Lhx1 in the proximal region of the optic vesicle permits neural retina development in the chicken

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Summary
How the eye forms has been one of the fundamental issues in developmental biology. The retinal anlage first appears as the optic vesicle (OV) evaginating from the forebrain. Subsequently, its distal portion invaginates to form the two-walled optic cup, which develops into the outer pigmented and inner neurosensory layers of the retina. Recent work has shown that this optic-cup morphogenesis proceeds as a self-organizing activity without any extrinsic molecules. However, intrinsic factors that regulate this process have not been elucidated. Here we show that a LIM-homeobox gene, Lhx1, normally expressed in the proximal region of the nascent OV, induces a second neurosensory retina formation from the outer pigmented retina when overexpressed in the chicken OV. Lhx2, another LIM-homeobox gene supposed to be involved in early OV formation, could not substitute this function of Lhx1, while Lhx5, closely related to Lhx1, could replace it. Conversely, knockdown of Lhx1 expression by RNA interference resulted in the formation of a small or pigmented vesicle. These results suggest that the proximal region demarcated by Lhx1 expression permits OV development, eventually dividing the two retinal domains.

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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 (Tsushida 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 subtype-specific neurite morphogenesis of horizontal cells in chicken (Suga et al., 2009). However, a role for Lhx1 in early eye development has not yet been studied.

In this study, we show that Lhx1 is expressed in the proximal region of the nascent OV. Gain-of-function experiments show that Lhx1 is sufficient to elicit NR development in vivo. Lhx1 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

#### cDNA isolation

Chicken Lhx1/Lmx1 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/Lmx2 cDNA was isolated from stage 22 head cDNA using PCR primers (5′-atgatgtgcattgtgcgggctgcg-3′, 5′-ctacaacacctgctgectg -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 unless otherwise stated. As shown in supplementary material Fig. S1A, a platinum tungsten needle (CUY614T; Unique Medical Imada) was used as a cathode, which was inserted into the lumen of the right OV. After the DNA solution (70 nl) with fast green (0.1%) was injected into the OV, electric pulses (7 V, 30-millisecond duration, 50-millisecond interval, 2 pulses) were applied, using the pulse generator CUY21EDIT (BEX, Tokyo, Japan). We also constructed a 2A peptide mediated bicistronic expression vectors for RFP and Lhx1, verifying simultaneous expression of RFP and Lhx1 (Trichas et al., 2008) (supplementary material Figs S1B, S2). Using this vector, we confirmed that the ectopic NR was induced by overexpression of Lhx1 in the RFP-positive outer layer of the optic cup, while normal eye formation was observed when using an RFP-2A peptide construct without Lhx1 (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 CUY21Viro-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 pRFPRNAiA 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. S5A) and used more effective constructs. The Lhx1, Lhx5 or control/pRFPRNAi 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). After 24, 36, 48, 60, and 96 hours of incubation, the chicken embryos were harvested and processed for further analysis.

#### RNA in situ hybridization (ISH)

Digoxigenin-labeled RNA probes were synthesized according to standard procedures. After fixation in 4% PFA in PBS containing 0.1% Tween 20, the embryos were dehydrated and stored in 100% methanol at −20 °C until further analysis. All experimental steps for whole mount ISH were carried out using an automated ISH apparatus (HS-5100; Aloka, Tokyo, Japan), except pre-staining and staining steps. For selected embryos, frozen sections (10 μm) were prepared after ISH to further examine the gene expression domains. The ISH experiments with sections were carried out manually according to standard procedures. Information about ISH probes is shown in supplementary material Table S1.

#### 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-Control (supplementary material Fig. S5C). 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′-AACAGCAATACTGCGCAAAGAAAC-3′ (forward) and 5′-GGCGGCTTGGGACTCTTT-3′ (reverse), for β-actin 5′-GGG-CCTGCTTGGGACTT-3′ (forward) and 5′-CATACCCACACGGCGTCTTT-3′ (reverse), for Lhx1 5′-TCTGAGATGCTTGGCTGAC-3′ (forward) and 5′-GACCCAGTCTGTGAGTTGACG-3′ (reverse), and for Lhx2 5′-GGCA-

| Gene/Construct name | Sequences |
|---------------------|-----------|
| Lhx1-1-551          | GACCCACCATCAAGGCCAACA |
| Lhx1-1-471          | AAGGCCAACAGTGTCGGCACA |
| Lhx1-Control        | CAACCCGGACAGCAGACCTAACC |
| Lhx5-1-382          | AAGGCCACGCTCAACTCAGTGT |
| Lhx5-3-1019         | AAGGTACAGGGATATGATCTC |

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

| Antigen     | Antibody | Dilution | Animal | Origin         |
|-------------|----------|----------|--------|----------------|
| Beta3 tubulin | TUJ1     | 500      | mouse IgG | Covance        |
| Hu/C/D      | 16A11    | 500      | mouse IgG | Invitrogen     |
| Isl1        | 39.4D5   | 100      | mouse IgG | DSHB           |
| Lhx1/Lhx5   | 4F2      | Not diluted | mouse IgG | DSHB           |
| N-cadherin  | GC-4     | 1000     | mouse IgG | Sigma          |
| Phosphorylated histone H3 | Ser10 | 1000 | rabbit IgG | Rockland Immunochemicals |
| RF          | RF       | 200      | rabbit IgG | Millipore     |
| Sox2        | Sox2     | 1000     | mouse IgG | DSHB           |
| Visinin     | 7G4      | 100      | mouse IgG | DSHB           |

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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 analyses were performed with the SYBR Green PCR Master Mix using the ABI

Expression of Lhx1, which is expressed in the ventral diencephalon (arrow in J–L), but not in the presumptive neural retina (arrowhead in L). Scale bars: 100 µm.

