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.[1-5] 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.[9,10] 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.[6] 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.[6,7,9,11,12] 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.[13,14] 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[17,18] 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;13)(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,[23,24] 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.[22,25,26]

Despite their well-established role in leukemogenesis, the physiological 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, 5' 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 $Cdx4^{F}$). The $Cdx4^{F}$ allele was back-crossed for at least six generations with C57/B6 wild-type animals (Stock # 000664, The Jackson Laboratory). $Cdx4^{F/WT}$ were then bred with *Mx1-Cre* transgenic animals and interbred to obtain $Cdx4^{F/F}$ *Mx1-Cre* (abbreviated $Cdx4^{F/F}$-Cre^{+}) animals and $Cdx4^{F/F}$ control (abbreviated $Cdx4^{F/F}$-Cre^{-} or *F/F-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) $Cdx4^{-/-}$ mice: WT-F: 5'-GCA CCT GCG GTA TAA ATT CT-3'; WT-R: 5'-GCA ACT CAG AAC AGG TCC TT-3'; GFP-F: 5'-TCA TCT GCA CCA CCG GCA A-3'; GFP-R: 5'-GTT GTA GTT GTA CTC CAG CT-3'. Wild-type and mutant alleles gave 200-bp and 300-bp PCR products, respectively; (ii) $Cdx4^{F/F}$ mice: WT-F5: 5'-CTT TAC GGA TGG TTG TGA GC-3'; WT-R5: 5'-AGG ACA GGA ACT CAT GGA GTT T-3'; Exc-R1: 5'-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. Mice bearing a germline deletion of *Cdx4* exon 1 (termed $Cdx4^{-}$ allele) were described previously.[6] 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.[25] 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, $Lin^{-}Sca1^{+}cKit^{+}$ (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/Bl6 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, Sca-1 PE-Cy7 conjugated antibody (BD Pharmingen) and Fcγ-RII/III PE conjugated antibody (Abcam). Cells were flow sorted using a double laser (488 nm/350 nm Enterprise II and 647nm Pectrum) FACS (FACSAria, BD Biosciences). For T-cell populations, thymocytes were obtained from 6- to 8-week old wild-type C57/Bl6 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/Bl6 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 flow sorted using a double laser (488 nm/350 nm Enterprise II and 647nm Pectrum) FACS (FACSAria, BD Biosciences) and analyses were carried out on a four-color FACSCalibur (Becton Dickinson). Raw data were analyzed using FlowJo software.

