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

Takumi Kawaue[1], Mayumi Okamoto[1], Akane Matsuyo[1], Junji Inoue[1], Yuhki Ueda[1], Sayuri Tomonari[1], Sumihare Noji[1] and Hideyo Ohuchi[1,2,*]

[1]Department of Life Systems, Institute of Technology and Science, The University of Tokushima Graduate School, 2-1 Minami-Josanjima-cho, Tokushima 770-8506, Japan
[2]Department of Cytology and Histology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama City, 700-8558, Japan

*Author for correspondence (hohuchi@okayama-u.ac.jp)

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.

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; Martínez-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; Martínez-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*,

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

Gene/Construct name	Sequences
Lhx1-1-551	GCACCACCATCAAAGCCAAACA
Lhx1-N-471	AAGCGCCAACGTGTCCGACAA
Lhx1-Control	CAACACCGAGACACAACTAACC
Lhx5-1-382	AAGGCAGCCTCAACTCAGTGT
Lhx5-3-1019	AAGGTACACGGATATGATCTC

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 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/Lim1* and *Lhx2* cDNAs were kindly provided by Thomas Jessell (Columbia University, USA) and Tsutomu Nohno (Kawasaki Medical School, Japan). The *Eco*RI-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′-atgatggtgcattgtgcgggctgcg -3′, 5′- ctaccacaccgctgcctcgctgagc -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℃. 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 wire electrode (1 mm long, CUY611P3-1; Unique Medical Imada, Miyagi, Japan), which acts as an anode, was placed lateral to the optic vesicle. A sharpened tungsten needle (CUY614T; Unique Medical Imada) was used as a cathode. 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 CUY21Vitro-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℃ 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℃ 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′-AACAGCAATACTGCCAAAGAAAAC-3′ (forward) and 5′-GGCGCTTTCCGAGTCCTT-3′ (reverse), for *β-actin* 5′-GCG-CTCGTTGTTGACAAT-3′ (forward) and 5′-CATCACCAACGTAGCTGTCT-TT-3′ (reverse), for *Lhx5* 5′-TCTGAGATGCTTGGCTCGAC-3′ (forward) and 5′-GAGCCAGGTCCTGAGTTCTGAAGG-3′ (reverse), and for *Lhx2* 5′- GGCA-

Table 2. Antibodies used in this study.

| Antigen	Antibody	Dilution	Animal	Origin
Beta3 tubulin	TUJ1	500	mouse IgG	Covance
HuC/D	16A11	500	mouse IgG	Invitrogen
Islet1	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	Upstate
RFP	RFP	200	rabbit IgG	Rockland Immunochemicals
Sox2	Sox2	1000	rabbit IgG	Millipore
Visinin	7G4	100	mouse IgG	DSHB
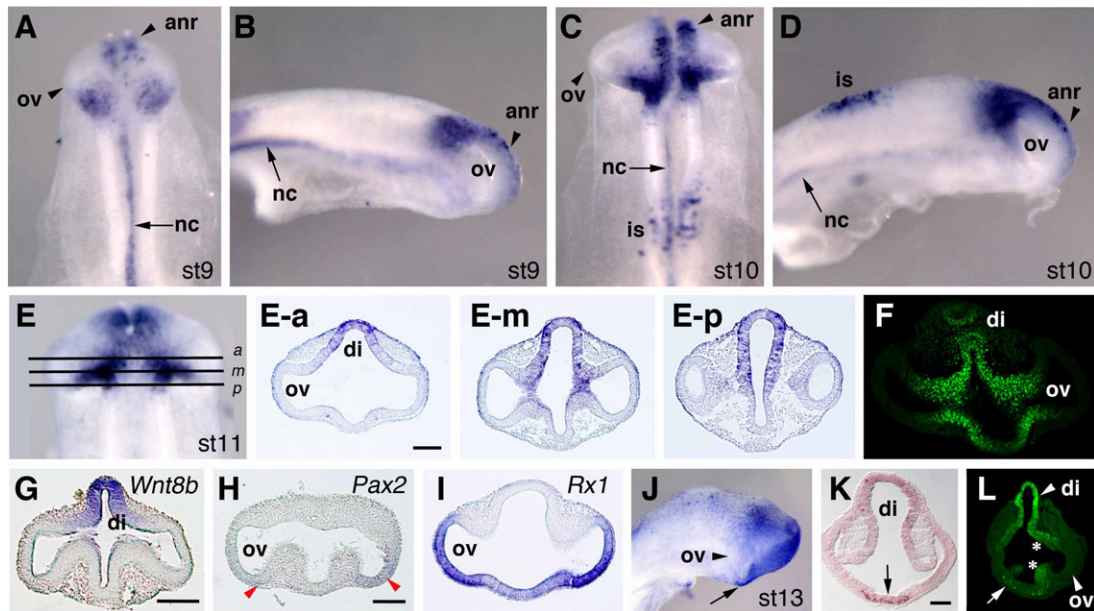
Fig. 1. *Lhx1* is expressed in the proximal region of the OV. Whole mount *in situ* hybridization (WISH) of embryos at Hamburger's and Hamilton's stages 9 (**A,B**), stage 10 (**C,D**), stage 11 (**E**), and stage 13 (**J**). (A,C,E) Dorsal views. (B,D,J) Lateral views. Transverse sections cut after 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′- GTTTGAATGATGTCCT-CATACGCTTTG -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℃, followed by 40 cycles of 15 seconds at 95℃, 30 seconds at 60℃. Efficiencies of amplification included: 0.942 ± 0.048 for E_{cLhx1}, 0.993 ± 0.036 for $E_{β\text{-}actin}$, 0.936 ± 0.054 for E_{cLhx5}, and 0.877 ± 0.023 for E_{cLhx2}. 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

