**Lhx2, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development**

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**SUMMARY**

We investigated the function of Lhx2, a LIM homeobox gene expressed in developing B-cells, forebrain and neural retina, by analyzing embryos deficient in functional Lhx2 protein. Lhx2 mutant embryos are anophthalmic, have malformations of the cerebral cortex, and die in utero due to severe anemia. In Lhx2−/− embryos specification of the optic vesicle occurs; however, development of the eye arrests prior to formation of an optic cup. Deficient cellular proliferation in the forebrain results in hypoplasia of the neocortex and aplasia of the hippocampal anlagen. In addition to the central nervous system malformations, a cell non-autonomous defect of definitive erythropoiesis causes severe anemia in Lhx2−/− embryos. Thus Lhx2 is necessary for normal development of the eye, cerebral cortex, and efficient definitive erythropoiesis.

Key words: LIM homeobox, eye, forebrain, erythropoiesis, mouse

**INTRODUCTION**

Transcription factors containing homeodomains play a major role in morphogenesis. The homeodomain is a highly conserved protein motif that binds DNA, and homeodomain proteins function as cell specific transcription factors. In addition to the prototypical homeobox genes characterized by the paralogous HoxA-D clusters, multiple sub-families of homeobox genes exist. These families have distinct homeodomains and often are found in association with other protein motifs (Gehring et al., 1994; Manak and Scott, 1994). The LIM homeodomain encoding genes are one of these families.

LIM homeobox genes encode a LIM type homeodomain and two zinc-finger like LIM domains. Recent reviews (Dawid et al., 1995; Sánchez-García and Rabbitts, 1994) describe the structure, function and nomenclature of LIM domain and the LIM homeodomain families. LIM domains bind zinc ions (Archer et al., 1994), and nuclear magnetic resonance studies showed that LIM domains have a secondary structure similar to the DNA binding domains of both the glucocorticoid receptor and GATA-1 (Perez-Alvarado et al., 1994). Unlike these zinc-finger containing transcription factors, LIM domains do not interact directly with DNA. Instead LIM domains are involved in protein-protein interactions (Crawford et al. 1992; Feuerstein et al., 1994; Schmeichel and Beckerle, 1994; Wadman et al., 1994; Wu and Gill, 1994; Agulnick et al., 1996; Jurata et al. 1996), and may modulate the function of the homeodomain (German et al., 1992; Sánchez-García et al., 1993; Xue et al., 1993; Taira et al., 1994; Agulnick et al., 1996).

The LIM homeobox genes are expressed widely in the developing central nervous system, and may be involved in specification of neurons. In the ventral spinal cord, pairs of LIM homeobox genes are expressed in specific subsets of motor neurons (Tsuchida et al., 1994). In the developing forebrain and hindbrain, specific domains of expression are reported for Lhx1 (LIM-1) (Barnes et al., 1994; Fujii et al., 1994), Lhx2 (LH-2) (Xu et al., 1993), Lhx3 (mLIM-3; pLIM) (Bach et al., 1995; Seidah et al., 1994; Zhadanov et al., 1995), Lhx4 (GSH-4) (Li et al., 1994), Lhx5 (LIM-2) (Sheng et al., 1997), and Isl-1 (Thor et al., 1991). The observations that Lhx4 mutant mice die during the perinatal period reportedly due to abnormal brainstem regulation of respiratory drive (Li et al., 1994), and that Isl1−/− mice have abnormal development of motor neurons in the ventral spinal cord (Pfaff et al., 1996) demonstrate the functional importance of LIM homeobox genes in CNS development.

LIM homeobox genes are required for morphogenesis of other organ systems. Lhx1 is required for renal and gonadal development, as well as prechordal mesoderm formation (Shawlot and Behringer, 1995). Lhx3 and Lhx4 are necessary for normal pituitary development (Sheng and Westphal; unpub-
were plated at a density of 2.5 per gram of body weight, and embryos were harvested 2 hours postinfection. Resistant colonies was begun 24 hours after plating. Double resistant colonies were selected in 0.1 mg of BrdU (Sigma #B5002, at a stock concentration of 10 mg/ml) and gancyclovir (2 \( \mu \)M, Syntex) into J1 embryonic stem (ES) cells (Li et al., 1992). Electroporated ES cells were plated at a density of 2.5x10^5 cells in 60 mm^2 dishes containing a layer of G418 resistant embryonic fibroblasts. Selection for G418 resistant (400 mcg/ml, Gibco BRL) and gancyclovir (2 \( \mu \)M, Syntex) resistant colonies was begun 24 hours after plating. Double resistant colonies were isolated and expanded after 5 days of selection. DNA was prepared as previously described (Lafrid et al., 1991).