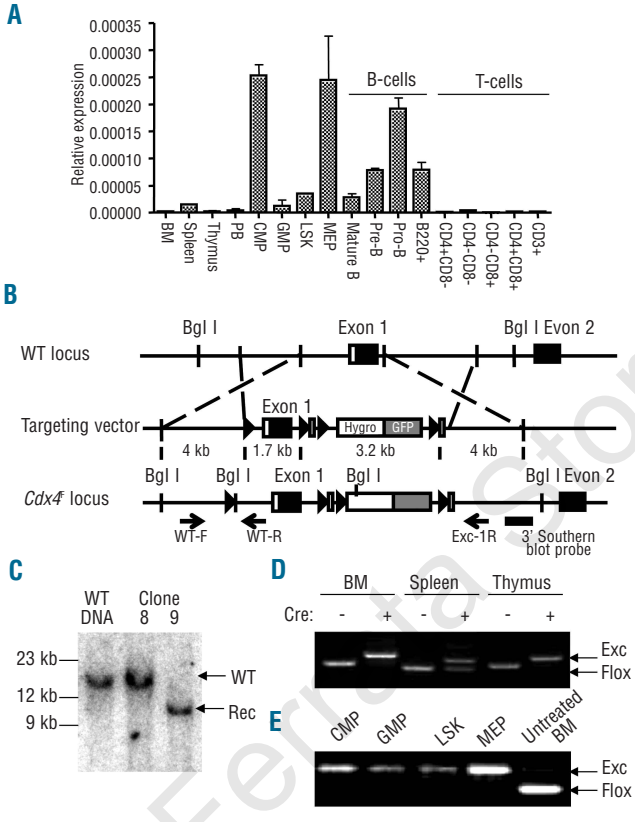

Figure 1. ***Cdx4*** **expression and conditional inactivation strategy. (A) Quantitative real-time RT-PCR was used to measure** ***Cdx4*** **mRNA expression levels relative to β-actin in normal hematopoietic tissues and flow sorted cells. BM: bone marrow; PB: peripheral blood; LSK, enriched for hematopoietic stem cells; CMP common myeloid progenitors; GMP, granulocyte-monocyte progenitors; MEP, megakaryocyte-erythroid progenitors. (B) Homologous recombination targeting strategy to obtain** ***Cdx4*** $^{F/F}$ **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 targeted, 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** ***Cdx4*** $^{F/F}$ **Mx1Cre^{+} or** ***Cdx4*** $^{F/F}$ **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** ***Cdx4*** $^{F/F}$ **Mx1Cre^{+} were flow sorted 6 weeks after pIpC treatment and DNA was extracted to assess excision efficiency as described in (D). DNA from untreated** ***Cdx4*** $^{F/F}$ **Mx1Cre^{+} total bone marrow cells was used as a control.**

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[27] using MSCV-MLL-AF9-GFP and MSCV-GFP. Briefly, 8-week old Cdx4$^{-/-}$ 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 at 2500 rpm and 33°C each time (spinfection) 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 1×10^{6} 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 *Cdx4F/F·Cre*$^{+}$ and control donor mice were injected into the lateral tail veins of lethally irradiated CD45.1^{+} C57/B6.SJL 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 in the peripheral blood. Bone marrow cells were obtained from recipient mice 16 weeks after transplantation for further analysis of the 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

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[6] (named the $Cdx4^{-}$ allele) or conditional knockout (named the $Cdx4^{F}$ 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 $Cdx4^{F}$ 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* ($Cdx4^{F/F}$-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

