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

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Running Title: *Hoxa* genes are required for HSC self-renewal

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Key Points:

- Depletion of *Hoxa* genes reduces the engraftment potential of adult HSCs
- Ectopic overexpression of Hoxa9 partially restores *Hoxa*$^{-/-}$ HSC activity

ABSTRACT

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 marked reduction of adult HSPC activity, both *in vitro* and *in vivo*. Specifically, *Hoxa*$^{-/-}$ HSPCs proliferate much less than wild type cells *in vitro* and they are also much less competitive *in vivo*. Notably loss of *Hoxa* genes had little impact on HSPC differentiation. Comparative RNA sequencing analyses of *Hoxa*$^{-/-}$ and wild type HSCs (CD150^{+}/CD48^{-} /Lineage^{-}/c-kit^{+}/Sca-1^{+} (LKS)) identified a large number of differentially expressed genes (P<0.004), three of which (*Nr4a3*, *Col1a1* and *Hnf4a*) showed >10-fold reduced levels. Engineered overexpression of *Hoxa9* in *Hoxa*$^{-/-}$ HSCs 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.

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, not the number, of the long-term (LT) 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 cells in transplantation assays [5]. Moreover Di Poi et al. previously reported that recipients of fetal liver-derived LT-HSCs in which *Hoxa* gene expression is reduced to 20% (called $HoxA^{c/-}$ 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 versus 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 definitively establish an essential function for *Hoxa* genes in proliferation, but not differentiation, of adult HSPCs.

METHODS

Mice and Hoxa gene deletion

To delete the *Hoxa* locus *Hoxa*$^{flox/flox(f/f)}$/MxCre mice[13] and MxCre controls received 7 injections of 10 µg 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 PCR.

Infection of primary BM cells

Lineage (LIN)$^-$ BM cells were purified from *Hoxa*$^{-/-}$ and control mice 4 weeks post pIpC, and transfected with MSCV-pgkGFP or MSCV-HOXA9-pgkGFP retroviral supernatants by spinoculation (2250 rpm) for 90 minutes. After 48 hours 0.25 x 10^6 sorted GFP$^+$ were intravenously injected without helper cells, in irradiated (8 Gy) adult B6SJL congenic recipient mice.

RNA isolation and RNA-Seq library preparation

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

RESULTS AND DISCUSSION

MxCre-induced conditional deletion of *Hoxa* genes in adult HSPCs resulted in significant reduction of white blood cells, red blood cells and platelets in the peripheral blood and moderate reductions in cellularity in all hematopoietic organs, primarily due to a severe decrease in B cells (Figure S1A-D). Flow cytometry and clonogenic assays also demonstrated reduced HSPCs (Figure 1A, S1E-G and confirmation of deletion in 1D), consistent with observations made in FL-derived *HoxA*$^{c/-}$ HSCs [12]. However unlike the fetal derived equivalents, adult *Hoxa*$^{-/-}$ HSCs (CD150^{+}/CD48^{-}/LKS) demonstrated reduced proliferation potential *in vitro* (Figure S1H), but were more in cycle *in vivo* (Figure S2B, C). In accordance with the *in vitro* results, *Hoxa*$^{-/-}$ BM cells demonstrated reduced ability in reconstituting irradiated congenic hosts compared to wild type controls (Figure 1B and S1I-J) that is partly due to an increase in apoptosis (Figure S2D, E), but not due to homing defects or non-*Hoxa*-deleted escapees (Figure S2F-H). Repopulation ability of *Hoxa*$^{-/-}$ cells was completely absent in secondary recipients, in contrast to control cells, suggesting an exhaustion of the HSC pool (Figure S1K). In this *in vivo* model B cells were also underrepresented, while *Hoxa*$^{-/-}$ cells reconstituted most other cell lineages proportionally (Figure 1C and S1L). These data confirm our earlier observation that adult HSC-derived B cells have an increased sensitivity for *Hoxa* gene levels [5], compared to 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 (supplementary Table S1). Functional annotation clustering

using Gene Ontology terms revealed that differentially expressed genes were associated with cell proliferation and differentiation, cell activation, signalling, regulation of gene expression, hematopoiesis, migration and apoptosis (Table S2). Moreover, differentially expressed genes were associated with several pathways according to the Kyoto Encyclopedia of Genes and Genomes (KEGG), which included hematopoietic cell lineage and cancer pathways, supporting a known role for *Hoxa* genes in hematopoiesis and leukemia (Table S3).

