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Cdx4 is dispensable for murine adult hematopoietic stem cells but promotes MLL-AF9-mediated leukemogenesis

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ABSTRACT

Background

Cdx4 is a homeobox gene essential for normal blood formation during embryonic development in the zebrafish, through activation of posterior Hox genes. However, its role in adult mammalian hematopoiesis has not been extensively studied and its requirement in leukemia associated with Hox gene expression alteration is unclear.

Design and Methods

We inactivated Cdx4 in mice through either a germline or conditional knockout approach and analyzed requirement for Cdx4 in both normal adult hematopoiesis and leukemogenesis initiated by the MLL-AF9 fusion oncogene.

Results

Here, we report that loss of Cdx4 had a minimal effect on adult hematopoiesis. Indeed, although an increase in white blood cell counts was observed, no significant differences in the distribution of mature blood cells, progenitors or stem cells were observed in Cdx4-deficient animals. In addition, long-term repopulating activity in competitive transplantation assays was not significantly altered. In vitro, B-cell progenitor clonogenic potential was reduced in Cdx4-deficient animals but no significant alteration of mature B cells was detected in vivo. Finally, induction of acute myeloid leukemia in mice by MLL-AF9 was significantly delayed in the absence of Cdx4 in a retroviral transduction/bone marrow transplant model.

Conclusions

These observations indicate that Cdx4 is dispensable for the establishment and maintenance of normal hematopoiesis in adult mammals. These results, therefore, outline substantial differences in the Cdx-Hox axis between mammals and zebrafish and support the hypothesis that Cdx factors are functionally redundant during mammalian hematopoietic development under homeostatic conditions. In addition, our results suggest that Cdx4 participates in MLL-AF9-mediated leukemogenesis supporting a role for Cdx factors in the pathogenesis of myeloid leukemia.

Key words: Cdx4, homeobox, leukemia, MLL-AF9, hematopoietic stem cell, Hox.


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The online version of this article has a Supplementary Appendix.
Introduction

The Cdx genes are the mammalian homolog of the Drosophila caudal gene and encode homeobox transcription factors that regulate axial elongation and anterior-posterior patterning during embryogenesis through modulation of Hox gene expression.\(^{1-6}\) The Cdx gene family consists of three members: Cdx1, Cdx2, and Cdx4.\(^{6-8}\)

Several studies have indicated a role for Cdx genes in normal hematopoiesis during embryonic development. In the zebrafish embryo, cdx1 and cdx4 are important for blood formation through activation of posterior Hox genes.\(^{6,8}\) In addition, during in vitro hematopoietic differentiation of murine embryonic stem cells (mESC), inactivation of Cdx4 results in reduced hematopoietic colony-forming potential, which is almost completely abolished upon compound Cdx1, Cdx2, and Cdx4 inactivation.\(^{5}\) Conversely, over-expression of Cdx1, Cdx2, or Cdx4 in mESC was shown to facilitate early hematopoietic progenitor formation through up-regulation of Hox gene expression.\(^{6,7,10}\) Of note, Cdx4 over-expression in this system resulted in enhanced formation of progenitors with lymphoid repopulation capacity, suggesting a specific role for Cdx4 during lymphopoiesis.\(^{11}\) Interestingly, several studies have suggested a connection between Cdx4 and the mixed lineage leukemia gene, MLL, a master regulator of Hox gene expression\(^{13-15}\) implicated in normal and malignant hematopoiesis.\(^{16}\) In support of this connection, a similar pattern of Hox gene dysregulation is observed in Mll\(^{-/-}\) and Cdx4\(^{-/-}\) embryos.\(^{15,14}\) In addition, in vitro hematopoietic differentiation of Mll\(^{-/-}\) mESC can be rescued by over-expression of Cdx4.\(^{14}\) Finally, Cdx4 has been found to interact with menin, a co-factor of MLL, in myeloid leukemogenesis,\(^{7,10}\) which participates in the control of Hox gene expression.\(^{19}\) Taken together, these observations suggest that Cdx4 plays an important role in the control of normal embryonic hematopoiesis, likely through a regulatory network involving Cdx4, Mll, and Hox genes; however, they do not directly establish the normal function of Cdx4 in adult mammalian hematopoiesis.

