Cdx gene deficiency compromises embryonic hematopoiesis in the mouse

Yuan Wang*, Akiko Yabuuchi†, Shannon McKinney-Freeman†, Danica M. K. Ducharme‡, Manas K. Ray§, Kallayanee Chawengsaksophak¶, Trevor K. Archer*‖, and George Q. Daley†‖**

*Laboratory of Molecular Carcinogenesis, ‡Microarray Group, §Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709; †Division of Hematology and Oncology, Children's Hospital Boston, **Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Harvard Stem Cell Institute, Howard Hughes Medical Institute, Harvard University, 300 Longwood Avenue, Boston, MA 02115; and ¶Institute for Molecular and Bioscience, University of Queensland, St. Lucia, Queensland 4067, Australia

Edited by Matthew P. Scott, Stanford University School of Medicine, Stanford, CA, and approved March 14, 2008 (received for review September 21, 2007)

***Cdx* genes (*Cdx1*, *Cdx2*, and *Cdx4*) encode a family of *caudal*-related transcription factors that mediate anterior–posterior patterning during embryogenesis through *Hox* gene regulation. Homologues in the zebrafish have been shown to play key roles in blood formation. To define the role of *Cdx* genes during embryonic hematopoiesis in a mammalian system, we examined the hematopoietic potential of *Cdx*-deficient mouse embryonic stem cells (ESCs) *in vitro* and *in vivo*. Individual *Cdx*-deficient ESCs exhibited impaired embryonic hematopoietic progenitor formation and altered *Hox* gene expression, most notably for *Cdx2* deficiency. A more severe hematopoietic defect was observed with compound *Cdx* deficiency than loss of function of any single *Cdx* gene. Reduced hematopoietic progenitor formation of ESCs deficient in multiple *Cdx* genes could be rescued by ectopic expression of *Cdx4*, concomitant with partially restored *Hox* gene expression. These results reveal an essential and partially redundant role for multiple *Cdx* genes during embryonic hematopoiesis in the mouse.**

Hox genes | embryonic stem cell | A-P patterning | development | embryoid body

Hematopoietic development occurs sequentially at distinct sites during vertebrate embryogenesis. In mice, the first wave of primitive yolk sac (YS) hematopoiesis occurs before 7.5 days postcoitum (dpc), followed by a second wave of definitive hematopoiesis in the aorta–gonad–mesonephros (AGM) region, which then relocates to fetal liver before birth (1). The underlying molecular pathways involved in the initiation and maintenance of hematopoiesis at different stages during embryogenesis are poorly understood.

Over the past several years, accumulating evidence suggests that the master regulators of *Hox* genes, Cdx family members, are involved in the proliferation and differentiation of hematopoietic cells. *Cdx* genes are *caudal*-related transcription factors that mediate anterior–posterior patterning through *Hox* gene regulation (2–5). Three *Cdx* genes (*Cdx1*, *Cdx2*, and *Cdx4*) have been identified so far in mammals. A potential role for *Cdx* genes in embryonic hematopoiesis initially came from studies in zebrafish. *Cdx4* mutant zebrafish have severe blood defects and altered *hox* gene expression during embryogenesis. *Cdx1* functions redundantly with *cdx4* to promote hematopoiesis in zebrafish (6, 7). In mouse, ectopic expression of *Cdx4* enhances hematopoietic mesoderm formation and further promotes blood progenitor specification and hematopoietic engraftment in adult mice from murine embryonic stem cells (ESCs) (8). In addition, *Cdx4* overexpression rescues defective blood progenitor formation in mouse ESCs deficient in the *Mll*, a *Hox* regulator involved in definitive hematopoiesis (9). During adult hematopoiesis, overexpression of human *CDX2* or murine *Cdx4* alone results in acute myeloid leukemia in mice (10, 11). Interestingly, despite all of the evidence suggesting an essential role for *Cdx* genes in hematopoietic development, no significant defects in hematopoiesis have been observed in *Cdx* single or compound deficient mice (2–5). This led us to hypothesize that *Cdx* genes may have overlapping or redundant functions during hematopoiesis, and that defects during embryonic stages of hematopoietic development may ultimately be masked by compensatory mechanisms in the adult.

Long-term culture of pluripotent ESCs has been established from a variety of mammalian species, including human and mouse. When induced to differentiate *in vitro*, ESCs form cystic embryonic bodies (EB) in which hematopoietic cells develop. Collective findings strongly suggest that the ESC/EB *in vitro* differentiation system recapitulates early hematopoiesis observed *in vivo* during embryonic development (1). As such, the ESC/EB system provides a powerful and convenient *in vitro* model to explore the molecular pathways that specify hematopoietic commitment, which otherwise would be difficult to examine in embryos.

In this study, using *Cdx*-deficient ESCs and murine models, we demonstrate a requirement for the *Cdx-Hox* gene pathway during primitive embryonic hematopoiesis. Deletion of any single *Cdx* gene led to impaired embryonic hematopoietic progenitor formation with disturbed *Hox* expression profiles. In particular, the *Cdx2* null state caused the most dramatic hematopoietic deficiency in a cell autonomous manner. In addition, ectopic expression of *Cdx4* rescued decreased blood progenitor formation caused by compound *Cdx* deficiency and partially restored *Hox* gene expression patterns. Furthermore, *HoxB4* transgene activation partially compensated for the impaired hematopoietic defects of *Cdx2* deficiency. These results reveal an essential role for the *Cdx-Hox* pathway during early primitive hematopoiesis in mammals and support our hypothesis that *Cdx* genes function redundantly in hematopoietic progenitor specification during embryogenesis.

Results

***Cdx4* Deficiency Causes Modest Defects in Embryonic Hematopoiesis.** To investigate the physiological function of *Cdx4* during embryonic hematopoiesis in mammals, we created *Cdx4*-deficient ESC lines and mice by replacing the first exon of *Cdx4* on the X-chromosome with a cDNA encoding GFP [supporting information (SI) Fig. S1 *A–D*].

We first evaluated the ability of *Cdx4*-deficient ($Cdx4^{GFP/y}$) ESCs to undergo hematopoietic differentiation *in vitro*. As shown in Fig. 1*A*, $Cdx4^{GFP/y}$ ESCs demonstrated a modest reduction in number of blood progenitors, primarily in erythroid and mixed progenitor

Author contributions: T.K.A. and G.Q.D. contributed equally to this work; Y.W., T.K.A., and G.Q.D. designed research; Y.W., A.Y., S.M.-F., D.M.K.D., and M.K.R. performed research; Y.W., A.Y., M.K.R., and K.C. contributed new reagents/analytic tools; Y.W. analyzed data; and Y.W., T.K.A., and G.Q.D. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

‖To whom correspondence may be addressed. E-mail: archer1@niehs.nih.gov or george.daley@childrens.harvard.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0708951105/DCSupplemental.

