Mice Bearing a Targeted Interruption of the Homeobox Gene *HOXA9* Have Defects in Myeloid, Erythroid, and Lymphoid Hematopoiesis

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Several homeobox genes of the *HOXA* and *HOXB* clusters are expressed in primitive blood cells, suggesting a role for *HOX* genes in normal hematopoiesis. The *HOXA9* gene is expressed in CD34$^+$ marrow cells and in developing lymphocytes. We examined blood-forming organs of mice homozygous for an interrupted *HOXA9* allele to determine if loss of *HOX* gene function is deleterious to hematopoiesis. *HOXA9*−/− mice have approximately 30% to 40% reductions in total leukocytes and lymphocytes ($P < .001$) and a blunted granulocytic response to granulocyte colony-stimulating factor (G-CSF). Homozygous mice have significantly smaller spleens and thymuses. Myeloid/erythroid and pre-B progen-

itors in the marrow are significantly reduced, but no significant decreases are noted in mixed colonies, day 12 colony-forming units-spleen (CFU-S), or long-term culture–initiating cells (LTC-IC), suggesting little or no perturbation in earlier progenitors. Heterozygous animals display no hematopoietic defects. The abnormalities in leukocyte production are transplantable, indicating that the defect resides in the hematopoietic cells. These studies demonstrate a physiologic role for a *HOX* gene in blood cell differentiation, with the greatest apparent influence of *HOXA9* at the level of the committed progenitor.

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MUCH DATA HAVE emerged on the role of the *HOX* family of homeobox genes during embryonic development.[1,2] The Hox proteins are transcription factors that have a major effect in specifying position and tissue fate in the embryo.[3] Mammalian *HOX* genes are organized in 4 clusters (*HOXA, HOXB, HOXC,* and *HOXD*), each containing 9 to 11 genes.[4] Individual *HOX* genes are expressed in precise temporal and spatial patterns along various axes in the embryo,[5] and targeted interruptions of *HOX* genes in mice often produce dramatic morphologic abnormalities of multiple tissues in specific regions of the embryo.[6-8] These studies indicate a key role for *HOX* genes in patterning during embryogenesis.

A number of studies indicate that *HOX* genes are also expressed in adult tissues, including blood cells.[9] Analysis of *HOX* gene expression in various subsets of normal marrow cells demonstrates that several members of the *HOXA* and *HOXB* loci are expressed in primitive CD34$^+$ marrow cells, and their expression is downregulated during differentiation.[10] Certain 5′ genes such as *HOXA9* and *HOXA10* are strongly expressed in CD34$^+$ cells and are inactivated as

cells leave the CD34$^+$ compartment. Other more 3′ genes such as *HOXB3* are downregulated within the CD34$^+$ compartment as cells progress to the committed progenitor stage. Other studies have confirmed that *HOXB3* is strongly expressed in normal marrow cells that are enriched for pluripotent progenitors, and that induction of differentiation is accompanied by an ordered 3′ to 5′ wave of *HOX* gene activation.[11] A similar wave of *HOX* gene expression has been reported during T-cell activation.[12] These studies suggest that sequential activation of certain *HOX* gene clusters may orchestrate, in part, the differentiation of various hematopoietic lineages.

A definitive physiologic role for *HOX* genes in blood cell development and differentiation remains to be established. Overexpression of *HOXB4*[13] or *HOXB8*[14] genes in normal murine cells results in marked expansion of primitive progenitors, suggesting that altered *HOX* gene expression can perturb early events in hematopoiesis. However, these observed phenotypic changes do not by themselves establish the normal function of homeobox genes in blood formation. To provide direct evidence for a role of *HOX* genes in the regulation of hematopoiesis, we examined the hematopoietic systems of *HOXA9*−/− knockout mice. These studies demonstrate that loss of function of *HOXA9* results in a reduction in peripheral blood leukocytes and contractions in committed myeloid and lymphoid progenitors.

MATERIALS AND METHODS

Fluorescence-activated cell sorting, cDNA generation and amplification, and Southern blotting. Normal low-density human marrow cells were isolated, stained with directly conjugated fluorescent antibodies to CD34, CD45RA, and CD71, and then sorted on a FACStarPlus (Becton Dickinson, San Jose, CA) as described previously.[10] A similar strategy was used to sort murine marrow cells and thymocytes using fluorescent antibodies to sca1, CD4, CD8, B220, CD43, IgM, and IgD. The methodology for generating and amplifying the cDNA from individual cell fractions is described in detail elsewhere.[15] *HOXA9* expression in sorted cell populations was determined by Southern blot analysis of amplified cDNAs using ^{32}P-labeled probes derived from the non–homeobox-containing 3′ flanking regions of the human or mouse *HOXA9* gene, respectively. Total cDNA synthesis was estimated by reprobing the blots with either a human or murine β-actin probe as previously described.[10]

Targeting a disruption of the HOXA9 gene in murine embryonic

stem cells. Mice bearing a targeted disruption of *HOXA9* were furnished by Dr Cynthia Peterson and Dr Mario Capecchi. The details of engineering embryonic stem (ES) cells with a disrupted *HOXA9* allele are described elsewhere (C. Peterson et al, manuscript submitted). In brief, a 1.8-kb *Bg1* II/*Eco*RI fragment (shown in brackets in Fig 2A) of the *HOXA9* gene was replaced with the MCI *neo* cassette. This fragment contains most of the homeobox plus 3′ noncoding sequences. The rearranged *HOXA9* allele in the resulting targeted cell line 3C-3 is shown below. 3C-3 cells were injected into C57B1 blastocysts. Resultant chimeric males were crossed with C57B1/6J females to generate heterozygous offspring, which were then back-crossed to C57B1/6J mice two to four times.

