Otx2 Is Involved in the Regional Specification of the Developing Retinal Pigment Epithelium by Preventing the Expression of Sox2 and Fgf8, Factors That Induce Neural Retina Differentiation

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Abstract

The retinal pigment epithelium (RPE) shares its developmental origin with the neural retina (NR). When RPE development is disrupted, cells in the presumptive RPE region abnormally differentiate into NR-like cells. Therefore, the prevention of NR differentiation in the presumptive RPE area seems to be essential for regionalizing the RPE during eye development. However, its molecular mechanisms are not fully understood. In this study, we conducted a functional inhibition of a transcription factor Otx2, which is required for RPE development, using early chick embryos. The functional inhibition of Otx2 in chick eyes, using a recombinant gene encoding a dominant negative form of Otx2, caused the outer layer of the optic cup (the region forming the RPE, when embryos normally develop) to abnormally form an ectopic NR. In that ectopic NR, the characteristics of the RPE did not appear and NR markers were ectopically expressed. Intriguingly, the repression of Otx2 function also caused the ectopic expression of Fgf8 and Sox2 in the outer layer of the optic cup (the presumptive RPE region of normally developing eyes). These two factors are known to be capable of inducing NR cell differentiation in the presumptive RPE region, and are not expressed in the normally developing RPE region. Here, we suggest that Otx2 prevents the presumptive RPE region from forming the NR by repressing the expression of both Fgf8 and Sox2 which induce the NR cell fate.

Introduction

The retinal pigment epithelium (RPE), one component of the vertebrate eye, consists of a monolayer of melanin-producing cells. Both the RPE and the neural retina (NR), which contains photoreceptors, retinal ganglion cells (RGC), horizontal cells, amacrines cells, bipolar cells and Muller glia cells, originate from the same eye primordium, called the optic vesicle (OV), which derives from the lateral wall of the forebrain. The inductive interactions between the OV and the surface ectoderm (the future lens) result in the invagination of the OV to form the bilayered optic cup (OC), in which the outer and inner layers are specified into the RPE and NR, respectively [1,2].

The development of the RPE is promoted by several transcription factors, which are specifically expressed in the presumptive RPE region; Microphthalmia-associated transcription factor (Mitf) and Orthodenticle homeobox 1 and 2 (Otx1 and 2). Mitf promotes melanin synthesis and regulates cell proliferation in the developing RPE [3]. In mutant mice with non-functional alleles of the Mitf gene, a non-pigmented NR-like tissue is ectopically formed in the outer layer of the OC [4,5]. The expression of Mitf in the presumptive RPE region requires the function of Otx genes [6]. Compound mutations in Otx1 and 2 (all Otx1−/−; Otx2−/− mice and 30% of Otx1+/−; Otx2−/− mice) result in the down-regulation of Mitf expression and the ectopic formation of NR-like tissue in the outer layer of the OC, although Otx1−/− mice do not display significant defects in the RPE [6]. Still, in spite of these key findings in mutant mice, it is unclear whether the loss-of-function of Otx2 affects RPE development, since the head region including the eyes is not formed in Otx2−/− mice [7,8]. However, previous reports have pointed out the roles of Otx2 as an upstream regulator of Mitf expression and the promotion of RPE differentiation [9,10]. In cultured quail retina cells, transfection of Otx2 induces a pigmented phenotype with Mitf expression [9].
In the chick NR, co-transfection of Otx2 and a constitutively active form of β-catenin induces the ectopic expression of Mif [10].

While RPE development requires the functions of Mif and Otx, NR differentiation is induced by several other transcription and growth factors, including Fibroblast growth factor 8 (Fgf8), Subgroup B1 SRY-box family genes (SoxB1) and Paired-box 6 (Pax6). When Fgf8-soaked beads are placed in the vicinity of the developing RPE, cells in the RPE change their fate to differentiate into NR cells [11]. As a result, some areas of the outer layer of the OC form a non-pigmented ‘ectopic NR’ which takes on a stratified structure and displays several differentiation markers of the NR [11]. Similarly, ectopic formation of the NR can also be caused by the misexpression of SoxB1 or Pax6 in the outer layer of the OC [12, 13]. The Fgf8 and SoxB1 genes are expressed in the NR, but not in the RPE [11, 12]. Pax6 also becomes absent from the presumptive RPE, although its expression is detected in the RPE during the early stages of eye development [14, 15]. Although the expression patterns of these factors are well known, it is noteworthy that it is still unclear how these factors are restricted to the NR region and disappear from the RPE region in normally developing eyes. Unveiling how the expression domains of Fgf8, SoxB1 and Pax6 are down-regulated in the outer layer of the OC may lead to understanding the mechanism(s) involved in the regionalization of the RPE.

