INTRODUCTION
The retina originates from a bilateral evagination of the forebrain, called the optic vesicle (OV). As the overlying lens ectoderm thickens and invaginates to form the lens vesicle, the distal part of the OV begins to invaginate to form a double-layered optic cup. The inner layer of the cup forms the neural retina (NR), where six types of neurons and one type of glial cell are generated. Conversely, the outer layer of the cup gives rise to the retinal pigmented epithelium (RPE), characterized as a melanin-containing simple cuboidal epithelium.

In vivo and in vitro studies have demonstrated that the early OV neuroepithelium is capable of differentiating into both RPE and NR cell types (reviewed by Moshiri et al., 2004). Patterning of the OV into these cell types depends on the exposure to extracellular signals from pericellular mesenchyme and surface ectoderm, which control the choice of OV cell fate. The former tissue, presumably mediated by BMP- or activin-like signals, promotes RPE development (Furhmann et al., 2000; Hyer et al., 2003; Müller et al., 2007), whereas the latter tissue, likely to be mediated by FGF signaling, inhibits RPE development and specifies the NR domain (Guillemot and Cepko, 1992; Hyer et al., 1998; Nguyen and Arnheiter, 2000; Opas and Dziak, 1994; Park and Hollenberg, 1991; Pittack et al., 1991; Pittack et al., 1997; Vogel-Höpker et al., 2000; Zhao et al., 2001). Misexpression of truncated FGF receptors suggests that FGF signaling is necessary for the NR to undergo normal specification of retinal cell types (McFarlane et al., 1998; Zhang et al., 2003) and proliferation of precursor cells (Dias da Silva et al., 2007).

Although several paracrine signals involved in OV patterning have been identified and studied extensively, little is known about transcriptional regulatory pathways that respond to paracrine signals and regulate morphogenesis and cytodifferentiation specific to each retinal subdomain. *Pax6*, if misexpressed, is capable of converting a pigmented RPE into a layered NR-like tissue (Azuma et al., 2005). However, its normal expression in the early optic cup does not precisely delineate the NR domain (Kamachi et al., 1998; Li et al., 1994) and it appears to play a role in the dorsoventral patterning (Reza et al., 2007). Subgroup B1 Sox family genes (SoxB1 genes) encode HMG transcription factors (reviewed by Kamachi et al., 2000; Pevny and Lovell-Badge, 1997) implicated in regulating epithelial morphology and neural progenitor state (Abu-Elmagd et al., 2001; Bylund et al., 2003; Dee et al., 2008; Ferri et al., 2004; Graham et al., 2003; Ishii et al., 2001; Kamachi et al., 1998; Kamachi et al., 2001; Köster et al., 2000; Taranova et al., 2006). Although SoxB1 genes show dynamic patterns of expression in the optic cup in chick (Kamachi et al., 1998; Le Rouëdec et al., 2002; Uchikawa et al., 1999), their roles in early retinal development remains to be determined.

In the present study, we demonstrate that all SoxB1 gene members are downregulated in the presumptive RPE by the time that NR and RPE domains become evident morphologically. Using in ovo electroporation, we demonstrate that the forced maintenance of SoxB1 expression inhibits epithelial thinning and pigmentation of the RPE. These phenotypes are associated with the ectopic expression of neural markers that are normally excluded from the RPE. We also demonstrate that SoxB1 genes lie downstream of Fgf4-activated signaling and mediate part of inhibitory effect of FGF on RPE molecular identity. These results suggest that SoxB1 downregulation is necessary for RPE morphogenesis and cytodifferentiation, and is dependent on the absence of exposure to FGF-like signals.

MATERIALS AND METHODS
Embryos
Chick (*Gallus gallus domesticus*) embryos were incubated at 38.5°C in a humidified incubator and were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).
**In situ hybridization**