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
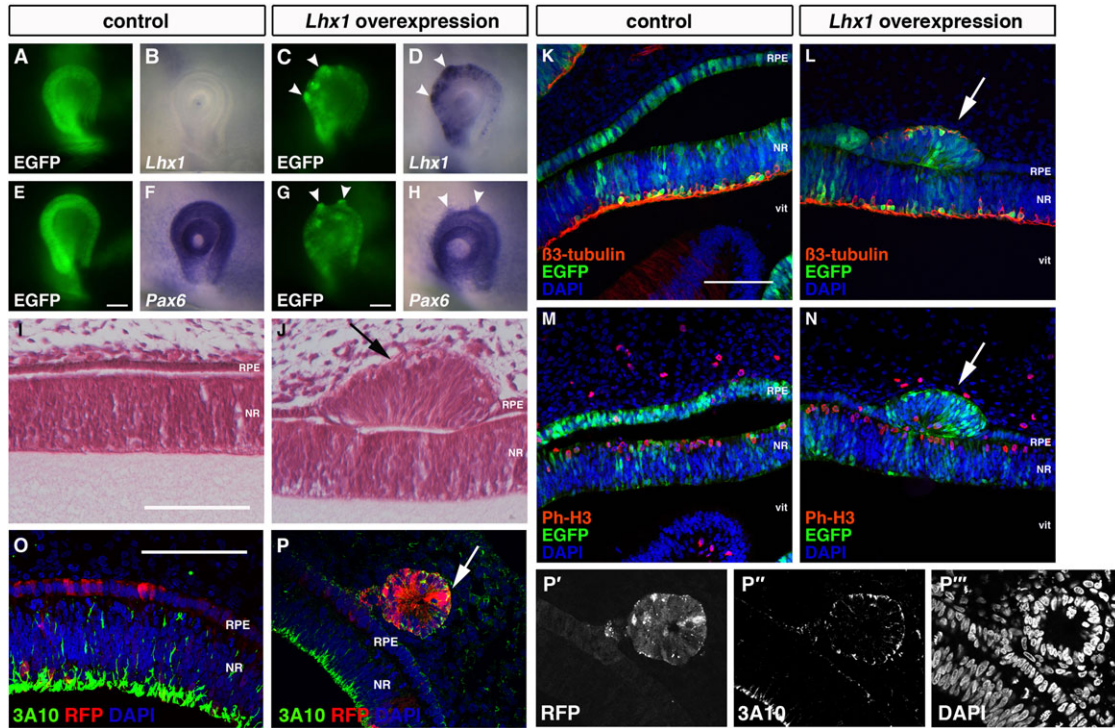
Fig. 2. *Lhx1* **overexpression induces a second NR formation from the presumptive RPE.** (**A,B**) In the EGFP-overexpressing control eye, *Lhx1* is not expressed at stage 15/16 (24 hours after electroporation). (**C,D**) In the *Lhx1*-overexpressing eye, *Lhx1* mRNA is strongly detected in the protruding 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 vitread 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. (**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.