Since Otx2 and Mif are specifically expressed in the outer layer of the OC (the region forming the RPE, when embryos normally develop), it is possible that these RPE-specific factors are involved in the down-regulation of Fgf8, SoxB1 and Pax6 in the outer layer of the OC. Here, we conducted a functional inhibition of Otx2 in the developing eye, using chick embryos. Chick embryos were transfected with a recombinant gene encoding a dominant negative form of Otx2 that was confirmed to repress the function of wild-type Otx2 in vitro. Functional inhibition of Otx2 in chick eyes caused the outer layer of the optic cup (the RPE region of normally developing eyes) to abnormally form an ectopic NR. In that ectopic NR, the characteristics of the RPE did not appear and NR markers were ectopically expressed. Intriguingly, the repression of Otx2 function also caused the ectopic expression of Fgf8 and Sox2 (one of the SoxB1 family members) in the outer layer of the OC, whereas the expression of Pax6 was reduced. Our data suggest that Otx2 prevents the outer layer of the OC from forming the NR by repressing the expression of Fgf8 and Sox2 which can forcibly induce NR differentiation [11, 12].

Results

Expression Pattern of Otx2 and the Dominant Negative Activity of EnR-Otx2

First, we compared the expression patterns of Otx2 with Mif in the OV and the OC stage. In HH10 chick embryos, Otx2 was expressed in a large part of the OV (asterisks in Figure 1A), although its expression was weak in the ventral part of the OV. Mif was not expressed in the OV in HH10 chick embryos (Figure 1B). From HH12-13, Mif expression could be detected in the dorsal part of the OV (arrowheads in Figure 1D). At the same stages, Otx2 was highly expressed in the dorsal part of the OV (arrowheads in Figure 1C), similar to the expression pattern of Mif. After the OC was formed, the expression of both Otx2 and Mif was apparent in the outer layer of the OC where the RPE formed (Figure 1E and F).

For the functional inhibition of Otx2, we used a recombinant gene encoding a dominant negative form of Otx2, called EnR-Otx2. EnR-Otx2 encodes chick Otx2 fused to the Drosophila Engrailed repressor domain (EnR). We confirmed the dominant negative activity of EnR-Otx2 with in vitro assays. Consistent with a previous study which showed that OTX2 activates the Dct gene promoter [16], chick wild-type Otx2 (wtOtx2) drove the Dct promoter (lanes 1 and 2 in Figure 1G) in D407 cultured cells. This function of wtOtx2 was blocked by EnR-Otx2 (lane 3 in Figure 1G), suggesting that EnR-Otx2 could be used for the functional inhibition of Otx2. As expected, the Dct promoter was not activated by EnR-Otx2 (lane 5 in Figure 1G). EnR-Otx2AC, which encodes EnR-Otx2 without its C-terminal DNA-binding domain, did not repress the activity of wtOtx2 (lane 4 in Figure 1G).

Loss of Characteristics of the RPE by EnR-Otx2 Transfection

To address how Otx2 contributes to RPE development in chick embryos, we conducted gene transfection experiments. The OV of HH19-11 chick embryos (incubated for 1.5 days) were transfected with pMiwIII-EnR-Otx2 and a GFP-expressing vector (pCAGGS-EGFP) using in ovo electroporation, carried out as described previously [3]. For controls, both the pMiwIII-empty vector and pCAGGS-EGFP were transfected. After incubation for 2 days, the transfected embryos were fixed and prepared for observation.

In the case of normally developing eyes, the control eyes had a blackish tinge except for the lens (n = 15), since the differentiated RPE cells synthesize melanin pigment (corresponding to HH20-22 embryos, Figure 2A-C). However, the EnR-Otx2-transfected eyes revealed a highly reduced pigmentation in the GFP-positive portion (n = 23, brackets in Figure 2D-F).

To address how EnR-Otx2 affects the state of differentiation and morphology of the outer layer of the OC (the presumptive RPE region of normally developing eyes), sections of EnR-Otx2-transfected or control eyes were stained for RPE differentiation markers. In the control eyes, no obvious morphological changes were observed (Figure 2G–I), and the outer layer of the OC (the presumptive RPE region) maintained the mono-layered structure (bracket in Figure 2G). In contrast, EnR-Otx2 caused an abnormally thickened tissue to be formed in the outer layer of the OC (compare brackets between Figure 2G and M). In this thickened outer layer (the areas sandwiched between the dashed lines), pigment granules were hardly detected. In addition, some of the EnR-Otx2 transfected eyes did not keep their cup-like structure to form an OV-like structure (Figure 2O–R), and the size of the lens seemed reduced in the EnR-Otx2-transfected eyes (compare the areas enclosed in the dashed lines in Figure 2M and O with Figure 2G).

In EnR-Otx2-transfected eyes (Figure 2M–R), the expression of Mif became weakened over a large part of the thickened outer layer (Figure 2M and N), and Mif signals were only detected in the GFP-negative areas in the thickened outer layer (arrowheads in Figure 2M and N). Differentiation markers of the RPE, dopachrome tautomerase (Dct, encoding a enzyme required for black melanin synthesis) and melanosomal matrix protein 115 (MMP115) [3, 17, 18], also could not be detected in these eyes (the areas between the dashed lines in Figure 2P and R). In contrast, Mif, Dct and MMP115 were specifically expressed in the outer layer of the OC (the presumptive RPE region) of control eyes (Figure 2G–L). Thus, proper morphogenesis and differentiation in the outer layer of the OC (the presumptive RPE region) is disrupted by EnR-Otx2 misexpression.