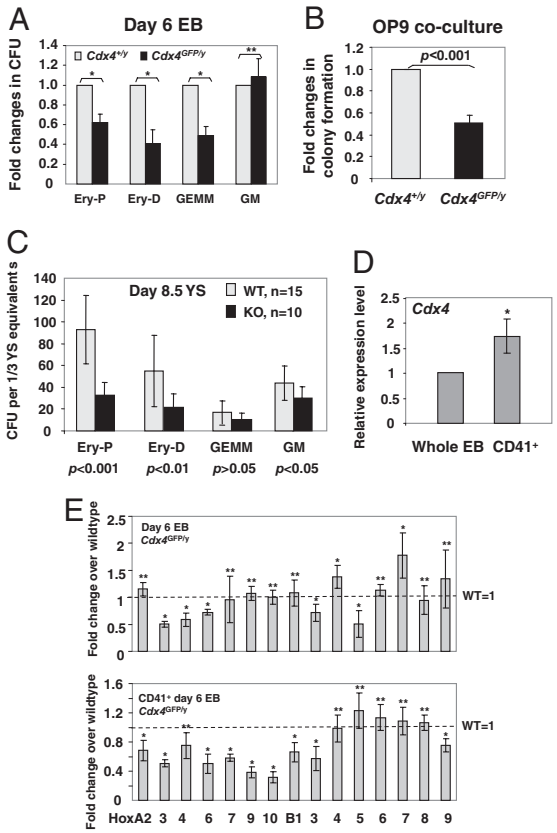

Fig. 1. *Cdx4* deficiency leads to reduced hematopoietic progenitor formation and altered *Hox* gene expression profiles. (*A*) Hematopoietic progenitor and (*B*) OP9 colony formation of day 6 EB-derived cells. Day 6 EB cells from $Cdx4^{+/y}$ or $Cdx4^{GFP/y}$ ESCs were plated into M3434 methylcult (*A*) or onto OP9 stromal cells (*B*), and colony number was assessed. CFU, colony forming unit; Ery-P, primitive erythroid; Ery-D, definitive erythroid; GEMM, multilineage progenitor of granulocyte, erythroid, macrophage, megakaryocyte; GM, multilineage progenitor of granulocyte and macrophage. (*C*) Hematopoietic colony formation of YS at 8.5 dpc from either *Cdx4*-deficient ($Cdx4^{GFP/GFP}$ and $Cdx4^{GFP/y}$: KO) embryos or their WT littermates ($Cdx4^{+/+}$ or $Cdx4^{+/y}$: WT). Data represent an average of five litters from two independent experiments. (*D*) Relative expression levels of *Cdx4* in $CD41^+$ or whole day 6 EBs, measured by real-time RT-PCR. (*E*) Expression levels of *Hox* genes in $CD41^+$ or whole day 6 EBs, assessed by real-time RT-PCR. (*A* and *B*, *D* and *E*) Data represent averaged fold changes relative to WT control from replicates in two to three independent experiments. Each data point denotes six biological replicates. *, *P* value <0.01; **, *P* value >0.05. For all experiments, error bars represent $\pm$ 1 SD.

colonies, when assayed for hematopoietic progenitor colony formation. In addition, day 6 $Cdx4^{GFP/y}$ EB-derived cells showed decreased hematopoietic expansion on OP9 stroma (Fig. 1*B*), a coculture system that allows the expansion of multipotential hematopoietic progenitors (12). The reduced hematopoietic progenitor formation *in vitro* caused by loss of function of *Cdx4* was further confirmed by analyzing hematopoietic tissues in *Cdx4*-deficient mice ($Cdx4^{GFP/y}$ and $Cdx4^{GFP/GFP}$). In this study, YS-derived primitive erythroid colony formation was reduced in *Cdx4*-deficient embryos from 7.5 to 9 dpc compared with their WT littermates (Fig. 1*C*, Fig. S1*F*). Taken together, these data suggest that primitive hematopoiesis is impaired, although modestly, upon *Cdx4* deficiency.

Because Cdx4 is a master regulator of *Hox* genes, and altered *hox* gene expression patterns due to *cdx4* deficiency is associated with blood formation defects in zebrafish (6), we next measured *Hox* gene expression levels by real-time RT-PCR from either $CD41^+$ or whole day 6 EBs derived from $Cdx4^{GFP/y}$ or $Cdx4^{+/y}$ ESCs. $CD41^+$ cells are enriched with early hematopoietic progenitors (9, 13), and expression of *Cdx4* was higher in CD41-sorted cells than whole day 6 EBs (Fig. 1*D*). Although *Hox* gene levels were not profoundly altered in whole EBs, the expression of posterior A cluster genes was notably reduced in $Cdx4^{GFP/y}$ $CD41^+$ cells (Fig. 1*E*). Taken together, these data suggest that *Cdx4* affects *Hox* gene expression patterns specifically in $CD41^+$ hematopoietic cells, which in turn affect hematopoietic colony forming potential and/or proliferation.

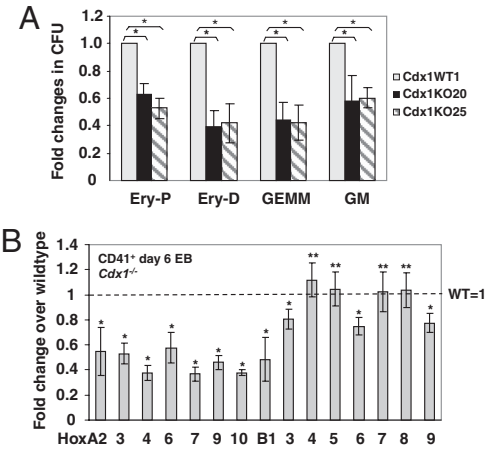

Fig. 2. *Cdx1* deficiency causes reduced hematopoietic progenitor formation and altered *Hox* gene expression profiles from ESCs. (*A*) Hematopoietic colony formation of day 6 EB cells from either WT or two $Cdx1^{-/-}$ ESC lines (KO20 and KO25). Data represent averaged fold changes ($\pm$1 SD) in CFU relative to WT control from triplicates in two independent experiments. (*B*) Expression levels of *Hox* genes in day 6 $CD41^+$ $Cdx1^{-/-}$ EBs, measured by real-time RT-PCR analysis and presented as $Cdx1^{-/-}$ relative to $Cdx1^{+/+}$ (WT). Data represent the averaged fold changes ($\pm$1 SD) from triplicates in two independent experiments. *, *P* value <0.01; **, *P* value >0.01.

