Brief Report

HEMATOPOIESIS AND STEM CELLS

Hoxa cluster genes determine the proliferative activity of adult mouse hematopoietic stem and progenitor cells

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Key Points

- Deletion of *Hoxa* genes reduces the engraftment potential of adult hematopoietic stem cells.
- Ectopic overexpression of *Hoxa9* partially restores *Hoxa*$^{-/-}$ hematopoietic stem cell activity.

Determination of defined roles for endogenous homeobox (*Hox*) genes in adult hematopoietic stem and progenitor cell (HSPC) activity has been hampered by a combination of embryonic defects and functional redundancy. Here we show that conditional homozygous deletion of the *Hoxa* cluster (*Hoxa*$^{-/-}$) results in a marked reduction of adult HSPC activity, both in vitro and in vivo. Specifically, proliferation of *Hoxa*$^{-/-}$ HSPCs is reduced compared with wild-type (WT) cells in vitro and they are less competitive in vivo. Notably, the loss of *Hoxa* genes had little impact on HSPC differentiation. Comparative RNA sequencing analyses of *Hoxa*$^{-/-}$ and WT hematopoietic stem cells (CD150^{+}/CD48^{-}/Lineage^{-}/c-kit^{+}/Sca-1^{+}) identified a large number of differentially expressed genes, three of which (*Nr4a3*, *Col1a1*, and *Hnf4a*) showed >10-fold reduced levels. Engineered overexpression of *Hoxa9* in *Hoxa*$^{-/-}$ HSPCs resulted in partial phenotypic rescue in vivo with associated recovery in expression of a large proportion of deregulated genes. Together, these results provide definitive evidence linking *Hoxa* gene expression to proliferation of adult HSPCs. (*Blood*. 2016;127(1):87-90)

Introduction

Specific developmental phenotypes are associated with loss of individual *Hoxa* cluster genes in the mouse.[1-4] Interestingly, most of these *Hoxa* genes are expressed in hematopoietic cells, with the highest levels in primitive hematopoietic stem and progenitor cells (HSPC) and much lower levels in mature cells.[5-8]

Evaluation of individual *Hoxa* gene mutant mice has provided only limited insight into their role in adult hematopoiesis, indicative of a level of functional redundancy of cluster genes in this tissue.[1,9,10] The *Hoxa9* homozygous null mice display the most overt hematopoietic phenotype characterized by mild leucopenia resulting from bone marrow (BM) hypocellularity, particularly of myeloid and B cells. This phenotype is associated with impairment in activity, but not the number of the long-term (LT) hematopoietic stem cells (HSCs).[11]

Further supporting the importance of *Hoxa* genes in hematopoiesis, we previously showed that heterozygous *Hoxa* cluster (*Hoxa*$^{+/-}$) adult LT-HSCs are less competitive than wild-type (WT) cells in transplantation assays.[5] Moreover, Di Poï et al previously reported that recipients of fetal liver-derived LT-HSCs in which *Hoxa* gene expression is reduced to 20% (*HoxA*$^{c/-}$ cells)[12] have impaired ability to repopulate adult mice, particularly in the output of early erythrocytes. This phenotype was much less obvious in *HoxA*$^{c/-}$ newborn mice, possibly indicating that *Hoxa* cluster genes are more important in activity of adult vs fetal HSPCs.

To address this point, we now assess the impact of the complete ablation of *Hoxa* cluster genes in adult HSPCs and explore the consequence of this deletion on the transcriptome using RNA sequencing (RNASeq). Results clearly establish an essential function for *Hoxa* genes in proliferation, but not differentiation, of adult HSPCs.

Study design

Mice and *Hoxa* gene deletion

To delete the *Hoxa* locus *Hoxa*$^{flox/flox}$ / MxCre mice[13] (referred to as *Hoxa*$^{-/-}$ after deletion), MxCre controls received 7 injections of 10 μg poly I/poly C (pIpC) (GE Healthcare Life Sciences, Baie-d'Urfé, QC, Canada) per gram body weight for a maximum of 250 μg per mice intraperitoneally every 2 days. *Hoxa* cluster deletion was confirmed by polymerase chain reaction. All our animal studies were approved by the local Animal Care Committee under legislation of the Canadian Council on Animal Care.