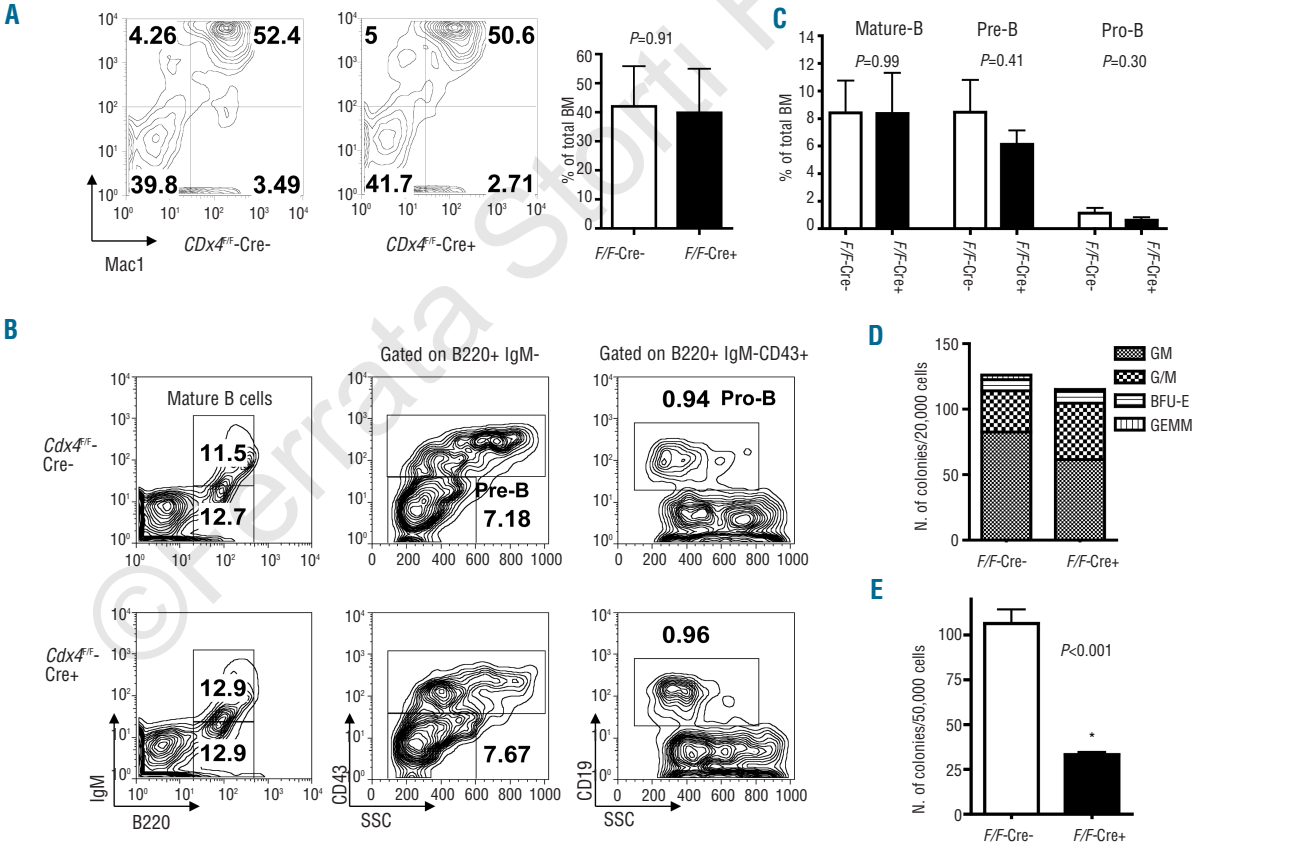

Figure 2. Analysis of the hematopoietic compartment in $Cdx4^{F/F}$-Cre^{+} mice. Flow cytometric analysis of the myeloid lineage (A) and B-cell lineage (B) analyzed on bone marrow cells (n=4). Except where otherwise indicated, analyses were gated on total bone marrow cells. (C) Histogram representation 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*$^{-/-}$ and *Cdx4*$^{F/F}$-*Cre*$^{+}$ 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*$^{-/-}$ or *Cdx4*$^{F/F}$-*Cre*$^{+}$) and their respective littermate controls (*Online Supplementary Table S2*). 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 clonogenic potential of hematopoietic progenitor populations, we performed *in vitro* 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 germline 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,[9] 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^{+} *Cdx4*$^{F/F}$-*Cre*$^{+}$ or control *Cdx4*$^{F/F}$-*Cre*$^{-}$ bone marrow cells were transplanted into lethally irradiated CD45.1^{+}CD45.2^{-} B6/SJL recipients with or without wild-type CD45.1^{+}CD45.2^{+} competitor bone marrow cells (Figure 3C). The contribution of the *Cdx4*-deficient and control bone marrow cells to hematopoiesis in the recipients (percentage of CD45.1^{-}CD45.2^{+} 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^{-}CD45.2^{+} 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.

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

	Cdx4$^{F/F}$-Cre- (n=10)	*Cdx4*$^{F/F}$-Cre+ (n=10)	t-test (*P* value)
White blood cells (×10^{9}/L)	7.090±0.5695	8.918±0.865	0.0015
Lymphocytes (×10^{9}/L)	4.002±0.4485	5.372±0.592	0.0011
Granulocytes (×10^{9}/L)	1.635±0.2755	1.835±0.283	0.2431
Monocytes (×10^{9}/L)	0.3362±0.0572	0.2962±0.042	0.1974
Red blood cells (×10^{12}/L)	9.238±0.5336	9.502±0.5608	0.4230
Hematocrit (%)	48.81±2.884	52.02±2.722	0.0755
Platelets (×10^{9}/L)	443.4±47.69	481.8±42.94	0.1734

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 *in vitro* differentiation of *Mll*$^{-/-}$ ESC into blood cells could be rescued by over-expressing *Cdx4*, suggesting that *Cdx4* might be epistatic to, or a critical downstream effector of MLL.[14] 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*$^{-/-}$ bone marrow cells developed acute leukemia with a significantly longer latency of 63 days (P=0.0005) (Figure 4B). Histological analysis identified no morphological differences between leukemias derived from wild-type and *Cdx4*$^{-/-}$ cells (*Online Supplementary Figure S5*). However, flow cytometric analysis revealed that while leukemias arising in both backgrounds showed a similar expansion of Mac1^{+} cells in the bone marrow (Figure 4C), leukemic cells generated on the *Cdx4*$^{-/-}$ background also displayed low levels of expression

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*.[9,11,19] 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,[9] it is important to note that mammals have

