Introduction

The vertebrate retina is part of the nervous system that develops through a highly organized process during embryogenesis. During the initial stage of eye development, the eye primordium emerges as an evagination from the forebrain, the optic vesicle (OV), which subsequently invaginates to become the optic cup. The optic cup consists of two layers: the inner and outer layers, which develop into the neural retina (NR) and the retinal pigmented epithelium (RPE), respectively.

The basic molecular mechanisms underlying the initial regionalization in the OV have been elucidated. At the early stage of OV development, TGFβ-like molecules, including activin and BMPs, from the surrounding mesenchyme are thought to promote RPE specification by inducing the expression of microphthalmia-associated transcription factor (Mitf), which is a basic helix–loop–helix gene involved in the acquisition and maintenance of RPE identity (Fuhrmann et al., 2000; Martinez-Morales et al., 2004; Müller et al., 2007). Otx2, which is induced by Mitf, is also required for RPE specification (Martinez-Morales et al., 2001; Martinez-Morales et al., 2003). On the other hand, the surface ectoderm, which is located adjacent to the OV, expresses several Fgf genes that ensure NR development at the distal portion of the OV (Pittack et al., 1997; Hyer et al., 1998; Nguyen and Arnheiter, 2000; Chow and Lang, 2001). Regionalization of the OV into the NR and RPE domains is promoted by FGF signaling and mediated by upregulation of Chx10 expression (Nguyen and Arnheiter, 2000; Rowan et al., 2004), which in turn represses Mitf in the NR region (Rowan et al., 2004; Horsford et al., 2005). Furthermore, the prospective NR itself expresses Fgf genes after contact with the surface ectoderm to regulate the boundary between the NR and RPE by maintaining the Chx10 expression (Müller et al., 2007; Vogel-Höpker et al., 2000; Zhao et al., 2001). The antagonistic interaction between Mitf and Chx10 ensures the differentiation of the RPE and NR during early retinal development (Müller et al., 2007).

While regionalization in the OV and optic-cup morphogenesis are affected by the neighboring tissues, recent work using three-dimensional culture of mouse embryonic stem cells has shown that this process proceeds as a self-organizing activity without any extrinsic molecules (Eiraku et al., 2011). However, little attention has been paid to intrinsic factors that regulate the expression of these retinal specification genes.

Herein, we focus on LIM class homeodomain (LIM-HD) transcription factors. The LIM-HD factor family has been identified in organisms ranging from humans to nematodes, in which it establishes neuronal cell subtype identity (for a review, see Hobert and Westphal, 2000). A LIM-homeobox gene, Lhx1/Lim1,
known for its head organizing activity in mouse (Shawlot and Behringer, 1995), is expressed by a subset of developing motor neurons of the spinal cord and guides its axons along specific trajectories (Tsuchida et al., 1994; Kania et al., 2000). In the developing retina, Lhx1 is required for correct laminar positioning of mouse horizontal cells (Poché et al., 2007), and it contributes to normal eye formation when overexpression converts cells of the prospective RPE into NR. By contrast, interfering with Lhx1 expression at OV stages inhibits NR formation, and in severe cases a pigmented vesicle forms in place of the optic cup. Thus, we provide evidence that during OV stages, Lhx1 in the proximal region of the OV permits NR development and concomitant separation of the OV into the two domains, NR and RPE.

### Materials and Methods

#### Cdiolation

Chicken Lhx1/Lim1 and Lhx2 cDNAs were kindly provided by Thomas Jessell (Columbia University, USA) and Tsutomu Nohno (Kawasaki Medical School, Japan). The EcoRI-cleaved cDNAs from the original vectors were ligated into the pCAGGS expression vector (Niwa et al., 1991). The chicken Lhx5/Lim2 cDNA was isolated from stage 22 head cDNA using PCR primers (5'-atgatgtagcttgcagctggtcgccggcctgcg -3', 5'-ctaccaacacctgcatctcgtggtggg -3') designed based on the public database.

#### Vector construction and in ovo electroporation

Fertilized chicken eggs (Goto Co., Gifu, Japan) were used in this study. Chicken embryos were grown in a humidified incubator at 37.5˚C. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1992; reprint of 1951 paper) and harvested after a specified period of time post-fertilization. For Lhx1 overexpression experiments, Lhx1:pCAGGS (3 mg/ml) and EGFP:pCAGGS (3 mg/ml) vectors were co-electroporated into the right OV at stages 9+ to 10 (1 mg/ml) was electroporated into the right OV at stage 9— When the RNAi or expression vector was electroporated into the OV at stage 12, neither the small eye phenotype nor the ectopic NR formation phenotype was observed (not shown).