***Cdx1* Deficiency Reduces Hematopoietic Formation from ESCs.** *Cdx1*-deficient ($Cdx1^{-/-}$) mice were established (4). Analysis of the early YS hematopoiesis at 7.5–9.5 dpc or adult hematopoiesis from $Cdx1^{-/-}$ mice did not reveal any obvious blood defects compared with $Cdx1^{+/+}$ controls (Fig. S2).

To investigate whether subtle defects in hematopoietic specification could be detected with the ESC/EB *in vitro* system, we generated $Cdx1^{-/-}$ and $Cdx1^{+/+}$ ESC lines from these mice (Fig. S2*A*). The growth and appearance of $Cdx1^{-/-}$ EBs were indistinguishable from WT controls (data not shown). However, hematopoietic progenitor formation from day 6 $Cdx1^{-/-}$ EB cells was notably reduced compared with $Cdx1^{+/+}$, as measured by progenitor colony-forming assay (Fig. 2*A*). These results indicate that *Cdx1* may play a role in early hematopoiesis, but compensatory mechanisms *in vivo* may mask subtle phenotypes caused by *Cdx1* deficiency. The expression of several posterior *Hox* A cluster genes was down-regulated in both $CD41^+$ hematopoietic cells and whole day 6 EBs from $Cdx1^{-/-}$ ESCs (Fig. 2*B*, Fig. S2*C*). These findings correlate altered *Hox* expression patterns with decreased blood formation in $Cdx1^{-/-}$ ESCs.

***Cdx2* Deficiency Results in Severe Blood Defects During Embryonic Hematopoiesis.** *Cdx2* null embryos die between 3.5 and 5.5 dpc because of defects in trophectoderm differentiation (2, 14). Analysis of *in vitro* differentiation of *Cdx2*-deficient ESCs (3) provides an opportunity to survey potentially later stages of embryonic development in hematopoiesis.

As shown in Fig. 3*A*, the number of multipotential blood progenitor colonies was reduced in $Cdx2^{-/-}$ day 6 EBs, suggesting that hematopoietic development was impaired in the absence of Cdx2 activity. We next explored hematopoietic differentiation in $Cdx2^{-/-}$

DEVELOPMENTAL BIOLOGY

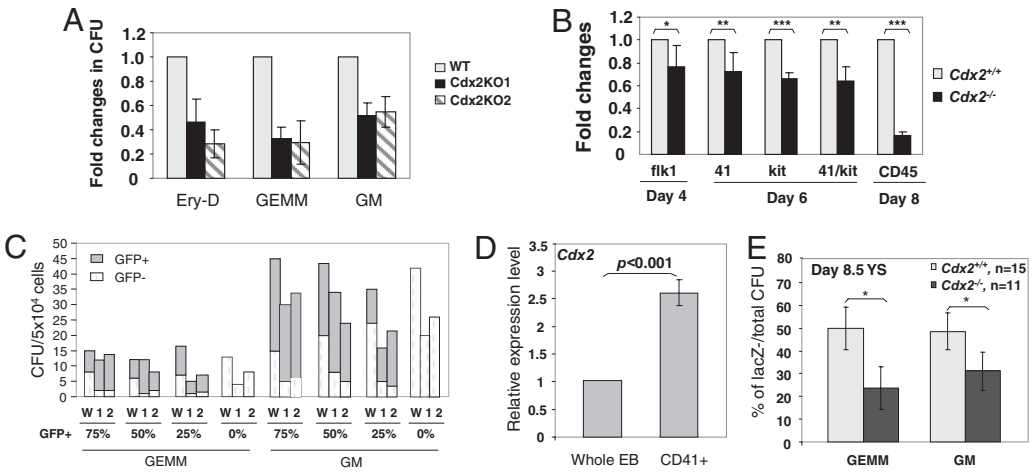

Fig. 3. *Cdx2* deficiency results in severe blood defects during embryonic hematopoiesis. (*A*) Hematopoietic colony formation of day 6 EB cells from $Cdx2^{+/+}$ (WT) or two $Cdx2^{-/-}$ ESC lines, KO1 and KO2. *P* value <0.01 for all comparison of $Cdx2^{-/-}$ to WT ESC lines. (*B*) Surface antigens analyzed by flow cytometry at different time points during EB development. *, *P* value >0.1; **, *P* value = 0.01–0.04; ***, *P* value <0.01. (*C*) Hematopoietic colony formation of day 6 EB cells from mixed population of either $Cdx2^{+/+}$ (W) or two $Cdx2^{-/-}$ ESC lines (1 and 2) with normal GFP^+ ESCs. Percentage of GFP^+ ESC composition is indicated below the *x* axis. Data are averaged CFU from duplicates in one representative experiment and reproduced from two independent experiments. (*D*) Relative expression level of *Cdx2* in $CD41^+$ or whole day 6 EBs, measured by real-time RT-PCR. (*A*, *B*, and *D*) Data are averaged fold changes (±1 SD) relative to WT control from replicates in two or three independent experiments. Each data point denotes six to eight biological replicates. (*E*) Progenitor colony activity of YS from chimeric embryos at 8.5 dpc generated with either $Cdx2^{-/-}$ or $Cdx2^{+/+}$ ESCs. Donor blastocysts are $lacZ^+$. The percentage of $lacZ^-$ (ESC-contributed)/total CFU was measured. Data represent average ± 1 SD. * *P* values <0.001.

EBs by conducting flow cytometry analysis on surface antigens that are enriched for hematopoietic precursors. Flk1 is a marker for multipotential mesodermal progenitors, which develop into hematopoietic and endothelial lineages (15). As for CD41, c-kit$^+$ cells are also enriched for early hematopoietic precursors, and the majority of EB-derived hematopoietic progenitors are CD41$^+$/c-kit$^+$ (9, 13). CD45 is a panhematopoietic marker. As shown in Fig. 3*B*, the overall hematopoietic compartment was reduced in $Cdx2^{-/-}$ day 6 EBs, as reflected by surface antigen analysis. Notably, the frequency of CD41$^+$, c-kit$^+$, or CD41$^+$/c-kit$^+$ cells showed only a moderate decrease, in contrast with more significant reductions in CD45$^+$ from $Cdx2^{-/-}$ EBs, suggesting that *Cdx2* may largely affect the differentiation or proliferation of hematopoietic progenitors. Taken together, these data support an essential role for *Cdx2* in early embryonic hematopoiesis.