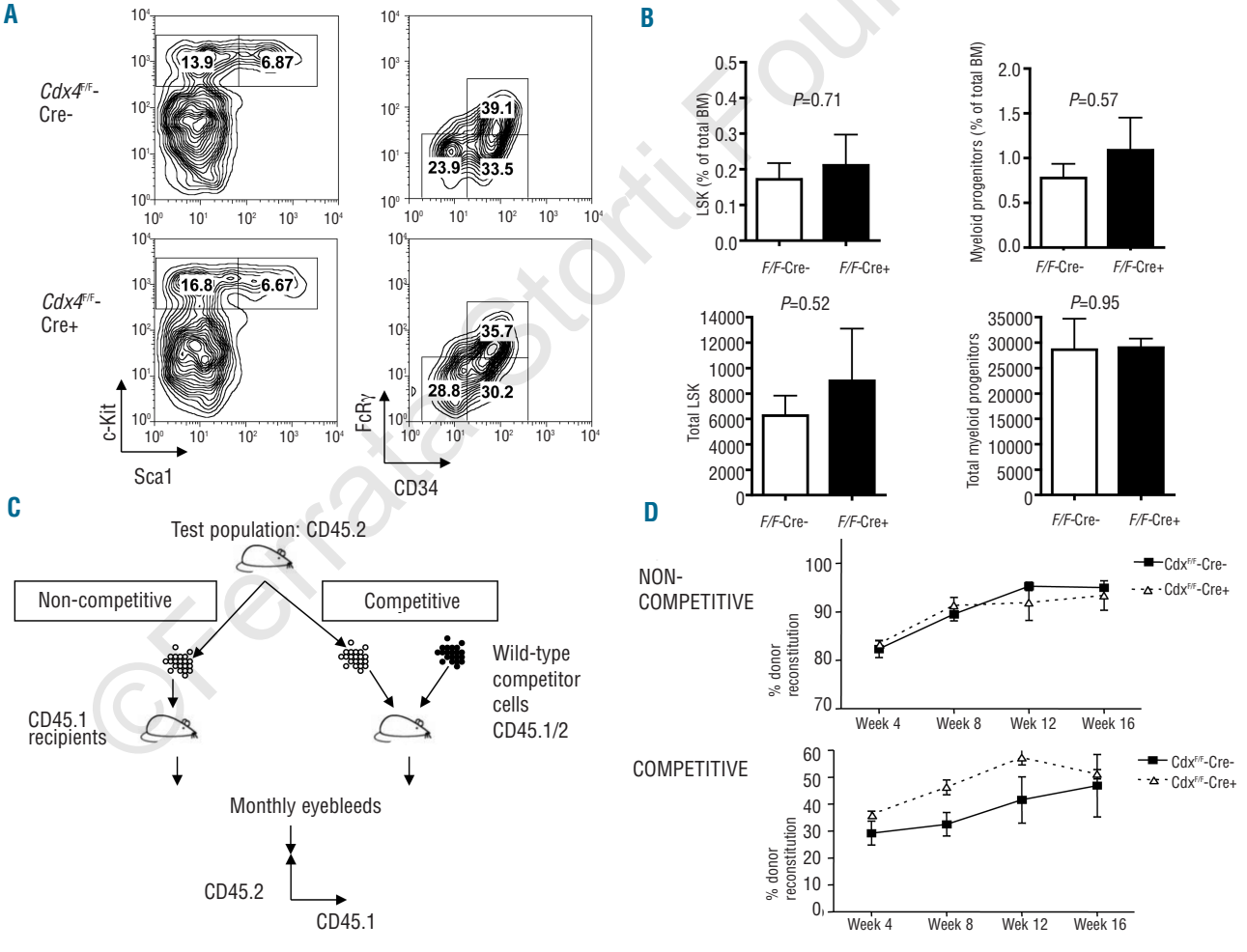

Figure 3. Analysis of the hematopoietic stem cell compartment in *Cdx4*$^{F/F}$-*Cre*$^{+}$ mice. (A) Flow cytometric analysis of the LSK (Lin^{-}c-Kit^{+}Sca1^{+}) and myeloid progenitor compartments 4-6 weeks after pIpC treatment. Left panels show 10000 cells gated on viable Lin^{-} cells. Right panels show 10000 cells gated on viable Lin^{-} c-Kit^{+}Sca1^{-} cells (B) Histogram representation of results presented in (A). Mean±SD are shown (n=4). (C) Diagram showing non-competitive and competitive bone marrow repopulation assays. (D) Contribution of donor cells (CD45.2^{+}CD45.1^{-}) to the hematopoiesis of lethally irradiated recipients in non-competitive and competitive transplants. The percentages of CD45.2^{+} and CD45.1^{+} cells in the peripheral blood of recipient mice were measured by flow cytometric analysis at 4, 8, 12 and 16 weeks after transplantations (n=5). Mean±SD of the percentage of CD45.2^{+} cells are shown.

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,[9] knockdown of *cdx1* in a *cdx4* mutant background results in a complete failure to specify blood,[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.[6] Furthermore, only *Cdx2/4* compound mutants, but not *Cdx4*-deficient or *Cdx1/4* double mutants, present axial elongation defects during mouse development.[2] 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 for 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-