Only 13 genes expressed at Reads Per Kilobase of transcript per Million mapped reads (RPKM) values >1 showed greater than 10-fold differential expression between the 2 conditions (Figure 1E, Table S1). Genes downregulated in *Hoxa*$^{-/-}$ cells were mostly from the *Hoxa* cluster (Figure 1E and F), but also included *Nr4a3*, *Col1a1* and *Hnf4a*. *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 AML [15], while 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 *Mir196a*-1, were significantly upregulated (≥10-fold) in *Hoxa*$^{-/-}$ HSCs (Figure 1E and G), possibly pointing to cross-regulation between *Hoxa* and *Hoxb* cluster genes and their inter-cluster-located Mir's [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. With the exception of the B cell lineage, we found that *Hoxa9* overexpression indeed restored HSPC engraftment to levels found with control wild type

cells (Figure 2A-D). However and in line with our previous observations [5], B cell numbers remained persistently lower in these mice (Figure 2C). Most importantly, overexpression of *Hoxa9* provided a recovery at the molecular level of over 70% (14 of 19) deregulated genes found in *Hoxa*$^{-/-}$ mutant (Figure 2E).

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

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AUTHORSHIP CONTRIBUTIONS

CELG designed and performed experiments, analysed data, mounted the figures and wrote the paper. MF contributed in execution of experiments, analysed RNASeq data

and mounted figures. LK was involved in the writing of the paper. AT and GS were involved in the experimental design and writing of the paper. JB designed the experiments, analysed data, mounted figures and wrote the paper.

DISCLOSURE OF CONFLICTS OF INTEREST

The authors declare no competing financial interest.

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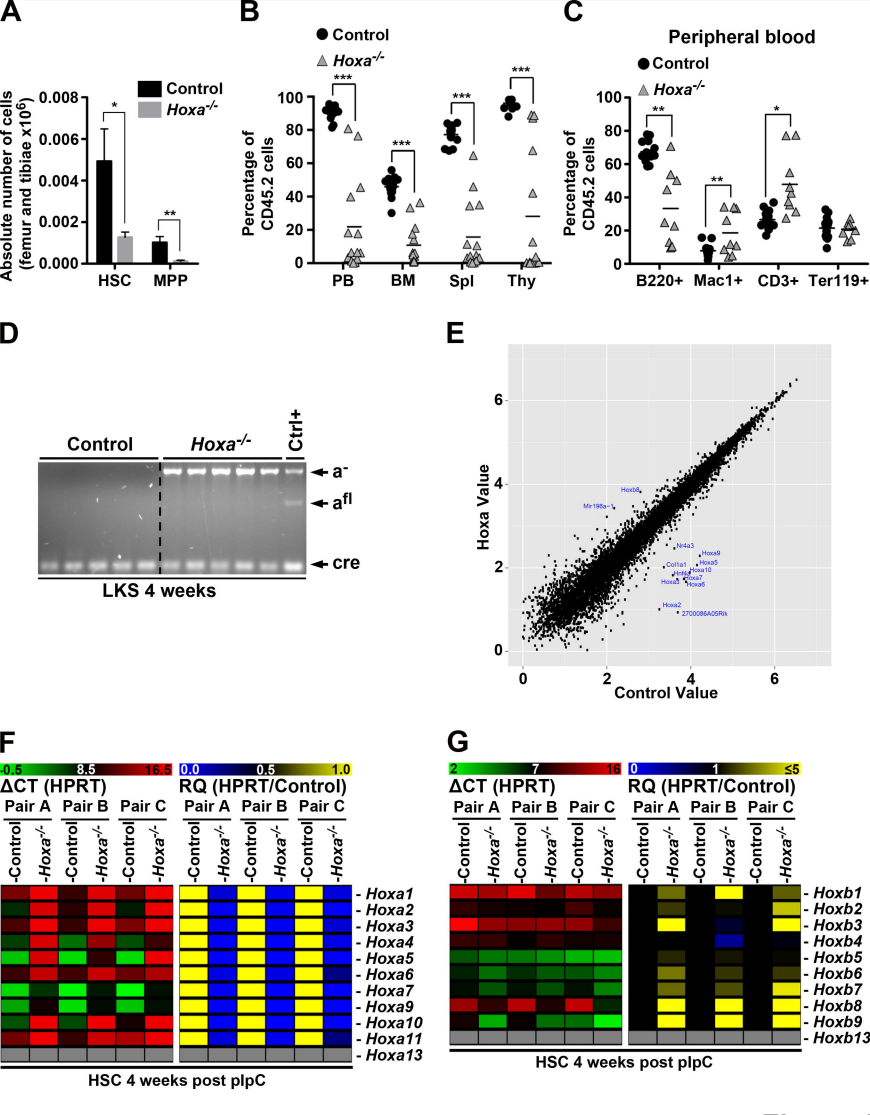
LEGENDS TO THE FIGURES