Infection of primary BM cells

Lineage (Lin)$^{-}$ BM cells were purified from *Hoxa*$^{-/-}$ and control mice 4 weeks post-pIpC, and transfected with murine stem cell virus-pgk green fluorescent

Submitted February 26, 2015; accepted October 26, 2015. Prepublished online as *Blood* First Edition paper, November 19, 2015; DOI 10.1182/blood-2015-02-626390.

The online version of the article contains a data supplement.

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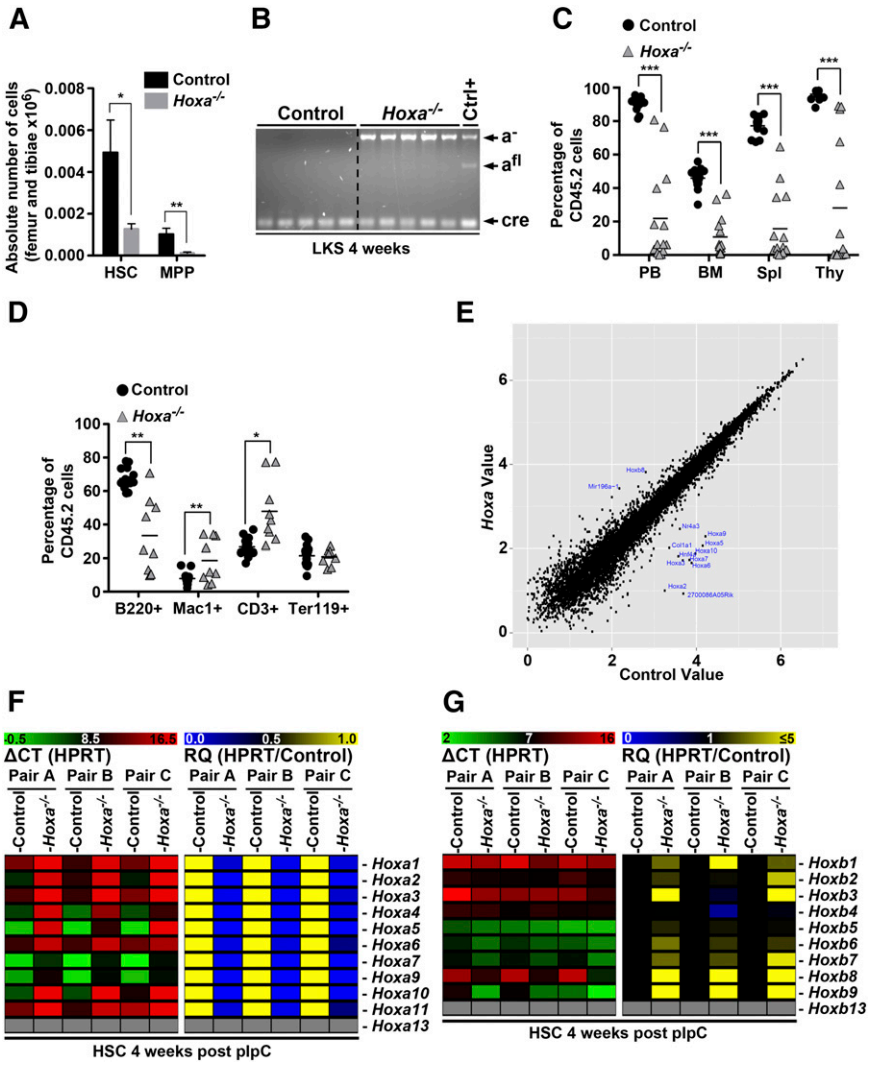