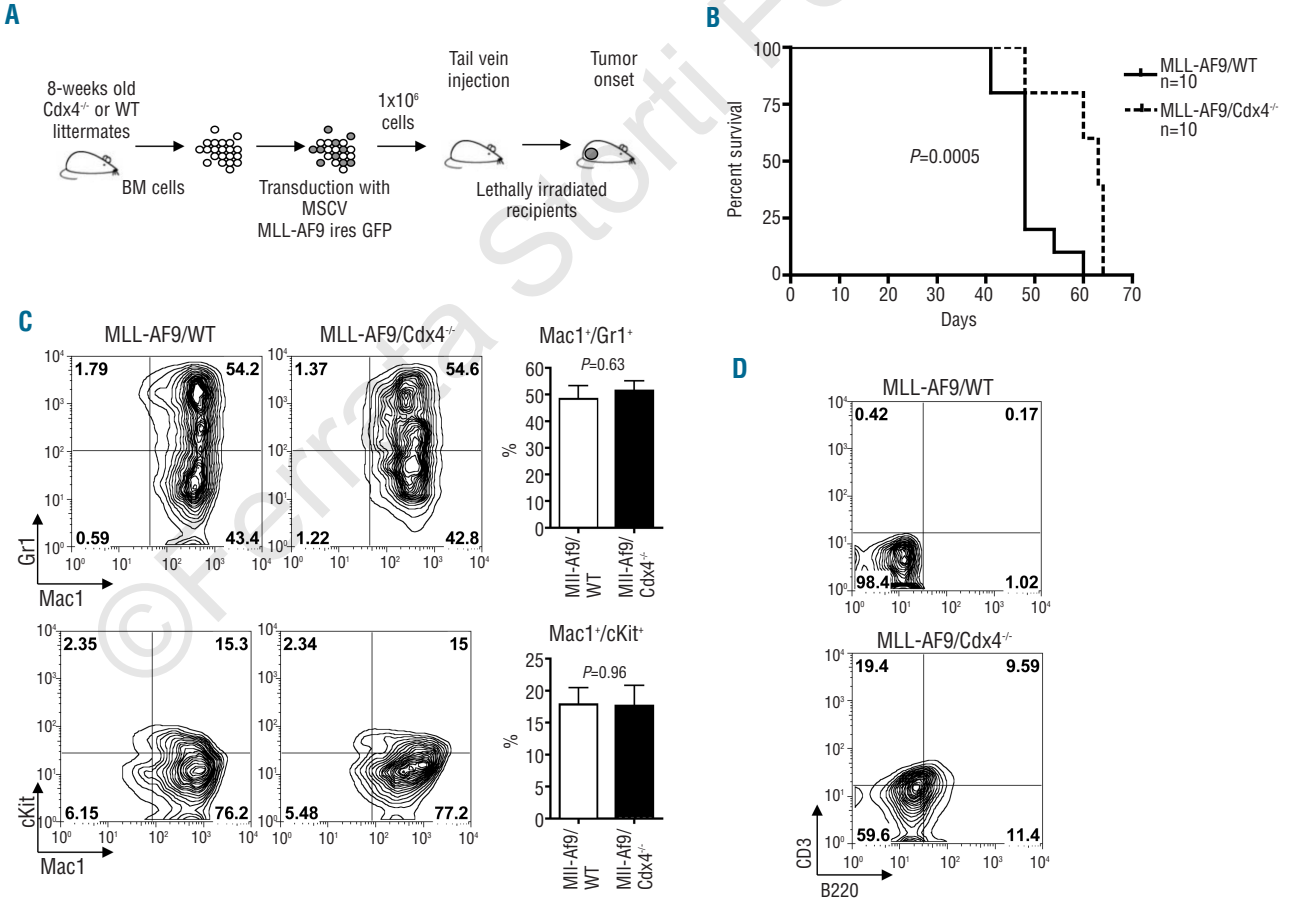

Figure 4. Loss of *Cdx4* delays *MLL-AF9*-induced leukemia. (A) Schematic of the retroviral transduction/bone marrow transplant protocol. (B) Survival curves for cohorts of mice injected with wild-type (*Cdx*$^{+/+}$) or *Cdx*$^{-/-}$ bone marrow cells transduced with *MLL-AF9*. Ten animals were used 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.

cient animals. However, we also observed that lymphocyte blood counts were consistently higher in *Cdx4*-deficient animals and that *Cdx4*-deficient *MLL-AF9*-transformed blasts aberrantly express some lymphoid markers, suggesting that Cdx4 deficiency accelerates and promotes lymphoid differentiation. This latter hypothesis suggests that Cdx4 restricts lymphoid identity. Although these two hypotheses may not be mutually exclusive, further studies are required to understand the precise role of Cdx factors during lymphopoiesis.

Aberrant expression of *CDX2* or *CDX4* genes has recently been implicated in the pathogenesis of human AML, and it has been suggested that CDX proteins may, at least in part, be responsible for the deregulated *HOX* gene expression observed in the majority of AML cases.[8,22] In addition, *Cdx4* can rescue the differentiation of *Mll*-deficient ESC *in vitro*[14] and has been shown to interact with menin in the up-regulation of *Hoxa* cluster genes during *Mll* fusion-induced leukemogenesis,[19] suggesting the possibility that oncogenic transformation by *MLL* fusions may require *CDX4*. In support of this hypothesis, we found that although *Cdx4* is not absolutely required for leukemia induction by *MLL-AF9*, its absence significantly prolonged the latency of disease development. Moreover, the phenotype of the resultant disease was altered with increased expression of B- and T-lymphoid markers subsequent to *Cdx4* loss. Taken together, this suggests a role for *Cdx4* in *MLL*-induced leukemogenesis. On