Study animals and genotyping of progeny. Both *HOXA9*+/− and *HOXA9*−/− mice appear normal and are fertile. For some experiments, heterozygotes were intermated to generate homozygotes and wild-type littermates as controls. In other experiments, *HOXA9*−/− mice were compared with animals of the parental strains to rule out a contribution from a mixed genetic background, since the targeted 3C-3 cell line is of 129 Sv/Ev origin. In these studies, the control mice included C57B1/6J (Jackson Laboratory, Bar Harbor, ME), 129 Sv/Ev (Taconic Farms, Germantown, NY), and C57B1/129 F1 hybrid mice, which were bred and maintained at the Animal Research Facility of the Veterans Affairs Medical Center, San Francisco. *HOXA9*−/− animals for these experiments were generated by interbreeding homozygous mice. Mice used as marrow recipients in colony-forming units-spleen (CFU-S) assays and long-term transplantation studies were 8- to 14-week-old female C57B1/6J animals. All animals were housed in microisolator cages and provided with sterilized food and acidified water.

The progeny of various intercrosses were genotyped by polymerase chain reaction (PCR) using DNA isolated from tail or toe clips[16] of weanlings and a three-primer system to distinguish wild-type from mutant *HOXA9* alleles. The two *HOXA9*-specific primers were hoxa-9#1 (CGCTGGAACTGGAGAAGGAGTTTCTG) and hoxa-9#2 (ATCCTGCGGTTCTGGAACCAGATC); the Neo-specific primer was Neo (TCTATCGCCTTCTTGACGAGTTC) (Fig 2A). The primers hoxa-9#1 and hoxa-9#2 generate a 123-bp band representing the wild-type allele, whereas hoxa-9#1 and Neo produce a 200-bp band representing the mutant allele (Fig 2B).

Fluorescence-activated cell sorting (FACS) analysis. FACS analysis was performed using a FACScan machine (Becton Dickinson). The antibodies used included TER119 (Pharmingen, San Diego, CA) for erythroid cells; Gr-1 (Pharmingen) and Mac-1 (CalTag, South San Francisco, CA) for granulocytes and monocytes; CD3, CD4, and CD8 (CalTag) for T lymphocytes; B220 (CalTag) and CD43 (Pharmingen) for B cells; and CD34 and c-kit (Pharmingen) for primitive cells.

Assays for committed progenitors. Myeloid/erythroid progenitor assays of marrow and spleen cells from adult mice were performed in 0.8% methylcellulose in alpha medium containing 30% fetal calf serum (FCS), 3 U/mL human urinary erythropoietin, and 2% spleen cell–conditioned medium (StemCell Technologies, Vancouver, Canada) as previously described.[13] To determine the distribution of progenitors, colonies from six experiments were plucked for cytospin preparations and scored for colony type. Pre-B cell colony assays of adult marrow were performed using 0.8% methylcellulose in Iscove's medium with 30% FCS, 10^{-4} mol/L 2-mercaptoethanol (2-ME), and 5 ng/mL interleukin-7 (Sandolfi-Winthrop, College Park, PA). Marrow cells were plated at 50,000 and 100,000 per dish, and colonies were counted at days 5 to 7. Representative colonies were plucked to confirm that the cells had lymphoid morphology.

B-lymphoid progenitor cell assay. A monolayer of the stromal cell line S17[17] was established by plating 5×10^4 S17 cells on 6-cm^2 tissue culture dishes in RPMI with 5% FCS and 50 mmol/L 2-ME, following previously described methods.[18] When cells reached near-confluence (2 to 3 days), 1.5×10^6 unfractionated marrow cells were plated on the stromal layer. A 70% media change was performed every 3 days. After 5 weeks, nonadherent cells were washed off the dish, counted, stained with anti-B220 and anti-CD43, and evaluated by FACS analysis. B220$^+$CD43$^+$ cells were scored as pro-B cells, and B220$^+$CD43$^-$ cells as pre-B cells.

Day 12 CFU-S. Marrow cells were harvested from the femora of adult *HOXA9*−/− mice and either wild-type littermates and C57B1 mice. Recipient C57B1 mice were lethally irradiated (800 cGy, 100 cGy/min, x-ray) and then injected with 50,000 or 100,000 unfractionated marrow cells. Untransplanted irradiated mice were tested in each experiment for endogenous CFU-S and consistently showed no spleen colonies. Twelve days after injection, animals were killed by cervical dislocation, and the number of macroscopic colonies on the spleen were evaluated after fixation in Telleyesniczky's solution.

Long-term culture–initiating cell (LTC-IC) assays. LTC-IC assays were performed as previously described.[19] In brief, S17 feeder layers were established in 96-well plates, and 1 week later these were overlaid with eight different dilutions (range, .23 to 3.0×10^3 cells per well, 12 replicates of each dilution) of marrow cells harvested from adult *HOXA9*−/− mice and control animals. Cultures were maintained at 33°C for 4 weeks with weekly half-media exchanges. Cells were then harvested and plated in myeloid colony assays in methylcellulose, as described earlier, to determine the total clonogenic cell content of each LTC. The number of negative wells (ie, no clonogenic progenitors) was determined for each cell concentration, and the frequency of LTC-IC in the starting population was calculated using Poisson statistics and the weighted mean method.

Statistical analysis. All statistical comparisons of blood cell counts and colony numbers were performed using a two-tailed *t* test, using the Primer of Biostatistics Version 3.0 software for Apple Macintosh by Dr S.A. Glantz (McGraw-Hill, New York, NY). Standard errors are indicated in all tables and figures.