#### DNA isolation

Chicken embryo heads were fixed in 4% PFA/PBS for one hour at room temperature. Messenger RNA was extracted from stage 11 (at 7–8 hours after RNAi) embryo heads (supplementary material Fig. S3). To analyze Lhx1-overexpressing retina at stage 29 (96 hours post electroporation), 4 mg/ml of RFP-2A-Lhx1:pCAGGS was used with another pulse generator CUY21Vitrino-EX (BEX, Tokyo, Japan) under a different condition (50 V, poration pulse; 8 V, driving pulse, 2 pulses). For Lhx1-RNAi experiments, we used the pRFPNRAA vector (Das et al., 2006). The Lhx1 or Lhx5 short hairpin RNA (shRNA) target sequences were designed by GenScript (http://www.genscript.com) (Table 1) and constructed according to Das et al. (Das et al., 2006). Briefly, we examined two target sequences against one gene, Lhx1 or Lhx5, (supplementary material Fig. SAA) and used more effective constructs. The Lhx1, Lhx5 or control pRFPNRAA vector (1 mg/ml) was electroporated into the right OV at stage 9—. When the RNAi or expression vector was electroporated into the OV at stage 12, neither the small eye phenotype nor the ectopic NR formation phenotype was observed (not shown).

#### Immunofluorescence, TUNEL staining, and microscopy

Chicken embryo heads were fixed in 4% PFA/PBS for one hour at room temperature. After washing, the tissue was equilibrated in 30% sucrose in PBS containing 0.2% Triton X-100 (PBST), embedded in OCT compound, and 16 μm cryosections were cut. The sections were treated with PBST and incubated with 5% normal goat serum in PBST for 30 minutes. Sections were incubated overnight at 4˚C with primary antibodies, diluted in the blocking reagent. Information about primary antibodies is shown in Table 2. After washing in PBST, the sections were incubated with Cy3- (Jackson ImmunoResearch, 1:1500) or Alexa Fluor 488-conjugated secondary antibody (Invitrogen, 1:750) for three hours at room temperature. After washing, the sections were mounted in Vectashield containing DAPI (Vector Laboratories, UK). For TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining, Click-It TUNEL Alexa Fluor 488 Imaging Assay (Invitrogen) was used. Immunostained sections were analyzed with a confocal laser microscope (Eclipse C1si Confocal, Nikon, Japan or Leica TCS-SP5, Leica, Germany).

#### Quantitative PCR (Q-PCR) analysis

Messenger RNA was extracted from stage 11 (at 7–8 hours after RNAi) embryo heads (supplementary material Fig. S5B) using the QuickPrep Micro mRNA Purification Kit (GE Healthcare). The total number of collected embryos included 22 for Lhx1-1-551 and 29 for Lhx1-1- (supplementary material Fig. SBC). One hundred fifty ng of mRNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Primer sequences for Lhx1 included 5' -AACAGCAGAACATCTGCAAAAGAAAC-3' (forward) and 5' -GGCCCGTTCCCGAGCTCTTT-3' (reverse), for β-actin 5'-GGG-CTCTGTCGTCGAAAT-3' (forward) and 5'-CATACCCAGCTAGCTGTCCTTT-3' (reverse), for Cux1 5’-CTTGAGATCTCGGTCGAC-3’ (forward) and 5’-GAGCCGGTCTCGAGTCGAAGG-3’ (reverse), and for Lhx2 5’-GGCA-

### Table 1. Target or control sequences for RNAi used in this study.

<table>
<thead>
<tr>
<th>Gene/Construct name</th>
<th>Sequences</th>
</tr>
</thead>
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<tr>
<td>Lhx1-1-551</td>
<td>GACCAACATCAAGGCACAAACA</td>
</tr>
<tr>
<td>Lhx1-4-71</td>
<td>AAGGGCCACGCTGTCCGACAA</td>
</tr>
<tr>
<td>Lhx1-Control</td>
<td>CAACAACGGACAGCACAATCACC</td>
</tr>
<tr>
<td>Lhx5-1-382</td>
<td>AGGGACACCTCTACATCTG</td>
</tr>
<tr>
<td>Lhx5-3-1019</td>
<td>AAGGTCACCGGATGATGTC</td>
</tr>
</tbody>
</table>

### Table 2. Antibodies used in this study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Animal</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta3 tubulin</td>
<td>TUIJ</td>
<td>500</td>
<td>mouse IgG</td>
</tr>
<tr>
<td>Hu/C/D</td>
<td>16A11</td>
<td>500</td>
<td>mouse IgG</td>
</tr>
<tr>
<td>Isl1</td>
<td>39.4D5</td>
<td>100</td>
<td>mouse IgG</td>
</tr>
<tr>
<td>Lhx1/Lhx5</td>
<td>4F2</td>
<td>Not diluted</td>
<td>mouse IgG</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>GC-4</td>
<td>1000</td>
<td>mouse IgG</td>
</tr>
<tr>
<td>Phosphorylated histone H3</td>
<td>Ser10</td>
<td>1000</td>
<td>rabbit IgG</td>
</tr>
<tr>
<td>Phosphorylated histone H3</td>
<td>RFP</td>
<td>200</td>
<td>rabbit IgG</td>
</tr>
<tr>
<td>Sox2</td>
<td>Sox2</td>
<td>1000</td>
<td>rabbit IgG</td>
</tr>
<tr>
<td>Visinin</td>
<td>7G4</td>
<td>100</td>
<td>mouse IgG</td>
</tr>
</tbody>
</table>
Lhx5, and Lhx2 are 117 bp, 151 bp, 155 bp, and 123 bp, respectively. Q-PCR by incubation for 10 minutes at 95˚C, followed by 40 cycles of 15 seconds at 95˚C, 7900 Real Time PCR System (Applied Biosystems). AmpliTaq Gold was activated E method.

