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Morphogenesis and cytodifferentiation of the avian retinal pigmented epithelium require downregulation of Group B1 Sox genes

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The optic vesicle is a multipotential primordium of the retina, which becomes subdivided into the neural retina and retinal pigmented epithelium domains. Although the roles of several paracrine factors in patterning the optic vesicle have been studied extensively, little is known about cell-autonomous mechanisms that regulate coordinated cell morphogenesis and cytodifferentiation of the retinal pigmented epithelium. Here we demonstrate that members of the SoxB1 gene family, *Sox1*, *Sox2* and *Sox3*, are all downregulated in the presumptive retinal pigmented epithelium. Constitutive maintenance of SoxB1 expression in the presumptive retinal pigmented epithelium both in vivo and in vitro resulted in the absence of cuboidal morphology and pigmentation, and in concomitant induction of neural differentiation markers. We also demonstrate that exogenous Fgf4 inhibits downregulation all SoxB1 family members in the presumptive retinal pigment epithelium. These results suggest that retinal pigment epithelium morphogenesis and cytodifferentiation requires SoxB1 downregulation, which depends on the absence of exposure to an FGF-like signal.

KEY WORDS: Eye, Retina, Pigmented epithelium, Electroporation, Sox genes, Chick embryo

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 periocular 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).

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In situ hybridization

Sox1, ∂-crystallin, *Mitf*, *Otx2*, *Six3*, *Rx1*, *Chx10* and *Optx2* cDNAs were amplified by RT-PCR from embryonic day (E) 3 whole chick embryo cDNA (*Sox1*, ∂-crystallin), E3.5 NR cDNA (*Rx1* and *Opix2*), E3.5 RPE cDNA (*Otx2*) or E5 whole eye cDNA (*Mitf* and *Chx10*). The following primers were used for PCR: *Sox1*, 5′-CCCTTGACGCACATCTGAGCG-3′ and 5′-AACTATGTACAGTCTGGGTTCC-3′; ∂-crystallin, 5′-GAGCAAAACGTCGTCCGAAATG-3′ and 5′-CTCTGGATTAGTGAGATAAGCA-3′; *Mitf*, 5′-CTTCCCACAGCAATTCCGAGC-3′ and 5′-ACACTGGGCTACCGATGAAGCAC-3′; *Otx2*, 5′-GATATCCAACTTTAGCATGATGTCTTATCT-3′ and 5′-TCTAGAGTCTGAGCAGGAAATGAGTCTG-3′ (first round), 5′-GATATCCAACTTTAGCATGATGTCTTATCT-3′ and 5′-TCTAGATCACAAAACCTGGAACTTCCATGAG-3′ (second round); *Six3*, 5′-ACGAAGAGTTGTCAATGTTTCAGC-3′ and 5′-TCTAGATATCATACATCACATTCCGAGTC-3′ (first round), 5′-ACGAAGAGTTGTCAATGTTTCAGC-3′ and 5′-GCTCTTTCTGTCAAACTGGAGAC-3′ (second round); *Rx1*, 5′-GATATCACCAAGATGTTCCTCAATAAGTGT-3′ and 5′-TCTAGAGCGTTCATCAAATGGGCTGCCAGGT-3′ (first round), 5′-GATATCACCAAGATGTTCCTCAATAAGTGT-3′ and 5′-TCTAGATCAAATGGGCTGCCAGGTCTTGTC-3′ (second round); *Chx10*, 5′-GGCTTCGGCATCCAGGAGATC-3′ and 5′-TTCTGTGATGCACTGGACTTC-3′; *Optx2*, 5′-GATATCGGAACCGCCACCATGTTCCAGCT-3′ and 5′-CGGGGACAGCGATAGGCACTG-3′. Nested PCR was carried out to amplify *Otx2*, *Six3* and *Rx1*. Amplified cDNA fragments were ligated into pCRII-TOPO vector (Invitrogen, CA, USA), sequenced and used as templates for synthesis of digoxigenin-labeled riboprobes (Hurtado and Mikawa, 2006). Plasmids used to synthesize riboprobes for *Sox2* and *Sox3* (Uwanogho et al., 1995) were gifts from Dr Scotting (University of Nottingham, Nottingham, UK). Section in situ hybridization was performed as described previously (Ishii et al., 1998).