Sox1, 5'-crystallin, Mitf, Otx2, Sox3, Sox1, Sox2 and Sox3 were amplified by RT-PCR (from embryonic day (E) 3 whole chick embryo cDNA (Sox1, 5'-crystallin), E3.5 NR cDNA (Otx1 and Otx2), E3.5 RPE cDNA (Otx2) or E5 whole eye cDNA (Mitf and Chxl0)). The following primers were used for PCR: Sox1, 5'-CCCTTGCAGCCACATCAGGC-3' and 5'-AACATGTACAGTCTGGGTTCC-3'; 5'-crystallin, 5'-GACGAAAACGTCGTCGCAAGAATG-3' and 5'-CTCCTGAGTATGAGTAAGAACA-3'; Mitf, 5'-CTTCCCCACGACAATTTCCGACG-3' and 5'-ACACTGGGCTACGATGGACAC-3'; Otx2, 5'-GATATCAACATTTACTTGATGTTATCTTAC-3' and 5'-TCTAGATCACAAACACTGGAATCCATTAG-3' (second round); Sox3, 5'-ACAGAAGAGTTGTCAATGTTTCAGC-3' and 5'-TCTAGATACTACATACTACATTCCGAGTC-3' (first round); 5'-ACGAAGAGTTGTCAATGTTTCAGC-3' and 5'-GCTCTTCTTCTGTAACACTGGAGACG-3' (second round); Sox1, 5'-GATATCACAAGAGATGTCTCTCATAAAGTGT-3' and 5'-TCTAGAGGCTTCTCATAAAGTGGCGGCA-GGT-3' (first round), 5'-GATATCACAAGAGATGTCTCTCATAAAGTGT-3' and 5'-TCTAGAGGCTTCTCATAAAGTGGCGGCA-GGT-3' (second round); Sox2, 5'-GATATCACAAGAGATGTCTCTCATAAAGTGT-3' and 5'-TCTAGAGGCTTCTCATAAAGTGGCGGCA-GGT-3'. Amplified cDNAs were ligated into pCRII-TOPO vector (Invitrogen, CA, USA), sequenced and used as templates for synthesis of digoxigenin-labeled riboprobes for in situ hybridization (Hurtado and Mikawa, 2006). Plasmids used to synthesize riboprobes (Hurtado and Mikawa, 2006) were gifts from Dr. Mima et al. (Mima et al., 1995); Das et al. (2000); Ishii et al., (1998). The Sox3 gene (Mikawa, 1995; Das et al., 2000; Ishii et al., 1998) was inserted into the site of pBluescript KS+, and SmaI and RsrI. These cDNAs were digested with EcoRV and Xhol, RT-PCR was used to amplify Sox2 and Sox3 genes from E3 whole chick embryo cDNA. The following primers were used: Sox2, 5'-AAAGATATCGGGCTTGGGACTTCGCCGCCGCC-3' and 5'-GCTCTAGAGTCATCTCCATGATGAGGGGAGAGAGGAG-3'; Sox3, 5'-CTCGATATCACCCCTCCGCCCTCCATCG-3' and 5'-GCTCTAGATATCACCCCTCCGCCCTCCATCG-3'. The amplified cDNAs were ligated into the pCRII-TOPO vector (Invitrogen, CA, USA), sequenced and excised with EcoRV and Xhol. All the above fragments were inserted into Smal-Xhol sites of pCXZ. The vectors used for constitutive expression of Fgf4 and a repressor form of Sox1 were proteins have been published in Mima et al. (Mima et al., 1995a; Mima et al., 1995b) and Bylund et al. (Bylund et al., 2003), respectively.

**In ovo electroporation**

In ovo electroporation was carried out according to Ishii and Mikawa (Ishii and Mikawa, 2005). Briefly, after windowing the shell, 100-200 μl of ink (Black India; Rotring, Germany; diluted 1:40 in phosphate buffered saline, PBS) was injected beneath the embryo to render the embryo visible. A gold-plated wire electrode (2 mm long), which acts as an anode, was placed lateral to the stage 10-11 right eye. A sharpened tungsten needle was used as a cathode, which was inserted into the lumen of the right OV. After the DNA solution (2 μl/μl, 50 nl) was injected into the OV, electric pulses (5 V, 50-millisecond duration, 150-millisecond interval) were applied, using the pulse generator ECM 830 (BTX, San Diego, CA, USA). Eggs were sealed with Parafilm and reincubated. The embryos were fixed and stained with X-Gal staining, in situ hybridization or immunohistochemistry.

**In vitro electroporation of the RPE**

The RPE at E6 were isolated together with the underlying mesodermal tissue and electroporated as described (Fukuda et al., 2000), with some modifications. A vessel made of 1% agarose gel was placed between two electrodes (Gene-paddles, Harvard Apparatus, MA, USA; 7 mm distance), which were placed on a 10 mm plastic dish filled with PBS. RPE fragments were mixed with 200 ng/ml DNA solution in the gel vessel and electroporated (60 V, 50 milliseconds, 5 pulses, 150-millisecond interval), using ECM 830. After electroporation, the tissues were embedded in collagen gel and cultured in Medium 199 containing 10% fetal bovine serum.