cDNA from two independent assays was determined using the comparative Ct standard to normalize for variability in mRNA quality, and the amount of input Lhx1 was expressed in the early OV stage to late optic cup stage. At stage 9, Lhx1 is expressed in the proximal region of the early OV (Fig. 1F), although this antibody also detects Lhx5, which is a closely related cognate of Lhx1. Similar to the expression domain of Wnt8b (Fig. 1G), a dorsal diencephalon marker (Garcia-Lopez et al., 2004; Hollyday et al., 1995), Lhx1 was expressed in the dorsal diencephalon as well (Fig. 1E-p). When compared with Pax2 and Rx1 expression domains (Fig. 1H,I), Lhx1 was not expressed in the emerging optic stalk or in the ventro-distal region of the OV. Lhx1 expression in the proximal OV appeared highest at stages 10–11. At stage 13, Lhx1 was expressed in the diencephalon alar region and in subsets of cells in the ventral midline region, which is the prospective hypothalamus (Fig. 1J–L). In later stages, Lhx1 was not expressed in the inner layer of the OV or optic cup until it became expressed by retinal horizontal precursors by stage 24 (Okamoto et al., 2009) (supplementary material Fig. S6B,E). Thus, Lhx1 is expressed in the proximal region of the early OV, the boundary between the diencephalon and OV, and the expression level appeared highest around stage 11.

Overexpression of Lhx1 in the OV induces NR formation from the outer layer of the optic cup

To examine the function of Lhx1, we overexpressed Lhx1 in the OV by in ovo electroporation at stages 9–10. We found that at 24 hours post-electroporation, the outer layer of the optic cup (around stage 15/16) protruded and appeared knotty (Fig. 2C; Fig. 2A as control) (n=23/23). We confirmed marked ectopic expression of Lhx1 in the protruded regions (Fig. 2D; Fig. 2B as control), and induction of Pax6 expression demonstrated that the knotty regions were part of the OV (Fig. 2H; Fig. 2F as control). At stage 24 (after 60 hours), the pigmented epithelium was partly thickened and pigment was lost (Fig. 2J; Fig. 2I as control). To characterize the thickened outer epithelium of the optic cup, the expression of retinal marker genes was examined. Immunostaining of the electroporated eye (stage 24) showed that β3-tubulin and a

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**Fig. 1. Lhx1 is expressed in the proximal region of the OV.** Whole mount in situ hybridization (WISH) of embryos at Hamburger’s and Hamilton’s stages 9 (A,B), stage 10 (C,D), stage 11 (E), and stage 13 (J, A-C,E) Dorsal views. (B,D,J) Lateral views. Transverse sections cut after WISH are shown in (E-a-E-m,E-p). At the middle OV level (E-m), Lhx1 is distinctly expressed in the proximal region of the OV and dorsal diencephalon (di). Outside these regions, Lhx1 is also expressed in the anterior neural ridge (anr) and notochord (nc). (F-L) Immunohistochemistry of Lhx1/Lhx5 protein. Transverse sections at the OV level of stage 11 (F), and stage 13 (L). (G–I) Expression patterns of Wnt8b (G), Pax2 (H), and Rx1 (I) at stage 11. Transverse sections at the OV level are shown for comparison. (J-L) At stage 13, Lhx1 is expressed in the dorsal diencephalon and by a subset of cell in the ventral diencephalon (arrow in J–L), but not in the presumptive neural retina (arrowhead in L). Scale bars: 100 μm.

GATCTGGCAGCCTACAAC -3’ (forward) and 5’- GTTTGAATGATGTCCCTCATACGGTGTG -3’ (reverse). The amplicon sizes for chicken Lhx1, β-actin, Lhx5, and Lhx2 are 117 bp, 151 bp, 155 bp, and 123 bp, respectively. Q-PCR analyses were performed with the SYBR Green PCR Master Mix using the ABI 7900 Real Time PCR System (Applied Biosystems). AmpliTaq Gold was activated by incubation for 10 minutes at 95˚C, followed by 40 cycles of 15 seconds at 95˚C, 30 seconds at 60˚C. Efficiencies of amplification included: 0.942±0.048 for E-Lhx1, 0.993±0.036 for E-β-actin, 0.936±0.054 for E-Lhx5, and 0.877±0.023 for E-Lhx2. Relative gene expression levels were computed using β-actin as the internal standard to normalize for variability in mRNA quality, and the amount of input cDNA from two independent assays was determined using the comparative Ct method.