RESULTS

Expression of HOXA9 in normal mammalian blood cells. In previous studies using leukemic cell lines, we observed that some *HOXA* genes appeared to be preferentially expressed in myelomonocytic cells.[20,21] To determine more accurately the expression patterns of *HOXA9* in normal hematopoietic cells, we performed studies of fractionated human marrow cells using reverse transcriptase–PCR and Southern blotting, and observed that *HOXA9* is expressed at high levels in CD34$^+$ cells but only faintly if at all in CD34$^-$ cells (Fig 1A). Expression levels appear particularly high in CD34$^+$ cells enriched for LTC-ICs (lane 1) and for myeloid progenitors (lane 2), although there is detectable message in the erythroid progenitor fraction (lane 3) as well. *HOXA9* is also expressed in developing murine blood cells (Fig 1B). In sorted murine marrow, *HOXA9* expression is strong in primitive sca1$^+$/Lin$^-$ cells (lanes 2 and 4) but is not detectable in total marrow. In sorted lymphoid populations, *HOXA9* message is detectable in developing lymphocytes of both T-cell (lanes 7 and 8) and B-cell (lanes 12 and 13) lineages. These studies indicate that this gene is active in normal primitive hematopoietic cells and various lymphoid populations.

Generation of HOXA9−/− mice. A targeted interruption of the *HOXA9* gene was introduced into ES cells by homologous recombination as described in the methods. The resulting *HOXA9*+/− and *HOXA9*−/− mice appear entirely
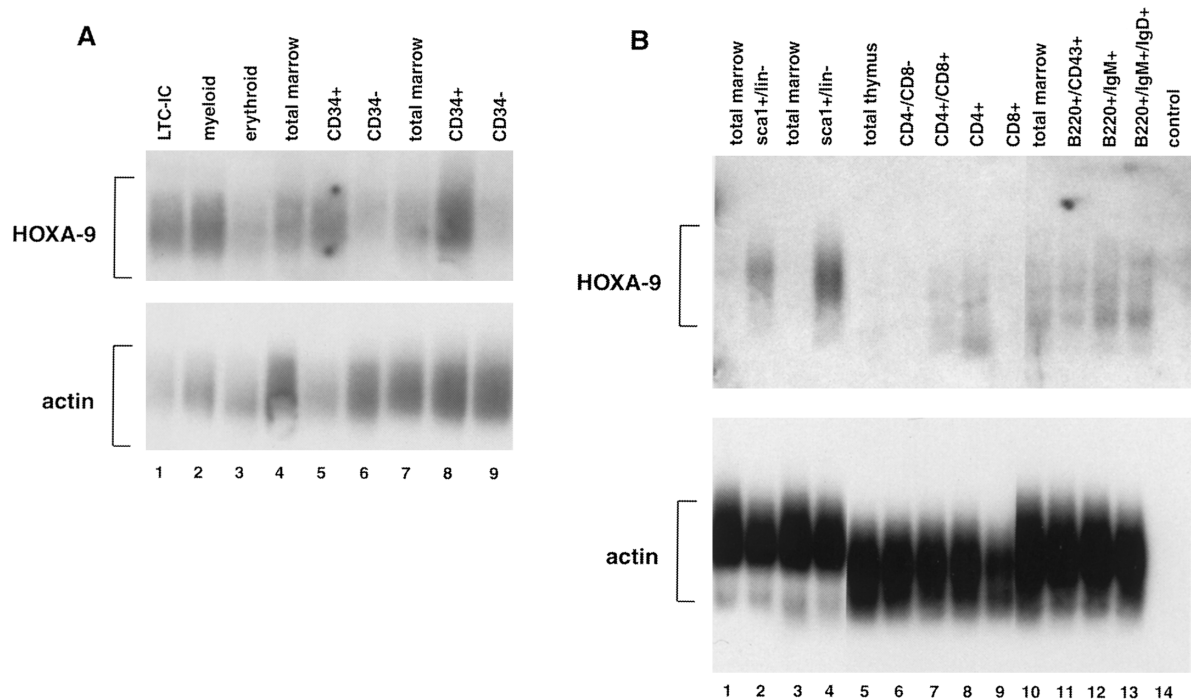
A

B

Fig 1. Expression of *HOXA9* in normal marrow cells and lymphocytes. (A) Southern blot analysis of total amplified cDNA derived from various fractions of normal human bone marrow. Lane 1, CD34⁺Lin⁻ cells enriched for LTC-IC; lane 2, CD34⁺CD45RA⁺ cells enriched for myeloid progenitors; lane 3, CD34⁺CD71⁺ cells enriched for erythroid progenitors; lanes 4 and 7, total marrow cells; lanes 5 and 8, CD34⁺ marrow cells; lanes 6 and 9, CD34⁻ marrow cells. *HOXA9* message is clearly detectable in CD34⁺ cell fractions, particularly those enriched for LTC-IC and myeloid progenitors, and not in CD34⁻ cells. (B) Southern blot analysis of total amplified cDNA from fractionated murine bone marrow, T cells, and B cells. *HOXA9* is clearly detectable in primitive Sca1⁺/lin⁻ marrow cells (lanes 2 and 4), but not in total marrow (lanes 1, 3, and 10). Within the T-cell fractions, there is detectable *HOXA9* message in CD4⁺/CD8⁺ (lane 7) and in CD4⁺/CD8⁻ (lane 8) cells. In B220⁺ B-cell populations, *HOXA9* is expressed weakly in CD43⁺ cells (lane 11) and more strongly in IgM⁺ (lane 12) and IgM⁺/IgD⁺ (lane 13) cells.

healthy, and weigh the same as their wild-type littermates and age-matched animals of the parental strains. Moreover, the mutant animals do not appear to be predisposed to infection or leukemia after periods of observation of more than 1 year. Figure 2B shows the genotyping of *HOXA9* mutant animals using a three-primer PCR strategy. A systematic analysis of the blood and blood-forming organs of these animals was undertaken to ascertain if loss of *HOXA9* function had any discernible effects on hematopoiesis.

HOXA9−/− mice have reduced numbers of peripheral blood granulocytes and lymphocytes. The white blood cell counts of homozygous animals are reduced by roughly one third compared with the counts in normal mice and heterozygous mutants, with statistically significant reductions in both lymphocytes and granulocytes ($P < .001$). Similar reductions in white blood cell counts are seen in *HOXA9−/−* mice when matched to their wild-type siblings, although the reduction in granulocytes does not achieve statistical significance (data not shown). It is notable that the *HOXA9−/−* animals have entirely normal red blood cell counts, hematocrits, and platelet counts (Table 1).

