Lhx2 links the intrinsic and extrinsic factors that control optic cup formation

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A crucial step in eye organogenesis is the transition of the optic vesicle into the optic cup. Several transcription factors and extracellular signals mediate this transition, but whether a single factor links them into a common genetic network is unclear. Here, we provide evidence that the LIM homeobox gene Lhx2, which is expressed in the optic neuroepithelium, fulfils such a role. In Lhx2−/− mouse embryos, eye field specification and optic vesicle morphogenesis occur, but development arrests prior to optic cup formation in both the optic neuroepithelium and lens ectoderm. This is accompanied by failure to maintain or initiate the expression patterns of optic-vesicle-patterning and lens-inducing determinants. Of the signaling pathways examined, only BMP signaling is noticeably altered and expression patterns of optic-vesicle-patterning and lens-inducing determinants. Our data indicate that Lhx2 is required for optic vesicle patterning and lens formation in part by regulating BMP signaling in an autocrine manner in the optic neuroepithelium and in a paracrine manner in the lens ectoderm. We propose a model in which Lhx2 is a central link in a genetic network that coordinates the multiple pathways leading to optic cup formation.

KEY WORDS: BMP, Anophthalmia, Eye field transcription factor, Lens, Optic vesicle, Retina

INTRODUCTION

Vertebrate early eye formation follows a conserved sequence of events (Fig. 1A). Soon after gastrulation begins, the eye field is specified in the anterior neural plate. The first morphological landmarks are bilateral indentations (optic sulci) in the eye field, followed by evagination of the lateral walls of the diencephalon, giving rise to the optic vesicles (OVs). Interaction between the OV and surface ectoderm (SE) induces the lens placode, and a concerted invagination of the lens placode and OV produces the optic cup (OC). Coincident with these events are lens morphogenesis, the establishment of dorsoventral polarity of the OV, and regional patterning of the optic neuroepithelium into neural retina, retinal pigment epithelium (RPE) and optic stalk. Disruptions in these early steps lead to severe congenital anomalies, including absent eyes (anophthalmia), small eyes (microphthalmia) and optic fissure (anophthalmia), small eyes (microphthalmia) and optic fissure closure defects (coloboma) (Fitzpatrick and van Heyningen, 2005).

The homeobox genes Rx (Rx), Pax6, Six3 and Optx2 (Six6), the T-box gene ET, the orphan nuclear receptor Tll, and the LIM homeobox gene Lhx2 are expressed in dynamic and overlapping patterns in the Xenopus eye field and are collectively defined as eye field transcription factors (EFTFs) (Zuber et al., 2003). EFTF overexpression in toto induces ectopic eye fields that lead to well-formed eyes, and sufficiency experiments suggest that EFTFs participate in a network analogous to the retinal determination gene network in Drosophila (Pappu and Mardon, 2004; Silver and Rebay, 2005; Zuber et al., 2003). That EFTFs are required for early eye organogenesis is revealed by their loss-of-function mutations in human and several model systems (Bailey et al., 2004; Fitzpatrick and van Heyningen, 2005; Gräf, 2003).

It is not clear, however, whether the factors that regulate the subsequent events of eye organogenesis, namely those occurring during the OV-to-OC transition, are linked into a common genetic network. These events, which include regional and axial patterning of the optic neuroepithelium and lens induction in the SE, are highly dependent on multiple signals, including sonic hedgehog (Shh) from the ventral midline, FGFs from the presumptive lens ectoderm and OV, TGFβ superfamily ligands from the mesenchyme, and BMPs from the OV (Fig. 1A) (Bharti et al., 2006; Chow and Lang, 2001; Martinez-Morales et al., 2004; Yang, 2004). An important outcome of these signals is the establishment of defined expression domains for several EFTFs and other transcription factors, including Pax2, Vxs2 (Chx10), Mif, Thx5, Vax2 and Sox2 (Behesti et al., 2006; Fuhrmann et al., 2000; Furuta and Hogan, 1998; Gotoh et al., 2004; Hyer et al., 1998; Jensen, 2005; Kim and Lemke, 2006; Macdonald et al., 1995; Morcillo et al., 2006; Murari et al., 2005; Nguyen and Arnheiter, 2000).

Because multiple pathways function simultaneously during the OV-to-OC transition, their coordination seems likely. EFTFs, and Pax6 and Lhx2 in particular, are the best candidates for accomplishing this. In contrast to Rx and Six3 mutants (Carl et al., 2002; Mathers et al., 1997), which arrest prior to OV formation, and Six6 and Tll mutants, which progress past OC formation (Holleman et al., 1998; Li et al., 2002), eye morphogenesis in Pax6 and Lhx2 mutants arrests at the OC stage (Hill et al., 1991; Porter et al., 1997). However, regionalization of the OV occurs in the Pax6 mutant (Baumer et al., 2003), suggesting that Pax6 does not act as a central coordinating factor. Whether Lhx2 acts in this manner has not been addressed. Overexpression of EFTF combinations that do not include Lhx2 but are still able to induce ectopic eyes always activates endogenous Lhx2 expression in the ectopic eye fields, whereas combinations that are unable to induce

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eyes also fail to activate \textit{Lhx2} (Zuber et al., 2003). Where examined, expression of the vertebrate \textit{Lhx2} orthologs initiates in the presumptive eye field and continues through early eye organogenesis (Seth et al., 2006; Tetreault et al., 2008; Viczian et al., 2006) (S.Y. and E.M.L., unpublished).