Cdx genes have also been implicated in human hematopoietic malignancies. Cdx2 was found to be fused to the ETV6 gene in the rare chromosomal translocation t(12;15)(p13;q12) associated with acute myeloid leukemia (AML), resulting in its deregulated expression.\(^{20}\) Subsequent studies identified aberrant Cdx2 expression in the majority of adult AML patients regardless of karyotype.\(^{21,22}\) In addition, Cdx2 expression correlates with persistence of minimal residual disease and has been proposed as a negative prognostic marker in acute lymphoblastic leukemia.\(^{21,24}\) Suggesting that CDX2 could be involved in both myeloid and lymphoid malignancies. Similarly, aberrant expression of Cdx4, located on chromosome X, was also observed in patients with AML.\(^{25}\) Consistent with a causal role in malignant transformation of myeloid stem and progenitor cells, over-expression of Cdx2 or Cdx4 in murine bone marrow transplant models alters Hox gene expression and results in AML.\(^{21,25,26}\)

Despite their well-established role in leukemogenesis, the physiologic function of Cdx family members during normal mammalian hematopoiesis remains incompletely understood. We, therefore, assessed the consequences of germline and conditional Cdx4 deletion in mice. Furthermore, we investigated the impact of Cdx4 loss on the initiation and maintenance of AML induced by the chimeric MLL-MLLT3 (also known and hereafter referred to as MLL-AF9) fusion gene in a murine bone marrow transplantation model.

Design and Methods

Generation of Cdx4 knockout mice

To create the conditional Cdx4 mouse strain, a targeting vector containing loxP sites flanking the first exon, S' untranslated region and the proximal promoter region of the Cdx4 gene was generated (Figure 1B). This exon encodes the majority of the coding sequence of Cdx4 (165 out of 282 amino acids including the homeodomain) and its excision would preclude expression of a stable, functional protein. This construct was transfected into mESC, and ESC clones were selected with hygromycin and screened by polymerase chain reaction (PCR) and Southern blot analyses. Correctly targeted ESC (i.e. clone 9 shown in Figure 1) were injected into Balb/C blastocysts to obtain chimeric animals that were crossed with C57/B6 wild-type mice to obtain germline transmission of the floxed Cdx4 allele (termed Cdx4\(^{fl/+}\)). The Cdx4\(^{fl/+}\) allele was back-crossed for at least six generations with C57/B6 wild-type animals (Stock # 000664, The Jackson Laboratory). Cdx4\(^{fl/+}\) were then bred with Mix1-Cre transgenic animals and interbred to obtain Cdx4\(^{fl/+}\) Mix1-Cre (abbreviated Cdx4\(^{fl/+}\)-Cre\(^{-}\)) animals and Cdx4\(^{fl/+}\) control (abbreviated Cdx4\(^{fl/+}\)-Cre\(^{-}\)) animals that were treated with polyinosinic-polycytidylic acid (pIpc; Sigma, St. Louis, MO, USA; five intraperitoneal injections of 500 μg every other day). Excision efficiency was assessed by PCR with the following primers: (i) Cdx4\(^{fl/+}\) mice: WT-F: 5'-GCA CCT GCG GTG TAA TAA ATT CT-3'; GFP-F: 5'-TCA TCT GCA CCA CCG GCA A-3'; GFP-R: 5'-GCA ACT CAG AAC AGG TCC GGC CGC TCT AGA ACT AGT GGA-3'. Wild-type, floxed, and excised alleles generated 200-bp, 250-bp, and 300-bp PCR products, respectively; (ii) Cdx4\(^{fl/+}\) mice: WT-F5: 5'-GCA ACT CAG CAT GGA TGG TCG TTT GTT TGG TTA ATT CT-3'; WT-R: 5'-GCA ACT CAG CAT GGA TGG TCG TTT GTT TGG TTA ATT CT-3'; GFP-F: 5'-TCA TCT GCA CCA CCG GCA A-3'; GFP-R: 5'-GTA GTA GTT GTA GTC CAT CAG CT-3'. Wild-type and mutant alleles gave 200-bp and 300-bp PCR products, respectively; (iii) Cdx4\(^{fl/+}\) mice: WT-F5: 5'-GCA ACT CAG CAT GGA TGG TCG TTT GTT TGG TTA ATT CT-3'; WT-R: 5'-GCA ACT CAG CAT GGA TGG TCG TTT GTT TGG TTA ATT CT-3'; Exc-R1: 5'-GCA ACT CAG CAT GGA TGG TCG TTT GTT TGG TTA ATT CT-3'. Wild-type, floxed, and excised alleles generated 200-bp, 250-bp, and 300-bp PCR products, respectively. Mice bearing a germline deletion of Cdx4 exon 1 (termed Cdx4\(^{ex1}\) allele) were described previously.\(^{26}\) Approval for the use of animals in this study was granted by the Children’s Hospital Boston Animal Care and Use Committee.

