulating potential (average 18%) compared with ATRA-treated wild-type LKS^{+} cells (Fig. 6). These data show that RARγ, but not RARα, is essential for the maintenance of HSCs in ATRA-treated ex vivo cultures.

RARγ signaling impacts on Notch1 expression

The aforementioned observations suggest that loss of RARγ results in an imbalance in HSC self-renewal decisions, resulting in increased commitment/differentiation of HSCs into more mature progenitor cells. To determine if loss of RARγ altered the expression of genes previously shown to influence HSC self-renewal, we investigated the expression of such genes in mRNA from freshly isolated RARγ$^{+/+}$ and RARγ$^{-/-}$ LKS^{+} cells. We chose to investigate the possible deregulation of three different candidate genes (p21$^{Cip1/Waf1}$, Notch1, and Hoxb4) based on both their known involvement in HSC self-renewal (3, 23, 24) and also their previous links with retinoids in other settings (25–27).

Loss of RARγ did not alter expression of either p21$^{Cip1/Waf1}$ or Hoxb4 in LKS^{+} cells (Table I A). In contrast, there was a significant reduction in the expression of Notch1 in RARγ$^{-/-}$ LKS^{+} cells (Table I A, $P < 0.004$), accompanied by reduced expression of hairy and enhancer of split 1 (Hes1), a measure of activated Notch1 (28) in these cells (Table I A).

To determine if activation of RARγ altered the expression of these genes, we compared the expression of p21$^{Cip1/Waf1}$, Hoxb4, Notch1, and Hes1 in 3-d cultures of RARγ$^{+/+}$ LKS^{+} cells treated for 3 d with or without ATRA. There were no changes in the expression of p21$^{Cip1/Waf1}$ in LKS^{+} cells cultured with or without ATRA for 3 d (Table I B). In contrast, the expression of both Hoxb4 and Notch1 were significantly up-regulated in ATRA-treated LKS^{+} cells compared with LKS^{+} cells cultured without ATRA (Table I B, $P < 0.001$). Interestingly, despite the absence of exogenous Notch1 ligands in these cultures, expression of Hes1 was also significantly increased in the 3-d ATRA-treated LKS^{+} cells compared with the LKS^{+} cells cultured without ATRA (Table I B, $P < 0.005$).

We also compared the expression of these genes in cultures of ATRA-treated RARγ$^{+/+}$ and RARγ$^{-/-}$ LKS^{+} cells. The expression of p21$^{Cip1/Waf1}$ and Hoxb4 was similar for both populations. In contrast, both Notch1 and Hes1 were significantly down-regulated in ATRA-treated RARγ$^{-/-}$ LKS^{+} cells compared with RARγ$^{+/+}$ LKS^{+} cells (Table I C, $P < 0.05$). In marked contrast, studies using RARα$^{-/-}$ LKS^{+} cell populations showed that Notch1 and Hes1 were not down-regulated in RARα$^{-/-}$ LKS^{+} cells (Table I C).

Finally, we examined the expression of Notch1 and Hes1 in 2-d cultures of immature BM cells transduced with RARγ1. Both Notch1 and Hes1 were significantly up-regulated in RARγ1-overexpressing BM compared with those expressing the control vector (Table I D, $P < 0.02$). Interestingly, overexpression of RARα did increase Notch1 expression compared with the control vector, but not to the magnitude of RARγ1 (Table I D, $P < 0.05$). Furthermore, Hes1 was not significantly increased in RARα-overexpressing BM compared with the control vector (Table I D).

ATRA enhances the self-renewal of HSCs

Given that there was no difference in survival or cell cycle properties of RARγ$^{-/-}$ LKS^{+} cells (Fig. 4), whereas both RARγ null LKS^{+} cells and unfractionated BM populations had increased numbers of progenitors accompanied by reduced numbers of HSCs (Figs. 1–4), our data are consistent with RARγ null HSCs undergoing reduced self-renewal and more differentiation decisions. Furthermore, Notch1 and Hes1, a measure of activated Notch1, which has previously been shown to enhance HSC self-renewal (3), were significantly reduced in RARγ$^{-/-}$ LKS^{+} cell populations (Table I), further supporting that possibility. In addition, there was increased expression of two genes previously associated with HSC self-renewal (Hoxb4 and Notch1) after 3 d of culture of wild-type LKS^{+} cells with ATRA compared with LKS^{+} cells cultured without ATRA (Table I B). In this study (Fig. 6) and in a previous report, we have also shown that there is retention