Although \textit{Lhx2} is known to be essential for mouse OC morphogenesis, how it controls early eye development is unresolved. Zebrafish \textit{lhx2} (\textit{belladonna}) mutants do not have anophthalmia (Seth et al., 2006), probably because of redundancy with other LIM homeobox genes (Kikuchi et al., 1997). A recent study of the mouse \textit{Lhx2} mutant suggested that there is a delay in eye field specification and identified \textit{Six6} as a transcriptional target of \textit{Lhx2} (Tetreault et al., 2008), but a mechanistic explanation of how \textit{Lhx2} regulates early eye development was not forthcoming, especially as \textit{Six6} mutants have microphthalmia and reduced retinal progenitor cell proliferation rather than anophthalmia (Li et al., 2002). Here, we provide evidence in mouse that the eye field and OV form on schedule in the absence of \textit{Lhx2}, but patterning of the optic neuroepithelium arrests prior to regionalization and the establishment of dorsoventral polarity. We also elucidate that \textit{Lhx2} acts to link the multiple pathways needed for the OV-to-OC transition.

\textbf{MATERIALS AND METHODS}

\textbf{Animals}

Mouse strains are listed in Table 1. \textit{Lhx2} \textit{−−} eyes are indistinguishable from \textit{Lhx2} \textit{++} eyes and were used as controls. \textit{Hes1croERT2} mice were produced by homologous recombination of the \textit{creERT2} cDNA into the \textit{Hes1} locus (D.K. and L.C.M., unpublished). Primers used for PCR genotyping are listed in Table S1 in the supplementary material. Embryos were staged and matched for comparison by somite number. Somite stage (ss) and embryonic day (E) are indicated for all samples; ss is used in the text for samples up to ss22, which covers eye field specification and early OV stages, and E is used for samples ss23 and older (see Fig. 1B). Animal protocols were approved by the University of Utah IACUC.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
Allele & Targeting region & Reference \\
\hline
\textit{Lhx2} & Deletion in exon 2 and 3 & (Porter et al., 1997) \\
\textit{Lhx2} & \textit{loxP} sites in intron 1 and 3 & (Mangale et al., 2008) \\
\textit{Fgf8} & \textit{loxP} sites in intron 4 and 5; GFP insertion in 3'UTR & (Macatee et al., 2003) \\
\textit{ShhGFP-cre} & \textit{GFPcre} knock-in & (Harfe et al., 2004) \\
\textit{Rosa26} & \textit{creERT2} knock-in & (Badea et al., 2003) \\
\textit{Tg(Le-cre)} & \textit{cre} is under the control of \textit{Pax6} regulatory sequence (transgenic) & (Ashery-Padan et al., 2000) \\
\textit{Rosa26} & \textit{loxP-neo pA; lacZ knock-in} & (Soriano, 1999) \\
\hline
\end{tabular}
\caption{Mouse lines}
\end{table}
Organotypic head cultures

Heads from ss18-24 embryos were transsected at the midbrain and washed in cold DMEM/10 mM HEPES, then placed into holes made in 1.5% agarose and cultured in DMEM supplemented with N-2 (Invitrogen) for 48 hours in the presence or absence of 1 μg/ml BMP7 (R&D Systems). BMP4 beads were prepared as previously described (Furuta et al., 1997). Briefly, Affi-Gel Blue beads (BioRad, 100-200 mesh, #153-7302) of 25-50 μm diameter were rinsed with water. One hundred beads were incubated overnight (4°C) in 5 μl of 1 μg/ml BMP4 (R&D Systems). Bovine serum albumin (1 μg/ml in PBS) served as a control. Beads were placed into dorsal mesenchyme. mRNA was detected by whole-mount in situ hybridization. Sections were prepared post-staining.

Quantification and statistical analysis

For quantification, comparable regions of optic neuroepithela in Lhx2 mutants and controls were captured using a Fluoview 1000 confocal microscope for cell counting. The percentage of the total (DAPI-positive) cells that was pH3-positive was quantified and statistical significance between mutants and controls determined by Student’s t-test; error bars indicate s.d.

RESULTS

Eye field specification and OV identity are initiated, but not maintained, in the absence of Lhx2