Real-time quantitative reverse transcription polymerase chain reaction

Total RNA from sorted cells or hematopoietic tissues was isolated using the Trizol reagent (Invitrogen). RNA samples were reverse-transcribed with the Superscript II kit (Invitrogen). Real-time quantitative PCR assays for Cdx4 and β-actin (reference gene) were obtained from Applied Biosystems (Mm00432451_m1 and Mm01205647_g1, respectively). PCR were performed on an ABI-7000 sequence detection system. The ΔΔCt method was used to calculate expression of Cdx4 relative to β-actin. Hox genes were quantified as described previously.\(^{27}\) All reactions were performed on an ABI-7000 sequence detection system using SYBR Green PCR Master Mix or Taqman Universal PCR Master Mix reagents (Applied Biosystems).

Flow cytometry and cell sorting analyses

Single-cell suspensions were prepared from bone marrow cells after red blood cell lysis (Puregene). Cells were stained following standard procedures using antibodies purchased from BD-Pharmingen. Briefly, LinSca1 c’Klt (LSK) cells and myeloid progenitors, including common myeloid progenitors (CMP), granulo-
cyte-macrophage progenitors (GMP), and megakaryocyte-erythrocyte progenitors (MEP), were purified as follows. Total bone marrow cells from 6- to 8-week old wild-type C57/B6 animals were obtained after flushing the femora, tibiae and humeri and red blood cells were lysed (Puregene RBC lysis buffer, Qiagen). Cells were then stained with a cocktail of rat-anti-mouse antibodies against mature cells (lineage-positive cells), including Ter119, B220, CD3, CD4, CD8, IL7-R, CD19, and Gr1. After incubation with sheep anti-rat antibody coated magnetic beads (Dynabeads M-450, Dynal, Invitrogen), lineage-positive cells were physically depleted using a magnet (Invitrogen). Cells were then incubated with goat-anti rat PE-Cy5.5 conjugated antibody. After washing, cells were blocked with rat IgG prior to incubation with anti-CD34 FITC conjugated antibody, c-Kit APC conjugated antibody, and Foxp3 PE-Cy7 conjugated antibody (BD Pharmingen) and Fc-RIA/III PE conjugated antibody (Abcam). Cells were flow sorted using a double laser (488 nm/550 nm Enterprise II and 647 nm Pectrum) FACS (FACSAnia, BD Biosciences). For T-cell populations, thymocytes were obtained from 6- to 8-week-old wild-type C57/B6 animals. Cells were stained with anti-CD4, anti-CD8 and anti-CD3 antibodies (BD Pharmingen) and populations were purified as indicated in Figure 1A. For B-cell populations, total bone marrow cells from 6- to 8-week old wild-type C57/B6 animals were stained with anti-B220, anti-CD19, anti-IgM and anti-CD43 and were purified using the gates exemplified in Figure 2B. Cells were then sorted using a double laser (488 nm/550 nm Enterprise II and 647 nm Pectrum) FACS (FACSAnia, BD Biosciences) and analyses were carried out on a four-color FACS CALIBUR (Becton Dickinson). Raw data were analyzed using FlowJo software.