**Immunostaining**

Embryos were fixed in 4% paraformaldehyde in PBS, embedded in OCT compound (Sakura Finetek, CA, USA) and sectioned in a cryostat at 12-14 μm intervals. The sections were rehydrated in PBS, permeabilized in 0.2% Triton X-100 in PBS, blocked in 1% bovine serum albumin in PBS (blocking solution) and incubated with primary antibodies diluted with blocking solution. The following antibodies were used: anti-Sox2 (1:1000), rabbit polyclonal; Millipore, MA, USA), monoclonal anti-β-galactosidase (1:500; GAL-13; Sigma, MO, USA), rabbit polyclonal anti-β-galactosidase (1:800; 5-Prime-3Prime, CO, USA), anti-Iso1 (1:200); 39.ADS, concentrate; Developmental Studies Hybridoma Bank, IA, USA), anti-villinin (1:1000; GC-C; Sigma, MO, USA), anti-class-III β-tubulin (1:500; TuJ1, Covance, CA, USA), anti-neurofilament (1:500; 3A10, concentrate; Developmental Studies Hybridoma Bank, IA, USA), anti-laminin (1:200; Developmental Studies Hybridoma Bank, IA, USA), anti-laminin (1:200; L9393; Sigma, MO, USA) and HNK-1 (1:100; CD57; Becton Dickinson, CA, USA), anti-neurofilament (1:500; 3A10, concentrate; Developmental Studies Hybridoma Bank, IA, USA), anti-villinin (1:1000; GC-C; Sigma, MO, USA), anti-class-III β-tubulin (1:500; TuJ1, Covance, CA, USA), anti-neurofilament (1:500; 3A10, concentrate; Developmental Studies Hybridoma Bank, IA, USA), anti-laminin (1:200; L9393; Sigma, MO, USA) and HNK-1 (1:100; CD57; Becton Dickinson, CA, USA). After overnight incubation at 4°C, the sections were washed with PBS. Primary antibodies were detected with Alexa 488- or 594-conjugated secondary antibodies (Invitrogen, CA, USA) as described previously (Ishii et al., 2007). For detection of F-actin, Texas Red-X phallolidin (Invitrogen, CA, USA) was added at 1:80 to the secondary antibody solution. For BrdU labeling, 30 μg of bromodeoxyuridine (BrdU) was applied on chick embryos in ovo. After 30 minutes of incubation, the embryos were fixed in 4% paraformaldehyde and cryosectioned. The sections were treated with DNAse (20 Kunits/ml; Sigma, MO, USA), double immunostained with anti-BrdU (1:100; Becton Dickinson, CA, USA) and anti-Sox2 (1:1000; Millipore, MA, USA), and photographed under a Zeiss 510 Meta Confocal Microscope. TUNEL staining was performed using the In Situ Cell Death Detection Kit, TMR red (Roche, IN, USA) according to the manufacturer’s instructions. All sections were counterstained with 4’,6-diamidino-2-phenylindole (DAPI).

**Expression vectors**

Full coding regions of Sox1, Sox2, Sox3 and Sox9 were inserted into the dicistronic pCXZ vector, which allows the simultaneous expression of a test gene and the reporter LacZ gene (Mikawa, 1995; Das et al., 2000; Ishii et al., 2003, Ishii and Mikawa, 2005). The Sox1 fragment was excised from pCMV-SV2-cSox1 (a gift from Dr. Kondoh, Osaka University, Suita, Osaka, Japan) with HindIII and Xhol. The Sox1 fragment was excised from pBluescript SK-cSox3 (Uwanogho et al., 1995) with XhoRI and DraI. These fragments were blunt-end ligated into the Smal site of pBluescript KS+, and excised with EcoRV and Xhol. RT-PCR was used to amplify Sox2 and Sox9 genes from E3 whole chick embryo cDNA. The following primers were used: Sox2, 5'-AAAGATATCGGGCTTGGGACTTCGCCGCCGCC-3' and 5'-GCTCTAGAGTCATCTCCATGATGAGGGGAGAGGAG-3'; Sox3, 5'-CTCGATATCACCCCTCCGCCCTCCATCG-3' and 5'-GCTCTAGATATCACCCCTCCGCCCTCCATCG-3'. The amplified cDNAs were ligated into the pCRII-TOPO vector (Invitrogen, CA, USA), sequenced and excised with EcoRV and Xhol. All the above fragments were inserted into Smal-Xhol sites of pCXZ. The vectors used for constitutive expression of Fgf4 and a repressor form of Sox1 were proteins have been published in Mima et al. (Mima et al., 1995a; Mima et al., 1995b) and Bylund et al. (Bylund et al., 2003), respectively.
RESULTS

Expression patterns of subgroup B1 Sox genes in the eye primordium

To investigate the roles of the SoxB1 gene family, we examined their expression patterns during early retinal development in avian embryos. In situ hybridization analysis of all known SoxB1 family members, Sox1, Sox2 and Sox3, revealed both member-specific and shared features in their expression patterns. At stage 10, Sox1 transcripts were detectable only in the ventral region of the forebrain (Fig. 1A), and Sox2 and Sox3 transcripts were detectable more broadly in the forebrain, including in the OV (Fig. 1H,O). At stage 13, shortly after the optic stalk is constricted, all three members showed a graded expression pattern, with a strong staining in the brain and optic stalk that declines gradually towards the distal part of the OV (Fig. 1B,I,P). As the bilaminar optic cup began to form, a strong signal for Sox2 remained detectable in the presumptive NR (Fig. 1J-N), whereas signals for Sox1 and Sox3 declined both in the NR and the RPE (Fig. 1C,G,Q). Expression of Sox1 and Sox3 became detectable again in the optic cup by stage 19, but the signals were restricted to the presumptive NR (Fig. 1D,G,R-U). Importantly, no expression of three SoxB1 genes was detectable in the presumptive RPE after the onset of the optic cup invagination (Fig. 1C-G,J-N,Q-U).