As Lhx2 is expressed from the earliest stages of eye formation, it is possible that specification of the eye field is altered in its absence. This was recently suggested to be the case (see Tetreault et al., 2009). However, OV formation is apparent in Lhx2 mutants at ss18 (~E8.75; see Fig. S1A,B in the supplementary material), which suggests that the functional process of eye field specification still occurs. To address this more directly, we examined the expression of Rx, an EFTF that is essential for eye field specification and OV formation (Mathers et al., 1997; Medina-Martinez et al., 2009), in ss6 and ss12 mouse embryos (Fig. 2A-D’). ss6 corresponds to the anterior neural plate stage, when eye field specification occurs. Optic sulci are formed by ss12, and are the earliest morphological indications of OV formation. Although Rx mRNA may be slightly reduced in the mutant compared with the control at ss6 (Fig. 2A,B), it was still readily detected. At ss12, Rx expression was robust (Fig. 2C-D’). Furthermore, the Rx expression domain was bifurcated at the midline (arrowheads in Fig. 2C’D’), suggesting that the anterior neural ectoderm responds appropriately to midline signals that split the eye field. At ss18, Pax6 expression (Fig. 2E,F) and the relative proportion of cells positive for phosphorylated histone H3 (pHH3) occurs. To address this more directly, we examined the expression of Rx, an EFTF that is essential for eye field specification and OV formation (Mathers et al., 1997; Medina-Martinez et al., 2009), in ss6 and ss12 mouse embryos (Fig. 2A-D’). ss6 corresponds to the anterior neural plate stage, when eye field specification occurs. Optic sulci are formed by ss12, and are the earliest morphological indications of OV formation. Although Rx mRNA may be slightly reduced in the mutant compared with the control at ss6 (Fig. 2A,B), it was still readily detected. At ss12, Rx expression was robust (Fig. 2C-D’). Furthermore, the Rx expression domain was bifurcated at the midline (arrowheads in Fig. 2C’D’), suggesting that the anterior neural ectoderm responds appropriately to midline signals that split the eye field. At ss18, Pax6 expression (Fig. 2E,F) and the relative proportion of cells positive for phosphorylated histone H3 (pHH3) are similar in mutant and control (see Fig. S1A,B in the supplementary material). By E9 (ss20-22), however, expression of Rx (Fig. 2G,H) and Pax2 (Fig. 2I,J) was reduced in the Lhx2−/− OV, which failed to keep pace with the growth of the wild-type OV as indicated by its reduced size and the reduced proportion of pH3-positive cells (see Fig. S1C-G in the supplementary material). These data suggest that eye field specification and initiation of OV morphogenesis are relatively normal in the absence of Lhx2, but once the OV has formed, Lhx2 is required to maintain growth and gene expression in the optic neuroepithelium.

To better understand the extent of the molecular changes occurring in the optic neuroepithelium, we examined the expression patterns of EFTFs at later stages. At E9.5, Pax6 was expressed in the OV in a dorsoventral gradient, with highest expression dorsally (Fig. 2K). In the newly formed OC (E10.25), Pax6 was highly expressed throughout most of the retina and RPE and weakly in the optic stalk (Fig. 2M). In the E9.5 Lhx2−/− OV, Pax6 was highly expressed ventrally (Fig. 2L, arrowheads). The same pattern was
observed in E10.25 Lhx2−/− optic neuroepithelium, except at the distal tip (Fig. 2N). The normal pattern of OTX2 and OTX1 (OTX2/1) expression also exhibited a dorsoventral gradient (Fig. 2O,Q), and, similar to the change in PAX6, OTX2/1 staining was high in the ventral Lhx2−/− optic neuroepithelium (Fig. 2P,R, arrowheads). By contrast, the distribution of SIX3 and of SOX2 was similar in the Lhx2−/− and control optic neuroepithelia at E9.5 (Fig. 2S,T,W,X), although their expression levels appeared reduced in the mutant at E10.25 (Fig. 2U,V,Y,Z). These data indicate that Lhx2 inactivation does not eliminate the expression of all transcription factors important for eye development, but is required to maintain their proper spatial distribution or expression levels.

Pax6, Six3 and Sox2 are also expressed in the SE and are essential for lens development (Ashery-Padan et al., 2000; Furuta and Hogan, 1998; Liu et al., 2006). Whereas PAX6 was abundantly expressed in the Lhx2−/− SE at E9.5, its expression was downregulated by E10.25 (Fig. 2K–L′, red arrows). SIX3 and SOX2 were weakly expressed or absent at both stages (Fig. 2S′–Z′, red arrows). Since Lhx2 is not detected in the lens ectoderm, and OC morphogenesis is unaffected by conditional deletion of Lhx2 in this region (see Fig. S2 in the supplementary material), the changes in lens development in the germline Lhx2 mutant indicate that Lhx2 is upstream of neuroepithelium-derived signals required for lens induction.

Lhx2 is required for regionalization and dorsoventral patterning of the optic neuroepithelium

Regionalization of the optic neuroepithelium slightly precedes the OV-to-OC transition (Ashery-Padan and Gruss, 2001; Chow and Lang, 2001; Martinez-Morales et al., 2004). Vsx2 expression is activated by E9.5 and marks the presumptive neural retina. Mitf and Pax2, which are expressed broadly in the early OV (~E8.5), become restricted by the late OV stage to domains that correspond to RPE (Mitf) and optic stalk and ventral neural retina (Pax2). In addition to marking these domains, these factors are essential for maintaining regional identity (Baumer et al., 2003; Martinez-Morales et al., 2004; Horsford et al., 2005; Rowan et al., 2004). Interestingly, VSX2 and MITF expression failed to initiate in the Lhx2−/− OV (Fig. 3A–H), and PAX2, although expressed initially (Fig. 2J), was downregulated by E9.5 and largely absent by E10.25 (Fig. 3I–L). These observations suggest that the absence of Lhx2 causes an arrest in eye development prior to regionalization.