**Colony assays**

Myeloid and pre-B colony-forming assays were performed by plating 20,000 and 50,000 bone marrow cells per dish in duplicate into methylcellulose medium M3434 and M3630 (Stem Cell Technologies, Vancouver, BC, Canada), respectively. Colonies were counted on day 7 and 10, respectively. Bone marrow cells from leukemic mice were plated in methylcellulose medium M3434 at 10,000 cells per dish in duplicate. Colonies were counted on day 7 and serially re-plated every 7 days at a density of 10,000 cells per dish.

**Retroviral production, bone marrow transduction and transplantation assays**

Retroviral supernatant production and bone marrow transplants were performed as previously described using MSCV-MLL-AF9-GFP and MSCV-GFP. Briefly, 8-week old Cdx4fl/fl or wild-type littermate donor mice were injected with 5-fluorouracil (5-FU: 150 mg/kg) 5 days prior to bone marrow collection. On day 0, primary bone marrow cells were obtained from femora and tibiae by flushing with PBS 1x supplemented with 2% FBS; red blood cells were lysed (Puregene, Qiagen) and cells were then cultured overnight in RPMI 1640 supplemented with 10% FBS and IL3 (10 ng/mL), IL6 (20 ng/mL), SCF (10 ng/mL). Cells were mixed with similar titer viral supernatants twice on day 1 and day 2, centrifuged for 90 min (20 ng/mL), SCF (10 ng/mL). Cells were mixed with similar titer viral supernatants twice on day 1 and day 2, centrifuged for 90 min and returned to the incubator for 2-3 hours. After the second spinfection, cells were washed and re-suspended in Hank’s balanced salt solution 1x (HBSS 1x) and 1x10⁶ cells were injected into the tail veins of each lethally irradiated recipient. Animals were monitored daily for disease development and sacrificed according to institutional guidelines.

Non-competitive and competitive transplants were carried out with two sets of donor mice in two independent experiments, with five recipient mice per group in each experiment. CD45.2- bone marrow cells from Cdx4fl/fl-Cre + and control donor mice were injected into the lateral tail veins of lethally irradiated CD45.1+ C57/B6.SJ recipient mice, either alone for non-competitive transplants, or mixed with competitor bone marrow cells from wild-type F1 C57/B6 mice (CD45.1/2-) for competitive transplants (Figure 3c). Recipient mice were bled from the eyes every 4 weeks up to 16 weeks after transplantation for analysis of CD45.2+ donor contribution.

**Histopathology and microscopy**

Peripheral blood was collected through non-lethal eye-bleeds under anesthesia with isoflurane in accordance with institutional guidelines. Complete blood counts were determined using a Hemavet 950 cell counter (Drew Scientific, Oxford, CT, USA). Paraffin-embedded tissue sections were prepared at the Dana