neurofilament 3A10 antigen, which are early neuronal differentiation markers detected in the emerging retinal ganglion cells, were expressed in the transformed epithelium (Fig. 2L,P–P′′′; Fig. 2K,O as control). Phosphorylated histone H3, which marks mitotic cells usually found on the apical side of the neuroepithelium, was also detected in the thickened epithelium (Fig. 2N; Fig. 2M as control). We further examined the expression of neural retina differentiation makers such as 3A10, N-cadherin, HuC/D, Islet1, visinin, and AP2α finding that all these markers were ectopically induced in the thickened neuroepithelium after *Lhx1* overexpression (96 hours, stage 29) (Fig. 3). These results indicated that the thickened outer layer of the optic cup exhibited characteristics of a second NR initiating early neural differentiation, which was a symmetrical mirror-image to the authentic NR.

Since we observed that *Lhx1*-overexpressing eyes exhibited a round shape, rather than a typical elliptical shape, the expression of *Vax* and *Pax2* was examined to see whether the ventral portion of the OV, which develops the optic fissure and stalk, was affected by *Lhx1* overexpression. We found that *Vax* was expressed in the ventral half of the optic cup following *Lhx1* overexpression and the expression domain appeared to be largely unaffected (supplementary material Fig. S4C; supplementary

material Fig. S4A as control). In contrast, *Pax2* expression was decreased in this region after *Lhx1* overexpression (supplementary material Fig. S4G,H; supplementary material Fig. S4E,F as control). Thus, *Lhx1* likely repressed *Pax2* expression in the future optic fissure and stalk regions.

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; Martínez-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
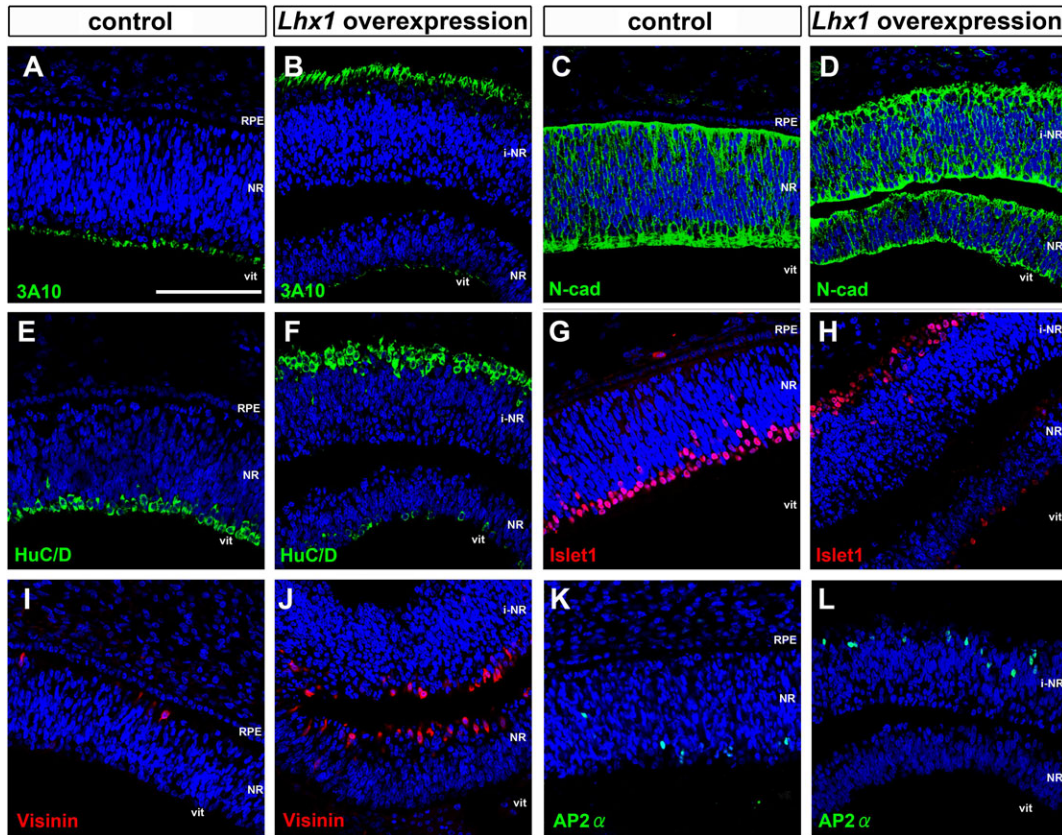
Fig. 3. Immunostaining for neural differentiation makers of the stage 29 optic cup. Immunoreactive signals are shown in green or red. Control (**A,C,E,G,I,K**) and *Lhx1*-overexpressing eyes (**B,D,F,H,J,L**). In these experiments, a bicistronic vector of pCAGGS-RFP-2A-Lhx1 was used. (A,B) 3A10, (C,D) 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.