Figure 1. Deletion of *Hoxa* cluster genes in adult mice and transcriptome analysis of *Hoxa*$^{-/-}$ HSCs. (A) Bar graphs depicting absolute numbers of CD150^{+}/CD48^{-}/CD244^{-}/LKS HSCs and CD150^{+}/CD48^{-}/CD244^{+}/LKS MPPs in BM of *Hoxa*$^{-/-}$ and control mice (n = 4). BM cells isolated from femur and tibia 4 weeks after pIpC injection. (B) Polymerase chain reaction analysis showing the deletion of the *Hoxa* cluster in purified LKS cells at 4 weeks following the last pIpC injection (n = 5). (C) Engraftment of *Hoxa*$^{-/-}$ (n = 14) and control (n = 15) cells in the PB, BM, spleen, and thymus of recipient mice at ~24 weeks after transplantation. Each dot represents a CD45.1 recipient mouse transplanted with 10^6 congenic CD45.2 BM cells. (D) Proportions of B (B220^{+}), myeloid (Mac1^{+}), T (CD3^{+}), and erythroid (Ter119^{+}) cells in PB of reconstituted mice shown in (C). (E) Scatterplot showing transcriptome analysis of genes that were up- (above axis) or downregulated (below axis) in *Hoxa*$^{-/-}$ vs WT control CD150^{+}/CD48^{-}/LKS cells sorted 1 month after the last pIpC injection. Values are expressed as average (log10 [(average RPKM)*1000 + 1]). Cut-off limit for showing gene identify was set at 3, representing a RPKM value of ~0.1. Highlighted genes show 10-fold differences between the 2 groups. (F-G) Comparative expression of *Hoxa* (F) and *Hoxb* (G) genes in pairs of *Hoxa*$^{-/-}$ and control HSC samples (n = 3). $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$. Cre, causes recombination; CT, cycle time; HPRT, hypoxanthine-guanine phosphoribosyltransferase; MPP, multipotent progenitors; RQ, relative quantification; Spl, spleen; Thy, thymus.

protein (GFP) or murine stem cell virus-*HOXA9*-pgk GFP retroviral supernatants by spinoculation (2250 rpm) for 90 minutes. After 48 hours, 0.25×10^6 sorted GFP^{+} were IV injected without helper cells, in irradiated (8 Gy) adult B6SJL congenic recipient mice.

RNA isolation and RNASeq library preparation

Total RNA was extracted from 60 000 to 100 000 *Hoxa*$^{-/-}$ and control LT-HSC (CD150^{+}/CD48^{-}/Lin^{-}/c-kit^{+}/Sca-1^{+} [LKS]) and used to generate transcriptome libraries. Paired end (2×100 bp) sequencing was performed using an Illumina HiSeq2000 (Illumina, San Diego, CA). RNASeq data were analyzed with Cuffdiff or the DeSeq R package.[14]

Results and discussion

MxCre-induced conditional deletion of *Hoxa* genes in adult HSPCs resulted in a significant reduction of white blood cells, red blood cells, and platelets in the peripheral blood (PB), and moderate reductions in cellularity in all hematopoietic organs, primarily due to a severe decrease in B cells (see supplemental Figure 1A-D, available on the *Blood* Web site). Flow cytometry and clonogenic assays also demonstrated reduced HSPCs (Figure 1A and supplemental Figure 1E-G, and confirmation of deletion in LKS cells in Figure 1B), consistent with observations made in fetal liver-derived *HoxA*$^{c/-}$ HSCs.[12] However, unlike the fetal-derived equivalents, adult *Hoxa*$^{-/-}$ HSCs demonstrated reduced proliferation potential in vitro (supplemental Figure 1H), but were more in cycle in vivo (supplemental Figure 2A-C). In accordance with the in vitro results, *Hoxa*$^{-/-}$ BM cells demonstrated a reduced ability in reconstituting irradiated congenic hosts compared with WT controls (Figure 1C and supplemental Figure 1I-J) that is partly due to an increase in apoptosis (supplemental Figure 2D-E), but not due to homing defects or non–*Hoxa*-deleted escapees (supplemental Figure 2F-H). Repopulation ability of *Hoxa*$^{-/-}$ cells was completely absent in secondary recipients, in contrast to control cells, suggesting an exhaustion of the HSC pool (supplemental Figure 1K). In this in vivo model, B cells were also underrepresented, whereas *Hoxa*$^{-/-}$ cells reconstituted most other cell lineages proportionally (Figure 1D and supplemental Figure 1L). These data confirm our earlier observation that adult HSC-derived B cells have an increased sensitivity for *Hoxa* gene levels[5], compared with fetal HSC-derived B cells.[12]

Comparative RNASeq transcriptome data from *Hoxa*$^{-/-}$ and control HSCs identified 881 significantly differentially expressed genes, the vast majority of which (614) were downregulated (supplemental Table 1). Functional annotation clustering using Gene Ontology terms revealed that differentially expressed genes were associated with cell proliferation and differentiation, cell activation, signaling, regulation of gene expression, hematopoiesis, migration,