**Genotyping of ES cell clones and Lhx2+/− mice**

Southern blot analyses of individual clones were performed using standard procedures. Genomic DNA was digested overnight with either XhoI or HindIII, separated on a 0.8% TAE agarose gel, and blotted onto Gene Screen Plus (NEN Research Products) membranes. The probe shown in Fig. 1A was labeled using a random primed DNA labeling kit (Boehringer Mannheim). Autoradiographs were utilized to identify the wild-type and mutant alleles as demonstrated in Fig. 1B. Lhx2+/− ES clones were injected into C57/B6 blastocysts as previously described (Bradley, 1987). Progeny with a Lhx2+/− genotype were identified by Southern blot analysis of tail DNA. Hematocrits as previously described (Bradley, 1987). Five million /− Lhx2 ES cells were injected into blastocysts as previously described (Bradley, 1987). 500,000 cells were injected in each reaction. Stained cells were analyzed with a FACScan (Becton-Dickinson) using the CellQuest software. PE-conjugated anti-CD4, anti-B220 antibodies, FITC-conjugated anti-CD8 antibody, and biotinylated anti-Ter-119 antibodies were obtained from Pharmingen. Biotinylated anti-Gr-1 antibody was obtained from Southern Biotechnology Associate, Inc. Biotinylated derivatives were revealed with FITC-conjugated streptavidin.

**RESULTS**

**Targeted disruption of Lhx2**

We inactivated the Lhx2 gene using homologous recombination in embryonic stem cells (ES cells). A replacement targeting vector, pEGneo, was designed to delete the two exons encoding the LIM domains and the domain linking the LIM domains and homeodomain of Lhx2, and replace them with a neomycin-resistance gene (PGK-neo). Potential splicing between exons one and four would introduce a frameshift mutation that would preclude expression of a homeodomain containing peptide. We used herpes simplex thymidine kinase, under control of the phosphoglycerate kinase promoter (PGK-
from a Lhx2 +/- of the genomic region containing the Lhx2 gene. Both clones produced germline transmitting chimeras after injection into C57BL/6 blastocysts.

Heterozygous Lhx2 mutant mice were phenotypically normal, thus we intercrossed Lhx2+/− mice to determine if the Lhx2 mutant allele had a recessive phenotype. Genotyping of 191 weaned offspring of these matings identified 71 Lhx2+/+ (37%) and 120 Lhx2+/- (63%) progeny, but no Lhx2−/− mice. Some stillborn Lhx2−/− pups were found. Genotyping of 242 embryos, E15.5 and younger, yielded numbers closer to the expected Mendelian ratio for an autosomal recessive mutation (+/+ 24%, +/- 55%, −/− 21%). Fig. 1C shows the genotyping of these embryos and the presence of homozygous mutant embryos. The lower than expected number of homozygous mutant embryos may represent early in utero death of some Lhx2−/− embryos. When reabsorbing embryos were genotyped, a disproportionately high number of homozygous mutants were found (data not shown). Likewise, the Lhx2 mutation was recessive and embryonic lethal on an inbred 129/Sv background.

We observed three major phenotypic abnormalities in E13.5 Lhx2−/− embryos. They were anophthalmic, had flattening of the forehead region due to cerebral cortex abnormalities, and had small livers (Fig. 2). After E13.5 the Lhx2−/− embryos were notably paler than their littermates. Stillborn pups were pale and hydropic. The observations that the Lhx2−/− embryos were pale, hydropic, and had small fetal livers suggested that the in utero death was due to a defect in definitive erythropoiesis.

**Lhx2 is necessary for eye development**

Using in situ analysis, we observed Lhx2 expression in the region of the optic vesicle as early as E8.5 (data not shown). Prior to birth we saw Lhx2 expression throughout the neural retina; while postnatally, Lhx2 expression became restricted to the inner nuclear layer of the retina (Xu et al., 1993; data not shown). Mice homozygous for the Lhx2 mutant allele were anophthalmic. By E13.5 no lens, globe, or retina were seen in Lhx2−/− embryos (Fig. 3A,B). However, the eyelid folds and, in some older embryos, the extraocular muscles persisted. Analysis of hematoxylin and eosin stained sections from E9.5 embryos demonstrated that eye development arrested after formation of an optic vesicle but prior to formation of an optic cup (Fig. 3C,D). Eye development did not proceed further in the mutant embryos, and by E10.5 only a small remnant of the optic vesicle was observed (data not shown).