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
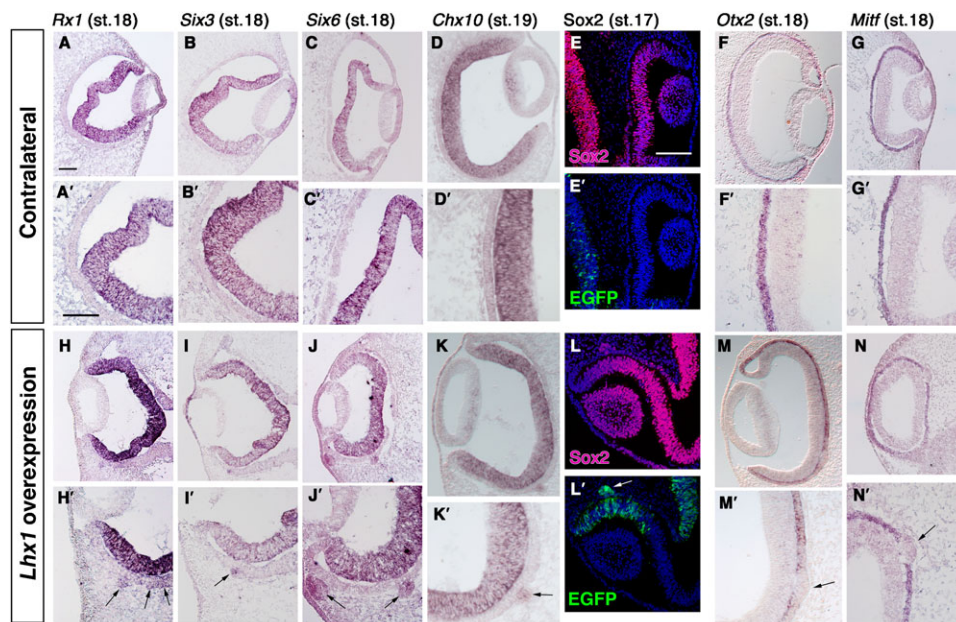

Fig. 4. *Lhx1* overexpression activates NR specification genes and suppresses RPE specification genes in the presumptive RPE. Expression of *Rx1* (**A,A′,H,H′**), *Six3* (**B,B′,I,I′**), *Six6* (**C,C′,J,J′**), *Chx10* (**D,D′,K,K′**), Sox2 (**E,E′,L,L′**), *Otx2* (**F,F′,M,M′**), and *Mitf* (**G,G′,N,N′**). 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 vise 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
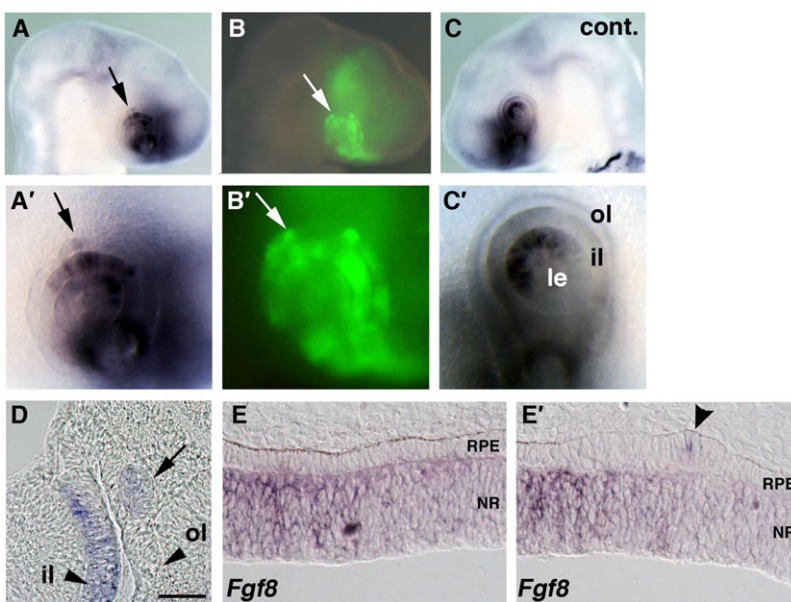