Table I. Notch1 expression is altered by RARγ signaling

A	Freshly isolated LKS$^+$		
	RARγ$^{+/+}$	RARγ$^{-/-}$	RARα$^{-/-}$
p21	28.07 ± 3.12	26.99 ± 2.77	ND
Hoxb4	3.12 ± 0.46	3.73 ± 0.23	ND
Notch1	315.4 ± 45.1	192.59 ± 21.6[a]	1,065 ± 90.8[b]
Hes1	96.74 ± 5.26	70.00 ± 12.7[a]	83.44 ± 9.35

B	3-d cultured wild-type LKS$^+$	
	No ATRA	ATRA
p21	5.38 ± 0.38	4.79 ± 0.55
Hoxb4	4.05 ± 0.48	12.39 ± 0.67[c]
Notch1	122.59 ± 2.19	290.55 ± 14.2[c]
Hes1	1.39 ± 0.23	8.02 ± 1.39[c]

C	3-d ATRA-treated LKS$^+$		
	RARγ$^{+/+}$	RARγ$^{-/-}$	RARα$^{-/-}$
p21	4.79 ± 0.55	4.25 ± 0.27	ND
Hoxb4	12.39 ± 0.67	11.27 ± 1.43	ND
Notch1	290.55 ± 14.2	205.94 ± 14.2[a]	361.9 ± 32.1
Hes1	8.02 ± 1.39	5.32 ± 0.57[a]	11.48 ± 1.00[b]

D	Gene overexpression studies		
	Control	RARγ1	RARα
Notch1	122.12 ± 8.93	352.94 ± 55.7[d,e]	202.42 ± 18.7[d]
Hes1	10.59 ± 0.65	16.49 ± 2.51[d,e]	10.80 ± 1.37

The levels of expression of the different genes were quantitated by Q-RT-PCR and are given as arbitrary units relative to β_2-microglobulin for each of the genes. Shown are the relative expression of HSC self-renewal–associated genes in (A) freshly isolated RARγ$^{+/+}$, RARγ$^{-/-}$, and RARα$^{-/-}$ LKS$^+$ cells; (B) 3-d cultured wild-type LKS$^+$ cell populations; (C) 3-d ATRA-treated RARγ$^{+/+}$, RARγ$^{-/-}$, and RARα$^{-/-}$ LKS$^+$ cells; and (D) control, RARγ1-, or RARα-overexpressing primitive hematopoietic cells. LKS$^+$, lineage$^-$, c-*kit*$^+$, Sca-1$^+$ (HSC enriched). The data represent the mean ± SEM of three different experiments for each population tested. ND, not determined.

[a]$P < 0.05$ RARγ$^{-/-}$ LKS$^+$ vs. RARγ$^{+/+}$ LKS$^+$ and RARα$^{-/-}$ LKS$^+$ cells.

[b]$P < 0.05$ RARα$^{-/-}$ LKS$^+$ vs. RARγ$^{+/+}$ LKS$^+$ cells.

[c]$P < 0.05$ ATRA vs. No ATRA treatment.

[d]$P < 0.05$ RARγ1 or RARα vs. control overexpressing cells.

[e]$P < 0.05$ RARγ1 vs. RARα-overexpressing cells.

of repopulating potential in wild-type LKS$^+$ cells cultured with ATRA for 14 d compared with loss of repopulating ability in LKS$^+$ cells cultured in the same conditions but without ATRA (8). We now show that RARγ is required for these effects (Fig. 6). Therefore, we wished to determine if ATRA was enhancing the self-renewal of wild-type HSCs.

The HSC compartment has been shown to be heterogeneous, comprised of a hierarchy of HSCs that can be identified by their functional capacity. The most immature HSC in this hierarchy is capable of sustaining hematopoiesis throughout serial transplantation (Fig. 7 A) (29, 30). Therefore, to determine if ATRA enhanced self-renewal of these highly primitive, serially transplantable HSCs, we investigated the serial BMT potential of LKS$^+$ cells cultured with or without ATRA for 3 d compared with noncultured LKS$^+$ cells using competitive repopulation assays.