Consistent with the in situ hybridization data, our RT-PCR analysis (Fig. 1V) detected expression of all three SoxB1 family members in the OV prior to overt RPE domain specification (stage 10), judged by the absence of detectable expression of two RPE markers, Mitf and tyrosinase. Once the bilaminar optic cup formed, both Mitf and tyrosinase were detectable in the presumptive RPE, whereas their expression remained detectable in the presumptive NR through stages 19-25. These data clearly demonstrate that all three members of the SoxB1 gene family are expressed in the OV and in the NR of the optic cup, but are downregulated in the RPE.

SoxB1 expression inhibits RPE morphogenesis and differentiation

Mature RPE is a melanin-containing simple cuboidal epithelium. To address the role of SoxB1 downregulation in RPE morphogenesis and cytodifferentiation, vectors for constitutive expression of SoxB1 gene family members were introduced into stage 10-11 OVs by in ovo electroporation (Fig. 2A,B). Our dicistronic expression vectors coexpress a Sox protein and the
reporter β-galactosidase (β-Gal) thus allowing detection of transfected cells by X-Gal staining (Fig. 2C-H), or immunostaining for β-Gal (Fig. 2I,K) or Sox2 (Fig. 2I,N).

Sections of X-Gal-stained optic cups at stage 19-20 (40 hours after electroporation) revealed that a control vector, which encodes only β-Gal, causes no obvious morphological abnormality (sample number n=26; Fig. 2C,D). By contrast, a Sox1/LacZ vector dramatically increased the thickness of the presumptive RPE layer (n=11; Fig. 2E,F). A Sox2/LacZ vector (n=15; Fig. 2G-O), as well as a Sox3/LacZ vector (n=25; data not shown), caused the same thickening phenotype. Coexpression of SoxB1 protein and the reporter β-Gal in transfected cells was confirmed by double immunostaining (Fig. 2I). Endogenous Sox2 protein was detected in nuclei of NR cells but not in β-Gal-negative RPE cells, consistent with the above in situ hybridization data (Fig. 1K-N). Transfected RPE cells expressing β-Gal contained Sox2-positive nuclei and exhibited a thickened morphology.

A detailed inspection of cellular morphology showed that the majority of SoxB1-transfected RPE cells were elongated across the presumptive RPE layer (Fig. 2J). Clusters of these cells were more constricted at the apical side (Fig. 2J,K), often exhibiting a local invagination (Fig. 2K). Consistent with this morphogenetic event, much denser accumulation of actin was evident at the apical side of transfected cells compared with the basal side (Fig. 2L,M). In addition, nuclei of transfected cells were distributed at different apicobasal levels in the thickened epithelial layer (Fig. 2L), in sharp contrast with nuclei in neighboring untransfected regions that were arranged in a single row at the middle of cuboidal or low columnar cells (Fig. 2M). Thus, maintained expression of SoxB1 inhibits formation of a simple cuboidal epithelium. No increase in cell proliferation (n=8, see Fig. S1A-C in the supplementary material) or altered apoptosis (n=4; see Fig. S1D,E1 in the supplementary material) was detectable in thickened SoxB1-transfected cells.
We next tested whether SoxB1 expression affects RPE cytodifferentiation by probing for pigmentation 72 hours after electroporation (stage 21-22). RPE cells electroporated with a β-Gal-only vector accumulated melanin pigments (Fig. 2N; n=5), whereas the cells transfected with Sox1 (n=4; Fig. 2O), Sox2 (n=5; Fig. 2P) or Sox3 (n=6; Fig. 2Q) vectors did not. Thus, maintained SoxB1 expression inhibits both RPE cell morphogenesis and cytodifferentiation in a cell-autonomous fashion.

**Maintained SoxB1 expression induces ectopic neural marker expression**

Previous studies have shown that the misexpression of Sox genes in the ectoderm promotes development of the lens (Kamachi et al., 2001), neural crest (Cheung and Briscoe, 2003; Cheung et al., 2005) and neuroectoderm (Dee et al., 2008; Kishi et al., 2000; Mizuseki et al., 1998). We tested whether the maintained expression of SoxB1 changes the fate of the presumptive RPE into any of these tissue types.