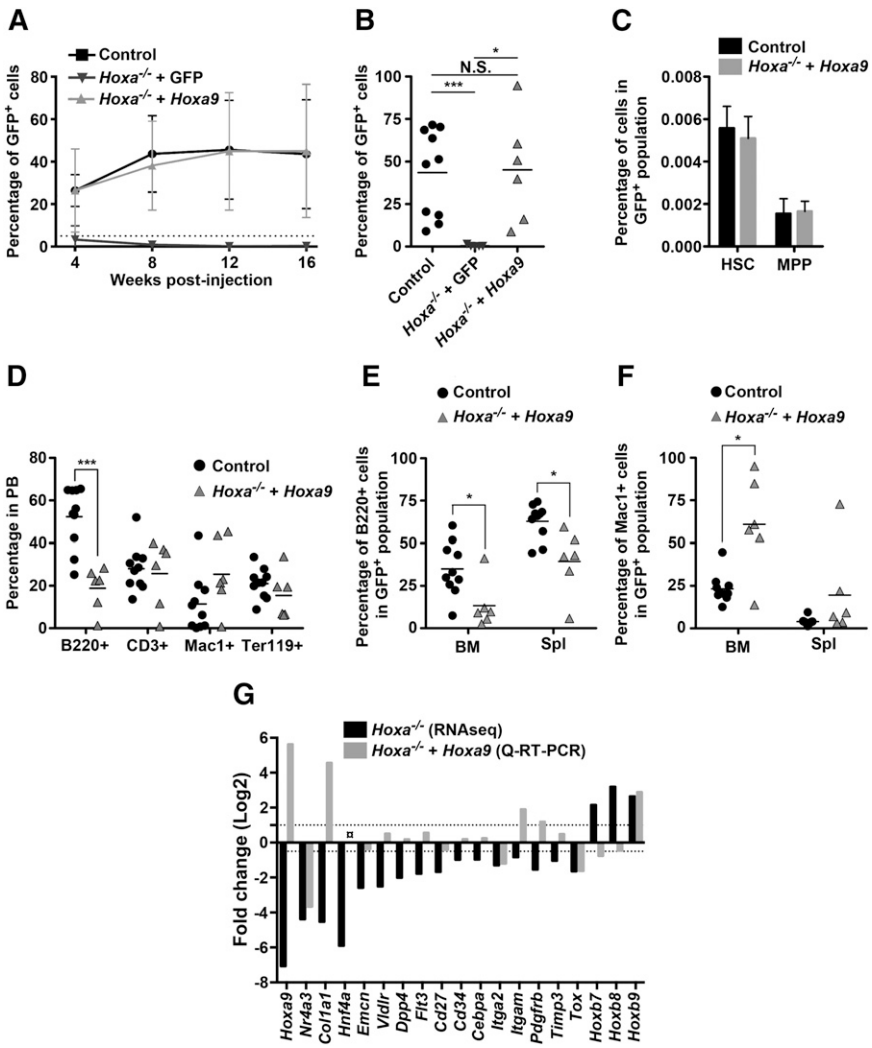

Figure 2. Rescue of *Hoxa*$^{-/-}$ HSC engraftment by overexpression of *Hoxa9*. (A) Average kinetics of engraftment in mice receiving 0.2×10^6 of either control *Hoxa*$^{+/+}$ + GFP (n = 13), *Hoxa*$^{-/-}$ + GFP (n = 5), and *Hoxa*$^{-/-}$ + *Hoxa9*-GFP (n = 6) BM cells. Percentage of donor cells are measured by flow cytometry for GFP$^+$ in the PB at indicated times. (B) LT engraftment of HSCs with indicated genotype in PB of individual mice 16 weeks posttransplantation. (C) Bar graphs depicting percentage of *Hoxa*$^{+/+}$ + GFP and *Hoxa*$^{-/-}$ + *Hoxa9* CD150$^+$/CD48$^-$/LKS HSCs (n = 3) and CD150$^-$/CD48$^-$/LKS MPPs (n = 3) in GFP$^+$ cell population. (D) Percentage of *Hoxa*$^{+/+}$ + GFP and *Hoxa*$^{-/-}$ + *Hoxa9*-derived B cells (B220$^+$), T cells (CD3$^+$), myeloid cells (Mac1$^+$), and erythroid cells (Ter119$^+$) in the PB of individual mice 16 weeks posttransplantation. (E-F) Percentage of B cells (B220$^+$) (E) and myeloid cells (Mac1$^+$) (F) of GFP$^+$ cells in BM and spleen. (G) Gene expression analysis in RNASeq HSC and Lin$^-$ cells overexpressing *Hoxa9*. ○ indicates quantity not sufficient defined as corrected CT value >36. *$P < .05$; ***$P < .001$. N.S., non-significant; MPP, multipotent progenitors; Q-RT-PCR, quantitative-reverse transcription-polymerase chain reaction; Spl, spleen.