To examine whether the requirement of *Cdx2* for hematopoiesis was cell autonomous, we conducted an *in vitro* chimera study. In this analysis, $Cdx2^{+/+}$ or $Cdx2^{-/-}$ ESCs were mixed with WT GFP^+ ESCs at different ratios, and the hematopoietic potential of these mixed populations was measured. Because GFP was not well expressed in erythroid colonies, only mixed progenitor colonies were counted. As shown in Fig. 3*C*, $Cdx2^{-/-}$ ESCs generated a reduced number of blood progenitors, indicating that the blood defects could not be rescued by mixing with normal GFP^+ ESCs. These results suggest an intrinsic requirement for *Cdx2* in hematopoiesis. Consistent with this finding, higher *Cdx2* expression was observed in the CD41$^+$ hematopoietic fraction compared with whole day 6 EBs (Fig. 3*D*).

We further investigated whether *Cdx2* was required for embryonic hematopoiesis in mice by an *in vivo* chimera study. $Cdx2^{-/-}$ or $Cdx2^{+/+}$ ESCs were injected into normal $lacZ^+$ blastocysts. The overall contribution of ESCs was then determined by X-Gal staining. Because a high contribution of $Cdx2^{-/-}$ ESCs resulted in embryonic lethality, we limited our analysis to embryos with overall contribution of 40–60% of $Cdx2^{-/-}$ or $Cdx2^{+/+}$ ESCs. In this case, the appearance of YS was indistinguishable in chimeras with $Cdx2^{-/-}$ ESCs compared to WT control. The hematopoietic activity of YS at 8.5 and 9 dpc was measured, and the ratio of $lacZ^-$ ($Cdx2^{-/-}$) progenitor colonies was determined. Consistent with our *in vitro* results, the percentage of $lacZ^-$ hematopoietic colonies was reduced in chimeric mice, indicating a reduced contribution of $Cdx2^{-/-}$ ESCs to YS-derived blood progenitors (Fig. 3*E*, Fig. S3*C*). In summary, our results demonstrate an intrinsic requirement for *Cdx2* during primitive embryonic hematopoiesis *in vitro* and *in vivo*.

Expression Analysis Implicates the Involvement of *Cdx2* in Embryonic Hematopoiesis. The biological functions of *Cdx2* during later embryonic development have not been understood completely because of the early lethality of *Cdx2* null embryos. To identify the target genes of Cdx2 during embryogenesis and to explore the molecular mechanism of reduced hematopoiesis caused by *Cdx2* deficiency, we performed a genome-wide microarray expression analysis on total RNA isolated from $Cdx2^{-/-}$ or $Cdx2^{+/+}$ day 6 EBs.

Using a 2-fold expression difference from WT control as a threshold, ingenuity pathway analysis (IPA) revealed >10 altered biological functions upon *Cdx2* deficiency. Among them, "cell growth and proliferation" and "hematopoietic development" scored as the most significant annotated function groups (Fig. 4*A*). As expected, the expression of *Hox* genes was significantly altered, and most of them displayed dramatic down-regulation upon *Cdx2* deficiency (Fig. 4*B*, Table 1). This observation was consistent with the role of *Cdx2* as a *Hox* gene regulator. In addition, the expression of several hematopoiesis-specific genes was decreased 2- to 20-fold, including *Scl*, *Gata1*, *Runx1*, and *β-H1* globin (Fig. 4*B*). Notably, *Scl*, *Gata1*, and *β-H1* are markers for primitive hematopoietic development (16). Dramatic decrease in their expression because of *Cdx2* deficiency thus supports our previous results that *Cdx2* is required for primitive hematopoiesis. Interestingly, the expression of several components of canonical signal transduction pathways was altered in $Cdx2^{-/-}$ EBs (Table 1, Fig. S3 *F* and G). Several of these pathways, such as the Jak/Stat and sonic hedgehog pathways, have been implicated in hematopoietic development through protein network interactions (17–19), suggesting that *Cdx2* deficiency may impair embryonic hematopoiesis indirectly through broad signal transduction networks. Taken together, our genome-wide expression analysis reveals that *Cdx2* deficiency perturbs expression profiles of *Hox* genes and genes involved in signal transduction and hematopoiesis, thus indicating a role for Cdx2 during hematopoietic development.

Functional Redundancy Among *Cdx* Genes During Embryonic Hematopoiesis. Although impaired hematopoietic progenitor activity was observed upon single *Cdx* gene deficiency, *Cdx* mutants are not totally bloodless, and *Cdx1* or *Cdx4* null mutations caused only modest defects in blood formation. Given the functional redundancy known to exist among Cdx family members during vertebral patterning and axis elongation (20), it is likely they also function redundantly during embryonic hematopoiesis. To examine this hypothesis, we investigated the effect of compound *Cdx* deficiency