Ectopic Expression of NR Markers in the Outer Layer of the OC Following EnR-Otx2 Transfection

Next, we examined the expression patterns of several NR markers. It is possible that EnR-Otx2 transfection caused the outer
layer of the OC to abnormally differentiate into the NR instead of the RPE, since ectopic NR-like tissues are formed in the outer layer of the OC of \( Otx1 \) and \( Otx2 \) compound mutant mice [6]. In addition, previous studies have shown that the RPE shares a common developmental origin (OV) with the NR and also has the potential to differentiate into the NR [11,12,13,19,20,21,22,23,24,25,26].

For analyzing the expression of NR markers, the embryos were transfected at embryonic stages HH9-11, and then were further incubated for 2 days to reach HH20-22 embryos.

In the developing NR, the transcription factor Islet1 is detected in postmitotic ganglion cells, migrating amacrine cells [11,12,27], while RNA-binding protein HuC/D is expressed in the differentiated neuronal cells [21,28,29]. In the control eyes, those differentiation markers of the NR were expressed on the vitreal surface (the surface facing the lens, described as (v) in Figure 3C and F) of the NR (arrowheads in Figure 3B and E), but could not be detected in the outer layer of the OC/presumptive RPE region (the area between the dashed lines in Figure 3B, C, E and F). In addition, phospho histone-H3 (PHH3)-positive mitotic cells were located at the sclera surface (the surface opposite the lens, described as (s) in Figure 3I) of the NR in the control eyes (arrowheads in Figure 3H), as in the case of normally developing eyes. In these eyes, only a small number of RPE cells were positive for PHH3 (the area between the dashed lines in Figure 3H and I).

In the \( EnR-Otx2 \)-transfected eyes, not only the NR but also the thickened outer layer were positive for HuC/D and Islet1 (Figure 3J–Q). Ectopic signals of Islet1 or HuC/D were detected in the developing NR, the transcription factor Islet1 is detected in postmitotic ganglion cells, migrating amacrine cells [11,12,27], while RNA-binding protein HuC/D is expressed in the differentiated neuronal cells [21,28,29]. In the control eyes, those differentiation markers of the NR were expressed on the vitreal surface (the surface facing the lens, described as (v) in Figure 3C and F) of the NR (arrowheads in Figure 3B and E), but could not be detected in the outer layer of the OC/presumptive RPE region (the area between the dashed lines in Figure 3B, C, E and F). In addition, phospho histone-H3 (PHH3)-positive mitotic cells were located at the sclera surface (the surface opposite the lens, described as (s) in Figure 3I) of the NR in the control eyes (arrowheads in Figure 3H), as in the case of normally developing eyes. In these eyes, only a small number of RPE cells were positive for PHH3 (the area between the dashed lines in Figure 3H and I).

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sections of eyes transfected with the empty vector (G–L) or pigmentation was reduced by and B), or F) Lateral views of embryos transfected with either the empty vector (A and D indicate GFP signals (green), B and E are bright field images, (G–R) Staining of RPE markers in sections of eyes transfected with the empty vector (G–L) or EnR-Otx2 (M–R). Immunohistological analyses of Mitf expression (H and N) and in situ hybridization analyses of transcripts of Dct (J and P) or MMP115 (L and R). G, I, K, M, O and Q indicate GFP signals (green). Brackets in G and M indicate the thickness of the outer layer of the OC, which are transfected with the empty vector (G) or EnR-Otx2 (M), respectively. Dashed lines in N, P and R highlight the thickened outer layer of EnR-Otx2-transfected eyes. Arrowheads in M and N indicate sites where Mitf expression remains. In M–R, ‘RPE’ refers to the abnormally thickened outer layer caused by EnR-Otx2 transfection. G and H are the same sections, so are M and N. Each set of I and J, K and L, O and P and Q and R are serial sections. Upper and lower sides of the panels correspond to dorsal and ventral sides of embryos, respectively. GFP, retinal pigment epithelium, NR, neural retina. Le, lens. Scale bars: 100 μm. doi:10.1371/journal.pone.0048879.g002

on the sclera surface (the surface opposite the lens, described as (s) in Figure 3L and Q) of the thickened outer layer (arrowheads in Figure 3K and N). Further, on the vitreal surface (the surface facing the lens, described as (v) in Figure 3R) of the thickened outer layer, most PHH3-positive cells were detected (arrowheads in Figure 3Q). The distribution of PHH3 on the opposite side of Huc/C/D and Islet1 in the thickened outer layer topologically mimicked the distribution of those factors in the normally developing NR. These data suggest that EnR-Otx2 misexpression results in the formation of an ‘ectopic NR’ in the outer layer of the OC.