the other hand, the subtlety of the phenotype and the absence of a difference in *Hox* gene expression between *MLL-AF9*-transduced wild-type and *Cdx4*$^{-/-}$ bone marrow cells, also point to a degree of functional redundancy among Cdx factors in the context of leukemogenesis. In addition, it is conceivable that there are context-specific differences in the requirement for *Cdx4* during AML pathogenesis, depending on the underlying mechanism that drives the leukemic phenotype. For example, mutant kinases, such as BCR-ABL1, which do not have the potential for activating self-renewal programs in hematopoietic cells, may be more reliant on *Cdx4*-controlled pathways than alleles such as the *MLL* fusion genes that can confer self-renewal properties to committed progenitors.[27-29]

The minimal hematopoietic impairment upon loss of *Cdx4* along with its aberrant expression in acute leukemias make CDX proteins, in principle, attractive therapeutic targets. Indeed the therapeutic utility of targeting *CDX2* was suggested by knockdown experiments in AML cell lines.[22] Although targeting transcription factors is very challenging and has not yet been clinically realized, critical protein-protein interactions associated with transcription factor function have recently been successfully targeted with small molecule inhibitors making transcription factors potentially "druggable" targets.[30] In this study, we have shown that loss of *Cdx4* significantly prolongs the latency of disease onset in a mouse model of *MLL-AF9*-induced AML. Although further studies will be necessary to understand the full degree of redundancy between *Cdx* genes in both normal and malignant hematopoiesis and the precise role of these proteins in the context of other *MLL*- or *HOX*-related leukemias, specific drug targeting of CDX factors could be of value in AML.

Authorship and Disclosures

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