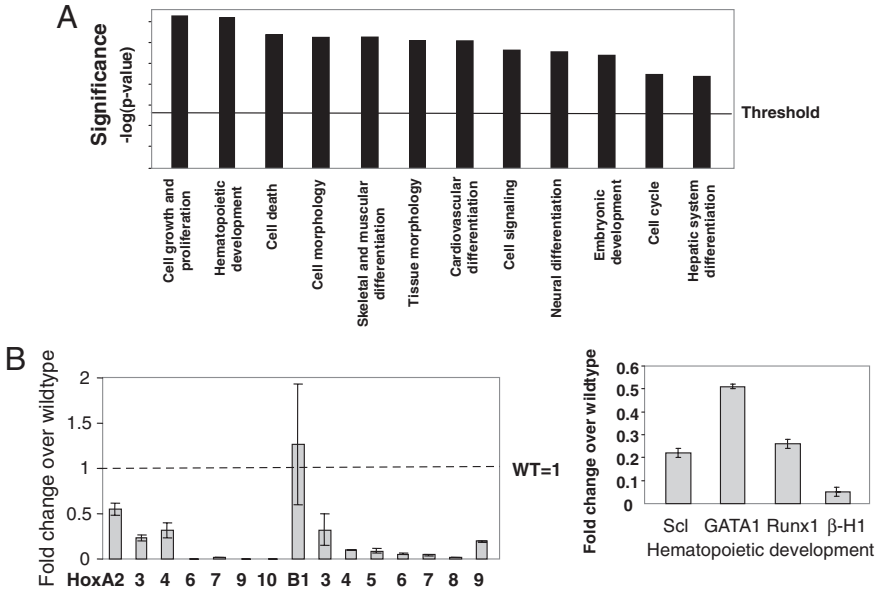

Fig. 4. Microarray expression analysis of day 6 EBs from *Cdx2*$^{-/-}$ or *Cdx2*$^{+/+}$ ESCs. (*A*) Functional analysis with IPA software. Genes included in this analysis all had *P* values <0.001 and a >2-fold expression alteration upon *Cdx2* deficiency compared with WT control. These genes were associated with biological functions through IPA analysis. Significance (−log *P* value) is reverse to the *P* value to demonstrate the most relevant biological functions beyond the threshold. (*B*) Partial verification of the results from microarray analysis by real-time RT-PCR in day 6 EBs from *Cdx2*-deficient ESCs. Data represent fold changes (±1 SD) in *Cdx*$^{-/-}$ EBs, relative to WT control. All results were reproduced from two independent experiments.

using RNA interference technology to knock down *Cdx1* or *Cdx2* in the *Cdx4* null background.

The combination of *cdx4* and *cdx1* deficiency in zebrafish leads to a completely bloodless embryo (6, 7). However, simultaneous reduction of *Cdx1* and *Cdx4* expression by *Cdx1* knockdown in the *Cdx4*$^{+/y}$ or *Cdx4*$^{GFP/y}$ ESCs decreased only the formation of erythroid progenitors more significantly than single *Cdx1* knockdown or *Cdx4* knockout (Fig. S4), demonstrating functional differences of *Cdx* genes in mammals from zebrafish. We thus examined the effect of compound *Cdx* deficiency including *Cdx2* reduction by knocking down *Cdx2* alone or both *Cdx1* and *Cdx2* in the *Cdx4*$^{+/y}$ or *Cdx4*$^{GFP/y}$ ESCs (KO3). Consistent with our results on *Cdx2*$^{-/-}$ ESCs, *Cdx2* knockdown resulted in significantly reduced blood progenitor formation (Fig. 5*A*, Fig. S4). In addition, the combination of *Cdx4* knockout with *Cdx1* and *Cdx2* knockdown almost abolished blood formation from day 6 EBs (Fig. 5*A*, Fig. S4). Taken together, these results support an essential role of *Cdx2* in murine hematopoiesis and suggest that the dosage of *Cdx* genes is an important permissive factor in hematopoietic specification.

Because our *Cdx4*$^{GFP/y}$ ESC line KO3 was derived from a tetracycline-inducible *Cdx4* ESC line, ectopic *Cdx4* expression could be induced upon doxycycline (dox) treatment, even though its endogenous *Cdx4* locus had been disrupted by *GFP* cDNA insertion (*SI Text*). Therefore, this cell line was used to investigate whether activation of a *Cdx4* transgene could rescue the blood defects caused by *Cdx* gene deficiency. Interestingly, ectopic *Cdx4* expression not only rescued the blood forming defects caused by loss of function of *Cdx4* but also compensated for the reduced blood formation because of *Cdx2* or compound *Cdx* gene deficiency (Fig. 5*A*, Fig. S4). Thus, these results support our hypothesis that Cdx family members function redundantly during embryonic hematopoiesis.

Because the *Hox* genes are good candidates for mediating the effects of the *Cdx* genes in hematopoiesis, we further examined whether *Hox* gene expression levels could be restored in *Cdx*-deficient cells by activation of a *Cdx4* transgene. As shown in Fig. 5*B*, the expression level of most *Hox* genes was indeed restored to normal, with some posterior *Hox* genes being up-regulated significantly by ectopic *Cdx4* expression (e.g., HoxA7, -A9, -B8, -B9). Compared to a luciferase (Luc) shRNA control, the expression levels of *HoxB1* and *HoxB3* appeared to be higher in the *Cdx*-deficient cells (Fig. 5*B*). Because *Cdx* genes typically do not influence the expression of the most anterior Hox genes, we suspect the effects are most likely due to inappropriate expression level or timing of the dox-driven *Cdx4* transgene.

Finally, to determine whether *Hox* genes act downstream of Cdx family members in hematopoiesis, we examined whether ectopic *HoxB4* expression could rescue the impaired hematopoietic commitment caused by *Cdx2* deficiency. We chose *HoxB4* in this analysis, because its expression was significantly reduced in *Cdx2* null cells. In addition, it has been shown that *Cdx4* activates *HoxB4* in hematopoietic progenitors, and *HoxB4* promotes hematopoietic specification from ESCs (8, 21). We thus introduced a shRNA construct against *Cdx2* into inducible *HoxB4* ESCs (21) and evaluated hematopoietic progenitor formation after dox induction of *HoxB4* expression. We observed that overexpression of the *HoxB4* transgene indeed rescued the impaired blood progenitor formation due to *Cdx2* deficiency, suggesting *HoxB4* is downstream of *Cdx2* (Fig. 5*C*). Taken together, these data suggest that proper *Hox* gene expression patterns, as regulated by *Cdx* genes, contribute to embryonic hematopoietic specification in mammals.

Table 1. Gene expression changes in *Cdx2* null EBs in microarray analysis

Gene class	Up-regulated	Down-regulated
Hox genes	*B1*	*A7, 9–11, 13, B2–5, 7–9, C6, 8, 10, D1, 3, 4, 8–11. 13*
Hematopoiesis		*Scl, GATA1, Runx1, Vav1, pu.1, β-H1, β-major, Fcrh3, Ddx3y, Tox, Tgtp, Gimap3, ifi204*
Cell signaling	*Socs2, Edil3, Gbp1, Shh, Fzd2, Cacna1d, Ppp2r2b, PDGFD*	*Jak3, Stat1–3, Stat5a, 5b, Stat6, Gpr123, FGFR1*

Discussion

Although the functional requirement for *cdx4* and *cdx1* during hematopoiesis has been demonstrated in zebrafish, the roles of *Cdx* genes during hematopoietic commitment are poorly understood in mammals. In this study, using the ESC/EB *in vitro* differentiation system and mouse models, we demonstrate that deficiency of *Cdx* genes perturbs hematopoiesis to variable extents, with *Cdx2* deficiency most substantially compromising primitive embryonic hematopoiesis. These data establish the functional conservation of the role of *Cdx* genes in hematopoietic development between fish and mammals, but that a combination of *Cdx* gene deficiencies is