Fig. 5. *Lhx1* overexpression induces ectopic *Fgf* gene expressions. (A–E′) Expression of *Fgf19* (A,A′,C,C′) and EGFP fluorescence (B,B′) was examined at 24 hours post-electroporation. (A′,C′) High magnification of (A,C), respectively. *Lhx1*-overexpressing (A,B,A′,B′) and contralateral (C,C′) eyes. (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.
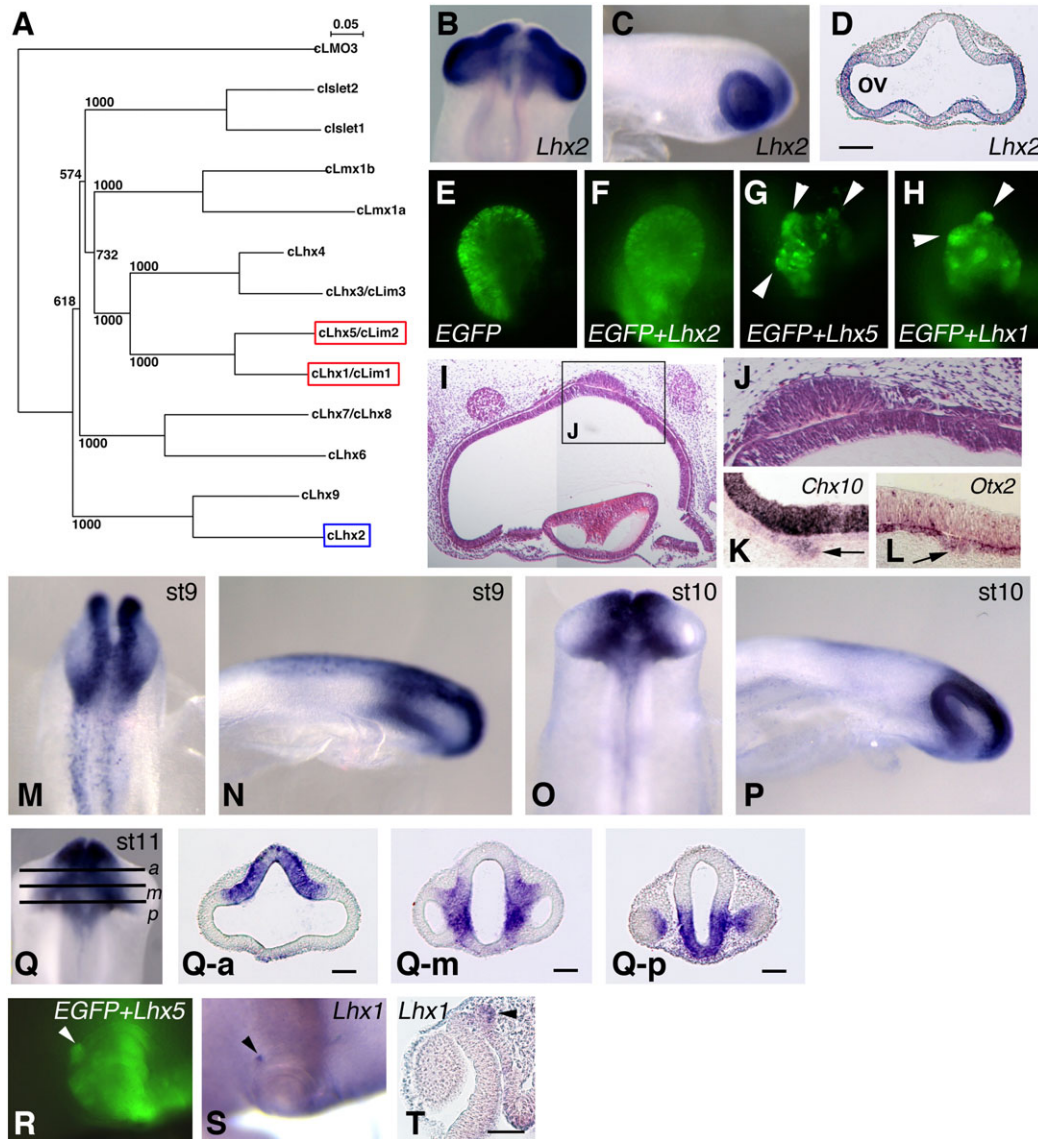
Fig. 6. **Differential effects of *Lhx2* and *Lhx5* on the OV and genetic interactions between *Lhx1* and *Lhx5*.** (**A**) Phylogenetic tree of chicken LIM-homeodomain (LIM-HD) factors. The LIM-HD factors are depicted by the NJ (neighbor-joining) method. Chicken LMO3 was used as an outgroup. The scale 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.