Dorsoventral patterning also initiates during OV stages (Chow and Lang, 2001; Furimsky and Wallace, 2006). Inversion of dorsoventral polarity leads to disruption of OV development and failure of OC formation in chick embryos (Uemonsa et al., 2002). The failure of OC formation combined with the expansion of PAX6 and loss of PAX2 in the ventral optic neuroepithelium imply defects...
in dorsoventral patterning. To address this, we examined the expression pattern of Vax2, a homeobox gene required for ventral identity and for repression of Pax6 expression in the ventral OV (Barberi et al., 1999; Chow and Lang, 2001; Mui et al., 2005). Like Pax2, Vax2 was expressed in the entire OV at early stages and became restricted to the ventral OV and OC (Fig. 3M,O). In the Lhx2–/– OV, Vax2 was expressed at E9.0, but not at E10.25 (Fig. 3N,P). The extensive apoptosis that occurs in the wild-type optic neuroepithelium: (1) that the ventral OV domain has acquired dorsal OV identity; or (2) that the entire OV has acquired an identity more akin to the dorsal diencephalon. If the former possibility were true, we would expect the dorsal marker Tbx5 to expand ventrally. However, Tbx5 mRNA was not detected at all in the Lhx2+/– OV (Fig. 3U-X). If the latter possibility were true, we would expect ectopic expression of genes that mark the dorsal diencephalon, such as Wnt3a, Aixin2 and Dbx1, but this was also not observed (see Fig. S3A-L in the supplementary material). These observations indicate that the optic neuroepithelium is not respecified. Rather, they suggest a developmental arrest and that Lhx2 is required to link eye field specification and OV formation with lens induction and OV patterning.

**Hedgehog and FGF signaling pathways are active in the Lhx2+/– OV and LHX2 expression is not dependent on Shh or Fgf8**

Signals emanating from the forebrain, extraocular mesenchyme, SE and OV arbitrate the regionalization and dorsoventral polarity of the optic neuroepithelium (Ashery-Padan and Gruss, 2001; Chow and Lang, 2001; Fuhrmann et al., 2000; Martinez-Morales et al., 2004). The expansion of Pax6 and loss of PAX2 in the Lhx2+/– optic neuroepithelium are similar to that observed in zebrafish when Hedgehog (Hh) signaling is perturbed and in Shh mutant mice (Chiang et al., 1996; Ekker et al., 1995; Macdonald et al., 1995). We therefore sought to determine whether a regulatory relationship exists between Hh signaling and Lhx2. SHH expression was still observed in the Lhx2+/– ventral diencephalon (Fig. 4A,B) and Gli1, a read-out of Hh signaling (Sigulinsky et al., 2008), was also expressed (Fig. 4C,D, arrows and arrowheads). LHX2 was also expressed in the OV of ShhGFP-cre/GFP-cre mice (Fig. 4E,F), which lack midline Hh signaling, resulting in cyclopia and eye patterning defects (Harfe et al., 2004). These findings indicate that Hh pathway activation and Lhx2 expression are not directly dependent on each other.

In the optic stalk of zebrafish belladonna mutants, the expression of fgf8, pax2 and vax2 is diminished, and blocking FGF signaling reduces lhx2 expression (Seth et al., 2006). These data suggest a mechanism of reciprocal regulation between FGF8 signaling and Lhx2 expression. However, Fgf8, Erm [Etv5; a downstream target of FGF8 signaling (Roehl and Nusslein-Volhard, 2001)] and phosphorylated ERK1/2 ([MAPK3/1] pERK; downstream effectors of FGF-MAPK signaling (Pan et al., 2006)] were expressed in the mutant OV and SE in a manner similar to that observed in controls (Fig. 4G-P). To address the possibility that Fgf8 regulates expression of Lhx2, we examined Lhx2 expression in Rosa26creER+/–; Fgf8f/f mice, in which Fgf8 begins to be ablated at E7.5 and the ablation is complete by E9.0 (Fig. 4Q,R). Although Rosa26creER+/–; Fgf8f/f mutants showed morphological defects in the telencephalon and OV, LHX2 was still expressed in the OV, as was PAX2 (Fig. 4S-V). These data indicate that FGF signaling is not Lhx2 dependent and argue against Lhx2 and Fgf8 regulating each other’s expression.

**Lhx2 is required for BMP signaling**

BMP signaling mediated by Bmp4 and Bmp7 is essential for several aspects of eye development, including lens induction and dorsoventral patterning of the optic neuroepithelium (Chow and Lang, 2001; Furuta and Hogan, 1998; Wawersik et al., 1999; Yang, 2004). Bmp4 is first detected in the distal OV and SE between E8.5 and E9.0, and its expression is maintained at a high level in the dorsal OV and OC (Furuta and Hogan, 1998). Bmp7 is widely expressed in the OV and is progressively restricted to the ventral region of the late OV and to the optic stalk after OC.
formation (Morcillo et al., 2006). Although Bmp7–/– mice exhibit a range of eye anomalies, from microphthalmia to anophthalmia, the anophthalmic phenotype of Bmp7–/– mice is morphologically similar to that of Lhx2–/– mice (Dudley et al., 1995; Luo et al., 1995). Interestingly, we found that Bmp7 and Bmp4 mRNA expression levels are downregulated in the Lhx2–/– OV (Fig. 5A-J). To assess whether BMP signaling is affected, we examined the phosphorylation status of SMAD1, 5 and 8 (pSMAD1/5/8), a read-out of BMP signaling (Massague, 1998; Murali et al., 2005). Whereas pSMAD1/5/8 was present in the optic neuroepithelium in controls at E9.5 and E10.25 (Fig. 5K,M), it was not detectable in the Lhx2–/– optic neuroepithelium (Fig. 5L,N), indicating disrupted BMP signaling. In the lens-forming region of the SE, pSMAD1/5/8 was detected in controls at both stages and in the Lhx2–/– embryo at E9.5, but was absent in the mutant at E10.25 (Fig. 5O-R, regions between dashed lines are SE or lens placode). The persistence of Bmp4 in the nasal placode (Fig. 5I,J, arrowheads) and of pSMAD1/5/8 in the extraocular mesenchyme (Mes; Fig. 5K-R) during these stages reveal that BMP signaling in the optic neuroepithelium and in the E10.25 lens ectoderm are dependent on Lhx2.