DEVELOPMENTAL BIOLOGY

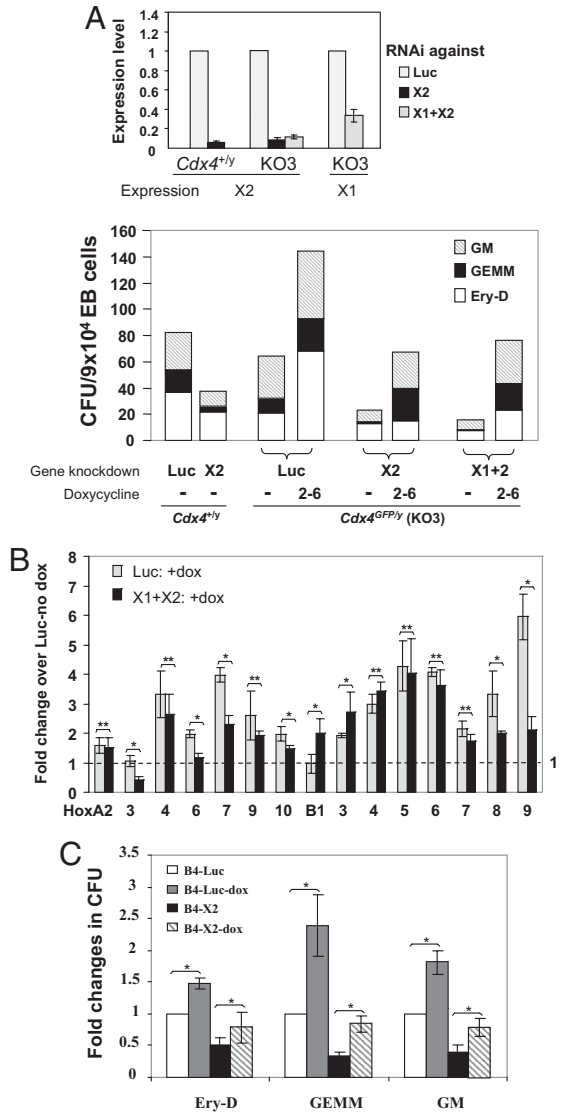

Fig. 5. Functional redundancy among Cdx family members. (*A*) Hematopoietic colony formation upon gene knockdown of *Cdx2* alone or *Cdx1* and *Cdx2* in *Cdx4*$^{+/y}$ or *Cdx4*$^{GFP/y}$ (KO3) ESCs. (*Upper*) Expression of *Cdx1* and *Cdx2* was analyzed by real-time RT-PCR on day 4 EBs upon gene knockdown of *Cdx1* (X1) and/or *Cdx2* (X2) compared with control shRNA against Luc. (*Lower*) Hematopoietic colony formation from day 6 EBs upon gene knockdown of *Cdx2* (X2), *Cdx1* and *Cdx2* (X1+X2), or Luc in *Cdx4*$^{+/y}$ or *Cdx4*$^{GFP/y}$ (KO3) ESCs. Cells were treated without (−) or with dox from day 2–6 of EB development (as indicated below *x* axis). Data are averaged CFU from triplicates in one representative experiment reproduced from three additional experiments. (*B*) Relative *Hox* gene expression level as measured with real-time RT-PCR analysis. Samples were *Cdx4*$^{GFP/y}$ day 6 EBs with either control shRNA against Luc or shRNA against *Cdx1* and *Cdx2* (X1+X2). Both samples were treated with dox from day 2–6 of EB development, and their *Hox* gene expression level was relative to *Cdx4*$^{+/y}$ day 6 EBs with control shRNA against Luc without dox treatment. Data represent the averaged fold changes of gene expression from triplicates in two independent experiments. (*C*) Ectopic *HoxB4* expression rescues impaired hematopoietic progenitor formation caused by *Cdx2* deficiency. Hematopoietic colony formation was examined from tetracycline-inducible *HoxB4* ESCs with shRNA against either Luc or *Cdx2* (X2) in the absence or presence of dox. Data represent as averaged fold change in cfu from triplicates in two independent experiments. (*A–C*) Error bars represent ±1 SD. *, *P* value <0.01; **, *P* value >0.01.

required to produce substantial deficits implies a more complex and redundant role in mammals.

Our study reveals a previously undescribed role for *Cdx2* during embryonic hematopoiesis. Because *Cdx2* deficiency results in embryonic lethality in mice, future investigation of the specific role of *Cdx2* in hematopoietic development will require construction of a conditional knockout. Our data demonstrated only modest hematopoietic defects in *Cdx1* or *Cdx4* null cells but severe deficiency due to loss of function of *Cdx2* in the murine system. A zebrafish orthologue of *cdx2* has not been identified; thus, it is possible that the critical function of *Cdx4* in zebrafish hematopoiesis is shared by *Cdx2* in mammals.

All *Cdx* genes can be detected in the posterior primitive streak of mouse embryos ≈7 dpc (14, 22–25), where the highest frequency of the first hematopoietic cells, the hemangioblast, is found before blood island formation in YS (26). In EBs, peak *Cdx* expression occurs from day 2 to 5 of ESC differentiation (data not shown), which corresponds to the window of primitive hematopoietic fate specification (1). In addition, the expression of *Cdx2* and *Cdx4* is enriched in CD41-sorted hematopoietic cells. These findings suggest an intrinsic requirement for *Cdx* genes in embryonic hematopoiesis. Consistent with this hypothesis, our chimera studies showed that the reduced hematopoietic colony formation of *Cdx2* null ESCs could not be rescued by mixing with normal ESCs or injecting into normal blastocysts. Although deficiencies of *Cdx* genes might alter a number of different tissue fates, our observations suggest that the *Cdx* genes act cell autonomously, at least in part, during blood development.

Recently, van Nes *et al.* reported that *Cdx1* and *Cdx4* double mutant mice were viable, and no hematopoietic defects were noted (5). In our study, the blood formation defects caused by *Cdx* deficiency were transient, primarily at the stage of YS hematopoiesis before 9 dpc. No significant blood alteration was observed in YS after 9 dpc (when circulation is established in embryos), in fetal liver at 14.5 dpc, in adult bone marrow, or in peripheral blood from *Cdx1* or *Cdx4* null mice (Figs. S1 and S2 and Dataset S1). The hematopoietic contribution from *Cdx2*-deficient ESCs at the fetal liver stage appeared to be indistinguishable from the WT control (data not shown). Thus, *Cdx* genes primarily affect primitive hematopoiesis during embryonic development, which explains why published accounts of *Cdx* knockout mice have not reported hematopoietic defects. It is possible that definitive hematopoiesis is less sensitive to the dosage of *Cdx* gene expression and has a more efficient compensation mechanism than YS hematopoiesis.