Quantitative analysis of small eye

Sixty hours after in ovo electroporation of Lhx1-1-551/pRFPRNAi construct or Lhx1-Control/pRFPRNAi, both eyes of the embryos at stage 24 were photographed at the same magnification. The size of the eye was measured by using Photoshop CS4 (Adobe) and the ratio of the right eye area to the left was calculated. The small eye phenotypes were categorized into three classes: 1) Normal, less than 10% reduction, 2) Class I, 10 to 40% reduction, 3) Class II, 41 to 60% reduction, and 4) Class III, more than 61% reduction.

**Results**

Lhx1 is expressed in the proximal region of the nascent OV

As a first step to know the role for Lhx1 during early eye development, we examined the expression pattern of Lhx1 from the early OV stage to late optic cup stage. At stage 9, Lhx1 was expressed in the anterior neural ridge toward the posterior region of the nascent OV (Fig. 1A,B). At stage 10, Lhx1 expression became more distinct in the proximal region of the OV and in the dorsal forebrain (Fig. 1C–E). Immunostaining with anti-Lhx1 antibody verified that Lhx1 protein localized to the proximal region of the OV (Fig. 1F), although this antibody also detects Lhx5, which is a closely related cognate of Lhx1. Similar to the expression domain of Wnt8b (Fig. 1G), a dorsal diencephalon
Fig. 2. Lhx1 overexpression induces a second NR formation from the presumptive RPE. (A,B) In the EGFP-overexpressing control eye, Lhx1 is not expressed at stage 15/16 (24 hours after electroporation). (C,D) In the Lhx1-overexpressing eye, Lhx1 mRNA is strongly detected in the protruding region, as indicated by arrowheads and intense EGFP fluorescence. (E,F) In the EGFP-overexpressing control eye, Pax6 is expressed in the optic cup and lens vesicle. (G,H) In the Lhx1-overexpressing eye, Pax6 is ectopically expressed in the protruding region of the optic cup, as indicated by arrowheads and intense EGFP fluorescence.

Lhx1 is localized to the thickened epithelium (arrow in L) in a mirror-image symmetrical pattern when compared to the authentic retina. (M,N) Immunostaining for phosphohistone-H3 (ph-H3; red), which marks mitotic cells on the apical side of the neuroepithelium. Phosphohistone-H3 is localized to the thickened epithelium (arrow in N) in a mirror-image symmetrical pattern when compared to the authentic retina. In (K–N), EGFP fluorescence is shown in green, and nuclei were stained with DAPI (blue). (O,P) Immunostaining for a neurofilament 3A10 antigen (green), which is an early neuronal differentiation marker. In this experiment, a bicistronic vector, RFP-2A-Lhx1/pCAGGS (P) or a mock vector, RFP-2A/pCAGGS (O) was electroporated. Within 48 hours after electroporation, only RFP-positive cells (red) in the outer layer of the OV are thickened to form rosette that express 3A10 antigen. (P′–P′″) Single-channel confocal images of (P). NR, neural retina; RPE, retinal pigment epithelium; and vit, vitreous. Scale bars: 100 μm.

Lhx1 activates the expression of NR specification genes in ectopic NR formation

We next examined whether ectopic NR formation after Lhx1 overexpression was mediated by expression of NR specification genes such as Rx1, Six3, Six6, Chx10 and Sox2 (Ishii et al., 2009; for a review, see Locker et al., 2009). We found that the expression of all these genes was induced ectopically in the thickened outer epithelium by 24 hours after Lhx1 overexpression (Fig. 4H–L; Fig. 4A–E′ as control). In contrast, the expression of Otx2 and Mitf, which regulate RPE specification, disappeared in this region (Fig. 4M–N′; Fig. 4F–G′ as control).

FGFs secreted from the surface ectoderm and within the OV are known to promote NR formation (for a review, see Nguyen and Arnheiter, 2000; Chow and Lang, 2001; Martinez-Morales et al., 2004). Therefore, it was tempting to determine whether the ectopic NR formation induced by Lhx1 overexpression was mediated by the induction of Fgf expression. Among the 22 FGF
members, Fgf8 is expressed in the distal tip of the chicken OV and the ventral stalk region by stage 12 (Vogel-Höpker et al., 2000; Crossley et al., 2001; Müller et al., 2007). Fgf19 is distinctly expressed in the distal region of the OV at stage 11 (Kurose et al., 2004; Kurose et al., 2005). Therefore, we examined whether the expression of the Fgf genes was induced...
when *Lhx1* was overexpressed in the OV. By 24 hours post-electroporation, the expression of *Fgf8* was not induced in the protruded region of the outer optic cup (not shown). In contrast, *Fgf19* expression was ectopically induced in the protruded region of the optic cup (Fig. 5A,A’,D; Fig. 5C,C’ as control) (*n*=4/5). As it was reported that *Fgf8* expression in the central retina triggers the wave of retinal ganglion cell (RGC) differentiation (Martinez-Morales et al., 2005), we examined whether *Fgf8* expression was induced at later stages. Following 48 hours of *Lhx1* overexpression, *Fgf8* expression was induced in the center of the second NR (Fig. 5E; Fig. 5E as control), suggesting that ectopically induced *Fgf8* likely triggered the second RGC differentiation.