introduced while endogenous apoptosis was detected in the hindbrain region as reported (supplementary material Fig. S7A,B; supplementary material Fig. S7C,D as control) (*n*=3) (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
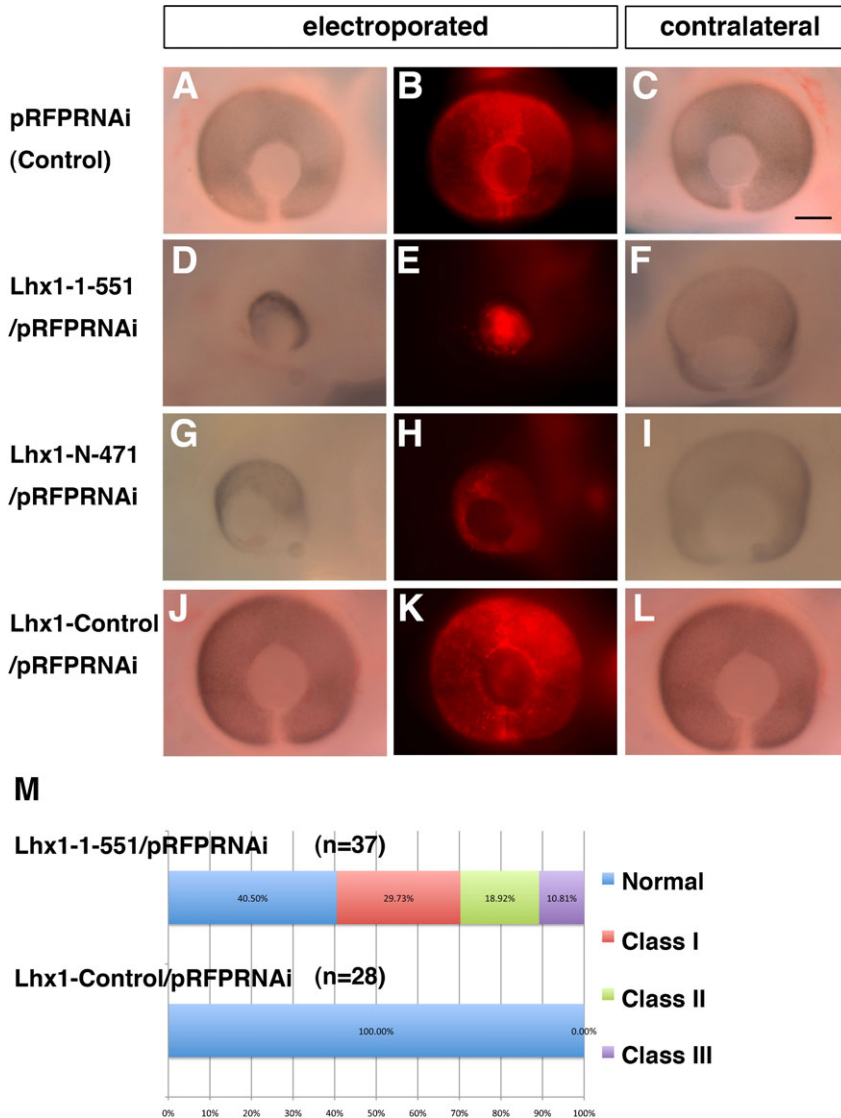
Fig. 7. *Lhx1*-RNAi reduces the eye size. (**A–L**) *Lhx1*-RNAi resulted in a small eye phenotype at stage 24. Electroporated right eyes are shown in the left two columns, and contralateral left eyes are shown in the right column. RFP fluorescence images are shown in the middle column. All images are 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.