Mice with mutations in Pax6 (Sey) are anophthalmic (Hill et al., 1991) and Pax6 has been hypothesized to be a ‘master control gene’ for eye development (Halder et al., 1995). We thus hypothesized that a regulatory relationship between Pax6 and Lhx2 might exist. Using in situ hybridization analysis, we found that Pax6 continued to be expressed in the arrested optic vesicle of Lhx2−/− embryos. Fig. 3E demonstrates Pax6 expression in the nascent optic vesicle of an E9.5 Lhx2+/+ embryo, while Fig. 3F shows the persistence of Pax6 expression in the arrested optic vesicle of a Lhx2−/− littermate. Conversely, homozygous Sey embryos continue to express Lhx2 in the developing optic vesicle/cup (Richard Maas, personal communication). Pax6 is also normally expressed in the lens placode (Hill et al., 1991; Walther and Gruss, 1991). We did not observe a lens placode in hematoxylin and eosin stained sections from Lhx2−/− embryos (Fig. 3D), and we did not see specific hybridization of a Pax6 probe to the ectoderm overlying the arrested optic vesicle of Lhx2−/− embryos (Fig. 3F).

**Lhx2 is necessary for efficient definitive erythropoiesis**

We found a significant decrease in the hematocrits of Lhx2−/− embryos between E13.5 and E15.5 (Fig. 4A). Lhx2+/+ and +/− embryos were phenotypically normal, thus we intercrossed Lhx2+/− mice to determine if the Lhx2 mutant allele had a recessive phenotype. Genotyping of 191 weaned offspring of these matings identified 71 Lhx2+/+ (37%) and 120 Lhx2+/- (63%) progeny, but no Lhx2−/− mice. Some stillborn Lhx2−/− pups were found. Genotyping of 242 embryos, E15.5 and younger, yielded numbers closer to the expected Mendelian ratio for an autosomal recessive mutation (+/+ 24%, +/- 55%, −/− 21%). Fig. 1C shows the genotyping of these embryos and the presence of homozygous mutant embryos. The lower than expected number of homozygous mutant embryos may represent early in utero death of some Lhx2−/− embryos. When reabsorbing embryos were genotyped, a disproportionately high number of homozygous mutants were found (data not shown). Likewise, the Lhx2 mutation was recessive and embryonic lethal on an inbred 129/Sv background.

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**Lhx2 is necessary for efficient definitive erythropoiesis**

We found a significant decrease in the hematocrits of Lhx2−/− embryos between E13.5 and E15.5 (Fig. 4A). Lhx2+/+ and +/−
embryos had hematocrits in the 30-40% range; however, by E15 the hematocrits of Lhx2−/− mice decreased to about 10%. This time course was consistent with normal yolk sac erythropoiesis, but impaired definitive erythropoiesis (Russell, 1979).

Definitive erythropoiesis was not completely blocked in the mutant embryos. Microscopic analysis of Wright-Giemsa stained blood smears from E12.5-E15.5 embryos showed that mature enucleated erythrocytes were present in Lhx2−/− embryos (Fig. 4C). However, we did note variation in the size of the Lhx2−/− erythrocytes. The percentage of enucleated erythrocytes in Lhx2−/− embryos progressively increased from E12.5 to E15.5 (Fig. 4B). Thus, although the hematocrit of mutant embryos was severely reduced by E15.5, mature erythrocytes were the most common type of erythrocytes present in the mutant embryos. Although Lhx2−/− livers were smaller than normal, histological examination of liver sections stained with hematoxylin and eosin demonstrated hematopoietic foci of normal appearance in the parenchyma of the mutant livers (data not shown). Thus definitive erythropoiesis was inefficient rather than completely blocked in the mutant embryos.