*Lhx5, but not Lhx2, can substitute for the function of Lhx1 in ectopic NR formation*

We next asked whether the effect of *Lhx1* overexpression on the OV is specific to *Lhx1* or redundant among LIM-HD factors (Fig. 6A). As another LIM-HD member, *Lhx2*, is expressed in the OV at stage 11 (Fig. 6B–D) (Nohno et al., 1997) and has been shown to contribute to OV invagination (Porter et al., 1997), we overexpressed *Lhx2* in the chicken OV to determine its effect. We found that overexpression of *Lhx2* did not induce protrusion of the optic cup (Fig. 6F). In contrast, overexpression of *Lhx5* (*Lim2*), which is in the same paralogue group as *Lhx1* (*Lim1*) (Fig. 6A) (Hobert and Westphal, 2000), induced a marked protrusion of the optic cup at 24 hours post-electroporation (Fig. 6G). Histological and molecular analyses showed that NR formation was partly observed in the outer layer of the optic cup, similar to that observed following *Lhx1* overexpression (Fig. 6I–L).

To determine whether *Lhx5* is expressed during early eye development, the expression pattern of *Lhx5* was examined in the OV stage. At stage 9, chicken *Lhx5* was already expressed intensely in the anterior neural ridge (ANR) toward the posterior proximal region of the OV (Fig. 6M,N). At stages 10–11, *Lhx5* expression was confined to the ANR and dorsal forebrain, (Fig. 6O–Q,Q-a), and the proximal region of the OV (Fig. 6Q-m,Q-p). Although the overall expression pattern of *Lhx5* resembled that of *Lhx1*, the level of *Lhx5* expression in the forebrain appeared much higher than that of *Lhx1* (compare Fig. 6M–Q with Fig. 1A–E). To characterize genetic interactions between *Lhx1* and *Lhx5*, we carried out experiments to determine whether *Lhx1* overexpression could induce *Lhx5* expression, and vice versa. We found that *Lhx1* overexpression did not induce *Lhx5* expression (not shown), whereas *Lhx5* overexpression could induce *Lhx1* expression in the OV at 24 hours post-electroporation (*n*=3/3) (Fig. 6R–T). These results showed that *Lhx1* expression is positively regulated by its most related cognate, *Lhx5*, during early chicken eye development.

**Decreased Lhx1 expression inhibits NR formation and optic cup morphogenesis**

To further clarify the role of *Lhx1* during early eye development, we downregulated *Lhx1* expression by *in ovo* RNAi with an shRNA vector (Das et al., 2006). When the *Lhx1*-RNAi vector was introduced at stages 9 to 10, the size of the eye was decreased (Fig. 7D,E; Fig. 7F as control), and small eye phenotype was observed in about 60 percent when using the *Lhx1*-1-551 RNAi-construct (Fig. 7M) (*n*=37). This RNAi construct was targeted against the 5’-region of the homeobox (supplementary material Fig. S5A). The other RNAi constructs, which were targeted outside the homeobox, also induced mild small eye phenotypes (*Lhx1*-N-471) (Fig. 7G,H; Fig. 7I as control). A control RNAi construct (*Lhx1*-Control) that produced a scrambled version of the *Lhx1*-1-551 RNA had no effect on eye development (Fig. 7J,K; Fig. 7L as control). *Lhx5* overexpression did not induce cell death in the site where the RNAi vector was injected. A control vector (supplementary material Fig. S5B,C). Since it was reported that apoptosis could be induced after RNAi in early chicken embryos (Mende et al., 2008), we performed TUNEL staining to exclude the possibility that cell death causes small eyes or disturbs eye development. We confirmed that no ectopic cell death was observed in the site where the RNAi vector was injected.

![Fig. 5. Lhx1 overexpression induces ectopic Fgf gene expressions.](http://bio.biologists.org/)

(A–E') Expression of *Fgf19* (A,A’,C,C’) and EGFP fluorescence (B,B’) was examined at 24 hours post-electroporation. (A’,C’) High magnification of (A,C), respectively. *Lhx1*-overexpressing (A,B,A’,B’) and contralateral (C,C’) eyes. (D) Section of the optic cup after *Lhx1*-overexpression, showing ectopic induction of *Fgf19*. (E,E’) Expression of *Fgf8* was examined by *in situ* hybridization in the eye overexpressing *Lhx1* at 48 hours post-electroporation. Control (E), and in (E’) arrowhead shows ectopic induction of *Fgf8* in the center of the second NR. Scale bar: 50 µm.
introduced while endogenous apoptosis was detected in the hindbrain region as reported (supplementary material Fig. S7A,B; supplementary material Fig. S7C,D as control) (Graham et al., 1993).