Effects of EnR-Otx2 on Factors Involved in NR Differentiation or other Aspects of Eye Development

The ectopic NR formation in the outer layer of the OC (the presumptive RPE region) could also be induced by some transcription factors or secreted factors, which are expressed in the OC. For example, misexpression of transcription factors Sox1, 2 or 3 (SoxB1) or Pax6 causes ectopic formation of the NR in the outer layer of the OC (the presumptive RPE region) [12,13]. The application of FGF8-soaked beads in the vicinity of the developing RPE also results in the ectopic NR formation [11].

In normally developing eyes, the expression of Sox1, 2 and 3 (SoxB1), Pax6 and Fgf8 is detected in the NR [11,12,14,15]. It is noteworthy that SoxB1 and Fgf8 are not expressed in the normally developing RPE after the OC is formed [11,12]. Although Pax6 is expressed in the normally developing RPE after the OC formation, its expression disappears from the presumptive RPE region as eye development further proceeds [14,15].

In other words, the expression domains of SoxB1, Pax6 and Fgf8, which induce NR differentiation eventually become restricted to the presumptive NR region during normal eye development. In this point of view, we tested whether expression of these factors was induced in the ectopic NR by EnR-Otx2 transfection.

For this analysis, the embryos were transfected in embryonic stages HH9-11, and then were further incubated for 2 days to reach HH20-22 embryos. In the control eyes, Sox2 was not expressed in a large part of the outer layer of the OC (the RPE region, the areas between the dashed lines in Figure 4B and C), but its expression was detected in the NR (Figure 4A–C). In these eyes, Sox2 was not expressed in the peripheral (arrow in Figure 4A) or central RPE, although only a small part of the proximal RPE (asterisk in Figure 4A) was positive for Sox2 signals (arrowheads in Figure 4B). In contrast, transfection of EnR-Otx2 caused the ectopic expression of Sox2 in the thickened outer layer of the OC/ectopic NR (the areas between the dashed lines in Figure 4K and L). The expression of Sox2 was detected in the peripheral, central and proximal regions of the thickened outer layer/ectopic NR following EnR-Otx2 transfection (Figure 4J–L). Moreover, unlike in the cases of Islet1 and Huc/D, Sox2 was not only expressed in the basal side but over the whole range of the ectopic NR across the apical-basal axis (Figure 4K and L).

Similar to Sox2, EnR-Otx2 induced the ectopic expression of Fgf8 in the thickened outer layer of the OC/ectopic NR (Figure 5B and D–F). In the normal eyes, Fgf8 was expressed in the central part of the NR but not in the outer layer of the OC/presumptive RPE region (Figure 5A and C). Interestingly, ectopic expression of Fgf8 was also detected in the dorsal and ventral parts of the NR by

Figure 2. Loss of RPE characteristics and morphological changes in the chick eye following EnR-Otx2 transfection. (A–F) Lateral views of embryos transfected with either the empty vector (A and B), or EnR-Otx2 (D and E). Illustrations in C and F correspond to B and E, respectively. Brackets in D and E indicate sites where pigmentation was reduced by EnR-Otx2. A and D indicate GFP signals (green), B and E are bright field images, (G–R) Staining of RPE markers in sections of eyes transfected with the empty vector (G–L) or EnR -Otx2 (M–R). Immunohistological analyses of Mitf expression (H and N) and in situ hybridization analyses of transcripts of Dct (J and P) or MMP115 (L and R). G, I, K, M, O and Q indicate GFP signals (green). Brackets in G and M indicate the thickness of the outer layer of the OC, which are transfected with the empty vector (G) or EnR-Otx2 (M), respectively. Dashed lines in N, P and R highlight the thickened outer layer of EnR-Otx2-transfected eyes. Arrowheads in M and N indicate sites where Mitf expression remains. In M–R, ‘RPE’ refers to the abnormally thickened outer layer caused by EnR-Otx2 transfection. G and H are the same sections, so are M and N. Each set of I and J, K and L, O and P and Q and R are serial sections. Upper and lower sides of the panels correspond to dorsal and ventral sides of embryos, respectively. GFP, retinal pigment epithelium, NR, neural retina. Le, lens. Scale bars: 100 μm. doi:10.1371/journal.pone.0048879.g002

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Control

Islet1

HuC/D

PHH3

EnR-Otx2

Islet1

HuC/D

PHH3

A

RPE

Le

NR

B

RPE

C

RPE

(S)

D

RPE

E

RPE

(F)

RPE

(S)

G

RPE

H

RPE

(I)

RPE

J

RPE

K

RPE

(L)

RPE

M

RPE

N

RPE

(Q)

RPE

P

RPE

Q

RPE

(R)

RPE

(V)
EnR-Otx2, although only a few NR cells were transfected with EnR-Otx2 (Figure 5B).