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 (n=4) and CD150^{+}/CD48^{-}/CD244^{+}/LKS MPPs in BM of *Hoxa*$^{-/-}$ and control mice. BM cells isolated from femur and tibia 4 weeks after pIpC injection. (B) 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. (C) Proportions of B (B220^{+}), myeloid (Mac1^{+}), T (CD3^{+}) and erythroid (Ter119^{+}) cells in PB of reconstituted mice shown in (B). (D) PCR analysis showing the deletion of the *Hoxa* cluster in purified LKS cells at 4 weeks following the last pIpC injection (n=5). (E) Scatterplot showing transcriptome analysis of genes that were up- (above axis) or down-regulated (below axis) in *Hoxa*$^{-/-}$ versus wild type 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, approximately 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<0.05, **P<0.01, ***P<0.001, N.S., non-significant; MPP, multi-potent progenitors; HSC, hematopoietic stem cells; BM, Bone marrow; PB, peripheral blood.

Figure 2. Rescue of *Hoxa*$^{-/-}$ HSC engraftment by overexpression of *Hoxa9*.

(A) Average kinetics of engraftment in mice receiving 0.2 x 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) Long-term engraftment of HSCs with indicated genotype in individual mice 16 weeks post-transplantation. (C) 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 post-transplantation. (D) Bar graphs depicting percentage of *Hoxa*$^{+/+}$ + GFP and *Hoxa*$^{-/-}$ + *Hoxa9* CD150$^+$/CD48$^-$ /LKS HSCs (n=3) and MPPs (n=3) in GFP$^+$ cell population. (E) Gene expression analysis in RNAseq HSC and Lin$^-$ cells overexpressing *Hoxa9.* ¤ indicates quantity not sufficient defined as corrected CT value >36. **P*<0.05, ***P*<0.01, ****P*<0.001, N.S., non-significant; MPP, multi-potent progenitors; HSC, hematopoietic stem cells; BM, Bone marrow; PB, peripheral blood; CT, cycle time.


A
Absolute number of cells (femur and tibiae x10^6)
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0.006
0.004
0.002
0.000
Control
Hoxa-/-
*
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HSC
MPP
B
Control
Hoxa-/-
Percentage of CD45.2 cells
***
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***
100
80
60
40
20
0
PB
BM
Spl
Thy
C
Peripheral blood
Control
Hoxa-/-
Percentage of CD45.2 cells
**
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*
100
80
60
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20
0
B220+
Mac1+
CD3+
Ter119+
D
Control
Hoxa-/-
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Hoxa Value
Control Value
6
4
2
0
0
2
4
6
Hoxb4
Mir196a-1
Nrip3
Hoxa9
Hoxa5
Col1a1
Hoxa10
Hoxa7
Hoxa6
Hoxa2
2700086A05Rik
F
-0.5
8.5
16.5
ΔCT (HPRT)
0.0
0.5
1.0
RQ (HPRT/Control)
Pair A
Pair B
Pair C
-Control
-Hoxa-/-
Hoxa1
Hoxa2
Hoxa3
Hoxa4
Hoxa5
Hoxa6
Hoxa7
Hoxa9
Hoxa10
Hoxa11
Hoxa13
HSC 4 weeks post pIpC
G
2
7
16
ΔCT (HPRT)
0
1
≤5
RQ (HPRT/Control)
Pair A
Pair B
Pair C
-Control
-Hoxa-/-
Hoxb1
Hoxb2
Hoxb3
Hoxb4
Hoxb5
Hoxb6
Hoxb7
Hoxb8
Hoxb9
Hoxb13
HSC 4 weeks post pIpC