Lhx2 utilizes non-cell-autonomous and cell-autonomous mechanisms to regulate OV patterning

To determine whether the patterning defects in the Lhx2–/– OV are attributable to disrupted BMP signaling, we treated E9.0 heads with combinations of BMP7 and BMP4 for 2 days (Fig. 6A). As Bmp7 is widely expressed in the OV, BMP7 was added directly to the medium. BMP7-treated wild-type OVs exhibited strong expression of Pax2 (n=11/11), Vsx2 and Mitf (Fig. 6B,D; data not shown). In the Lhx2–/– OV, BMP7 treatment restored Pax2 expression (n=8/8), although at reduced levels compared with the wild type (Fig. 6C,E). However, it was not sufficient to initiate expression of Vsx2 or Mitf (data not shown).

Fgf15 is a potential downstream target of BMP4-mediated signaling in the neural retina (Murali et al., 2005). As Fgf15 expression is enhanced in wild-type optic neuroepithelium cultured with BMP4 (n=5/5; Fig. 6F,H,J), we did not detect Fgf15 in Lhx2–/– OVs (n=0/3; Fig. 6G,I,K). These
observations suggest that there are defects in BMP4-mediated signaling in addition to those induced by the absence of BMP4 expression.

As both BMP4 and BMP7 are downregulated in the Lhx2−/− OV, we tested the effect of adding BMP4 and BMP7 together to the cultures. Surprisingly, this condition elicited a strong enhancement of BMP4 and BMP7 signaling in eye development is unlikely as a concentration over a threshold, functional redundancy of BMP7 and possibility that adding both ligands simply raises the BMP neuroepithelia (Fig. 6L-Q). Although we cannot rule out the expression.

signaling in addition to those induced by the absence of Lhx2, Tbx5

Surprisingly, this condition elicited a strong enhancement of tested the effect of adding BMP4 and BMP7 together to the cultures. As BMP signaling is needed for lens induction (Furuta and Hogan, 1998; Wawersik et al., 1999), we asked whether BMP paradigms we tried (data not shown).

Whereas

Sox2 expression in the SE of the Lhx2 mutant. We utilized a newly generated tamoxifen-inducible locus. Under

creERT2 driver in which creER T2 is knocked into the Hes1 locus. Under low-dose tamoxifen administration at E8.5, rare cells in the OC express a recombination reporter by E10.5 (see Fig. S5 in the supplementary material), indicating that this approach is suitable for generating Lhx2 mutant cells at low frequency. As predicted, we found that Lhx2 mutant cells in the neural retina do not express VSX2 (Fig. 7A-D; n=17/17), nor do they express MITF in the RPE (Fig. 7E-H; n=23/23). We also examined PAX2 expression (Fig. 7I-P) as its response to BMP treatment was relatively weak, and observed that most Lhx2 mutant cells were also PAX2 negative (Fig. 7I-L; n=48/50), which suggests that Lhx2 utilizes both cell-autonomous and non-cell-autonomous mechanisms to regulate PAX2 expression. By contrast, PAX6 was expressed in all Lhx2 mutant cells analyzed (Fig. 7M-P; n=33/33), consistent with our data and the work of others indicating that PAX6 expression is independent of Lhx2 function.

**DISCUSSION**

In addition to the morphological changes occurring during the OV-to-OC transition, complex patterning and inductive interactions are executed during this period. In this study, we found that Lhx2 has a unique role in mediating this transition. Even though other EFTFs continue to be expressed in the Lhx2−/− OV, loss of Lhx2 results in a failure of the optic neuroepithelium to become regionalized and patterned along the dorsoventral axis. In addition, the interaction between the optic neuroepithelium and SE that leads to lens formation is severely compromised. We propose that Lhx2 acts as a central factor in eye organogenesis by coordinating several of the crucial events that occur during the transition of the OV to OC, in part through its role in establishing a BMP signaling center in the optic neuroepithelium.