Since the reduction in granulocytes under basal conditions was small, we wished to determine if the defect in granulocyte production in *HOXA9−/−* mice could be exaggerated by stress. Therefore, *HOXA9−/−* and wild-type sibling animals were treated with granulocyte colony-stimulating factor

(G-CSF) twice daily for 96 hours and then bled for hematologic evaluation, as described previously.[22] The G-CSF–induced granulocytosis in *HOXA9−/−* animals is only one third that seen in their wild-type siblings ($P < .001$) (Fig 3). Identical differences were seen in experiments comparing *HOXA9−/−* mice with normal C57 animals; control animals treated only with phosphate-buffered saline injections show no change in granulocyte counts (data not shown).

FACS analysis was performed to ascertain the distribution of T and B lymphocytes in *HOXA9−/−* mice compared with heterozygous animals and with C57B1, 129 Sv/Ev, and C57B1/129 Sv/Ev F1 hybrid animals. Figure 4 demonstrates that homozygous mutants have reductions in B220⁺ B cells (~40%) compared with all the control groups (not statistically significant *v* 129 Sv/Ev mice) and reduction in CD3⁺ T cells (~35%) compared with all the controls except C57. It should be noted that there are significant strain differences in T-/B-cell ratios between C57 and 129 Sv/Ev animals. These data demonstrate that the leukocyte abnormalities seen in the mutant animals require interruption of both alleles of *HOXA9* and are not explained by differences in genetic background.

HOXA9−/− mice have smaller spleens and thymuses than normal animals. The different hematopoietic organs of the homozygous mutant mice were compared with those of heterozygous animals and normal parental strains (Fig 5).
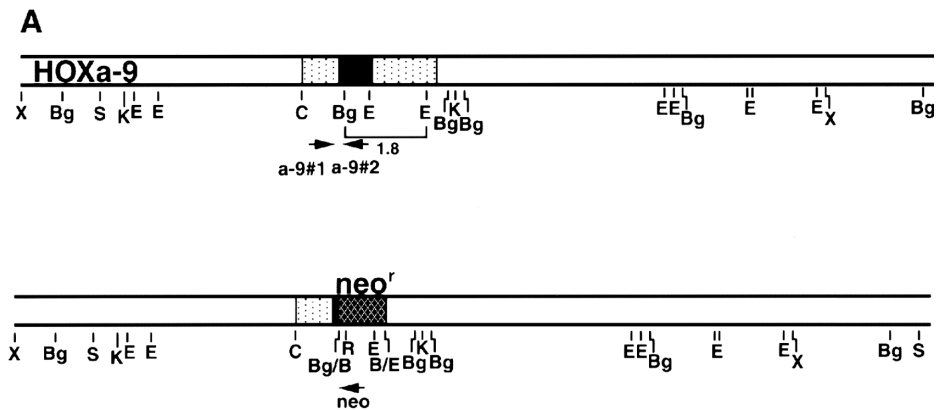
Fig 2. HOXA9 targeting and genotypic analysis. (A) Normal HOXA9 genomic locus is shown on top and the targeted locus on the bottom. Arrowheads marked a-9#1, a-9#2, and neo denote primer sites for genotyping by PCR. (B) PCR products from tail or toe DNA, amplified using the 3 primers shown in (A). Amplification of the wild-type allele with primers a-9#1 and a-9#2 results in the lower 123-nt band, whereas amplification of the targeted allele with primers a-9#1 and neo results in the upper 200-nt band (arrows). The gel shows results from normal (lanes 1, 2, 3, and 8), HOXA9+/− (lane 4), and HOXA9−/− (lanes 5 and 6) animals. Lane 7 is a control (C) lane with no DNA.

Spleens of homozygous animals are significantly smaller than those of control mice, including wild-type siblings, and grossly appear thinner and shorter in the longest diameter. The reduction in spleen size is evident in both sexes and in young (4 to 7 weeks), adult (8 to 19 weeks), and older (>20 weeks) groups and is not accounted for by a difference in the overall weight of the animals. FACS analysis of spleen cells from HOXA9−/− mice demonstrated a reduction in B cells (45% ± 7% v 61% ± 6% for C57 animals, $P < .001$)

with a commensurate increase in the percentage of T cells (42% ± 9% v 31% ± 7% for C57, $P < .01$). Histologic evaluation of mutant spleens showed a reduction in white pulp and indistinct germinal centers (data not shown). These findings taken together indicate that the reduction in spleen size is largely accounted for by diminished numbers of B lymphocytes. The overall size of the thymus is also significantly reduced in HOXA9−/− mice (Fig 5); however, FACS analysis of thymic lymphocytes showed no difference in the percentages of CD4 and CD8 double- or single-positive T cells (data not shown).

The bone marrow of HOXA9−/−-mice displayed no evident histologic abnormalities, and the number of nucleated cells per femur is normal (mean, $17.4 ± 1.9 × 10^6$). FACS analysis of marrow cells showed a normal 2.5:1 ratio of myeloid (Gr-1/Mac-1) to erythroid (TER119) elements and a small statistically insignificant reduction in B lymphocytes as compared with HOXA9+/+ sibling controls (21% v 27%). The percentages of CD34$^+$ cells (7.3% v 6.4%), c-kit$^+$ cells (7.1% v 6.8%), and double-positive cells (3.1% v 2.6%) were comparable between HOXA9−/− animals (n = 7) and their wild-type siblings (n = 5). Mesenteric lymph nodes appeared grossly normal and showed normal ratios of T and B cells (data not shown).