At stage 24, the normal eye primordium reaches the optic cup stage: the inner layer of the optic cup forms a thickened pseudostratified NR, while the outer layer of the optic cup develops into a single-cell layered pigmented epithelium (Fig. 8A,B). Histological examination of the severe small eye case after Lhx1-RNAi (stage 24) showed that optic cup formation was perturbed and that there was no NR formation from the inner layer. The vesicle consisted of pseudostratified pigmented cells, that is a pigmented vesicle (Fig. 8C–E). Furthermore, in situ hybridization after Lhx1-RNAi (stage 15/16) showed lower expression of Chx10 (Fig. 8J; Fig. 8F as control). In contrast, Otx2 expression expanded to the inner layer of the optic cup, which usually develops into the NR, after Lhx1-RNAi (Fig. 8K; Fig. 8G as control). The expression of RPE marker genes, Mitf and Mmp115, was induced in the neuroepithelium abutting the lens.

Fig. 6. Differential effects of Lhx2 and Lhx5 on the OV and genetic interactions between Lhx1 and Lhx5. (A) Phylogenetic tree of chicken LIM-homeodomain (LIM-HD) factors. The LIM-HD factors are depicted by the NJ (neighbor-joining) method. Chicken LMO3 was used as an outgroup. The scale bar is calibrated in substitutions per site. Numbers show bootstrap confidence values. Amino acid sequences used in the tree construction were deduced from the nucleotide sequences listed in supplementary material Table S2. (B–D) Lhx2 is expressed in the forebrain and whole OV at stage 11. Dorsal (B) and lateral (C) views. (D) Transverse section of the OV. (E–H) EGFP fluorescence images at 24 hours post-electroporation. Overexpression of Lhx2 (F) did not induce transformation of the outer optic cup to the NR, whereas overexpression of Lhx5 (G) or Lhx1 (H) induced a protrusion of the OV. (I,J) Histology of an Lhx5-overexpressing retina at stage 24. A second NR transformed from the outer layer of the optic cup. (K,L) In situ hybridization of an Lhx5-overexpressing retina at stage 21. Arrows show activation of Chx10 (K) and suppression of Otx2 (L) in the developing second NR. (M–Q) Whole mount in situ hybridization (WISH) of embryos at Hamburger’s and Hamilton’s stages 9 (M,N), stage 10 (O,P), and stage 11 (Q). (M,O,Q) Dorsal views. (N,P) Lateral views. Transverse sections cut after WISH are shown in (Q-a,Q-m,Q-p). At the middle OV level (Q-m), Lhx5 is distinctly expressed in the proximal region of the OV, while it is expressed in the ventral diencephalon at the posterior OV level (Q-p). (R–T) EGFP fluorescence (R) and in situ hybridization of Lhx1 (S,T) at 24 hours post-electroporation. Lhx1 expression is ectopically induced in the protruding outer layer of the optic cup after Lhx5 overexpression (arrowhead in S,T). Scale bars: 100 μm.
vesicle, indicating that the prospective NR region might be transformed to differentiate into RPE following Lhx1-RNAi (Fig. 8L,M; Fig. 8H,I as control). These results indicate that decreased Lhx1 expression promotes RPE differentiation while inhibits NR development, and thus Lhx1 in the proximal region of the OV is required for proper development of the OV into the optic cup.

We also performed RNAi against Lhx5 to discriminate the role for Lhx1 and Lhx5, but so far neither of the two constructs targeting against Lhx5 has caused marked small eyes (not shown). It is conceivable to think that the Lhx5-RNAi was ineffective because of the intense expression of Lhx5 from the earlier stages than Lhx1. In contrast, Lhx1-RNAi inevitably induced small eye phenotypes. Since exogenous Lhx5 induced Lhx1 expression (Fig. 6S,T), it is likely that ectopic NR formation after Lhx5 overexpression is mediated by Lhx1. Taken together, the ability to induce ectopic NR formation was mediated by expression of NR specification genes (Rx1, Six3, Six6, Chx10, Sox2) at least in early stages but also likely mediated by induction of unidentified secreted factors. This function of Lhx1 could be substituted by overexpression of Lhx5, which is in the same paralogue group as Lhx1. Cells with an RNAi-mediated decrease in Lhx1 expression in this region induced a small eye or a vesicle that differentiated into pseudostratified pigmented epithelium. These studies suggest that Lhx1 in the proximal region of the OV permits NR development and eventual separation of the two retinal domains.