The long-term, multilineage, competitive repopulating HSC potential (6 mo after BMT) of 1,000 freshly isolated LKS$^+$ cells or their cultured equivalents was first measured in primary lethally irradiated recipients. BM cells from these mice were then pooled within treatment groups and injected into secondary lethally irradiated recipients. The multilineage repopulating potential of HSCs in secondary recipients and each subsequent serial transplant recipient was measured at 3 mo after BMT, then BM from these mice were pooled and injected into the next serial transplant recipients.

The donor cell chimerism of HSCs cultured with or without ATRA for 3 d increased relative to the control (noncultured) LKS$^+$ cells (% donor cell chimerism, mean ± SEM control: 59.7 ± 17, no ATRA: 74.91 ± 10.75, ATRA: 73.98 ± 10.95, Fig. 7 B). Interestingly, in the secondary recipients of the HSCs cultured without ATRA, the donor cell chimerism was significantly reduced compared with the control, suggesting that there was enhanced differentiation into more mature HSCs within the HSC hierarchy. In contrast, the reconstituting ability of the 3-d ATRA-treated LKS$^+$ cells remained significantly higher in the secondary recipients compared with that of recipients of control (noncultured) LKS$^+$ cells, even during subsequent transplantation (Fig. 7 B). Of note, all quaternary recipients of the 3-d ATRA-treated

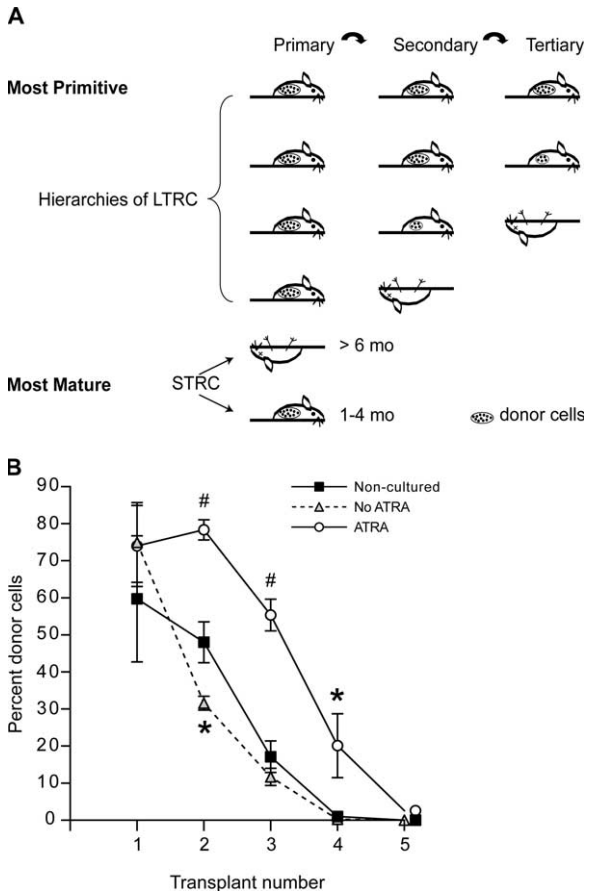


Figure 7. ATRA enhances the serial transplantability of cultured HSCs. (A) Experimental protocol for detecting the most primitive HSCs by serial transplantation. (B) The data show the increased serial transplantability of LKS^+ cells after 3 d of culture with ATRA compared with noncultured LKS^+ or LKS^+ cells cultured without ATRA for 3 d. Data are expressed as the mean ± SEM donor cell reconstitution in the peripheral blood of transplanted recipients (six mice per group) analyzed at 6 mo (primary transplant) and 3 mo (subsequent serial transplants) after BMT. *, $P < 0.05$; #, $P < 0.01$ compared with noncultured LKS^+ cells (paired Student's *t* test).

LKS^+ cells had significant multilineage donor reconstituting ability, with donor repopulating levels in quaternary BMT recipients of 20.1 ± 8.6% (Fig. 7 B). In marked contrast, no quaternary BMT recipient of noncultured LKS^+ cells or LKS^+ cells cultured without ATRA for 3 d had detectable donor cell reconstitution (Fig. 7 B). The strikingly increased serial transplantability of the ATRA-treated LKS^+ cells over that of the noncultured LKS^+ cells, which would have the highest serial transplant potential if self-renewal was not enhanced, indicated that pharmacological stimulation with ATRA enhanced the self-renewal of HSCs in the 3 d ex vivo cultures.