Cdx family members appear to function redundantly during embryonic hematopoiesis, because a more severe hematopoietic defect was observed with compound *Cdx* deficiency than loss of function of any single *Cdx* gene. Similar alterations in expression levels of posterior *Hox* A cluster genes (A6–10) were seen in each of the different *Cdx*-deficient ESC lines, demonstrating that the Cdx family members shared common posterior *Hox* gene targets. In addition, activation of the *Cdx4* transgene in *Cdx*-deficient EBs both rescued the hematopoietic defects and restored *Hox* gene expression. Therefore, it is reasonable to speculate that *Cdx* genes function redundantly during blood development by activating overlapping *Hox* gene targets. Although there is considerable evidence for functional redundancy, loss of function of specific *Cdx* genes showed some unique effects on hematopoiesis, perhaps mediated through the differential effects on the Hox gene targets.

Perturbation in the anterior expression boundaries of *Hox* genes alters cell fate. This finding has led to the "*Hox* code" hypothesis that a specific *Hox* gene expression pattern determines the tissue identity along the anterior–posterior axis (27). Ectopic expression and loss-of-function studies have implicated many *Hox* genes in normal hematopoiesis and leukemogenesis (28). *Cdx* genes have been shown to act directly as *Hox* gene regulators, and this is corroborated by the homeotic transformations in *Cdx* knockout studies (2, 4, 20, 29). Furthermore, blood defects due to *Cdx4* deficiency in zebrafish or loss of function of *Mll* in mouse ESCs can be rescued by ectopic expression of *Cdx4* or particular posterior *Hox* genes (6, 9). In this study, we observed that the *Hox* gene expression profile, possibly representing a "*Hox* code," was altered in CD41^{+}

hematopoietic cells upon *Cdx* deficiency, and that this alteration correlated with reduced blood formation. In addition, *Hox* gene expression levels were restored when blood formation was rescued by ectopic *Cdx4* expression in *Cdx* single or compound deficient cells, and activation of a *HoxB4* transgene partially compensated for the impaired blood progenitor formation caused by *Cdx2* deficiency. Together with previous studies, our data provide compelling evidence that a proper *Hox* gene code, initiated by *Cdx* genes, is important for embryonic hematopoietic specification in mammals.

The ESC/EB system is a powerful, sensitive, and convenient *in vitro* model to explore the molecular pathways that specify the earliest stages of hematopoiesis during embryonic development, especially in cases where gene deficiency leads to embryonic lethality. In this study, by using the ESC/EB in *vitro* differentiation system and mouse models, we demonstrated an intrinsic requirement or the *Cdx-Hox* gene pathway during primitive embryonic hematopoiesis in mouse. Our findings advance an understanding of the role of *Cdx* genes in normal hematopoiesis and, given the links between *Cdx* genes and leukemogenesis, illuminate how reactivation of embryonic pathways might contribute to disease.

Materials and Methods

Cell Culture. ESCs were maintained, differentiated, and harvested *in vitro* according to published protocols (8, 21). OP9 coculture was performed as described (8, 21), and the colony number was counted on day 6 after plating. Hematopoietic colony formation assay were performed according to published protocol (15).

Establishing and screening *Cdx4* knockout/GFP knockin ESC lines and mouse are described in details in *SI Text*.

Obtaining Cdx1-Deficient ESC Lines and Cdx1 Genotyping. $Cdx1^{-/-}$ and $Cdx1^{+/+}$ ESCs were obtained from blastocysts of intercrossed $Cdx1^{-/+}$ heterozygotes according to standard protocols (30). *Cdx1* genotyping primers: sense-5′-GGCTCCTTGGCCCGGCGG-3′, antisense-5′-CCGAGCTGGCTGCTAACC-3′. PCR from the WT allele gave a 1.5-kb band, whereas the mutant allele gave a 400-bp band.

Western Blot Analysis. Protein extract was prepared from dissociated $Cdx1^{-/-}$ and $Cdx1^{+/+}$ day 4 EB, and Western blot analysis of Cdx1 and β-actin was performed according to published protocol (24).

Embryo Dissection and Analysis. The age of embryo was defined as 0.5 dpc at 8:00 a.m. on the day of vaginal plug observation. In the chimera study, donor blastocysts were collected from C57BL/6 female mice intercrossed with ROSA26-lacZ male mice. Expression of lacZ in chimeric embryos was revealed according to published protocols (9). For lacZ expression in the colonies from YS, 400 μl of X-Gal staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml X-Gal) was added in drops into methylcult 9 days after plating of YS cells. Colonies were counted before and 12 h after X-Gal staining. All experimental procedures were conducted in accordance with the Animal Welfare Act and Public Health Service Policy.

Quantitative Real-Time PCR. Cells were harvested in TRIzol. Total RNA and cDNAs were prepared according to the manufacturer's instructions (Invitrogen). All RNA samples were treated with DNaseI and then purified by RNeasy MinElute kit (Qiagen). Real-time PCR was performed on Stratagene MX3000P instrument and analyzed as described (8). Primer sequences were listed in Dataset S2.

FACS Analysis. All antibodies were purchased from PharMingen, BD Biosciences. Propidium iodide was added to exclude dead cells. Samples were acquired on LSR II Flow Cytometer (BD Biosciences). CD41 sorting was done on Becton Dickinson FACSVantage SE Flow Cytometer with FITC-labeled CD41 antibody. The sorting purity was checked to ensure >90% purity for all sorted samples.

Microarray hybridization and data processing are described in detail in *SI Text*.

RNA Interference. Lentivirus-based shRNA against Luc, *Cdx1* or *Cdx2* were generated as described (31). Detailed shRNA sequences and selection procedure are described in *SI Text*.

Statistical Analysis. *P* values were calculated from Student's *t* test for all comparisons, except in Fig. 4*A*, Fisher's exact test was used to calculate the *P* values.