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
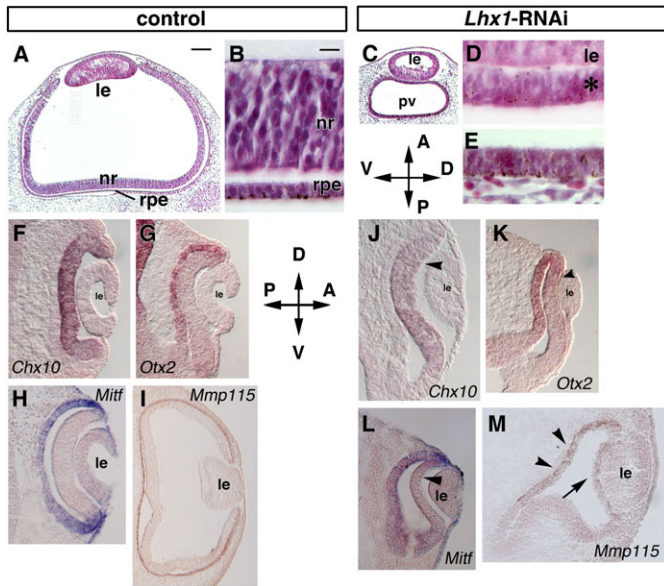
Fig. 8. ***Lhx1*-RNAi perturbs NR formation.** (A–E) Hematoxylin-Eosin staining at stage 24. (B) Magnified view of the posterior retina in (A); distinct pigmentation is observed on the basal side of the single-layered RPE. (C) Small eye phenotype after *Lhx1*-RNAi, in which a pigmented vesicle (pv) forms 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 *Chx10*, *Otx2*, *Mitf*, and *Mmp115*. Eyes were examined at 24 hours (F,G,J,K), 36 hours (H,L) or 60 hours post-electroporation (I,M). *In situ* hybridization was performed under the same conditions for controls and *Lhx1*-RNAi, depending on the probes. (J) *Chx10* expression in the inner layer of the optic cup is reduced (arrowhead). (K) The *Otx2* expression domain is expanded to the inner layer of the optic cup (arrowhead). (L) *Mitf* expression is detected in the inner layer as well as outer layer of the optic cup. (M) *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).

ventral OV and nevertheless the overexpression of *Lhx1* results in ectopic NR formation and reduction of *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 *Lhx1* in the optic vesicle can have such an influence on eye development is unsolved here. On the other hand, a recent sophisticated study has shown that NR differentiation from the OV is a default state and the proximal neuroepithelium is required for patterning the OV into NR and RPE domains (Eiraku et al., 2011). Thus, Lhx1 in the proximal region of the OV can be related to the default state of the OV that differentiates to the NR.

Since implantation of FGF8-soaked bead induces NR formation from the prospective RPE in the chicken, it was suggested that FGF8 defines the domain of NR or initiates neural differentiation (Vogel-Höpker et al., 2000; Martinez-Morales et al., 2005). *Lhx1* overexpression induced expression of *Fgf19, Chx10* and sustained other NR gene expressions, followed by *Fgf8* expression. However, *Fgf19* overexpression is not sufficient to induce ectopic NR formation (Kurose et al., 2005). Our preliminary data showed that the expression of *Wnt* pathway genes was altered after *Lhx1/Lhx5* overexpression (T.K. and H.O., unpublished observation). The *Fgf19* and *Wnt* pathway genes, therefore, may act synergistically to induce NR formation as reported in the case of otic induction (Ladher et al., 2000). In

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-Six6-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, where 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 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 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 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 LIM-HD factor, Lhx1/Lhx5 can induce the expression of both retinal stem cell marker cassettes, which is promising from the aspect of regenerative medicine as well.

Acknowledgements

Competing Interests

The authors have no competing interests to declare.

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