In contrast to Sox2 and Fgf8, Pax6 was strongly expressed in the outer layer of the OC (the presumptive RPE region) of control embryos, which were incubated for 2 days after electroporation (corresponding to HH20-22 embryos, Figure 4D-F). In the normally developing HH20-22 embryos, Pax6 was similarly detected in the outer layer of the OC (the presumptive RPE region). It was intriguing that EnR-Otx2 transfection resulted in the reduced expression of Pax6 in the thickened outer layer of the OC/ectopic NR (Figure 4M-O). Pax6 expression was apparently weakened in the proximal part of the thickened outer layer/ectopic NR (asterisks in Figure 4M and N), and expression of Pax6 also became weaker in the peripheral part of the thickened outer layer/ectopic NR (arrows in Figure 4M and N).

We also analyzed whether some other transcription factors changed their expression patterns in the outer layer of the OC following EnR-Otx2 transfection. We examined the expression of Six3, Lhx2 and Pax2, each of which is associated with multiple aspects of eye development.

During eye development, inactivation of Six3 causes cyclopia, small eyes or disrupted proximo-distal patterning of the OV in medaka embryos [30]. Over-expression of Six3 results in retinal hyperplasia or ectopic retinal primordia formation [31]. In the postnatal retina, Six3 is also involved in cell specification [32,33].

In the case of eye development in Lhx2, Lhx2^+/− mice have eye development that is arrested in theOV stage, and expression domains of various transcription factors are disrupted until the OV stages [34,35]. To analyze the function of Lhx2 in OV stages, Yun et al. generated genetic mosaic mice, in which Lhx2-mutant cells exist at low frequency among the wild-type cells [35]. In the OC of these mice, Mitf, Vsx2 and Pax2 were not expressed in the Lhx2-mutant cells, although Pax6 was expressed [35].

Pax2 is required for optic fissure closure and proper projection of the optic nerve [36,37]. Although Pax2 is not expressed in the presumptive RPE, its expression is detected in the optic stalk (OS), which is adjacent to the presumptive RPE [36,38]. Pax2-deficient mutant mice display expansion of the RPE domain toward the OS region [36,38]. Therefore, Pax2 is thought to repress RPE development to make a sharp boundary between the OS and the RPE [38].

In the control eyes, expression of Pax2 and Six3 was not detected in the outer layer of the OC (the presumptive RPE region) (the areas between the dashed lines in Figure 4H and I and described as ‘RPE’ in Figure 6A and B, respectively), although Lhx2 was expressed in this region (described as ‘RPE’ in Figure 6C). Similarly, the ectopic NR was negative for Pax2 and Six3 (the areas between the dashed lines in Figure 4Q and R and Figure 6E, respectively) but was positive for Lhx2 expression in the EnR-Otx2 transfected eyes (the area between the dashed lines in Figure 6F). In the control NR, Six3 and Lhx2 were expressed (Figure 6A-C), although Pax2 was highly expressed in the ventral NR but was only weakly expressed in the dorsal NR (Figure 4G-I). Similar expression patterns of Pax2, Six3 and Lhx2 were observed in the NR of EnR-Otx2 transfected eyes (Figure 4P-R and Figure 6D-F).

Our analyses of the transcription factors and a secreted factor suggest that EnR-Otx2 induces the ectopic expression of Sox2 and Fgf8 in the thickened outer layer of the OC/ectopic NR. It is notable that these two factors share two traits: 1) being able to forcibly induce NR differentiation in the outer layer of the OC/presumptive RPE region [11,12], and 2) being not expressed in the outer layer of the OC/presumptive RPE region but detected in the inner layer of the OC/presumptive NR region in normally developing eyes [11,12].

Increased Cell Proliferation and Apoptosis in EnR-Otx2-transfected Eyes

Martinez-Morales et al. reported that Otx1^-/-; Otx2^+/+ mice display increased cell proliferation and cell death in the retina [6]. Therefore, we also assessed the effects of EnR-Otx2 on cell proliferation and apoptosis in chick eyes. To analyze cell proliferation and apoptosis, anti-PHH3 and anti-single stranded DNA (ssDNA) antibodies were used, respectively.

For this analysis, the embryos were transfected in embryonic stages HH9-11, and then were further incubated for 2 days to reach HH20-22 embryos.

Although a small number of PHH3-positive or ssDNA-positive cells existed in the normal RPE and NR (Figure S1A, D, F and I), the number increased following EnR-Otx2 transfection (Figure S1B, C, E, G, H and J), as in the case of Otx1 mutant mice [6].

Ectopic Formation of Telencephalon-like Vesicles Following EnR-Otx2 Transfection

When embryos were incubated for about a week after EnR-Otx2 transfection, their eyes displayed a small eye phenotype (Figure S2A), as in the case of Otx1^-/-; Otx2^+/+ mice. Intriguingly, we noticed that some vesicle-like structures were ectopically formed following EnR-Otx2 transfection (arrow in Figure S2A). The ectopic vesicles were connected to the small eyes of EnR-Otx2 transfected embryos, but were not observed in control embryos (data not shown). The ectopic vesicles lacked the characteristics of the RPE (pigmentation and a monolayered-structure, Figure S2B, C, F, G, I and J) or the NR (expression of a photoreceptor marker visinin, Figure S2C).