ACKNOWLEDGMENTS. We thank the following people who provided reagents: *Cdx1* knockout mice and genotyping protocol from Dr. Jacqueline Deschamps (Netherlands Institute for Developmental Biology, Utrecht, The Netherlands), ROSA26-lacZ mice and PGK-dta vector from Dr. Philippe Soriano (Fred Hutchinson Cancer Research Center, Seattle), Cdx1 antibody from Dr. Peter Gruss (Max Planck Institute, Munich), parental *Cdx4* targeting vector from Dr. Brian Huntly (Children's Hospital Boston, Boston), and V6.5 ESC line from Dr. Rudolf Jaenisch (Whitehead Institute, Massachusetts Institute of Technology, Boston). We thank Dr. Yuji Mishina for advice and Gregory J. Scott and Toni Ward for technical support in the chimera study. We also thank Il-Ho Jang for help in the cfu assays. We appreciate the critical comments during manuscript preparation from Drs. Alan J. Davidson, Thomas Eling, Paul Wade, and Yuji Mishina. This study is supported by the intramural research program of the National Institute on Environmental Health Sciences. G.Q.D. was supported by grants from the National Institutes of Health and by the National Institutes of Health Director's Pioneer Award of the National Institutes of Health Roadmap for Medical Research. G.Q.D. is also the recipient of Clinical Scientist Awards in Translational Research from the Burroughs Wellcome Fund and the Leukemia and Lymphoma Society.

1. Lensch MW, Daley GQ (2004) Origins of mammalian hematopoiesis: *in vivo* paradigms and *in vitro* models. *Curr Top Dev Biol* 60:127–196.
2. Chawengsaksophak K, James R, Hammond VE, Kontgen F, Beck F (1997) Homeosis and intestinal tumours in Cdx2 mutant mice. *Nature* 386:84–87.
3. Chawengsaksophak K, de Graaff W, Rossant J, Deschamps J, Beck F (2004) Cdx2 is essential for axial elongation in mouse development. *Proc Natl Acad Sci USA* 101:7641–7645.
4. Subramanian V, Meyer BI, Gruss P (1995) Disruption of the murine homeobox gene Cdx1 affects axial skeletal identities by altering the mesodermal expression domains of Hox genes. *Cell* 83:641–653.
5. van Nes J, de Graaff W, Lebrin F, Gerhard M, Beck F, Deschamps J (2006) The Cdx4 mutation affects axial development and reveals an essential role of Cdx genes in the ontogenesis of the placental labyrinth in mice. *Development* 133:419–428.
6. Davidson AJ, *et al.* (2003) cdx4 mutants fail to specify blood progenitors and can be rescued by multiple hox genes. *Nature* 425:300–306.
7. Davidson AJ, Zon LI (2006) The caudal-related homeobox genes cdx1a and cdx4 act redundantly to regulate hox gene expression and the formation of putative hematopoietic stem cells during zebrafish embryogenesis. *Dev Biol* 292:506–518.
8. Wang Y, Yates F, Naveiras O, Ernst P, Daley GQ (2005) Embryonic stem cell-derived hematopoietic stem cells. *Proc Natl Acad Sci USA* 102:19081–19086.
9. Ernst P, Mabon M, Davidson AJ, Zon LI, Korsmeyer SJ (2004) An Mll-dependent Hox program drives hematopoietic progenitor expansion. *Curr Biol* 14:2063–2069.
10. Bansal D, *et al.* (2006) Cdx4 dysregulates Hox gene expression and generates acute myeloid leukemia alone and in cooperation with Meis1a in a murine model. *Proc Natl Acad Sci USA* 103:16924–16929.
11. Rawat VP, *et al.* (2004) Ectopic expression of the homeobox gene Cdx2 is the transforming event in a mouse model of t(12;13)(p13;q12) acute myeloid leukemia. *Proc Natl Acad Sci USA* 101:817–822.
12. Nakano T, Kodama H, Honjo T (1994) Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science* 265:1098–1101.
13. Mitjavila-Garcia MT, *et al.* (2002) Expression of CD41 on hematopoietic progenitors derived from embryonic hematopoietic cells. *Development* 129:2003–2013.
14. Beck F, Erler T, Russell A, James R (1995) Expression of Cdx-2 in the mouse embryo and placenta: possible role in patterning of the extra-embryonic membranes. *Dev Dyn* 204:219–227.
15. Kennedy M, *et al.* (1997) A common precursor for primitive erythropoiesis and definitive haematopoiesis. *Nature* 386:488–493.
16. Godin I, Cumano A (2002) The hare and the tortoise: an embryonic haematopoietic race. *Nat Rev Immunol* 2:593–604.
17. Neubauer H, *et al.* (1998) Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. *Cell* 93:397–409.
18. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF (1999) Fetal anemia and apoptosis of red cell progenitors in Stat5a−/−5b−/− mice: a direct role for Stat5 in Bcl-X(L) induction. *Cell* 98:181–191.
19. Ross J, Li L (2006) Recent advances in understanding extrinsic control of hematopoietic stem cell fate. *Curr Opin Hematol* 13:237–242.
20. van den Akker E, *et al.* (2002) Cdx1 and Cdx2 have overlapping functions in anteroposterior patterning and posterior axis elongation. *Development* 129:2181–2193.
21. Kyba M, Perlingeiro RC, Daley GQ (2002) HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell* 109:29–37.
22. Gamer LW, Wright CV (1993) Murine Cdx-4 bears striking similarities to the *Drosophila* caudal gene in its homeodomain sequence and early expression pattern. *Mech Dev* 43:71–81.
23. Gaunt SJ, Drage D, Trubshaw RC (2005) cdx4/lacZ and cdx2/lacZ protein gradients formed by decay during gastrulation in the mouse. *Int J Dev Biol* 49:901–908.
24. Meyer BI, Gruss P (1993) Mouse Cdx-1 expression during gastrulation. *Development* 117:191–203.
25. Lohnes D (2003) The Cdx1 homeodomain protein: an integrator of posterior signaling in the mouse. *BioEssays* 25:971–980.
26. Huber TL, Kouskoff V, Fehling HJ, Palis J, Keller G (2004) Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature* 432:625–630.
27. Krumlauf R (1994) Hox genes in vertebrate development. *Cell* 78:191–201.
28. Abramovich C, Humphries RK (2005) Hox regulation of normal and leukemic hematopoietic stem cells. *Curr Opin Hematol* 12:210–216.
29. Charite J, *et al.* (1998) Transducing positional information to the Hox genes: critical interaction of cdx gene products with position-sensitive regulatory elements. *Development* 125:4349–4358.
30. Abbondanzo SJ, Gadi I, Stewart CL (1993) Derivation of embryonic stem cell lines. *Methods Enzymol* 225:803–823.
31. Zaehres H, Daley GQ (2006) Transgene expression and RNA interference in embryonic stem cells. *Methods Enzymol* 420:49–64.