First, the co-presence of Sox2 and Pax6 has been shown to promote lens development in surface ectoderm (Kamachi et al., 2001). Since Pax6 is expressed in the OV (Fuhrmann et al., 2000; Kamachi et al., 1998; Vogel-Höpker et al., 2000), it is plausible that ectopic expression of SoxB1 might lead to ectopic lens development in the RPE. However, no β-crystallin signal was detected in Sox2-transfected RPE cells, despite the observation that an intense signal was evident in the authentic lens tissue (n=3; see Fig. S2A,B in the supplementary material).

Second, overexpression of Sox9, a GroupE Sox gene, can trigger ectopic neural crest-like development in the ventral spinal cord (Cheung and Briscoe, 2003; Cheung et al., 2005). Sox2-transfected optic cup, however, showed neither cell delamination nor ectopic immunoreactivity to the neural crest marker HNK-1 (n=3; see Fig. S2C-F in the supplementary material). Conversely, Sox9 misexpression caused disruption of the basal lamina and cell delamination (n=8; see Fig. S2G-L in the supplementary material). Most delaminating β-Gal-positive cells were immunoreactive to HNK-1.

Third, SoxB1 members play a role in early neural fate specification in both *Xenopus* and zebrafish (Dee et al., 2008; Kishi et al., 2000; Mizuseki et al., 1998). Furthermore, multiple Sox-site-dependent neural enhancers of the N-cadherin (N-Cad) gene have been identified (Matsumata et al., 2005). We tested whether maintained SoxB1 expression converts the identity of RPE cells to the neural fate, using a neural marker N-Cad; general neuronal differentiation markers class III β-tubulin (TuJ1) (Lee et al., 1990), neurofilament (3A10) (Serafini et al., 1996) and HuC/D (Marusich et al., 1994; Hyer et al., 1998); and markers for specific retinal cells islet-1 [Isl1, ganglion and amacrine cells (Vogel-Höpker et al., 2000)] and visinin [photoreceptor cells (Bruhn and Cepko, 1996; Vogel-Höpker et al., 2000), it is plausible that SoxB1 expression converts the identity of RPE cells into any of these tissue types. In untransfected eyes, strong N-Cad signal was detected in the RPE. However, no β-crystallin signal was detected in the authentic lens tissue (n=4; Fig. 3A-C). In transfected eyes, intense N-Cad staining was seen ectopically in the presumptive NR (nr) and the brain (br), but not in the presumptive RPE (rp). In transfected eyes (D-F), intense N-Cad staining was seen ectopically in the presumptive RPE. B,C and D,E are high magnification images of boxed regions in A and D, respectively. (G-P) Ectopic expression of neuronal differentiation markers (red) in the Sox2/LacZ-transfected presumptive RPE. Class III β-tubulin (TuJ1; G,H), neurofilament (3A10; I,J), HuC/D (K,L), Isl1 (M,N) and visinin (Q,P). Each section was co-stained with anti-β-Gal antibody (green) and DAPI (blue). (Q-T) Ectopic expression of class III β-tubulin in the presumptive RPE electroporated with Sox1/LacZ (Q,R) and Sox2/LacZ (S,T) expression vectors. H,J,N,P,R,T are high magnification images of boxed regions in G,I,K,M,O,Q,S, respectively. Arrowheads indicate cells positive for neuronal markers. All embryos are at stage 19-20, except for the embryo shown in M and N (stage 21-22). Scale bars: 100 μm. br, brain; le, lens vesicle; nr, presumptive neural retina; rp, presumptive RPE.
whether SoxB1 activity is capable of inducing neural characteristics in a pigmented RPE that is older than E4.5. In vitro electroporation was used to achieve efficient gene transfer into these tissues, which is difficult in ovo due in part to a narrowed optic cup lumen where injected DNA can be held. RPEs were isolated from E6 embryos together with associated mesenchymal tissues. They were then electroporated and explant-cultured for 2 days in vitro. Control RPEs electroporated with a LacZ-only plasmid maintained melanin pigments and thin cuboidal epithelial morphology \((n=4; \text{Fig. 4A,B}).\) Conversely, RPEs electroporated with a Sox2/LacZ vector exhibited an overall increase in epithelial thickness and reduced pigmentation \((n=4; \text{Fig. 4C,D}).\) Signals for HuC/D (Fig. 4E,F), Isl1 (Fig. 4G,H) and visinin (Fig. 4I,J) were detected in subpopulations of transfected RPE cells \((n=3).\) Although some of the electroporated explants contained dying cells, most of these cells were located in the mesenchymal layer and were seen both in control and Sox-transfected explants (Fig. 4A,C). Thus, exogenous SoxB1 expression is capable of suppressing RPE characteristics and activating the neurogenic program even in the pigmented RPE.