Table 1. Peripheral Blood Cell Counts of Adult HOXA9−/−, HOXA9+/−, and Normal Mice

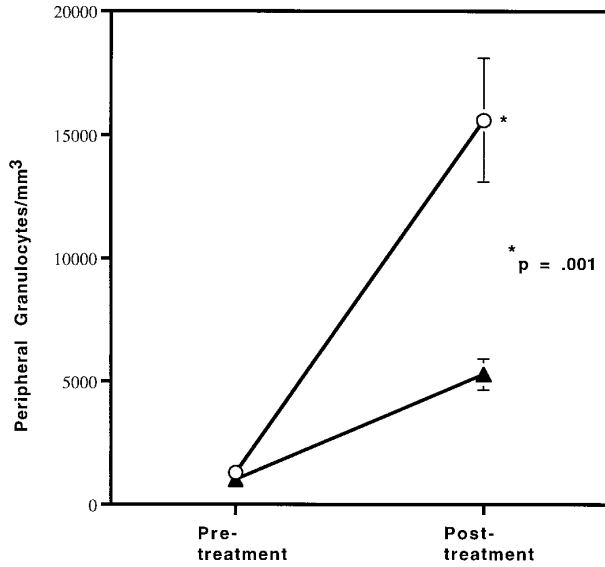| Cell Type	C57B1/6J (n = 50)	HOXA9+/− (n = 24)	HOXA9−/− (n = 79)
Red blood cells			
(per μL)	10,200,000	10,700,000	10,160,000
Hematocrit (%)	48.0	47.0	48.0
Platelets (per μL)	1,100,000	1,120,000	1,050,000
White blood cells			
(per μL)	9,924 ± 382	10,730 ± 550	6,850* ± 252
Lymphocytes	8,720 ± 554	8,988 ± 518	5,193* ± 405
Granulocytes	1,677 ± 124	1,928 ± 170	1,071* ± 126

*$P ≤ .001$ v C57B1 and HOXA9+/−.

Fig 3. HOXA9-/- mice have a blunted response to G-CSF. Cohorts of (▲) 3 HOXA9-/- mice and (○) 3 HOXA9+/+ siblings were treated with twice-daily subcutaneous human G-CSF (Amgen, Thousands Oaks, CA) for 96 hours and were then bled 3 hours after the last injection. White blood cell counts were determined on a Coulter counter, and 200-cell manual differential counts of granulocytes and lymphocytes were performed on Wright-stained blood smears.
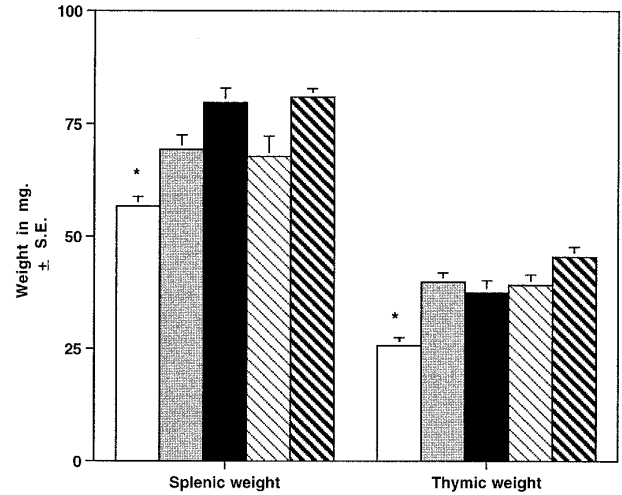

Fig 5. HOXA9-/- mice have small spleens and thymuses. Mean ± SE splenic and thymic weights in milligrams are shown for HOXA9-/- and 4 groups of control animals. (□) A9-/- (n = 39); (▤) A9 +/- (n = 16); (■) C57 (n = 39); (▨) 129 (n = 26); (▧) C57/129 (n = 27). *P < .01 for A9-/- versus all other groups.

HOXA9-/- marrows and spleens have reduced numbers of committed myeloid and erythroid progenitors. To determine if the decrease in peripheral blood leukocytes was due to an abnormality in progenitor populations, clonogenic
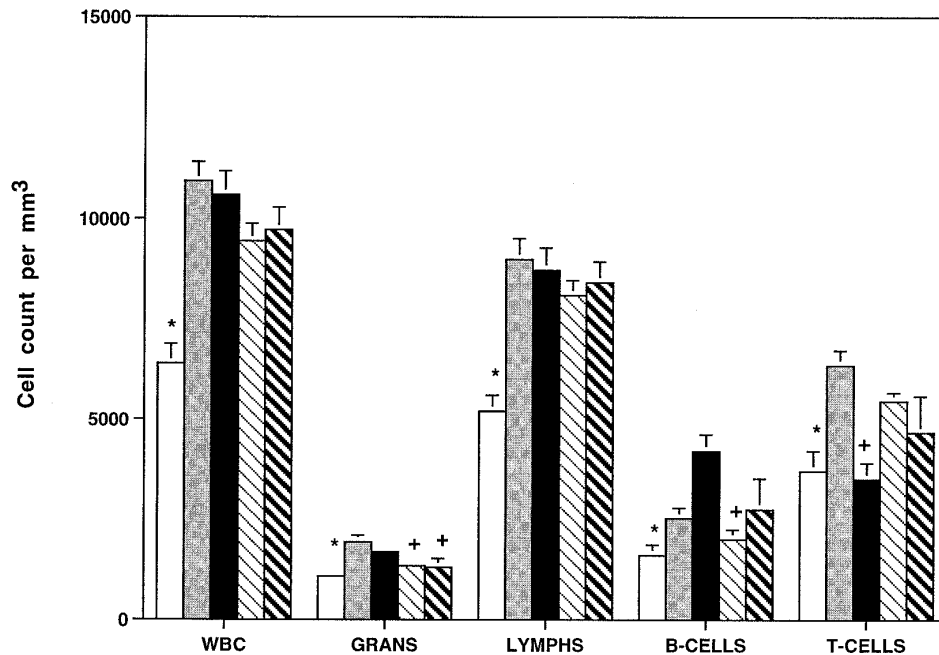