**Lhx2 links eye field specification to OV patterning**

Our analysis of eye development from ss6 to ss18 suggests that eye field specification in the anterior neural plate and initiation of OV morphogenesis occur on, or close to, schedule in the absence of Lhx2. Our conclusion differs from that of Tetreault et al., who concluded that Lhx2 is required for eye field specification and for the correct timing of EFTF expression, which was based on their observations of weak or absent expression of Rx, Six3 and Pax6 in the presumptive eye field of Lhx2 mutant embryos from E8.25 and
E8.5 litters (Tetreault et al., 2009). The lack of Rx and Six3 expression at E8.25 was surprising to us because the absence of either factor prevents OV morphogenesis (Carl et al., 2002; Mathers et al., 1997), whereas OV morphogenesis clearly occurs in the Lhx2 mutant, and embryo chimeras show that Rx mutant cells are excluded from the optic neuroepithelium as early as E8.0, demonstrating its cell-autonomous requirement (Medina-Martinez et al., 2009). We determined that Rx is expressed in ss6 and ss12 embryos. The discrepancy in Rx expression between the two studies is probably not due to genetic differences because we observed similar staining in two different genetic backgrounds (Lhx2+/– were backcrossed two generations with 129sv or Black Swiss mice) and both studies analyzed the same allele. One possibility is that Lhx2 is required for stable EFTF expression in the eye field and that their expression levels fluctuate in its absence. We consider this unlikely, however, as we observed robust Rx expression in every mutant embryo analyzed between ss4 and ss12 (n=5). Another possible explanation lies in how the mutant and control embryos were matched for comparison. Whereas Tetreault and colleagues reported the staging of embryos according to the time of vaginal plug (i.e. gestational age), our embryos were staged by counting somites and matched for comparison by equivalent somite stage. As embryogenesis proceeds rapidly during this period, comparing embryos at the same somite stage controls for the normal, but sometimes considerable, variation in developmental progression that occurs among such embryos (including among littermates). It also controls for the possibility of general developmental delays that might occur in Lhx2 mutants. Although we do not exclude the possibility that variability in EFTF expression might occur in the anterior neural plate of Lhx2 mutants, our analysis of somite-stage-matched embryos suggest that the initiation of Rx expression is not significantly delayed in the Lhx2–/– anterior neural plate. Furthermore, because OV morphology, PAX6 expression and the proportion of pHH3-positive cells are similar to those in the wild type as late as ss18, we propose that the functional output of eye field specification is operating in the absence of Lhx2. Rather, the marked differences in morphology and marker expression that occur from ss20 onward indicate that the absence of Lhx2 leads to a developmental arrest after OV formation, but prior to the patterning events that precede OC formation.

That the nature of the Lhx2 developmental arrest is unique is best exemplified by comparing it with the Pax6 mutant, which exhibits a remarkably similar morphological, but molecularly distinct, phenotype. In the absence of Pax6, the eye field is established and maintained through the OV stage as indicated by the persistent expression of Rx, Lhx2, Six3, Otx2 and Pax2 (Baumer et al., 2003; Bernier et al., 2001). Furthermore, regionalization of the optic neuroepithelium, an event that occurs late in the OV, still occurs, as

Fig. 6. BMP treatments restore the expression of optic-vesicle-patterning and lens-specification genes. (A) Organotypic culture method. Dashed line indicates approximate position at which heads were transected. Heads containing BSA- (control) and BMP4-soaked beads were cultured in medium containing BSA or BMP7. Wild-type (Lhx2+/+ or Lhx2+/–) and Lhx2–/– heads were cultured for 48 hours. (B-E) Pax2 response to BMP treatments. (F-Q) Fgf15 response to BMP treatments. Arrowheads (F,G,L,M) point to Fgf15 expression. Red arrows indicate BSA- and BMP4-coated beads. Dashed line delineates the optic neuroepithelium. Asterisk in K indicates an artifact. (R-Y) Sox2 response to BMP treatments. SE is located between the dashed lines. Coronal sections, except frontal views in B-G,L,M. Scale bars: 500 μm in B-G,L,M; 50 μm in H-K,N-Y.
indicated by the activation of Vsx2 and Mitf expression and by their segregation into distinct domains (Baumer et al., 2003). By contrast, Rx expression in the Lhx2−/− OV drops by E9.5, and expression of Vsx2, Mitf, Tbx5 and Pax2 fails to initiate or persist, indicating a failure of the neuroepithelium to maintain its ‘optic’ character and to initiate regionalization. Another notable difference is that whereas ventral retinal identity is not only maintained but also expanded at the expense of dorsal identity in the Pax6 mutant (Baumer et al., 2002), both dorsal and ventral retinal identity fail to be established in the Lhx2 mutant (see below). It appears, then, that the OV patterning progresses further in the Pax6 mutant, suggesting that there is a more profound and possibly earlier requirement for Lhx2 than for Pax6 in the maturation of the optic neuroepithelium during the OV stage.

The role of Lhx2 in OV patterning

In addition to failing to maintain PAX2 expression, other indicators of ventral identity, such as Vax2 expression and a high level of apoptosis in the optic stalk region, are diminished in the Lhx2 mutant. These findings, along with our observation that PAX6 expression is expanded ventrally, led us to suspect that the OV is dorsalized, as these types of changes correlate with dorsalization in other models [in Vax1; Vax2 double-knockout mice, BF-1 (FoxG1) mutant mice, and zebrafish with perturbed Hh signaling] and in OV cultures treated with BMP4 (Behesti et al., 2006; Ekker et al., 1995; Huh et al., 1999; Macdonald et al., 1995; Mui et al., 2005; Yang, 2004). Surprisingly, Tbx5 and Bmp4, which are markers for dorsal retinal identity, are not expressed in the Lhx2−/− OV, indicating a lack of dorsal identity as well. This outcome is markedly different from that which results from perturbing the expression or activation of other cell-intrinsic factors (Pax6, Pax2, Vax1, Vax2 and Tbx5) that regulate doroventral axis formation, which tends to cause expansion of one side of the axis at the expense of the other (Behesti et al., 2006; Huh et al., 1999; Macdonald et al., 1995; Mui et al., 2005). That dorsal and ventral determinants are lost or never activated in the Lhx2−/− OV supports a model by which Lhx2 contributes to the formation of the entire axis by regulating key determinants on both sides.