It was suggested that the ventral portion of the OV is required for the development of the NR (Uemono et al., 2002; Hirashima et al., 2008). Our study has shown that Lhx1 is normally expressed in the proximal OV, (Fig. 1A,C,E), but not in the

**Discussion**

The vertebrate OV must be subdivided into regions that differentiate into the NR and RPE. Here we have found that a LIM-HD transcription factor Lhx1 is expressed in the proximal region of the OV. When Lhx1 was overexpressed in the OV, an ectopic NR formed from the outer layer of the optic cup, which usually develops into the RPE. The ability to induce NR formation was mediated by expression of NR specification genes (Rx1, Six3, Six6, Chx10, Sox2) at least in early stages but also likely mediated by induction of unidentified secreted factors. This function of Lhx1 could be substituted by overexpression of Lhx5, which is in the same paralogue group as Lhx1. Cells with an RNAi-mediated decrease in Lhx1 expression in this region induced a small eye or a vesicle that differentiated into pseudostratified pigmented epithelium. These studies suggest that Lhx1 in the proximal region of the OV permits NR development and eventual separation of the two retinal domains.

It was suggested that the ventral portion of the OV is required for the development of the NR (Uemono et al., 2002; Hirashima et al., 2008). Our study has shown that Lhx1 is normally expressed in the proximal OV, (Fig. 1A,C,E), but not in the

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**Fig. 7. Lhx1-RNAi reduces the eye size.** (A–L) Lhx1-RNAi resulted in a small eye phenotype at stage 24. Electroporated right eyes are shown in the left two columns, and contralateral left eyes are shown in the right column. RFP fluorescence images are shown in the middle column. All images are shown in the same magnification. (M) Percentage of embryos with small eye phenotypes, Class I to Class III (see Materials and Methods), at stage 24. Scale bar: 300 μm.
hybridization was performed under the same conditions for controls and

ventral OV and nevertheless the overexpression of \textit{Lhx1} results in ectopic NR formation and reduction of \textit{Lhx1} mRNA perturbs NR formation. Although this study shows that the proximal region of the OV is essential for NR development, how the very restricted expression of \textit{Lhx1} in the optic vesicle can have such an influence on eye development is unsolved here. On the other hand, when \textit{Lhx1} expression is downregulated by RNAi, the promoting effect of \textit{Lhx1} on NR formation is compromised, and expression of \textit{Chx10} and other NR specification genes are perturbed, which leads to dominant expression of \textit{Mitf}/\textit{Otx2}, resulting in the formation of a pigmented vesicle in severe cases.

This study has focused on the role for a LIM-HD factor, \textit{Lhx1} in early eye development. There are 12 LIM-HD-containing genes in vertebrates (Fig. 6A), and most of them are involved in neural development, such as specification of motor neuron subtypes (Hobert and Westphal, 2000; Jessell, 2000). With regard to eye development, \textit{Lhx2} is expressed in the prospective retina field (supplementary material Fig. S9), the OV, and the NR (Xu et al., 1993; Tétreault et al., 2009) and essential for progression of the OV to the optic cup (Porter et al., 1997). Recent work has further shown that \textit{Lhx2} is required for dorso-ventral patterning and regionalization of the OV in mice (Yun et al., 2009). However, overexpression of \textit{Lhx2} did not induce NR formation from the prospective RPE in the chicken, indicating that the role for \textit{Lhx1} in early eye development is distinct from that of \textit{Lhx2}. \textit{Lhx1} and \textit{Lhx5} proteins belong to the LIN-11 group of the LIM-HD family (Hobert and Westphal, 2000). The LIM-HD factor consists of two protein-interacting LIM domains, LIM1 and LIM2, and a DNA-binding HD. The chicken \textit{Lhx1} and \textit{Lhx5} proteins exhibit more than 90% amino acid identity in all three domains, where \textit{Lhx1} and \textit{Lhx2} exhibit about 50% amino acid identity (supplementary material Fig. S8). This suggests that target genes and binding partners of \textit{Lhx1}/5 and \textit{Lhx2} may be different, and thus their functions are not compatible. \textit{Lhx1} was

any case, given that we observed formation of the second neural retina in large areas even after ectopic expression of \textit{Lhx1} was shut off (Fig. 3), indirect, non-cell autonomous mechanisms are also crucial downstream of \textit{Lhx1}, which should be clarified by future studies.