Fig 4. HOXA9-/- animals have reduced total white blood cell counts, granulocytes, and B and T lymphocytes. Peripheral blood cell counts were determined for (□) HOXA9 homozygotes (n = 22), (▤) heterozygotes (n = 12), and normal parental strains including (■) C57B1 (n = 24), (▨) 129 Sv/Ev (n = 18), and (▧) F1 hybrids of C57B1 and 129 Sv/Ev (n = 20). Animals were 9 to 12 weeks of age. WBC, total white blood cell counts; Grans, granulocytes; lymphs, lymphocytes. All counts expressed as cells/mm³ (mean ± SE). B- and T-cell counts were determined by FACS analysis using antibodies to B220 and CD3 on samples from 8 to 9 animals per group. Leukocyte counts of HOXA9-/- animals were compared with those of each group of control animals using a 2-tailed Student's t test. Control counts were significantly different from HOXA9-/- counts (P < .05), except those marked with a plus sign (+).
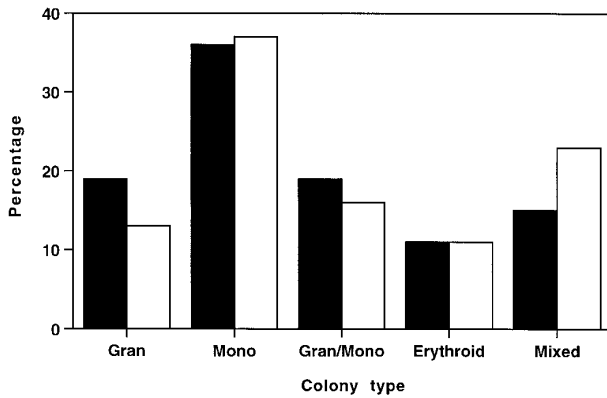
Fig 6. Distributions of myeloid/erythroid colony types in *HOXA9*−/− and normal marrow are similar. In 6 separate experiments, well-separated individual colonies were plucked from cultures of (□) *HOXA9*−/− (n = 166) and (■) normal C57 (n = 164) marrow after 7 to 10 days of incubation. Cytospin preparations were stained and scored as 1 of 5 colony types. All colony types were represented in *HOXA9*−/− marrow in virtually the same percentage as in normal marrow, except for a small reduction in the percentage of pure granulocyte colonies and an increased proportion of mixed erythroid/myeloid colonies.

assays in methylcellulose were performed. A 60% reduction in total myeloid/erythroid colonies (9,600 ± 770 per/femur v 24,800 ± 3,670 per/femur, $P < .001$) was observed in the marrow of homozygotes (n = 8) compared with wild-type littermates (n = 6). Similar twofold reductions were seen when *HOXA9*−/− mice were compared with heterozygotes and all normal parental strains (data not shown).

To ascertain whether this reduction in total myeloid/erythroid colonies was due to selective loss of specific colony types, individual colonies from six *HOXA9*−/− marrows and six normal marrows were plucked and scored by morphologic assessment of cytospin preparations. Figure 6 shows that the distribution of colony types from *HOXA9*−/− and normal marrow had virtually identical percentages of erythroid, monocytic, and granulocytic/monocytic colonies, and *HOXA9*−/− samples collectively showed a small contraction in the percentage of granulocytic colonies and an increase in the proportion of mixed erythroid/myeloid colonies. These results indicate that the decrease in total colonies was not due to selective loss of specific subsets of clonogenic progenitors, but rather to a global twofold reduction of all colony types, with the exception of mixed erythroid/myeloid colonies, which are not significantly diminished.

To identify possible defects in the proliferative potential of myeloid progenitors from *HOXA9* mutant animals, whole myeloid cultures were harvested 7 to 11 days after the initial plating, and various proportions were replated for secondary colony assays. In these studies, the number of secondary colonies formed per primary colony was not reduced in *HOXA9*−/− animals compared with normal controls (data not shown). Finally *HOXA9*−/− marrow cells showed a dose-response curve to interleukin-3–induced colony formation that overlapped the curve for normal cells (data not shown). These data taken together indicate that the decrease in *HOXA9*−/− myeloid colony formation was not due to

decreased proliferation or self-renewal or to reduced sensitivity to a hematopoietic growth factor, but rather to an absolute decrease in the number of progenitors in the marrow.

To ensure that myeloid progenitors in *HOXA9*−/− marrow are not simply redistributed to another hematopoietic organ, myeloid/erythroid colony assays were performed on spleen cells from *HOXA9*−/− and normal C57 animals. In these assays, *HOXA9*−/− mice showed a significant 3.5-fold reduction in total spleen-derived colonies (11.8 ± 1.6 × 10³ colonies per spleen v 3.3 ± 1.2 × 10³, $P = .002$) and a sixfold reduction in erythroid colonies (8.4 ± 1.5 × 10³ v 1.4 ± 0.8 × 10³, $P = .002$), with roughly twofold reductions in granulocyte-monocyte colonies (3.1 ± 0.3 × 10³ v 1.7 ± 0.4 × 10³, $P = .02$) and mixed colonies (.22 ± .11 × 10³ v .12 ± .04 × 10³, $P = .4$). Similar differences were seen when mutant animals were compared with 129 Sv/Ev mice (data not shown). Thus, myeloid and erythroid progenitors were not redistributed to the spleen, and in fact, spleen-derived erythroid progenitors were markedly contracted.