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 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.
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. (I,J) Hematoxylin-Eosin staining at stage 24 (60 hours after electroporation). Control EGFP-overexpressing (I) and Lhx1-overexpressing retina (J), where the outer layer of the optic cup is partly thickened (arrow). (K,L) Immunostaining for β3-tubulin (red), which is localized on the vitreal side of the neural retina. Beta3-tubulin is localized to the thickened epithelium (arrow in L) 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.

Fig. 3. Immunostaining for neural differentiation makers of the stage 29 optic cup. Immunoreactive signals are shown in green or red. Control (A,C,E,G,I,K) and Lhx1-overexpressing eyes (B,D,F,H,J,L). In these experiments, a bicistronic vector of pCAGGS-RFP-2A-Lhx1 was used. (A,B) 3A10, (C,D) N-cadherin, (E,F) HuC/D RNA-binding protein, (G,H) Islet1, (I,J) visinin (calcium-binding protein), and (K,L) AP2α. N-cadherin is localized to the neuroepithelium, visinin is localized to future cone photoreceptors, and others are localized to early retinal ganglion cells, and AP2α is localized to differentiating amacrine cells. Note that induced neural retina (iNR) is thicker than the authentic NR in this experimental condition. Scale bar: 100 μm.

Fig. 4. Lhx1 overexpression activates NR specification genes and suppresses RPE specification genes in the presumptive RPE. Expression of Rx1 (A,A’H,H’), Sox3 (B,B’L,L’), Six6 (C,C’J,J’), Chx10 (D,D’K,K’), Sox2 (E,F,G,G’), Otx2 (F,F’,M,M’), and Mitf (G,G’,N,N’). Frontal sections were shown. Eyes were examined at stage 15/16 (24 hours post-electroporation). Contralateral (A–G’) and Lhx1-overexpressing (H–N’) eyes are shown. (A–N’) High magnification of (A–N), respectively. Arrows in (H–N’) indicates Lhx1-overexpressing cells, as confirmed morphologically as thickened epithelial areas. Results were obtained by in situ hybridization, except for Sox2 by immunostaining. Scale bars: 100 μm.
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) (**n=28**). Quantitative RT-PCR analysis at 7–8 hours after RNAi (stage 11) showed that the level of Lhx1 mRNA in the head was decreased by approximately 44% with the Lhx1-1-551 RNAi relative to the 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.** (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 expression in the inner layer of the optic cup (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.
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 from presumptive RPE is characteristic to the Lhx1/Lhx5 group of the LIM-HD family. Collectively, the results of the current study provide the first report of a novel role for Lhx1 in early chicken eye development, which is distinct from that reported for Lhx2.

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

Within 24 hours, Lhx1 overexpression sustained expression of NR specification genes in addition to Chx10, such as Rx1, Six3, Six6, and Sox2. Sox2 is known to regulate retinal neural progenitor competence and its sustained expression in the presumptive RPE induces formation of a second NR mediated by ectopic expression of Six3 (Taranova et al., 2006; Ishii et al., 2009). However, Sox2 overexpression does not induce the expression of Rx1, Six6, or Chx10 (Ishii et al., 2009). In contrast, Lhx1 induces the expression of all of these NR specification genes. It is noteworthy that Lhx1 induces both of the two transcriptional NR gene cassettes: Rx1-Six6-Chx10 and Sox2-Six3. We therefore propose that Lhx1 primarily permits NR development by activating the two transcriptional NR cassettes, and eventually defines the RPE domain through indirect negative regulation of Mitf/Otx2. Since Lhx1 expression in the proximal region of the OV is rather transient, it seems that Lhx1 activates the NR cassettes as well as unidentified secreted factors and then the induced genes proceed with NR formation in place of Lhx1. For example, 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 Sox2 expression is induced in the ectopic NR by Lhx1 overexpression, it is likely that in the ventral proximal region, Lhx1 initiates neural differentiation by sustaining the expression of Sox2. After this stage, Sox2-expressing domain becomes NR as Lhx1 also activates Rx-Six-Chx10 cassette, while the RPE differentiates from the Sox2-negative dorsal domain.

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

This study has focused on the role for a LIM-HD factor, 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, 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 Lhx2 is required for dorso-ventral patterning and regionalization of the OV in mice (Yun et al., 2009). However, overexpression of Lhx2 did not induce NR formation from the prospective RPE in the chicken, indicating that the role for Lhx1 in early eye development is distinct from that of Lhx2. Lhx1 and 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 Lhx1 and Lhx5 proteins exhibit more than 90% amino acid identity in all three domains, whereas Lhx1 and Lhx2 exhibit about 50% amino acid identity (supplementary material Fig. S8). This suggests that target genes and binding partners of Lhx1/5 and Lhx2 may be different, and thus their functions are not compatible. Lhx1 was
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 hustle 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 mesonephros, 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 in the ciliary marginal zone, where a certain type of retinal stem cells also reside (for a review, see Locker et al., 2009), while Sox2 ciliary marginal zone, where a certain type of retinal stem cells also reside. Lhx1 and the zona limitans at the 10-somites stage in chick embryos. Dev. Bio. 268, 514-530.

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