Methylcellulose colony-forming assays, which allowed quantitation of erythrocyte progenitor cells in fetal livers, were undertaken to further characterize the erythropoietic defect. Similar results were observed with Lhx2+/+ and +/− embryos, thus these data were combined. Per 100,000 nucleated cells, there was a significant reduction of two types of erythroid precursors (Fig. 5). Blast Forming Unit-erythroid progenitors (BFU-e), the earliest pure erythroid precursor, and Colony Forming Unit-erythroid progenitors (CFU-e), a more mature erythroidic precursor, were both significantly decreased in the mutant livers. In contrast, the number of Colony Forming Unit-Granulocyte/Macrophage progenitors (CFU-GM) was not significantly reduced (Fig. 5). Enumeration of nucleated cells from control and mutant livers demonstrated a seven-fold reduction in the cellularity of the Lhx2−/− livers (1.15×10^6 versus 1.6×10^6). Therefore, the total numbers, per liver from Lhx2−/− embryos, of BFU-e and CFU-e progenitor cells were reduced 16-fold and 40-fold respectively. In contrast, the reduction (eight-fold) in the number of CFU-GM progenitor cells was proportional to the reduction in total liver cellularity. The multipotent progenitor cells, Colony Forming Unit-Gran-
uloctye/Erythrocyte/Macrophage (CFU-GEM), that give rise to both erythrocytic and myelocytic lineages were reduced 16- fold in the Lhx2−/− livers. However, a possible defect in the production of erythrocytes would impair our ability to identify CFU-GEM progenitors from mutant embryos. If the erythrocytic component of a multipotent CFU-GEM did not develop normally, these colonies would be enumerated as CFU-GM colonies. Thus, a firm conclusion about the status of the CFU- GEM progenitor cells was precluded.

The erythrocytic defect observed in Lhx2−/− embryos could have been due to an intrinsic defect in the development of the erythrocytic lineage itself (cell autonomous) or due to an extrinsic defect in the surrounding microenvironment (cell non-autonomous). To distinguish between these two possibilities we analyzed Lhx2 chimeric mice. We obtained a Lhx2−/− embryonic stem cell clone by exposing Lhx2+/− embryonic stem cells to elevated concentrations of G418 (Wu and Alt, unpublished). These Lhx2−/− embryonic stem cells were injected into C57BL/6 blastocysts to produce chimeric animals. The embryonic stem cells were derived from a 129/Sv mouse, thus erythrocytes derived from these cells express the hemoglobin-diffuse (Hb-d) allele; whereas, erythrocytes derived from C57BL/6 cells express the hemoglobin-single (Hb-s) allele. We took advantage of this hemoglobin polymorphism in order to determine the ability of Lhx2−/− embryonic stem cells to contribute to definitive erythropoiesis in chimeric animals. Our results are tabulated in Table 1. Lhx2−/− embryonic stem cells retained the ability to significantly contribute to erythropoiesis in chimeric animals. In seven highly chimeric animals, the level of Hb-d ranged from 40-80%. None of these chimeras were hydropic or pale. Thus, the effect of Lhx2 on erythropoiesis was cell non-autonomous. In contrast, given the high frequency of microphthalmia and anophthalmia seen in these chimeric pups, the effect of Lhx2 on eye development was judged to be cell autonomous.

To confirm the cell nonautonomous nature of the erythropoietic defect, fetal liver cells from Lhx2+/+ and Lhx2−/− embryos were transplanted into lethally irradiated recipient mice. Flow cytometric analysis was used to study the development of various hematopoietic lineages in these 'reconsti-}

**Fig. 4.** Blood analysis of progeny from Lhx2+/− matings. Hematocrits (A) and the percentage of enucleated erythrocytes (B) from Lhx2+/+ (□), −/+ (■), and −/− (▲) embryos were measured at the indicated gestational ages. The error bars represent one standard deviation. *Indicates P<0.001 using a Student t-test.

The number of embryos analyzed is indicated, in parenthesis, at the top of each bar. (C) Wright-Giemsa stained peripheral blood smears from Lhx2+/+ and −/− embryos. Yolk sac derived (PE) and hepatic derived (DE) erythrocytes are observed in both embryos.
Table 1. Chimeric analysis

<table>
<thead>
<tr>
<th>Pup</th>
<th>Eye phenotype</th>
<th>Hemoglobin (%)</th>
<th>Hb-s</th>
<th>Hb-d</th>
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<tr>
<td>1</td>
<td>Anophthalmic</td>
<td>33</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Normal</td>
<td>31</td>
<td>69</td>
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<tr>
<td>4</td>
<td>Normal</td>
<td>20</td>
<td>80</td>
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<tr>
<td>5</td>
<td>Left: anophthalmia</td>
<td>40</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Right: microphthalmia</td>
<td>40</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Normal</td>
<td>60</td>
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<tr>
<td>8</td>
<td>Normal</td>
<td>50</td>
<td>50</td>
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Lhx2−/− embryonic stem cells were injected into C57BL/6 blastocysts. The resulting chimeric pups were examined for ocular abnormalities, and the percentage of each hemoglobin variant was determined.