These ectopic vesicles were positive for markers of the developing brain, including Enx1, Nkx2.1 and Pax6 (Figure S2B and F-K), but were negative for a hindbrain marker, Ghx2 (data not shown). Enx1 is normally detected in the dorsal telencephalon [39,40], Nkx2.1 in the hypothalamus in the ventral portion of the diencephalon and telencephalon [41] and Pax6 in the dorsal telencephalon and diencephalon. These data suggest that the
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ectopic NR'. RPE, retinal pigment epithelium. NR, neural retina. Le, lens. Scale bars: 100

Figure 4. Alterations of expression patterns of transcription factors following EnR-Otx2 transfection. Immunohistological staining of Sox2, Pax6 and Pax2 in sections of eyes transfected with empty vector (A–I) or EnR-Otx2 (J–R). A–C and J–L indicate the expression of Sox2 (magenta). A and J are merged images with GFP (green). DAPI (blue) in C is used to ease observation of tissue structures of the RPE and NR. D–F and M–O indicate the expression of Pax6 (magenta). D and M are merged images with GFP (green). G–I and P–R indicate the expression of Pax2 (magenta). G and P are merged images with GFP (green). C, F, I, L, O and R are magnified images of the boxes in B, E, H, K, N and Q, respectively. Dashed lines highlight the RPE of control eyes (B–I) or the thickened outer layer of EnR-Otx2-transfected eyes (K–R). Arrows and asterisks in A, J, K, M and N indicate the peripheral and proximal areas of the outer layer of the OC, respectively. The central area of the outer layer of the OC corresponds to the area between the arrow and the asterisk. Arrowheads in B highlight the Sox2-positive small area of the RPE. The upper and lower sides of each image correspond to the dorsal and ventral sides of the specimen, respectively. In J–R, ‘RPE’ refers to the abnormally thickened outer layer, apparently ectopic NR, RPE, retinal pigment epithelium. NR, neural retina. Le, lens. Scale bars: 100 μm.

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Discussion
Repression of Otx2 Function in Chick Eyes
As in the case of Otx1 and Otx2 [7], chick eyes displayed the formation of an "ectopic NR" in the outer layer of the OC, as a result of the severely impaired function of Otx2. The outer layer of the OC began to form an unpigmented-thick structure following EnR-Otx2 transfection. In this tissue, the expression of some RPE-specific markers (Mif, Det and MMP115) was reduced but, instead, the expression of several differentiation markers of the NR (HuC/D and Islet1) was detected. In addition, the expression of EnR-Otx2 also caused increases in cell proliferation and apoptosis in the developing eye, similar to Otx1+/−; Otx2−/− mice [6].

Functions of Otx2 are Associated with Regional Specification of the RPE
Both the RPE and NR are derived from the same developmental origin. As eye development proceeds, the presumptive RPE and NR regions become subdivided into the outer and inner layers of the OC, respectively. One focus of this study was to elucidate how Otx2 functions during these regional specifications of the RPE and NR.

We analyzed the expression patterns of factors contributing to NR development. Among them, Sox2 and Fgf8 were ectopically expressed in the outer layer of the OC following EnR-Otx2 transfection. In contrast, the expression of Pax6 seemed to be decreased in the ectopic NR, and there were no obvious changes in Six3 or Lhx2. Considering that Sox2 and Fgf8 are known to induce NR cell fate in the outer layer of the OC (the presumptive RPE region) in vivo [11,12], it is possible that Otx2 is required to repress the expression of these factors (Sox2 and Fgf8) in the outer layer of the OC (the presumptive RPE region). Correspondingly, in normally developing eyes, Sox2 and Fgf8 are not detected in the outer layer of the OC (the presumptive RPE region) where Otx2 is expressed [11,12].

Considering these data, we would like to propose the following hypothesis regarding the regional specification of the RPE and NR (Figure 7). In the normally developing OC, Sox2 and Fgf8 function to induce NR differentiation. However, in the outer layer of the OC, the expression of Sox2 and Fgf8 is repressed by Otx2, and Sox2 and Fgf8 expression is restricted to and remains in the inner layer of the OC. As a result, NR differentiation is prevented in the outer layer of the OC and leads to formation of the RPE, whereas the NR is formed in the Sox2 and Fgf8-positive inner layer.