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**Figure 1. Cdx4 expression and conditional inactivation strategy.** (A) Quantitative real-time RT-PCR was used to measure Cdx4 mRNA expression levels relative toβ-actin in normal hematopoietic tissues and flow sorted cells. BM: bone marrow; PB: peripheral blood; LSK, enriched for hematopoietic stem cells; CMP common myeloid progenitors; GMP, granulocyte-monocyte progenitors; MEP, megakaryocyte-erythrocyte progenitors. (B) Homologous recombination targeting strategy to obtain Cdx4lox/lox mice. (C) Southern blot analysis using the 3’ probe indicated in Panel B. WT: wild-type Cdx4 locus, Rec: Cdx4 locus target by the conditional knock-out construct. Clone 9 showed homologous recombination and was used to generate the conditional knock-out mouse line. Clone 8 was not correctly target-ed, and demonstrates the germline configuration. (D) PCR to assess Cdx4 excision 5 weeks after pIpC treatment was performed on DNA from the indicated organs of Cdx4fl/fl Mx1Cre + or Cdx4fl/fl Mx1Cre animals using the three primers shown in Panel B (WT-F, WT-R, Exc-1R). Flox: unexcised allele, Exc: Excised allele. (E) LSK cells and myeloid progenitor populations from Cdx4fl/fl Mx1Cre + were flow sorted 6 weeks after pIpC treatment and DNA was extracted to assess excision efficiency as described in (D). DNA from untreated Cdx4fl/fl Mx1Cre + total bone marrow cells was used as a control.
Farber/Harvard Cancer Center Specialized Histopathology Services Core and stained with hematoxylin and eosin. Images were obtained using a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) and a SPOT RT color digital camera (Diagnostic Instruments, Sterling Heights, MI, USA).

**Statistical analysis**
Statistical significance of differences between the results was assessed by a two-tailed unpaired Student’s t-test using Prism software.

**Results**

**Cdx4 expression in normal murine hematopoeisis**
We first determined the expression pattern of Cdx4 during normal hematopoietic differentiation using quantitative reverse transcription PCR on RNA from wild-type C57/B6 murine hematopoietic tissues and flow-sorted progenitors. Cdx4 mRNA was preferentially expressed in myeloid progenitor cells (Figure 1A) and during B-cell differentiation.

**Generation of conditional Cdx4 knockout mice**
To establish the role of Cdx4 in normal hematopoiesis, we engineered two mouse models of Cdx4 inactivation, through either straight knockout (named the Cdx4 allele) or conditional knockout (named the Cdx4 allele) based on inducible deletion of the entire exon 1 of the Cdx4 gene (Figure 1B). This was achieved through homologous recombination in ESC; Southern blot analysis was performed to select clones correctly targeted at the endogenous Cdx4 locus on chromosome X (i.e. clone 9 but not clone 8 in Figure 1C and Online Supplementary Figure S1). After germline transmission of the Cdx4 and Cdx4 alleles, crosses were performed to obtain homozygous animals. Both Cdx4-/- and Cdx4 F/F knockout animals were born at Mendelian ratios, appeared normal, had a weight similar to their wild-type, age-matched littermates, and were fertile. Cdx4 F/F mice were next crossed with Mx1-Cre transgenic animals to allow for inducible Cre recombinase expression. Cdx4 excision in Cdx4 F/F-Mx1-Cre (Cdx4F/F-Cre+ or F/F-Cre+) mice was induced in 4- to 6-week old animals by pIpC treatment. Subsequent analyses were performed 4-6 weeks after pIpC treatment.

**Figure 2. Analysis of the hematopoietic compartment in Cdx4 F/F-Cre+ mice.** Flow cytometric analysis of the myeloid lineage (A) and B-cell lineage (B) analyzed on bone marrow cells (n=4). Except where otherwise indicated, analyses were gated on total bone marrow cells. (C) Histogram representation of results presented in Panel B (n=4). (D) Myeloid colony-forming potential of total bone marrow cells in SCF, IL3, IL6 and EPO-supplemented methylcellulose cultures (M3434, StemCells). Colonies were scored 7 days after plating and the mean ± SD is shown (n=4). GM, granulocyte macrophage; G/M, granulocyte or monocyte; GEMM, granulocyte erythroid macrophage megakaryocyte; BFU-E, burst-forming unit-erythroid. (E) B-cell colony-forming potential of total bone marrow cells in IL7-supplemented methylcellulose cultures (M3630, StemCells). Colonies were scored 10 days after plating and mean ± SD is shown (n=4).
unless otherwise indicated. Full Cdx4 excision was observed in whole bone marrow (Figure 1D) as well as in purified hematopoietic stem and progenitor-enriched populations (Figure 1E). Full excision of Cdx4 in the bone marrow was also demonstrated at 6 and 12 months (data not shown), indicating that under homeostatic conditions, there was no selective advantage for rare “escaper” cells in which excision had not occurred.