**SoxB1 mediates FGF-dependent inhibition of RPE development**

At early stages of eye development, the optic vesicle is bipotential, able to differentiate into both NR and RPE (Lopashov, 1963). We and others have previously shown that activation of FGF signaling facilitates NR development in retinal cells (Guillemot and Cepko, 1992; Hyer et al., 1998; Nguyen and Arnheiter, 2000; Opas and Dziak, 1994; Park and Hollenberg, 1991; Pittack et al., 1991; Pittack et al., 1997; Vogel-Höpker et al., 2000; Zhao et al., 2001). Since the above results demonstrate that SoxB1 regulates RPE development negatively and neural development positively, we asked whether SoxB1 mediates the fate conversion effects of FGF.

We first tested whether SoxB1 genes lie downstream of FGF signal by electroporating the OV with a Fgf4/LacZ expression vector. For easy comparison between transfected and untransfected areas, electrodes were oriented to electroporate the posterior region of the vesicles (Fig. 5A). After a 48-hour incubation, only the posterior region of the optic cup contained transfected cells, as demonstrated by immunostaining for β-Gal (Fig. 5B-D), and showed an ectopic increase in epithelial thickness in the presumptive RPE \((i-nr\) in Fig. 5D). After a 72-hour incubation (stage 21-22), β-Gal was no longer detectable (data not shown), but the posterior region of the presumptive RPE remained thick and was devoid of pigments as a result of the FGF-induced RPE-to-NR conversion, as reported previously (Guillemot and Cepko, 1992; Hyer et al., 1998; Park and Hollenberg, 1991). Importantly, ectopic expression of Sox1, Sox2 and Sox3 was detected in this thickened epithelial area \((n=4; \text{Fig. 5G,H,L,M,Q,R}).\) The expression of SoxB1 members was not detectable in the untransfected region where normal pigmentation took place (Fig. 5I,N,S). Thus, all three SoxB1 genes are downstream of FGF-activated signaling.

To determine the extent to which SoxB1 activity elicits the effect of FGF, we compared the genes downstream of Sox2 and Fgf4. Mitf and Otx2 encode transcription factors crucial for RPE fate specification (Bumsted and Barnstable, 2000; Martínez-Morales et al., 2001; Martínez-Morales et al., 2003; Mochii et al., 1998a; Mochii et al., 1998b; Nakayama et al., 1998; Nguyen and Arnheiter, 2000). Other regulatory genes we examined, Six3 (Bovolenta et al., 1998; Oliver et al., 1995), Rx1 (Furukawa et al., 1997; Mathers et al., 1997; Ohuchi et al., 1999), Chx10 (Chen and Cepko, 2000; Furmann et al., 2000; Liu et al., 1994) and Optx2 (López-Ríos et al., 1999; Jean et al., 1999; Toy and Sundin, 1999), are expressed predominantly in the presumptive NR. Consistent with previous studies, our in situ hybridization analysis shows that, in normal development, the strong signals of these genes become restricted to either the RPE or NR domain as a bilayered optic cup form (see Fig. S3 in the supplementary material). Sox2, when misexpressed in the presumptive RPE, downregulated Mitf and Otx2 (Fig. 6G,H,M,N), and upregulated Six3 (Fig. 6I,O). No upregulation of Rx1, Chx10 and Optx2 was detected in the transfected cells (Fig. 6L-P). Fgf4 similarly downregulated Mitf and Otx2 in transfected presumptive RPE (Fig. 6S,T,i-nr), but upregulated all NR marker genes \((i.e. \text{Six3, Rx1, Chx10 and Optx2; Fig. 6U-X}).\) Thus, whereas both Sox2 and Fgf4 suppress crucial regulators of RPE fate, Sox2 can regulate some, but not all, molecular markers associated with early NR identity.
Sox activity in the presumptive NR is necessary to repress RPE identity

As SoxB1 expression is maintained in the NR domain, we reasoned that endogenous Sox activity might have a role in suppressing RPE identity. To address this possibility, we misexpressed a repressor form of a SoxB1 protein, where the Sox3 HMG domain is fused to the repressor domain of the *D. melanogaster* Engrailed protein (HMG-EnR) (Bylund et al., 2003). In these eyes ectopic expression of *Mitf* and *Otx2* was detected in the presumptive NR (n=4; Fig. 7), implying that SoxB1 plays a role in suppressing RPE identity, possibly by activating transcription of downstream target genes.

DISCUSSION

Retinal patterning begins with partitioning the bipotential retinal primordium of the OV into the RPE and NR. Although several paracrine signals implicated in specifying RPE and NR fates in the OV have been studied extensively, little is known about the underlying transcriptional regulation in this developmental process. The present study provides the first experimental evidence that developmentally regulated downregulation of SoxB1 genes is crucial for normal OV patterning.