Reduction of Pax6 Expression by EnR-Otx2 transfection. Immunohistological staining of Sox2, Pax6 and Pax2 in sections of eyes transfected with empty vector (A–I) or EnR-Otx2 (J–R). A–C and J–L indicate the expression of Sox2 (magenta). A and J are merged images with GFP (green). DAPI (blue) in C is used to ease observation of tissue structures of the RPE and NR. D–F and M–O indicate the expression of Pax6 (magenta). D and M are merged images with GFP (green). G–I and P–R indicate the expression of Pax2 (magenta). G and P are merged images with GFP (green). C, F, I, L, O and R are magnified images of the boxes in B, E, H, K, N and Q, respectively. Dashed lines highlight the RPE of control eyes (B–I) or the thickened outer layer of EnR-Otx2-transfected eyes (K–R). Arrows and asterisks in A, J, K, M and N indicate the peripheral and proximal areas of the outer layer of the OC, respectively. The central area of the outer layer of the OC corresponds to the area between the arrow and the asterisk. Arrowheads in B highlight the Sox2-positive small area of the RPE. The upper and lower sides of each image correspond to the dorsal and ventral sides of the specimen, respectively. In J–R, ‘RPE’ refers to the abnormally thickened outer layer, apparently ectopic NR, RPE, retinal pigment epithelium. NR, neural retina. Le, lens. Scale bars: 100 μm.

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Our results show that the expression of Pax6 is reduced in the thickened outer layer/ectopic NR by EnR-Otx2 transfection. However, Pax6 is known to induce ectopic NR formation in the RPE region [13], as do Sox2 and Fgf8. In the case of ectopic NR formation by Fgf8, Pax6 expression is initially absent, but emerges in the ectopic NR at a later stage of differentiation [11]. Therefore, we cannot exclude the possibility that Pax6 would also be expressed in the ectopic NR if the EnR-Otx2-transfected embryos were incubated for a much longer term. In fact, Martinez-Morales et al. reported that the ectopic NR of Otx1−/−; Otx2−/− mice is positive for Pax6 expression [6]. Moreover, it has been revealed that Pax6 promotes NR development [13,42,43,44].

However, previous studies also indicate the requirement of Pax6 for RPE development, using chimeric mouse embryos composed of wild-type and Pax6−/−; Sry−/− mutant cells (both the Sry and Sry−/− alleles encode a non-functional Pax6 protein) [45]. In the outer layer of the OC of the chimera, the region occupied by Pax6-negative cells shows an abnormally thickened-layer, suggesting that loss of function mutations in Pax6 cause disruption of the mono-layered structure of the RPE. Pax6 is also required to initiate Mitf expression in the developing eye, in a redundant manner with Pax2 [46]. In addition, Pax6 is expressed in the presumptive RPE region (this study and [46]), and is also detected in cultured RPE cells which are derived from embryonic stem cells [47].

To elucidate the mechanisms of OC patterning in detail, it should be unveiled how Pax6 expression is regulated and how Pax6 switches its function according to the developmental context.

Future Prospects
By incubating the embryos for a long term after EnR-Otx2 transfection, ectopic vesicles were formed near the small eyes (Figure S2). Although the ectopic vesicles were continuously connected to the EnR-Otx2 transfected small eyes, they lacked the characteristics of the RPE or NR. Instead, the expression of Emx1, Pax6 and Nkx2.1 [39,40,41,48,49] suggests that the ectopic vesicles have the characteristics of the telencephalon. Although more detailed analyses on the molecular mechanisms involved are needed, our analyses of Otx2 function bring new insights into the relationships between eye and brain development.

EnR-Otx2 may bind to the Otx1 protein, since the structures of Otx1 and Otx2 are similar in their dimerization domain (homeodomain) and are able to bind the same DNA target sequences [50]. Moreover, the replacement of Otx1 with Otx2 rescues the phenotype of Otx1 knock-out mice, at least in part [51]. Therefore, our observations reinforce the requirement for Otx genes in the development of chick eyes. Other techniques that selectively reduce Otx2 or Otx1 expression, such as RNA interference, would dissect the functional divergence of Otx1 and Otx2 in eye development.

In the developing eye, patterning the polarity of the OV along the dorsal-ventral and posterior-anterior axes is required for proper regional specification of the presumptive RPE and NR regions [32,33,34,55]. In such a patterning process, BMP4 and Shh from the dorsal and ventral parts of the forebrain, respectively, are thought to be involved [36]. Moreover, Activin, BMP and Wnt from the extra-ocular mesenchyme or surface
Otx2 Is Involved in the Regionalization of the RPE
ectoderm are thought to regionalize the presumptive RPE [17,18,25,26], as well as FGFs from the surface ectoderm to regionalize the presumptive NR [5,21]. Future analyses should clarify how Otx2 mediates these signals from extra-ocular tissues to intrinsic molecular mechanisms in the OV and OC. By understanding how the expression domain of Otx2 is restricted to the presumptive RPE region, more details about the mechanisms responsible for regionalizing the RPE and NR in the developing eye will be unveiled.

Materials and Methods

Ethics Statement
All experiments involving animals were approved by the Nagahama Institute of Bio-Science and Technology (approval Id: 050).

Chick Embryos
White Leghorn chicken eggs were incubated at 38°C. Developmental stages of embryos were assigned according to Hamburger and Hamilton [57].