Within 24 hours, \textit{Lhx1} overexpression sustained expression of NR specification genes in addition to \textit{Chx10}, such as \textit{Rx1}, \textit{Six3}, \textit{Six6}, and \textit{Sox2}. \textit{Sox2} overexpression does not induce the expression of \textit{Rx1}, \textit{Six6}, or \textit{Chx10} (Ishii et al., 2009). In contrast, \textit{Lhx1} induces the expression of all of these NR specification genes. It is noteworthy that \textit{Lhx1} induces both of the two transcriptional NR gene cassettes: \textit{Rx1-Six6-Chx10} and \textit{Sox2-Six3}. We therefore propose that \textit{Lhx1} primarily permits NR development by activating the two transcriptional NR cassettes, and eventually defines the RPE domain through indirect negative regulation of \textit{Mitf}/\textit{Otx2}. Since \textit{Lhx1} expression in the proximal region of the OV is rather transient, it seems that \textit{Lhx1} activates the NR cassettes as well as unidentified secreted factors and then the induced genes proceed with NR formation in place of \textit{Lhx1}. For example, \textit{Sox2} is initially expressed in the proximal region of the nascent OV (Ishii et al., 2009) and continues to be expressed in the proximal OV and in the ventral NR domain. Since \textit{Sox2} expression is induced in the ectopic NR by \textit{Lhx1} overexpression, it is likely that in the ventral proximal region, \textit{Lhx1} initiates neural differentiation by sustaining the expression of \textit{Sox2}. After this stage, \textit{Sox2}-expressing domain becomes NR as \textit{Lhx1} also activates \textit{Rx-Six6-Chx10} cassette, while the RPE differentiates from the \textit{Sox2}-negative dorsal domain.

On the other hand, when \textit{Lhx1} expression is downregulated by RNAi, the promoting effect of \textit{Lhx1} on NR formation is compromised, and expression of \textit{Chx10} and other NR specification genes are perturbed, which leads to dominant expression of \textit{Mitf}/\textit{Otx2}, resulting in the formation of a pigmented vesicle in severe cases.

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**Fig. 8.** \textit{Lhx1}-RNAi perturbs NR formation. (A–E) Hematoxylin-Eosin staining at stage 24. (B) Magnified view of the posterior retina in (A); distinct pigmentation is observed on the basal side of the single-layered RPE. (C) Small eye phenotype after \textit{Lhx1}-RNAi, in which a pigmented vesicle (pv) forms and lens (le) development is perturbed. (D) The optic epithelium abutting the lens. (E) Posterior optic epithelium with distinct polarized pigmentation. (F–M) Expression of \textit{Chx10}, \textit{Otx2}, \textit{Mitf}, and \textit{Mmp115}. Eyes were examined at 24 hours (F,G,J,K), 36 hours (H,L) or 60 hours post-electroporation (LM). In situ hybridization was performed under the same conditions for controls and \textit{Lhx1}-RNAi, depending on the probes. (F,LM) \textit{Chx10} expression in the inner layer of the optic cup is reduced (arrowhead). (K) The \textit{Otx2} expression domain is expanded to the inner layer of the optic cup (arrowhead). (L) \textit{Mitf} expression is detected in the inner layer as well as outer layer of the optic cup. (M) \textit{Mmp115} expression is detected in the dorsal epithelium of the OV (arrowheads) and abutting the lens vesicle (arrow). The axis of the eye is depicted as follows: A, anterior; D, dorsal; P, posterior; V, ventral; nr, neural retina; and rpe, retinal pigmented epithelium. Scale bars, 100 μm (A,C), 10 μm (B,D,E).
originally identified as a gene expressed in the *Xenopus* organizer (Taira et al., 1994), and mouse *Lhx1* is expressed in the anterior mesendoderm (i.e. the head organizer) (Shawlot and Behringer, 1995). It was reported that expression of mouse *Lhx1* in the telencephalon and diencephalon is initiated by E10.5, but the *Lhx1* expression pattern associated with early OV development was not described previously (Fujii et al., 1994). Since *Lhx1* null mice exhibit a headless phenotype, whether mouse *Lhx1* functions during early OV development or not must be clarified by future studies using conditional inactivation of *Lhx1*.

Murine *Lhx5* is expressed in the forebrain and mesencephons, the expression domains of which exhibit significant overlap but also differences relative to those of *Lhx1* (Sheng et al., 1997). This is true for the chicken orthologs. Although the onset of mouse *Lhx5* expression in the prospective forebrain occurs earlier and the expression is more intense than *Lhx1* expression, *Lhx5* null mice exhibit much milder brain defects in hippocampal development, possibly due to functional compensation by *Lhx1* (Zhao et al., 1999). In contrast, *Lhx1* null mice exhibit a loss of *Lhx5* expression in the forebrain (Sheng et al., 1997), indicating that *Lhx1* is required for *Lhx5* expression and may be genetically upstream of *Lhx5* in mouse. However, *Lhx5* appeared to act upstream of *Lhx1* in the chicken, as *Lhx5* induced *Lhx1* expression but the opposite was not true. Despite these differences, it is noteworthy that there are genetic interactions between *Lhx1* and *Lhx5* in both species.

It is known that NR specification genes such as *Rx1, Chx10, Six6* and *Six3* referred in this study are also expressed in the ciliary marginal zone, where a certain type of retinal stem cells reside (for a review, see Locker et al., 2009), while *Sox2* is expressed by human Müller stem cells and required for its survival and maintenance of progenicity (Bhatia et al., 2011). Thus, the expression of these genes is thought to be molecular fingerprints of retinal stem cells. This study shows a LIN-11 class homeobox gene *Lhx1* in early eye development 1092

**Competing Interests**

The authors have no competing interests to declare.

**References**