DISCUSSION

In these studies, we show that the two major RARs expressed in hematopoietic cells, RARα and RARγ, have distinct roles in the regulation of immature progenitors and HSCs. We demonstrate that $RAR\gamma^{-/-}$ mice have increased numbers of committed hematopoietic progenitors (Fig. 1, C–F) and that this is accompanied by significantly reduced numbers of long-term repopulating HSCs (Figs. 2 and 4 C). These abnormalities are not observed in $RAR\alpha^{-/-}$ mice (Fig. 2 and Fig. S3). The survival and cell cycle properties of enriched populations of $RAR\gamma^{-/-}$ HSCs were not different to those of $RAR\gamma^{+/+}$ HSCs, suggesting that the impaired repopulating potential of the $RAR\gamma^{-/-}$ HSCs were not the result of altered apoptosis or cycling of these HSCs (Fig. 4 D). Furthermore, we show that ATRA enhances HSC self-renewal (Fig. 7 B) and that RARγ, but not RARα, is required for the potentiating effects of ATRA in extended ex vivo culture systems (Fig. 6).

These data collectively demonstrate that RARγ is a key physiological regulator of HSCs. In $RAR\gamma^{+/+}$ HSCs, there is a balance between HSC self-renewal and differentiation decisions. When treated with pharmacological levels of ATRA, the HSCs undergo more self-renewal divisions (Fig. 7 B). In contrast, our observations that $RAR\gamma^{-/-}$ mice display increased numbers of committed progenitors (Fig. 1, C–F, and Fig. 3 B) and reduced numbers of long-term repopulating HSCs (Figs. 2 and 4 C) suggests that the HSCs from $RAR\gamma^{-/-}$ mice display an imbalance between self-renewal and differentiation, favoring differentiation divisions. Furthermore, loss of RARγ signaling results in the inability of the HSCs to maintain repopulating ability in response to ATRA treatment in ex vivo culture (Fig. 6). In addition, the haplo-insufficiency shown by $RAR\gamma^{+/-}$ HSCs, which had an intermediate phenotype between that of $RAR\gamma^{-/-}$ and $RAR\gamma^{+/+}$ HSCs (Figs. 1–4), also underscores the importance of RARγ signaling in HSCs. These data demonstrate that RARγ is a key regulator of HSC homeostasis, influencing the balance between self-renewal and differentiation decisions of the HSC.

In a previous investigation, Labrecque et al. reported that RARγ null mice do not have altered numbers of progenitor cells (9). The major difference between the previous study and ours is that they used fetal BM (18.5 dpc), whereas we used BM from adult (8-wk-old) mice. In addition, in contrast with the increased numbers of progenitors in 8-wk-old mice, we observed significantly reduced numbers of progenitors in 12-mo-old RARγ null mice. These contrasting data therefore demonstrate that the age of the mice studied can also be crucial to the results obtained as can the subsequent interpretation of that data, when investigating hematopoietic defects such as that observed in $RAR\gamma^{-/-}$ mice.

The effects of RARγ were specific to this RAR, as RARα null mice did not display any HSC defects (Fig. 2), and RARα null HSCs retained their ability to self-renew in ex vivo culture in response to ATRA treatment (Fig. 6). RARβ2 was not apparently important to the HSC defect observed in $RAR\gamma^{-/-}$ mice as it was expressed at equal levels in both $RAR\gamma^{+/+}$ and $RAR\gamma^{-/-}$ LKS^+ cells (unpublished data). Collectively, these data highlight the importance and nonredundant role of RARγ in mediating the HSC self-renewing effects induced by ATRA treatment.

In hematopoiesis, ATRA is predominantly known as a differentiating agent, potently inducing the terminal granulocytic maturation of primary leukemia cells from patients with acute promyelocytic leukemia (31–33). We have also shown that, in contrast with its potentiating effects on HSC self-renewal, ATRA also has potent effects in enhancing normal primary granulocyte differentiation in vitro (11). RARα appears to be the most potent of the three receptors in inducing these effects, as shown by its genetic involvement in acute promyelocytic leukemia (34), and it has the most dramatic effects on neutrophil differentiation from both maturing committed granulocyte progenitors and more immature progenitors (35, 36).

In the current study, we have confirmed the predominant role of RARα in granulopoiesis, as RARα-overexpressing BM cells rapidly differentiated down the granulocytic pathway compared with both the control and RARγ1-overexpressing cells (Fig. S1). However, despite these profound effects of RARα in enhancing primitive cell differentiation down the granulocyte lineage, RARα does not appear to have a role in regulating HSCs, as we did not observe any defects in the numbers of primitive progenitors or HSCs in RARα null mice.