**Loss of Cdx4 results in minimal hematologic abnormalities**

We then performed analyses of the peripheral blood and hematopoietic organs. Both Cdx4<sup>-/-</sup> and Cdx4<sup>F/F-Cre</sup> mice showed a significant increase in the number of lymphocytes compared to their respective wild-type controls, as assessed by an automated cell counter (Table 1, Online Supplementary Table S1). However, there was no consistent difference in myeloid, erythroid and platelets counts, absolute numbers and distribution of bone marrow cells or spleen and liver weights between Cdx4 knockout mice (Cdx4<sup>-/-</sup> or Cdx4<sup>F/F-Cre</sup>) and their respective littermate controls (Online Supplementary Figures S2A-C). Flow cytometric analyses of bone marrow cells from Cdx4 knockout mice identified no significant differences in the major hematopoietic compartments including myeloid, erythroid, B- and T-cell lineages (Figure 2A-C, Online Supplementary Figures S2A-C and S3). Similar results were observed in a cohort of animals analyzed 8 and 12 months after pIpC treatment (Online Supplementary Figure S4A,B and data not shown).

To determine whether loss of Cdx4 affects the clonalogenic potential of hematopoietic progenitor populations, we performed in <i>vitro</i> colony-forming unit (CFU) assays. Compared to bone marrow from wild-type littermates, Cdx4 knockout bone marrow produced similar total numbers of myeloid colonies, and no significant differences in the distribution of colony types were observed (Figure 2D, Online Supplementary Figure S2D). We did, however, observe a significant decrease in pre-B colony-forming activity in bone marrow cells from Cdx4 knockout mice compared to bone marrow from their wild-type littermate controls in both the conditional as well as the germine knockout model (Figure 2E, Online Supplementary Figure S2E). Of note, we confirmed these observations in older mice that were analyzed 8 and 12 months after pIpC treatment. (Online Supplementary Figure S4C,D and data not shown).

**Loss of Cdx4 does not alter the number and repopulating activity of hematopoietic stem and progenitor cells**

Since Cdx4 is expressed in the hematopoietic stem and progenitor compartment, and was reported to be essential for normal hematopoiesis in zebrafish, we next assessed the effect of loss of Cdx4 specifically on hematopoietic stem cell function. We first performed multiparameter flow cytometry analyses on the hematopoietic stem and progenitor compartments. No significant differences in the number of LSK, CMP, GMP, or MEP were observed between both Cdx4 knockout mouse models and their respective wild-type controls (Figure 3A,B and data not shown).

We then performed non-competitive and competitive transplantation assays to assess the repopulating ability of Cdx4-deficient bone marrow cells. CD45.1 CD45.2<sup>-/-</sup>-<i>Cdx4<sup>F/F-Cre</sup></i> or control CD45.1 CD45.2<sup>-/-</sup>-<i>Cdx4<sup>F/F-Cre</sup></i> bone marrow cells were transplanted into lethally irradiated CD45.1<sup>+</sup>CD45.2<sup>+</sup>-<i>SigE</i>-<i>bone</i> recipients with or without wild-type CD45.1<sup>+</sup>CD45.2<sup>+</sup>-<i>bone</i> marrow cells (control). The contribution of the Cdx4-deficient and control bone marrow cells to hematopoiesis in the recipients (percentage of CD45.1<sup>+</sup>CD45.2<sup>+</sup>-<i>bone</i> cells) was assessed in the peripheral blood every 4 weeks over 16 weeks, and in the bone marrow after 16 weeks. We observed a similar contribution of donor-derived CD45.1<sup>+</sup>CD45.2<sup>+</sup>-<i>bone</i> cells from Cdx4-deficient and littermate control mice in the peripheral blood (Figure 3D) and bone marrow of recipient mice (data not shown). These results show that Cdx4-deficient bone marrow cells are not significantly altered in their normal long-term repopulating ability, indicating that Cdx4 is not essential for hematopoietic stem cell function even under the replicative stress associated with bone marrow reconstitution.