HOXA9−/− *animals have defects in B-cell production.* To assess B-cell progenitor frequency in the marrow, interleukin-7–responsive pre-B cell progenitors were measured, and a fourfold reduction (1,950 ± 450 calories per femur v 8,870 ± 930) was noted in *HOXA9*−/− marrows (n = 8) compared with wild-type sibling controls (n = 6, $P < .001$). Greater than twofold contractions in pre-B colonies were observed in *HOXA9*−/− marrows compared with marrows of heterozygotes and parental strain animals (data not shown). To measure the output of even younger B-cell precursors, bulk B-lymphoid progenitor cultures (LPC) were performed on S17 feeder layers. The output of total B cells, pro-B cells, and pre-B cells was reduced 2.5-fold to threefold in marrows of *HOXA9*−/− compared with both C57 and 129 Sv/Ev animals; the level of B-cell output by heterozygous mutants was intermediate between that of normal and homozygous animals (Fig 7).
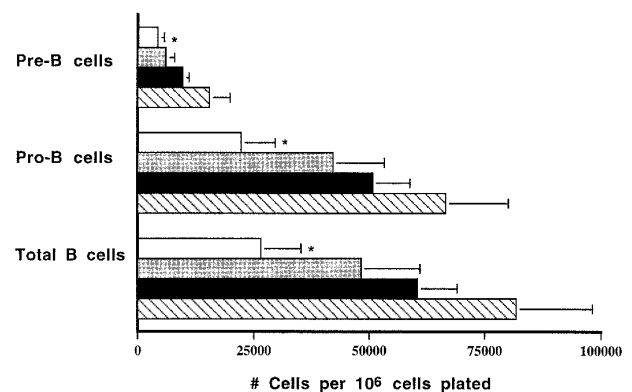

Fig 7. Output of B cells in LPC cultures is reduced in *HOXA9*−/− mice. Output of B cells from 5-week LPC cultures was determined. Total B cells, B220⁺ cells; pro-B cells, B220⁺CD43⁺ cells; pre-B cells, B220⁺CD43⁻ cells. Counts are expressed as the mean number of cells harvested at week 5 per 10⁶ marrow cells originally plated. Marrow samples from four animals were studied in each group. (□) A9−/−; (▦) A9+/−; (■) C57; (▨) 129. *$P < .05$ for A9−/− versus C57 and 129; not significant for A9−/− versus A9+/−.

Primitive progenitors are not significantly reduced in HOXA9−/− marrows. Having found a significant reduction in the committed progenitor population in *HOXA9−/−* mice, we wished to determine if there were defects earlier in the hematopoietic differentiation pathway. To measure the number of earlier multilineage precursors, day 12 CFU-S assays were performed. In two separate experiments using pooled donor marrow cells, there were small reductions in the number of CFU-S in *HOXA9−/−* marrows as compared with those of sibling controls or normal C57 samples (Fig 8). These differences were not significant using a two-tailed *t* test, although the second experiment was statistically significant using a one-tailed *t* test ($P < .05$). In one experiment injecting marrow from individual donor animals into separate cohorts of recipients, there was no difference in the number of CFU-S in *HOXA9−/−* and normal mice.

To assess the frequency of even earlier pluripotent progenitors, LTC-IC assays were performed. In numerous previous studies, the mean frequency of LTC-ICs in normal murine marrow was approximately one in 30,000, with 95% confidence intervals of about one in 20,000 to one in 55,000 cells. In two separate experiments, the mean LTC-IC frequency in three *HOXA9−/−* mice was one in 26,542, as compared with one in 36,030 for two normal mice and one in 34,051 in one heterozygote, with overlapping 95% confidence intervals for the three sets of animals. Thus, there is no evident contraction of LTC-ICs in the mutant mice.

The hematopoietic defect in HOXA9−/− mice is transplantable. The observation that *HOXA9* is normally expressed in hematopoietic cells does not preclude the possibility that the hematologic defects associated with the interruption of this gene are caused at least in part by defects in the microenvironment. We determined peripheral blood cell counts of animals transplanted with either 500,000 normal C57 marrow cells or 500,000 *HOXA9−/−* cells. After 2 months, the hematocrits and platelet counts of the two groups were identical. However, the total white blood cell counts, granulocyte counts, and lymphocyte counts were all reduced at least twofold in animals receiving *HOXA9−/−* marrow, reproducing the defect seen in primary animals with somewhat greater severity. These results are consistent with a defect in leukocyte production that is intrinsic to the hematopoietic cells.

DISCUSSION

Previous studies have demonstrated that several *HOX* genes are expressed in early hematopoietic cells,[10,11] and that overexpression of different *HOX* genes in normal marrow cells results in distinctive perturbations of stem cell pools and differentiation patterns.[13,14] Although these studies are intriguing, they do not prove a role for *HOX* genes in normal hematopoiesis. We now present data indicating that the loss of function of a *HOX* gene normally expressed in CD34+ blood cells and in certain subsets of developing lymphocytes does disturb hematopoiesis. In the case of *HOXA9,* there are defects in multiple lineages, with reductions in peripheral granulocyte and lymphocyte counts and in committed myeloid, erythroid, and B-cell progenitors. The lack of anemia

despite a reduction in erythroid progenitors may be due to the longer life span of mature red blood cells.

The involvement of several different lineages would suggest at first a defect in a shared progenitor. However, despite the obvious defects in committed progenitors, no major reductions were found in primitive pluripotent precursors (eg, day 12 CFU-S and LTC-IC). Although these experiments do not preclude the possibility of a mild reduction in CFU-S in *HOXA9−/−* marrow, they do indicate that there is not a progressively larger defect in the less mature progenitors. These findings contrast with the findings of marrow cells overexpressing the *HOXB4* gene, in which case primitive progenitors are selectively and markedly expanded with little or no change in the level of committed progenitors or in the output of mature cells.[13] The apparent lack of a major defect in the primitive progenitors of *HOXA9−/−* mice is puzzling, since *HOXA9* is normally expressed in this compartment and in the committed progenitor pools (Fig 1A). It is conceivable that 3′ *HOX* genes such as *HOXB4,* which are more selectively expressed in the primitive precursors, exert a dominant effect in the earlier stages of hematopoiesis.