It was of interest to determine whether RARγ signaling affected other genes known to induce HSC self-renewal (3, 23, 24). Despite having previously been reported to have an retinoic acid response element in its promoter region (37), expression of the cyclin-dependent kinase inhibitor $p21^{Cip1/Waf1}$ was not altered by ATRA treatment (Table I B), nor was it deregulated in RARγ$^{-/-}$ LKS$^+$ cells (Table I, A and C). We have also previously shown that $p27^{Kip1}$, which has roles in HSC homeostasis (38, 39), is in part required for RARα-, but not RARγ-, mediated responses (36); therefore $p27^{Kip1}$ is also unlikely to be involved in RARγ-induced HSC self-renewal.

The expression of Hoxb4 was markedly up-regulated in ATRA-treated LKS$^+$ cells (Table I B), consistent with previous studies showing that retinoids regulate Hox gene expression in other organs (40). Despite this increase, however, there were no differences in Hoxb4 expression between the RARγ$^{-/-}$ and RARγ$^{+/+}$ LKS$^+$ cells (Table I, A and C); hence, Hoxb4 is not likely a target gene of RARγ.

In contrast with Hoxb4 and $p21^{Cip1/Waf1}$, Notch1 expression was markedly increased in both ATRA-treated RARγ$^{+/+}$ LKS$^+$ cells (Table I B) and RARγ1-overexpressing BM cells (Table I D) and significantly decreased in RARγ$^{-/-}$ LKS$^+$ cells compared with wild-type LKS$^+$ cells (Table I, A and C). This was accompanied by similarly altered levels of expression of Hes1, a measure of Notch-mediated signaling, in these populations (Table I). In contrast, Notch1 and Hes1 were not significantly down-regulated in RARα$^{-/-}$ LKS$^+$ cells (Table I).

Of interest, it has recently been shown that Notch signaling is down-regulated as HSCs differentiate (41). Given both our observations of the reduced self-renewal/enhanced differentiation of RARγ$^{-/-}$ HSCs and the reduced expression of Notch1 in RARγ$^{-/-}$ LKS$^+$ cells, it is possible that altered Notch1 signaling was contributing to the HSC defects observed in RARγ null mice. Likewise, it is possible that increased signaling of Notch1 was contributing to the enhanced HSC self-renewal observed in ATRA-treated cultures of wild-type HSCs (Fig. 7 B). However, unlike RARγ null mice, Notch1 knockout mice do not have an HSC defect (7); hence, altered Notch1 is unlikely to be the main contributing factor to the HSC defects observed in RARγ null mice.

Of therapeutic relevance is that HSCs were induced to self-renew in simple ex vivo cultures using pharmacological levels of ATRA, which is approved for use. Interestingly, a recent report demonstrated the potential beneficial use of ATRA for the expansion of human HSCs (42). Our studies demonstrating the different roles of the RARs and especially the critical role for RARγ in regulating HSCs now suggest that specifically targeting RARγ could further improve on the ATRA-mediated ex vivo expansion of HSCs. Additional studies are therefore warranted to further investigate the effects of RAR-specific ligands in enhancing the expansion of HSCs for therapeutic purposes.

MATERIALS AND METHODS

Mice. For serial transplant studies, donor mice were C57BL/6J (CD45.2$^+$) and recipient mice were B6.SJL-Ptprc^{a}Pep3^b/BoyJ (herein referred to as Ptprca; CD45.1$^+$), both were obtained from Animal Resources Centre, Perth, WA, Australia. RARα (13) and RARγ (14) mutant mice were backcrossed 10 generations onto the C57BL/6J background for these studies. Except when otherwise described, all mice were used between 8 and 10 wk of age. All experiments performed were approved by the Peter MacCallum Cancer Centre animal experimentation ethics committee and were conducted in strict compliance to the regulatory standards of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Quantitation of progenitor cell content in RAR mutant mice. The number of CFU-GEMM per 5×10^4 BM cells and CFU-S from 5×10^5 BM and 500 LKS$^+$ cells were measured as described previously (11). The numbers of BM CMP and CLP were measured as described previously (38, 43, 44).