In situ Hybridization and Immunohistochemistry
In situ hybridization and immunohistochemistry were performed as previously described [3]. Primary antibodies used for immunohistochemistry include polyclonal antibodies against chicken Mitf (generated in our laboratory), Pax2 (COVANCE), phospho Histone-H3 (Upstate), Sox2 (MILLIPORE) and ssDNA (DAKO, Denmark), and monoclonal antibodies against HuC/D (Molecular Probes), TuJ1 (COVANCE), Islet1, neurofilaments, Pax6 and visinin (Developmental Studies Hybridoma Bank, DSHB, USA). Samples were observed using an Olympus BX51 microscope (Tokyo, Japan) with a cooled CCD camera.

Electroporation
In ovo electroporation was carried out as described previously [3] with the following modifications. White Leghorn chicken eggs were incubated at 38°C until the chick embryos reached stage 9–11, according to Hamburger and Hamilton [57]. The plasmid solution was then injected into the OV. An anode (0.5 mm in diameter, 1.0 mm in length; Unique Medical Imada, Japan) and a cathode (tungsten needle) were placed on the outside and inside of the embryo, respectively, across the OV (and surface ectoderm). Rectangular pulses (7 V, 30 ms) were then charged twice using an electroporator (CUY, Tokiwa Science, Japan).

Expression Vectors
The full-length chicken Otx2 cDNA was inserted in the pMiwIII vector. In this pMiwIII-wtOtx2 (wtOtx2) vector, Otx2 is fused to a nucleic acid encoding FLAG-tag. pMiwIII-Otx2-EnR (EnR-Otx2) is...
Figure S1 Increased cell proliferation and apoptosis in EnR-Otx2-transfected eyes. Immunohistochemical analyses of cell proliferation (A-E) and apoptosis (F-J) in sections of normal eyes (A, D, F and J) and EnR-Otx2-transfected eyes (B, G, E, G, H and J). A and C-E indicate PHH3-positive mitotic cells (magenta), and C and E are merged images with GFP (green). F and H-J indicate ssDNA-positive apoptotic cells (magenta), and H and J are merged images with GFP (green). D is a highly magnified image of the box in A, as well as E of C, I of F, and J of H. B and G are bright field images of C and H, respectively. Open arrows and arrowheads in A and D indicate PHH3-positive cells in the RPE and NR of the normal eye, respectively. Arrowheads and arrows in E indicate PHH3-positive cells in ectopic NR and NR of EnR-Otx2-transfected eyes, respectively. Arrows in F and I indicate ssDNA-positive cells in the normal eye. Arrows in H indicate ssDNA-positive cells which are located in the EnR-Otx2-transfected areas. Arrowheads and arrows in J indicate ssDNA-positive cells in ectopic NR and NR of EnR-Otx2-transfected eyes, respectively. RPE, retinal pigment epithelium. NR, neural retina. Le, lens. Scale bars: 100 μm in A [for A-C and F-H]; 50 μm in D [for D and I]; 10 μm in E [for E and J].

Figure S2 Ectopic formation of telencephalon-like vesicles following EnR-Otx2 transfection. (A) Lateral view of an embryo incubated for 1 week after EnR-Otx2 transfection. The right eye displays a ‘small eye’ compared to the untransfected-left eye. The arrow indicates a vesicle which is ectopically formed adjacent to the small eye. (B-K) Immunohistological and in situ hybridization analyses of eye and brain markers. Sections in B–K are sliced along the plane indicated by the white line in A. Sections in B, D, H and K are stained with anti-Pax6 antibody (green) and DAPI (blue). Sections in C and E are stained with anti-Visinin antibody (green) and DAPI (blue). B, C, H and K indicate the tissues around the small eye and ectopic vesicles formed by EnR-Otx2. D and E indicate parts of the normally developing eye. Sections in F and I are stained with an antisense probe for Nkx2.1 (violet-blue). Sections in G and J are stained with an antisense probe for Nkx2.1 (violet-blue). H and K are highly magnified images of boxes in B, as well as I of F, and J of G. F, G, I and J indicate tissues around the small eye (right eye), ectopic vesicle (Etc) and normally developing untransfected eye (left eye) of an EnR-Otx2-transfected embryo. Arrowheads in H and I indicate the dorsal area of ectopic vesicles in which both Pax6 and Emx1 signals are detected. Arrowheads in J indicate the ventral areas of ectopic vesicles in which the Nkx2.1 signal is detected. RPE, retinal pigment epithelium. NR, neural retina. Etc, Ectopic vesicle. inl, inner nuclear layer. onl, outer nuclear layer. gcl, glial cell layer.

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Author Contributions
Conceived and designed the experiments: DN IY TK KT HY. Performed the experiments: DN IY TK KT HY. Analyzed the data: DN IY NT KT SS. Contributed reagents/materials/analysis tools: DN IY NT TK KT SS. Wrote the paper: DN IY KT SS HY.

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