The superomedial cerebral cortex for control embryos was 0.29±0.06 (n=8) and for Lhx2 mutant embryos was 0.13±0.02 (n=6). The superomedial cortex values were 0.33±0.02 for controls and 0.15±0.02 for mutant embryos. Counts were bilaterally derived and were statistically significant (P<0.001). Analysis for cell death using the TUNEL stain in E13.5 embryos did not reveal any difference between mutant and control brains (data not shown). These data confirmed that the hypoplasia of the cerebral cortex was primarily due to a defect in precursor cell proliferation.

DISCUSSION

Targeted disruption of Lhx2, a LIM homeobox gene

We produced a functionless Lhx2 allele by homologous recombination in embryonic stem cells using a standard positive/negative targeting strategy. Lhx2−/− mice were phenotypically normal; however, Lhx2−/− embryos were anophthalmic, had forebrain malformations and were anemic. Thus Lhx2 plays an essential role in the development of the eye, the forebrain and definitive erythrocytes.

Lhx2 is essential for eye development

Eye development in the mouse begins on E8 with outgrowth of the optic vesicle from the neuroepithelium. On E9, the optic vesicle contacts the overlying ectoderm and begins to invaginate to form the optic cup. The inner layer of the optic cup will proliferate and give rise to the multi-layered neural retina; whereas, the outer layer of the optic cup will remain as a single cellular layer and become the retinal pigment epithelial layer. Concurrently, the overlying surface ectoderm thickens to form the lens placode, and then subsequently invaginates to form the lens vesicle. A series of reciprocal inductive interactions between the optic cup and lens vesicle are thought to control the development of the eye (Grainger, 1992; Saha et al., 1992).

Lhx2 expression has been observed in the optic vesicle as early as E8.5, and is highly expressed throughout the neural retina during embryonic development. Postnatally, expression of Lhx2 becomes restricted to the inner nuclear layer. This study has shown that Lhx2 is essential for progression of the optic vesicle to the optic cup stage. Specification of the optic vesicle in Lhx2−/− embryos appears normal; however, eye development arrests prior to formation of the optic cup. Thus Lhx2 is essential for proper outgrowth and invagination of the optic vesicle to form the optic cup.

Given this early defect in eye development, we were interested in determining whether Lhx2 was necessary for the expression of other genes important for eye development. One such gene is Pax6. Pax6 is normally expressed in both the lens placode and the optic vesicle. The Drosophila homolog of Pax6, eyeless, functions as a master regulatory gene for eye development (Halter et al., 1995), and mutations of Pax6 have been found in the Small eye (Sey) mouse and in cases of human aniridia (Hanson and van Heyningen, 1995). Eye development in the Sey mouse arrests after outgrowth of the optic vesicle (Hogan et al., 1986). Thus, conceivably, Lhx2 could regulate Pax6 expression in the developing eye. We found, using in situ analysis, that Pax6 expression continued in the arrested optic vesicles of Lhx2−/− embryos. Conversely, Lhx2 expression has been reported to be normal in Sey mice. These results suggest...
that $Lhx2$ and $Pax6$ are independently essential for normal development of the optic vesicle and cup.

In contrast to the continued expression of $Pax6$ in the mutant optic vesicles, $Pax6$ expression in the ectoderm overlying the optic vesicle was not observed in $Lhx2^{-/-}$ embryos. We observed $Pax6$ expression in the lens placode of $Lhx2^{+/+}$ embryos; however, we did not observe specific hybridization of a $Pax6$ probe in the ectoderm overlying the arrested optic vesicle of $Lhx2^{-/-}$ embryos. These data suggest that $Lhx2$ function in the optic vesicle is necessary for either induction or maintenance of $Pax6$ expression in the presumptive lens ectoderm.