Although the role of Lhx2 in regulating dorsoventral polarity is likely to involve several mechanisms, our data reveal a requirement for Lhx2 in extracellular signaling, which is crucial on both sides of the axis (Adler and Canto-Soler, 2007; Chow and Lang, 2001; Yang, 2004). On the ventral side, Lhx2 might couple signaling pathways with their context-dependent targets. For example, Shh signaling emanating from the ventral diencephalon is active in the Lhx2 mutant, and yet, Vax2 and Pax2, which are genetically downstream of Shh signaling (Ekker et al., 1995; Macdonald et al., 1995; Takeuchi et al., 2003), are not expressed. Similarly, Fgf8, which is predicted to be an important signal for ventral identity in zebrafish and Xenopus (Lupo et al., 2005; Shanmugalingam et al., 2000), is expressed in the Lhx2 mutant. Since Shh and Fgf8 do not regulate Lhx2 expression, Lhx2 seems to provide a key factor needed by these signaling pathways to promote ventral identity. Interestingly, reintroduction of BMP7 into Lhx2−/− OV reactivates Pax2 expression, although weakly. Not only does this reveal that the optic neuroepithelium retains some degree of competence to express Pax2, but also indicates that the control by Lhx2 of ventral identity involves BMP signaling, which is consistent with the recent finding that Bmp7 is required for ventral expression of Pax2 and apoptosis in the late OV/early OC (Morcillo et al., 2006).

Several studies have revealed the importance of BMP signaling in establishing dorsal polarity and the neural retina domain in the optic neuroepithelium. BMP signaling in the dorsal OV is required for the expression of Tbx5, Vsx2 and Fgf15 (Behesti et al., 2006; Murali et al., 2005), and Bmp4 misexpression in the ventral OC in chick induces ectopic Tbx5 expression (Koshiha-Takeuchi et al., 2000). Although the absence of detectable Bmp4 expression and signaling combined with the absence of Tbx5, Vsx2 and Fgf15 in the

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**Fig. 7.** Cell-autonomous regulation of VSX2, MITF and PAX2 expression by Lhx2. (A-P) Single-scan confocal images of LHX2, VSX2, MITF, PAX2 and PAX6 expression in mouse E10.5 optic neuroepithelium mosaic for Lhx2. LHX2-negative cells are outlined (dashed line). Images in each row are from the same section and the merged panels show the overlap of LHX2 with each marker. DAPI panels show that areas lacking LHX2 expression do contain cells. In contrast to VSX2, MITF and PAX2, PAX6 expression persisted in LHX2-negative cells. Scale bar: 40 μm.
Lhx2+/− OV indicates a problem with dorsal polarity and retina domain determination, reintroduction of BMP4 in culture did not stimulate their expression. This was not due to problems with the source of BMP4 protein because treatment enhanced Fgf15 expression in wild-type cultures. Rather, in addition to regulating BMP signaling through its effects on ligand expression, Lhx2 might also regulate other factors required for BMP4-mediated signaling. Interestingly, an increase in Fgf15 expression occurred when BMP4 and BMP7 were added simultaneously. These findings suggest that Lhx2, acting through Bmp7, could influence BMP4 signaling. Since Bmp7 expression overlaps with that of Bmp4, it is expressed at the right time and place to play such a role (Morcillo et al., 2006). Whether the enhanced effect of combining Bmp7 and BMP4 in culture is due to extracellular interactions (i.e. overcoming inhibition by BMPR antagonists), or whether BMP7 signaling provides a cell-intrinsic component needed for BMP4 signaling, is an open question. Regardless, our data suggest that Lhx2 acts by more than one mechanism to influence the expression of a battery of genes required for development of the optic neuropithelium. This is further exemplified by our genetic mosaic analysis, which indicates that Vsx2, Mitf and Pax2 (in many cells) require Lhx2 in a cell-autonomous manner. This could reflect a requirement for Lhx2 in BMP signaling at the intracellular level or a requirement for Lhx2 in other mechanisms, such as direct transcriptional regulation, as proposed for Six6 (Tetreault et al., 2008). Resolving these issues requires further study.

Lhx2 in the optic neuroepithelium is required for lens formation

Emergence of the lens from the SE occurs in a series of discrete steps termed competence, bias, specification and differentiation, initiating in the anterior neural plate and extending through OC formation (Donner et al., 2006; Henry and Grainger, 1987; Lang, 2004; Servetnick and Grainger, 1991). Pax6 is expressed in the lens-forming region throughout this process, but its expression is regulated in two temporal phases: preplacodal and placodal (Grindley et al., 1995; Lang, 2004). These phases are demarcated by the appearance of the lens placode, which follows the activation of Sox2 expression, a cell-autonomous factor required for lens specification (Kamachi et al., 1998). Their finding that Pax6 is expressed in the Lhx2+/− SE at stages prior to placode formation, but is not maintained at a time when the lens placode normally forms, suggests that the SE arrests at, or just prior to, specification. This is strongly supported by our observation that Sox2 is not detected in the Lhx2−/− SE.