The purpose of this study was to investigate the role of SoxB1 downregulation in RPE development. Downregulation of SoxB1 in the presumptive RPE begins in a specific time window prior to the initiation of optic cup morphogenesis. Since no ideal cis-element specific for this developmental window and region is currently available, it is difficult, if not impossible, to use a whole-organism/transgenic approach. Electroporation, used in the present study, allows the efficient introduction of exogenous genes in defined regions of the retinal primordium in this specific developmental window. Since expression of the transgene declines gradually due to the degradation and dilution of the introduced DNA, our analysis was limited to the early effects that became evident within 72 hours post electroporation. Abnormalities in cell morphogenesis and molecular markers observed in this short incubation period suggest that SoxB1 downregulation plays a role in early molecular events involved in RPE cell fate specification, although the long-term effect of maintained SoxB1 expression on retinal patterning remains to be explored.

The SoxB1 gene family consists of three members, *Sox1*, *Sox2* and *Sox3*, in multiple vertebrate species, including human, mouse, chick and *Xenopus* (Bowles et al., 2000; Nitta et al., 2006). Member-specific expression patterns in the eye and other organs (Collignon et al., 1996; Kamachi et al., 1998; Uchikawa et al., 1999; Wood and Episkopou, 1999) (Fig. 1), as well as differential effects of SoxB1 members on cultured neural progenitor cells (Kan et al., 2004), imply that each family member may play a different role in the developing eye. However, our data show that all chick SoxB1 genes are dramatically downregulated prior to the onset of RPE differentiation, and that misexpression of any single SoxB1 family member in the presumptive RPE suppresses RPE identity and promotes neural development. It is therefore likely that the absence of expression of all three SoxB1 genes is necessary for normal RPE development.
The precise mechanism underlying SoxB1 downregulation is currently unclear. Importantly, however, the present study suggests that the level of FGF signal plays a crucial role in this downregulation. We and others have shown previously that FGFs can act as a negative regulator of RPE development in vivo, overriding BMP and activin signaling that promotes RPE development (Furhmann et al., 2000; Hyer et al., 2003; Müller et al., 2007). Ectopic expression of all SoxB1 genes in Fgf4-stimulated presumptive RPE suggests that the absence of exposure to FGF-like signals is crucial for SoxB1 downregulation. A regulatory element of the Sox2 gene requires a Sox2 binding site for its transactivation in the OV (Inoue et al., 2007). Given that SoxB1 members appear to share similar transactivation properties (Kamachi et al., 1999; Okuda et al., 2006), downregulation of Sox2 might depend not only on the reduced activity of Sox2 itself but also on the reduced activities of Sox1 and Sox3. Determining the hierarchical relationship between SoxB1 members would provide a clue to resolve the mechanisms of their coordinated downregulation in the presumptive RPE.

The OV cells can differentiate into either pigmented or neural cells, even in the absence of the surface ectoderm (Hyer et al., 1998). Surface ectoderm organizes the placement of these cell types by instructing the fate of uncommitted bipotential OV cells, presumably through FGF or FGF-related factors (Hyer et al., 1998). Our data show that exogenous Fgf4 maintains SoxB1 expression in the presumptive RPE and that maintained expression of SoxB1 converts presumptive RPE into a neural-like tissue. These data are consistent with SoxB1 being downstream of FGF in a pathway that regulates this binary cell fate decision. A clear understanding of the molecular mechanisms underlying this fate decision awaits further study.

Fig. 6. SoxB1 mediates part of the FGF effect. (A-X) Expression of Mitf (A,G,M,S), Otx2 (B,H,N,T), Six3 (C,I,O,U), Rx1 (D,J,P,V), Chx10 (E,K,Q,W) and Optx2 (F,L,R,X) in untransfected eyes on the contralateral side (A-F) and in eyes transfected with Sox2 (G-R) and Fgf4 (S-X), as detected by in situ hybridization. Anterior, top; posterior, bottom. M,N,O,P,R are high magnification images of G,H,I,J,K,L, respectively. Arrowheads in M-R indicate transfected cells, as confirmed both morphologically, as thickened epithelial areas, and by β-Gal immunostaining on sister sections (data not shown). i-nr, induced neural retina; le, lens vesicle; nr, presumptive NR; rpe, presumptive RPE. Scale bar: 100 μm.