**Definitive erythropoiesis is inefficient in $Lhx2$ mutant embryos**

Erythrocyte development occurs in two distinct phases. In the mouse, primitive erythropoiesis is first observed on E7 in blood islands of the yolk sac. By E12, the major site of erythropoiesis shifts from the yolk sac to the fetal liver following migration of pluripotent hematopoietic stem cells into the fetal liver. The erythrocytes formed in the embryonic liver are distinct from primitive erythrocytes and are similar to those formed in the bone marrow. This phase of erythropoiesis is referred to as definitive erythropoiesis. The fetal liver remains the major site of erythropoiesis until after E15 when it shifts initially to the spleen and subsequently to the bone marrow (Russell, 1979).

Erythropoiesis appeared normal in $Lhx2^{-/-}$ embryos prior to E12; however, these embryos had a significant drop in their hematocrits between E13 and E15. This suggested a problem in definitive erythropoiesis. The fall in hematocrit could have been the result of either decreased erythrocyte formation, or increased erythrocyte destruction. The decreased liver size of $Lhx2^{-/-}$ embryos suggested a failure in erythrocyte production, and the decrease in erythrocyte progenitor cells confirmed that the anemia in the mutant mice was due to abnormal production of erythrocytes rather than increased destruction. By E15.5 most of the erythrocytes in $Lhx2^{-/-}$ embryos were of the mature type; however, their number, as reflected by the hematocrit, was significantly decreased. Thus definitive erythropoiesis was inefficient in $Lhx2^{-/-}$ embryos rather than completely blocked.

To further define $Lhx2$ function in hematopoiesis, methylcellulose cultures of hematopoietic progenitor cells from fetal liver were performed. $Lhx2^{-/-}$ embryos had a seven-fold reduction in the absolute number of nucleated cells present in E13.5 livers, and compared to control animals, $Lhx2^{-/-}$ embryos had a 16-fold decrease in BFU-e and a 40-fold decrease in CFU-e progenitor cells. In comparison, the decrease in the number of CFU-GM progenitor cells (8-fold) was just proportional to the decrease in total cellularity (7-fold). Thus, the lack of $Lhx2$ function had a disproportionate effect on erythropoiesis, and in particular impaired the progression of BFU-e to CFU-e progenitors.

A defect in the hepatic phase of erythropoiesis could be due to several causes. Either the migration of pluripotent hematopoietic stem cells to the fetal liver, the maturation of a multipotent progenitor cell, or the maturation of a lineage restricted progenitor cell could be impaired. The failure of progenitor cells to develop normally could be due to an intrinsic defect in the progenitor cell (cell autonomous), or a defect in the microenvironment in which the progenitor cell develops (cell non-autonomous). Our data can exclude a complete defect in migration of pluripotent hematopoietic stem cells to the fetal liver, but cannot exclude a partial defect at this level. Further studies to evaluate the number of pluripotent stem cells present in $Lhx2^{-/-}$ livers need to be completed to exclude a partial defect at this level. However, we have demonstrated a disproportionate effect on maturation of erythropoietic progenitors in the livers of $Lhx2^{-/-}$ embryos.

The onset and distribution of $Lhx2$ expression in the developing liver suggest that it is expressed in developing lymphoid cells (Xu et al., 1993). We did not detect $Lhx2$ expression in mouse erythroleukemia cells using northern blot analysis (data not show); however, expression of $Lhx2$ in human erythroleukemia cells was reported by Wu et al. (1996). To ascertain whether the defect in erythropoiesis was cell autonomous or cell non-autonomous, we evaluated the ability of $Lhx2^{-/-}$ embryonic stem cells to produce mature erythrocytes in chimeric animals. The proportion of hemoglobin derived from the $Lhx2^{-/-}$ embryonic stem cells ranged from 40-80% in eight chimeric

![Flow cytometric analysis of hematopoietic cells in lethally irradiated mice.](image)

**Fig. 6.** Flow cytometric analysis of hematopoietic cells in lethally irradiated mice. Fetal liver cells from $Lhx2^{+/+}$ and $Lhx2^{-/-}$ embryos were transplanted into lethally irradiated mice, and flow cytometric analysis was performed. The antibodies used for staining and tissues analyzed are indicated in the figure. In A,B and C cells residing in the lymphocyte gate were analyzed and the percentage of cells in a particular region is shown. In D bone marrow was analyzed for cells of the erythroid lineage (Ter-119) and granulocytic lineage (Gr-1).
The absence of the hippocampal anlagen and the reductions in the volumes of the forebrain (P<0.05), the cortical plate (P<0.05), and the basal ganglia anlagen (P<0.01) of Lhx2−/− embryos (n=4) compared to Lhx2+/+ embryos. The volumes of the ventricular system and the diencephalon did not differ significantly between Lhx2+/+ and −/− embryos at E12.5.