Although the role of the optic neuroepithelium in lens development has been debated, the prevailing model is that signals from the OV are required at around the time of specification (Furuta and Hogan, 1998). Since conditional deletion of Lhx2 in the SE has no obvious effect on eye development and Lhx2 is expressed in the optic neuroepithelium, we can conclude that Lhx2 regulates lens specification through a non-cell-autonomous mechanism, providing further support for the prevailing model. This distinguishes Lhx2 from other EFTFs, such as Six3, Pax6 and Sox2, which regulate lens formation cell-autonomously (Ashery-Padan et al., 2000; Furuta and Hogan, 1998; Lang, 2004; Liu et al., 2006; Ogin et al., 2008).

FGF-MAPK and BMP signaling, both of which depend on ligands produced in the OV, are required, but are not sufficient on their own, for lens specification (Faber et al., 2001; Furuta and Hogan, 1998; Gotoh et al., 2004). Whereas FGF-MAPK signaling is active in Lhx2+/− SE, BMP signaling is not by E10.25, consistent with the absence of Bmp4 and Bmp7 expression in the optic neuroepithelium. Interestingly, BMP7, or the combination of BMP4 and BMP7, was sufficient to induce Sox2 expression in the SE in Lhx2−/− head cultures, revealing that the SE of the mutant is competent to express Sox2, and that Sox2 expression in the SE depends on Lhx2 through its regulation of Bmp4 and/or Bmp7 expression and signaling. This proposed pathway is supported by the following findings: (1) Lhx2 is expressed in both Bmp7−/− and Bmpr1a; Bmpr1b double-knockout mice, the latter of which lack detectable BMP signaling in the optic neuroepithelium and SE; and (2) Sox2 is not expressed in the SE of Bmp4−/− or Bmp7−/− mice (Furuta and Hogan, 1998; Murali et al., 2005; Wawersik et al., 1999). Thus, the intersection of Lhx2 and BMP signaling extends to the formation of the lens, and our findings lend further support to the model that an interaction with the OV is essential for advancing the SE into the specification step and beyond.

Model for Lhx2 function in early eye development

We propose a model for how Lhx2 fits into the scheme of early eye organogenesis (Fig. 8). Although Lhx2 is activated during eye field specification, its requirement for advancing eye organogenesis does not manifest until after OV formation. Mechanistically, activation of Lhx2 expression is dependent on the EFTFT network (Zuber et al., 2003). The competence of the optic neuroepithelium to respond in a context-specific manner (i.e. the expression of tissue-specific gene targets) to the various signaling pathways (BMP, HH, FGF) is mediated by Lhx2 at the early OV stage. Lhx2 is required to induce or maintain the expression of genes required at the late OV stage for regionalization (Mitf, Vsx2, Pax2), establishment of retinal dorsoventral polarity (Tbx5 and Pax6), retinal progenitor cell properties (Fgf15) and lens specification (Sox2). Lhx2 regulates Vsx2 and Mitf cell-autonomously, whereas Pax2 is regulated by Lhx2 by cell-autonomous and non-cell-autonomous mechanisms. Several of these genes (Pax2, Fgf15, Sox2) are also linked to Lhx2
through its regulation of BMP signaling. This model places Lhx2 firmly at the center of a network that coordinates the development of the OV and SE to ensure the proper development of the OC.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/2/3885/DC1

References


### Table S1. Primer sequences

<table>
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<th>Allele</th>
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| Lhx2   | ACGGGGTCACTCGACGAAATGG (null For.)  
         | TTAAGGGCCAGCTCATTTCC (null Rev.)   
         | GAGTCAGGATTGGGCTCAAG (wt For.)    
         | GGTACCCACTGTACAACAAGC (wt Rev.)   |
| Lhx2f  | ACCGGTGAGGAAGACTTTT (For.)         
         | CAGCGGTCTAGTATGGGOCA (Rev.)       |
| Fgf8f  | TGTGGTTTGAGGAGGATTTAG (common)     
         | CCGTGCCTCCTGAGAGGAT (wt and flox) 
         | CTGGTACACAGCCACCTTCA (null)       |
| Shh<sup>GFP-cre</sup> | Jackson Lab protocol #1073 |
| Rosa26<sup>creER</sup> | Jackson Lab protocol #719 |
| Hes1<sup>ERT2</sup> | GGTCTTACCGGCTACATGC (common)      
                         | CAGCTTGAGAACATGACAGG (mut)        
                         | CTCTAGCTGCTGAGATGTG (wt)          |
| Tg(Le-cre) | TCGATGCAACGAGATGAG (For.)         
               | TCGGCTACATCGTAACAGGG (Rev.)       |
| Rosa26<sup>Z</sup> | Jackson Lab protocol #433 |
| Dbx1 (RT-PCR) | TTTCCGGCCACTCTAGTTTC (For.)       
                         | AACGCTTCTCCAGGCTT (Rev.)         |