Fig. 7. SoxB1-dependent transactivation plays a role in suppressing RPE marker genes. Ectopic expression of Mitf (A) and Otx2 (B) (arrowheads) in the NR at stage 18 after coelectroporation with HMG-EnR and EGFP vectors. Transfected regions were visualized by in situ hybridization for EGFP transcripts on sister sections (C). Arrows in B indicate endogenous Otx2 expression in differentiating retinal neurons. Anterior, left; posterior, right. le, lens vesicle; nr, presumptive NR; rpe, presumptive RPE. Scale bar: 100 μm.
of Mitf and Otx2 in SoxB1-transfected cells suggests that SoxB1-dependent fate conversion is mediated at least in part by the suppression of these genes. SoxB1 proteins function in both the activation and repression of gene expression (Ambrosetti et al., 2000; Cavallaro et al., 2008; Kamachi et al., 2000; Kondoh et al., 2004; Mansukhani et al., 2005). The different effects of SoxB1 on RPE and NR marker genes might depend on DNA-binding partner proteins (Kamachi et al., 1999; Kamachi et al., 2000; Kondoh et al., 2004). The distinct phenotypes caused by SoxB1 and Sox9 misexpression, demonstrated in the present study, are consistent with the involvement of such protein interactions, which are likely to be mediated by sequences outside of the conserved HMG DNA-binding domain (Kamachi et al., 1999). However, the pairing with one partner protein does not appear to fully explain how SoxB1 affects the fate of retinal cells. For example, despite the co-presence of Sox2 and Pax6 in normal and SoxB1-transfected eyes, α-crystallin is expressed only in the lens and not in the optic cup, even though Sox2 and Pax6 directly regulate the α-crystallin promoter (Kamachi et al., 2001). Nevertheless, the synergistic action of Sox2 and Bmf2 transactivates the nestin neural enhancer (Tanaka et al., 2004) and, more recently, Otx2 has been identified as a partner factor that can interact with Sox2 to transactivate an enhancer of the Rx gene (Danno et al., 2008). Although the in vivo roles of these protein interactions in retinal development are currently unknown, interactions with multiple partner proteins might underlie the transcriptional activities of SoxB1 on different downstream genes.

Mtf and Otx2 activities suppress NR identity and promote pigmentation both in vivo and in vitro (Tsukiji et al., 2009; Martinez-Morales et al., 2003; Mochii et al., 1998a). Our data suggest that the repression of SoxB1-dependent transactivation causes the ectopic expression of these RPE-inducing factors, suggesting that SoxB1 expression plays a role in suppressing RPE identity. Taranova et al. have demonstrated that Sox2 dosage is crucial for the temporal and spatial regulation of retinal progenitor cell differentiation, using null, hypomorphic and heterozygous mutant mice (Taranova et al., 2006). Although Sox2 null mice have smaller retinas that lack Notch1 expression, they still appear to retain distinct NR and RPE domains. This might be due to a functional redundancy of SoxB1 members and/or to the stage when Cre recombinase is activated. It would be interesting to test whether the presumptive NR is converted into RPE in mice lacking multiple SoxB1 member genes.

Retinal regeneration occurs in a variety of amphibians, some fish and in embryonic chick (reviewed by Reh and Pittack, 1995). This remarkable phenotypic change involves a fate conversion of RPE cells and is promoted by FGFs (Coulombre and Coulombre, 1965; Park and Hollenberg, 1989; Park and Hollenberg, 1991; Yoshii et al., 2007). Our data suggest that SoxB1 genes lie downstream of FGF-activated signaling and that their activity bypasses the requirement of paracrine factors and stage-dependent competence to respond to these factors. However, it is unlikely that SoxB1 mediates all the effects of exogenous FGF, as Sox2 activity did not induce detectable expression of Rxl, Chx10 or Otpx2. It is possible that FGF-induced retinal fate conversion involves multiple branches of gene regulatory pathways and that SoxB1 genes mediate some, but not all, of these branches. Consistent with this view, the misexpression of activated Mek1, which activates only part of FGF signaling, inhibits RPE pigmentation but does not induce a laminated NR (Galy et al., 2002). Further dissemination of the transcriptional regulatory pathways in the developing eye would provide insights into the mechanisms whereby multiple transcriptional pathways cooperate to generate this highly organized sense organ.

Acknowledgements
We thank Jeanette Hyer for critical reading of the manuscript; Hitato Kondoh for Sox1 plasmids; Paul Scotting for Sox2 and Sox3 plasmids, and Jonus Muhr for the HMGB-enr expression construct. Monoclonal antibodies against neuronfilament (3A10), Isil (39.D5) and visinin (7G4) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biological Sciences at The University of Iowa. This work was supported in part by the NIH. Y.J. was supported by fellowships from the Revson Foundation and CVRI at UCSF. Deposited in PMC for release after 12 months.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/15/2579/DC1

References
and organ culture techniques for the elucidation of mechanisms of epithelial-mesenchymal interaction in the developing gut. *Dev. Growth Differ.* **42**, 207-211.