and apoptosis (supplemental Table 2). Moreover, differentially expressed genes were associated with several pathways according to the Kyoto Encyclopedia of Genes and Genomes, which included hematopoietic cell lineage and cancer pathways, supporting a known role for *Hoxa* genes in hematopoiesis and leukemia (supplemental Table 3).

Only 13 genes expressed at reads per kilobase of transcript per million mapped reads (RPKM) values >1 showed a >10-fold differential expression between the 2 conditions (Figure 1E; supplemental Table 1). Genes downregulated in *Hoxa*$^{-/-}$ cells were mostly from the *Hoxa* cluster, but also included *Nr4a3*, *Col1a1*, and *Hnf4a* (Figure 1E-F). *Nr4a3,* also known as *Nor-1,* codes for an orphan nuclear receptor transcription factor highly homologous to *Nr4a1* (*Nur77*) and *Nr4a2* (*Nurr1*). Co-deficiency of *Nor-1* and *Nur77* is associated with an aggressive acute myeloid leukemia,[15] whereas individual mutants have only weak phenotypes both related to proliferation and apoptosis.[16,17] The latter phenotype corresponds with those for the *Hoxa*$^{-/-}$, indicating that *Nr4a3* may have contributed to the *Hoxa*$^{-/-}$ phenotype. Two genes, *Hoxb8* and microRNA *196a*-1, were significantly upregulated (>10-fold) in *Hoxa*$^{-/-}$ HSCs (Figure 1E-G), possibly pointing to cross-regulation between *Hoxa* and *Hoxb* cluster genes and their inter-cluster–located microRNAs.[18,19]

We next investigated if overexpression of a single *Hox* gene, *Hoxa9*, could at least in part, rescue the HSPC phenotype found in recipients of *Hoxa*$^{-/-}$ cells. *Hoxa*$^{-/-}$ HSPCs were transduced with *Hoxa9* or control GFP virus and transplanted into congenic animals. We found that *Hoxa9* overexpression restored HSPC engraftment to control WT levels (Figure 2A-C and supplemental Figure 3A). However, and in line with our previous observations,[5] the B and myeloid lineages are skewed and therefore do not recapitulate the normal distribution observed in control mice. Specifically, B cell numbers remained persistently lower and a substantial increase of Mac1$^+$ cells and myeloid progenitors (LKS$^-$) was observed in mice overexpressing *Hoxa9* (Figure 2D-F and supplemental Figure 3B-C). Notably, overexpression of *Hoxa9* did provide partial recovery at the molecular level of over 70% (14 of 19) of the deregulated genes found in the *Hoxa*$^{-/-}$ mutant (Figure 2G). Despite this, *Hoxa9* overexpression did not correct the decrease in B cells, and instead favored production of myeloid cells, indicating that qualitative contributions of individual *Hoxa* genes are vital for balanced hematopoiesis.

Together, the data identifies a critical role for *Hoxa* cluster genes in adult HSPC function, and furthermore, a *Hoxa* gene-dependent gene signature that underlies this function.

Acknowledgments

The authors gratefully acknowledge Martine Dupuis and Nathalie Henley for cell sorting, and in particular, Dr Denis Duboule

(University of Geneva, Geneva) and Dr Marie Kmita (Institut De Recherches Cliniques De Montreal) for providing the mice. The authors also thank the staff at the animal facility for taking care of the animals; Mona Hassawi, Gratianne Vaisson, and Gloria Giono for their help with the isolation of BM cells for the RNASeq; and Dr Julie Lessard and Dr Martin Guimond for critically reading the manuscript.

This work was supported by a grant from the Canadian Cancer Society (#20399). C.-E.L.-G. and M.F. are recipients of a department scholarship and award from the Cole foundation (M.F.).

Authorship

Contribution: C.-E.L.-G. performed experiments; C.-E.L.-G., M.F., and J.J.B. analyzed data and mounted the figures; C.-E.L.-G., A.T., G.S., and J.J.B. designed the research and wrote the paper; and L.K. was involved in the writing of the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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