**Cdx4 shortens disease latency but is not essential for MLL-AF9-induced leukemia in mice**

Although Cdx4 does not appear to be essential for homeostatic hematopoiesis, it was previously demonstrated that <i>vitro</i> differentiation of <i>Mll</i>-ESC into blood cells could be rescued by over-expressing Cdx4, suggesting that Cdx4 might be epistatic to, or a critical downstream effector of <i>Mll</i>. Furthermore, oncogenic fusion proteins involving MLL occur frequently in patients with acute myeloid or lymphoid leukemias. We, therefore, hypothesized that Cdx4 might play a role in the context of MLL-mediated leukemogenesis. To test this idea, we used a murine model in which transplantation of bone marrow cells transduced with MLL-AF9 into lethally irradiated recipients induces a fully penetrant myeloid leukemia (Figure 4A). Recipients of wild-type bone marrow cells transduced with MLL-AF9 developed acute leukemia with a median latency of 49 days. In contrast, recipients of MLL-AF9-transduced Cdx4<sup>-/-</sup> bone marrow cells developed acute leukemia with a significantly longer latency of 63 days (<i>P</i>=0.005) (Figure 4B). Histological analysis identified no morphological differences between leukemias derived from wild-type and Cdx4<sup>-/-</sup> cells (Online Supplementary Figure S5). However, flow cytometric analysis revealed that while leukemias arising in both backgrounds showed a similar expansion of Mac1<sup>+</sup> cells in the bone marrow (Figure 4C), leukemic cells generated on the Cdx4<sup>-/-</sup> background also displayed low levels of expression on the myeloid lineage.

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**Table 1. Peripheral blood counts of Cdx4<sup>-/-</sup>-<i>Cdx4<sup>F/F-Cre</sup></i> and control animals. Blood samples were taken 6-8 weeks after pIpC treatment from Cdx4<sup>-/-</sup>-<i>Cdx4<sup>F/F-Cre</sup></i> (n=10) and Cdx4<sup>-/-</sup>-<i>Cdx4<sup>F/F-Cre</sup></i> (n=10) post-pIpC treatment and complete blood counts were obtained with a Hemavet950 cell counter.**

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<th>Cdx4&lt;sup&gt;-/-&lt;/sup&gt;-&lt;i&gt;Cdx4&lt;sup&gt;F/F-Cre&lt;/sup&gt;&lt;/i&gt; (n=10)</th>
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of two lymphoid surface markers, CD3 and B220 (Figure 4D). Together, these studies suggest that Cdx4 is dispensable for leukemia induction by MLL-AF9, but its loss delays disease onset and alters the leukemic phenotype in a bone marrow transplantation model.

**Loss of Cdx4 does not significantly affect Hox gene expression**

Cdx4 has been reported to regulate the expression of Hox genes, including Hoxa9 and Hoxb4. To determine the effect of Cdx4 loss on Hox gene expression in murine hematopoiesis, real-time quantitative RT-PCR was used to measure the expression of several Hox genes in bone marrow cells from wild-type mice and Cdx4−/− mice, as well as in leukemic cells generated from MLL-AF9-transduced wild-type and Cdx4−/− bone marrow cells. This analysis showed that there were no significant differences in the expression of individual Hox genes between Cdx4−/− and wild-type controls (Online Supplementary Figure S6).