The contraction in progenitor pools in the *HOXA9−/−* mice is similar to that recently described for animals with targeted interruptions of the CD34 gene,[23] although we found no change in the expression of CD34 in the marrow of *HOXA9−/−* mice. In both cases, it is curious that a more profound abnormality is not seen, and it is not clear if the hematopoietic defects described would confer a selection
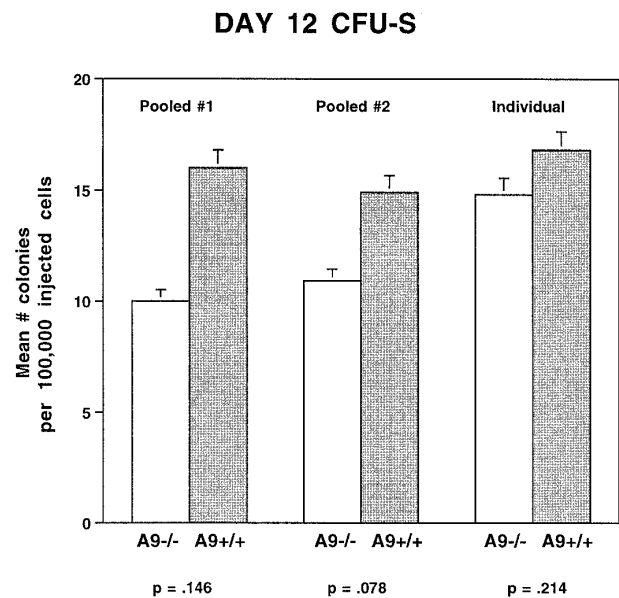
DAY 12 CFU-S

Fig 8. Day 12 CFU-S are not significantly reduced in *HOXA9−/−* mice. In experiments using pooled marrow samples, marrows from 2 to 3 *HOXA9−/−* mice were pooled and compared with pooled marrows from 2 to 3 *HOXA9+/+* mice. Recipient animals (3 to 4 mice per dose of cells) were injected with either 50,000 or 100,000 pooled cells. In the experiment using individual samples, single marrow samples from 3 homozygous and 3 C57 animals were injected into cohorts of lethally irradiated C57 animals, using the same doses of cells. In 2 of 3 experiments, there was a minor and statistically insignificant reduction in the frequency of day 12 CFU-S in *HOXA9−/−* mice.

disadvantage. *HOXA9−/−* animals make adequate numbers of leukocytes for basal needs, but the blunted response to G-CSF suggests that these animals would be vulnerable to infectious agents in the wild.

In some other *HOX* gene knockout experiments, the embryologic defects have been subtle and nonlethal,[24] and even examples resulting in severe abnormalities show remarkably variable penetrance even within the same animal, tempting speculation that there is redundancy in the *HOX* gene code.[6,7] Specifically, it is conjectured that *HOX* gene paralogs, ie, *HOX* genes in the same position of different *HOX* clusters, such as *HOXA9* and *HOXB9* partially compensate for each other. In this model, one could hypothesize that the *HOXB9* gene, a paralog of *HOXA9* that is also expressed in blood cells,[10] duplicates some of the functions of the interrupted gene. If this were the case, animals with interrupted alleles of both *HOXA9* and *HOXB9* may display a more severe hematopoietic defect; this is a prediction supported by the observation of combined mutations of other paralogous *HOX* genes, which result in much more profound skeletal defects than are seen with single mutations.[25,26] There are also data to indicate that adjacent genes in the same cluster may interact to influence phenotype.[27] Thus, a combined mutation of *HOXA9* and its neighbor *HOXA10,* also strongly expressed in normal CD34$^+$ marrow cells,[10] could produce more dramatic derangements in hematopoiesis.

These observations also have relevance to recent reports of the involvement of *HOXA9* in human and murine myeloid leukemias. Two groups have recently demonstrated that *HOXA9* is consistently involved with the t(7;11) translocation that occurs in a subset of human myeloid leukemias.[28,29] This translocation results in fusion of the *HOXA9* gene with the nucleoporin gene *NUP98* and a chimeric *NUP98/HOXA9* transcript, and establishes for the first time that *HOX* genes are involved in some spontaneous human leukemias. One group has shown that retrovirally induced myeloid leukemias in BXH-2 mice consistently involve proviral integration and activation of the murine *HOXA9* gene, with resultant overexpression of *HOXA9.*[30] In addition, we have observed that *HOXA9* is consistently activated in primary samples of acute myelogenous leukemia, and not in lymphoid leukemias (H.J. Lawrence, manuscript in preparation). Taken together, these findings imply that overexpression of *HOXA9* perturbs myeloid differentiation and perhaps enhances proliferation of myeloid cells, eventually leading to malignant transformation, whereas loss of *HOXA9* function inhibits myeloid proliferation without markedly altering differentiation or leading to leukemia.

The nature of the hematopoietic defect in these animals remains unclear, although it is transplantable, indicating that the lesion is intrinsic to the blood cells and is not microenvironmental. The reduction in committed progenitors is apparently not due to redistribution to other hematopoietic organs or to decreased sensitivity to growth factors. A summary of the experiments performed to date examining the influence of modulating *HOX* gene expression on hematopoiesis would indicate that a major effect appears to be on the size of various developing blood cell pools. Alterations of pool sizes could reflect a number of aspects of cell physiology such as proliferation, survival, and apoptosis. Although much of the focus of the effects of *HOX* genes has been on morphogenesis, an elegant argument has been made that the role of *HOX* genes in body patterning is based on regulation of cell proliferation.[31] An advantage of the hematopoietic system is the ease with which these cellular processes can be assayed. Thus, further studies using models of overexpression in marrow cells and analysis of the blood of knockout animals may provide insights into the general mechanisms by which *HOX* genes exert developmental regulatory properties.

ACKNOWLEDGMENT

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