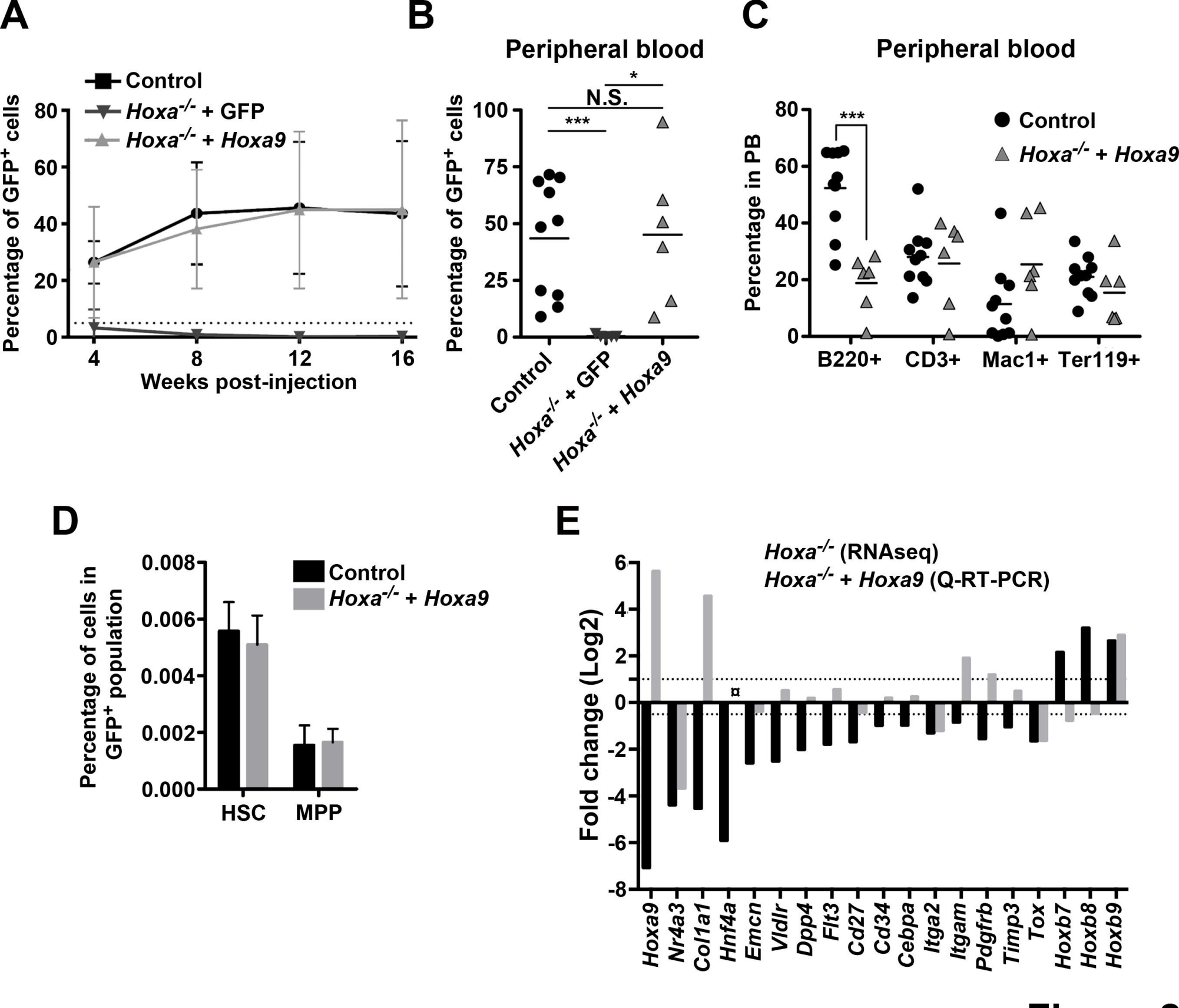
A
Control
Hoxa-/- + GFP
Hoxa-/- + Hoxa9
Percentage of GFP+ cells
Weeks post-injection
B
Peripheral blood
Percentage of GFP+ cells
N.S.
***
*
Control
Hoxa-/- + GFP
Hoxa-/- + Hoxa9
C
Peripheral blood
Percentage in PB
***
Control
Hoxa-/- + Hoxa9
B220+
CD3+
Mac1+
Ter119+
D
Percentage of cells in GFP+ population
Control
Hoxa-/- + Hoxa9
HSC
MPP
E
Hoxa-/- (RNAseq)
Hoxa-/- + Hoxa9 (Q-RT-PCR)
Fold change (Log2)
Hoxa9
Nr4a3
Col1a1
Hnf4a
Emcn
Vldlr
Dpp4
Flt3
Cd27
Cd34
Cebpa
Itga2
Itgam
Pdgfrb
Timp3
Tox
Hoxb7
Hoxb8
Hoxb9

Figure 2