Limiting dilution assay. The frequency of HSCs in whole BM obtained from RARγ+/+, +/−, or −/− mice was determined by Poisson statistics using the limiting dilution assay described previously (16). Recipient mice were lethally irradiated with a total dose of 10 Gy, given in two equal fractions 3 h apart, and delivered by two opposing ^{137}Cs sources (Gammacell 40, Atomic Energy of Canada) on the day of transplant. Recipient CD45.1$^+$ mice (8–10 mice per group) were injected with 10^4, 5×10^4, 2×10^5 or 2×10^6 BM cells obtained from the CD45.2$^+$ RARγ mice together with 2×10^5 Ptprca/C57BL/6J (CD45.2$^+$/CD45.1$^+$) congenic BM. Mice were assessed for CD45.2$^+$/CD45.1$^-$ donor cell peripheral blood reconstitution in lymphoid and myeloid lineages at 6 mo posttransplant as described previously (8, 38).

LKS$^+$ cell culture and transplantation studies. LKS$^+$ cells and LKS$^-$ cells were isolated and cultured in Iscove's modified essential medium containing 20% FBS, 100 ng/ml of each recombinant mouse (rm) SCF, recombinant human (rh) IL-6, rhFlt-3L, and 10 ng/ml rhIL-11 (Peprotech, Inc.) as described previously (11). Cultures were supplemented with or without 1 μM ATRA (Sigma-Aldrich).

Primary competitive repopulating potential of RARγ mutant LKS$^+$ cells was performed as described previously (8). The numbers of RU were assessed according to the method of Harrison et al. (18). In brief, donor RU = % donor x competitor RU/(100 − % donor), where competitor RU = number of competing BM ($\times 10^5$), in this situation = 1.

Primary competitive repopulating transplants of noncultured and 3-d cultured LKS$^+$ cells were performed as described previously (8). Donor mice were C57BL/6J (CD45.2$^+$), and recipient mice were Ptprca (CD45.1$^+$), both obtained from Animal Resources Centre. Multilineage peripheral blood cell reconstitution by CD45.2$^+$ donor cells was monitored at 6 mo posttransplant.

For serial transplant studies, the primary recipient mice were killed at 7 mo posttransplant and the contents of their femurs were pooled within the respective groups. Irradiated secondary Ptprca recipients received BM cells equivalent to one third of the femoral contents of the primary recipients. Donor (CD45.2$^+$) cell reconstitution was assessed at 3 mo posttransplant for the secondary and subsequent recipients, then these recipients were killed and the contents equivalent to one tenth of a femoral content were injected into new lethally irradiated Ptprca recipients.

Additional properties of LKS$^+$ cells obtained from RARγ null mice. Apoptosis was measured in LKS$^+$ cells by the annexin V–FITC apoptosis detection kit (Becton Dickinson), according to the manufacturer's instructions. Cell cycle analysis of LKS$^+$ cell populations was performed as described previously (38). The proportions of CD34 and Flt3 populations in LKS$^+$ cells were measured as described previously (17).

RNA extraction and quantitative RT-PCR. Extraction of mRNA and synthesis of cDNA were performed as described previously (45). All reactions were conducted using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). 8 μl cDNA (1:10 dilution) was combined with 10 μl SyBr green PCR Master Mix (Applied Biosystems) and 200 nm each of sense and antisense oligonucleotides (Geneworks). PCR conditions were as follows: 50°C for 2 min and 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. Expression of all genes were compared with β_2-microglobulin to determine relative levels of mRNA transcripts. All primers used in these studies are given in Table S2 (available at http://www.jem.org/cgi/content/full/jem.20052105/DC1).

Statistics. The results are expressed as the mean ± SEM for *n* given samples. Data were analyzed using the paired two-tailed Student's *t* test, with any p-value ≤0.05 being considered significant.

Online supplemental material. Fig. S1 shows the effects of overexpression of RARα or RARγ1 on primitive BM cells; Fig. S2 shows the limiting dilution graphs for calculation of HSC numbers in RARγ mutants; and Fig. S3 shows the repopulating potential of RARα$^{-/-}$ and RARα$^{+/+}$ LKS$^+$ cell populations. Table S1 shows the expression of RARs in LKS$^+$ and LKS$^-$ cell populations. Table S2 provides real-time primer sequences used in these studies. Online supplemental material, including supplemental Materials and methods, is available at http://www.jem.org/cgi/content/full/jem.20052105/DC1.

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