**Lack of Lhx2 function results in neocortex hypoplasia and aplasia of the hippocampal anlagen**

Lhx2 is expressed widely in the developing central nervous system. In the forebrain expression was observed in the neocortex, archicortex, the basal ganglia, the dorsal thalamus and the hypothalamus. We also observed Lhx2 expression in the dorsal midbrain and in the rostral hindbrain. In the cerebral cortex, Lhx2 transcripts are detected in the ventricular, intermediate, and mantle zones; whereas, in the midbrain and hindbrain Lhx2 expression is limited to the outer layers. In Lhx2−/− embryos, we observed a reduction in the size of the cerebral cortex. Specifically, we saw that the neocortex was hypoplastic, and the archicortex (which includes the hippocampal anlagen) was aplastic. BrdU labeling studies and TUNEL staining demonstrated that this decrease in cellular mass was due to decreased proliferation rather than increased cellular death. A similar defect in cellular proliferation of the anterior pituitary was observed in Lhx3−/− embryos (Sheng et al., 1996). It is not readily apparent why these two areas of the cerebral cortex are differentially affected in the Lhx2−/− embryos. One hypothesis is that a related gene is able to provide partial functional redundancy for Lhx2 in the neocortex, and that this second gene is not active in the archicortex. Such a situation has been observed with *engrailed-1* and *engrailed-2* in cerebellar development (Hanks et al., 1995), and also for Lhx3 and Lhx4 in pituitary development (Sheng and Westphal, unpublished observations). This hypothesis may also explain the apparent lack of a distinct phenotype in the midbrain, the hindbrain, the nasal epithelium and the limbs where Lhx2 is also expressed. A second hypothesis would be that the function of Lhx2 differs in these two architecturally distinct cortical structures. The aplasia of the hippocampal anlagen may therefore be
a reflection of the interaction of Lhx2 with a second protein that modulates its function and is exclusively expressed in the nascent hippocampus. The recent cloning of a novel family of proteins that binds to and activates LIM homeodomain proteins (Agulnick et al., 1996) supports the idea that Lhx2 in combination with regional specific interacting proteins may have different functions. Further studies to identify a related LIM homeobox gene and to further characterize the interactions of Lhx2 with Ldb family members are needed to differentiate between these two possibilities.

The differential effect of a mutation of Lhx2 on the development of the neocortex and archicortex provides functional support for the prosomeric model that has been proposed by Puelles and Rubenstein (1993). Their initial model proposed that the archicortex develops from the fourth prosomere, while the neocortex develops from the fifth prosomere. Rubenstein and colleagues (Bulfone et al., 1995) have subsequently advanced two alternative models that take into account the relatively homogeneous expression patterns of a number of genes in the developing cerebral cortex. In these models, both the archicortex and the neocortex are proposed to develop from a single prosomere. Although expression of Lhx2 across the proposed prosomeric boundaries, the development of these two CNS compartments differs in their requirement for Lhx2. Thus the forebrain phenotype of the Lhx2−/− embryos supports the initial model that allocates the archicortex and the neocortex to separate prosomeres. Analysis of the expression patterns of other genes proposed to be involved in specification of forebrain compartments will also be useful in further characterizing the possible functional difference for Lhx2 in the archicortex versus the neocortex.

This study shows that Lhx2 is essential for normal development of the cerebral cortex. The primary defect appears to be a proliferative block in neuronal precursor cells. Previously, it was hypothesized that the expression of Lhx2 in the outer layers of the developing cortex is consistent with a role for Lhx2 in neuronal differentiation (Xu et al., 1993); however, the presence of Lhx2 transcripts in the outer layer of the cerebral cortex may simply reflect the fact that neuronal precursor cells migrate rapidly after division, or that Lhx2 functions in both neuronal precursor cell proliferation and in neuronal differentiation. Due to the in utero death of these embryos prior to formation of a mature cortex, we currently cannot address the role of Lhx2 in the differentiation of neurons. This role and the function of Lhx2 in the adult cortex and hippocampus awaits development of a mouse model and in vitro systems in which Lhx2 function can be inhibited in a specific tissue and at a specific time of development.

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