**Discussion**

Here we report the first detailed analysis of the role of a caudal gene in adult homeostatic hematopoiesis in mammals and unexpectedly demonstrate that Cdx4 knock-out murine models do not present major hematopoietic defects. Indeed, in both germline and conditional Cdx4 knockout models, hematopoietic stem and progenitor cells as well as mature blood cells were not significantly affected by Cdx4 loss of function. Of note, our conditional Cdx4 knockout model bypasses any compensatory mechanism that might occur during embryonic development in the germline Cdx4 knockout model. These results demonstrate that Cdx4 is not essential for the establishment and maintenance of normal adult hematopoietic stem cell functions in mice. Although these results contrast with the severe hematopoietic defects observed in cdx4 mutant zebrafish, it is important to note that mammals have...
three Cdx genes (Cdx1, Cdx2, and Cdx4), whereas only two genes have been assigned to the Cdx family in zebrafish, Cdx1 and Cdx4. In addition, although zebrafish cdx4 mutants have a severe hematopoietic defect,10 suggesting a minor degree of redundancy between Cdx1 and Cdx4 during developmental hematopoiesis. It is likely that the critical function of cdx genes in zebrafish developmental hematopoiesis can be extended to the functionally redundant homolog Cdx2. In mammals, Cdx4 deficiency was reported to cause a modest hematopoietic defect during in vitro differentiation of mESC. In addition, although yolk sac hematopoiesis was transiently altered before 9 days post-conception, no significant blood alteration was observed in Cdx4 deficient embryos in the yolk sac 9 days post-conception or in fetal liver. In contrast, Cdx2 deficiency results in a more severe defect, and a combination of Cdx1, Cdx2, and Cdx4 deficiency almost abolished blood formation from mESC. Furthermore, only Cdx2/4 compound mutants, but not Cdx4-deficient or Cdx1/4 double mutants, present axial elongation defects during mouse development. Together, these observations suggest that Cdx4 may have a non-redundant role during very early hematopoietic development but that its function in adult hematopoietic stem cells in vivo is compensated by redundant mechanisms. This difference could be explained by a lower sensitivity to Cdx gene dosage in definitive compared to primitive hematopoiesis. An alternative possibility is that Cdx function is not essential for adult hematopoiesis. Indeed, it was previously shown that other genes essential for primitive hematopoiesis specification, such as SCL/Tal1, do not play an essential role in definitive adult stem cell function, supporting the existence of two distinct developmental pathways for the generation of embryonic hematopoietic stem cells and maintenance of adult hematopoietic stem cells.

Although Cdx4 deficiency was not associated with a gross hematopoietic phenotype, our results do suggest a previously unappreciated role for Cdx4 in lymphoid development. Indeed, we show that Cdx4 expression is up-regulated in pre-B, pro-B, and B220+ cells. We also observed a reduced capacity of bone marrow cells from Cdx4-deficient animals to form B-cell colonies in vitro. These observations are compatible with a positive role of Cdx4 in lymphopoiesis that would result in a partial block of differentiation during early B-cell development in Cdx4-defi-

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**Figure 4.** Loss of Cdx4 delays MLL-AF9-induced leukemia. (A) Schematic of the retroviral transduction/bone marrow transplant protocol. (B) Survival curves for cohorts of mice injected with wild-type (Cdx−/−) or Cdx−/− bone marrow cells transduced with MLL-AF9. Ten animals were used in each group. (C) Flow cytometric analysis of bone marrow cells from recipient animals. Histograms on the right side represent mean±SD of the percentage of the indicated population (n=3). (D) Flow cytometric analysis of expression of lymphoid markers on bone marrow cells from recipient animals.
Cdx4 is dispensable for murine hematopoiesis

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