RT-PCR

Total RNAs were isolated from the OV at stage 10, and from separated RPE and NR segments of the optic cup at stages 19-20 and 24-25 using the RNeasy Mini Kit (Qiagen, CA, USA). Collagenase (0.03%, for 5-10 minutes; Type 1; Worthington, NJ, USA) was used to remove surrounding mesenchyme from retinal tissue. Approximately 200 ng of RNAs were reverse transcribed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, CA, USA). For a negative control, reactions for cDNA synthesis were carried out in parallel without reverse transcriptase. The following primers were used for PCR: *Sox1*, 5′-AGAAGGTAACGGTGGCTTTACTGAC-3′ and 5′-GCGCGAGAACATCTACGGAAACTC-3′; *Sox2*, 5′-GGGCTGGTTCCAGGCTAAAGTAGT-3′ and 5′-AAGGGTCTCTTCTCCGCCTCGGAT-3′; *Sox3*, 5′-CGGCTCAGCAGACTCGATACTAAC-3′ and 5′-AACACAGATCAAACATCCATCC-3′; *Mitf*, 5′-GGCTTCGGCATCCAGGAGATC-3′ and 5′-TTCTGTGATGCACTGGACTTC-3′; *Chx10*, 5′-CTTCCCACAGCAATTCCGAGC-3′ and 5′-ACACTGGGCTACCGATGAAGCAC-3′; tyrosinase, 5′-TACTGCTGTGCTCTCTGGGCTCA-3′ and 5′-GTTACAAGAGGCACAAGGAGTGG-3′; glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), 5′-CAGCCTTCACTACCCTCTTG-3′ and 5′-ACGCCATCACTATCTTCCAG-3′. PCR was carried out under the following conditions: 1 cycle of denaturing at 94°C (30 seconds), 21 (*Gapdh*) or 29 (*Sox1*, *Sox2*, *Sox3*, *Mitf* and *Tyrosinase*) cycles of denaturing at 94°C (30 seconds), annealing at 58°C (*Gapdh*), 60°C (*Mitf* and tyrosinase), or 65°C (*Sox1*, *Sox2* and *Sox3*) (45 seconds) and elongation at 72°C (2 minutes). The amplified DNA fragments were separated on a 1% agarose gel containing ethidium bromide, and visualized and image captured with the FOTO/Analyst Investigator System (Fotodyne, WI, USA).

Expression vectors

Full coding regions of *Sox1*, *Sox2*, *Sox3* and *Sox9* were inserted into the dicistronic pCXIZ vector, which allows the simultaneous expression of a test gene and the reporter *LacZ* gene (Mikawa, 1995; Das et al., 2000; Ishii et al., 2004; Ishii and Mikawa, 2005). The *Sox1* fragment was excised from pCMV/SV2-*cSox1* (a gift from Dr Kondoh, Osaka University, Suita, Osaka, Japan) with *Hin*dIII and *Xho*I. The *Sox3* fragment was excised from pBluescript SK-*cSox3* (Uwanogho et al., 1995) with *Rsr*II and *Dra*I. These fragments were blunt-end ligated into the *Sma*I site of pBluescript KS$^+$, and excised with *Eco*RV and *Xba*I. RT-PCR was used to amplify *Sox2* and *Sox9* genes from E3 whole chick embryo cDNA. The following primers were used: *Sox2*, 5′-AAAGATATCGGCTTGGGACTTCGCCGCCGC-3′ and 5′-GCTCTAGAGTCTTACATATGTGATAGAGG-3′; *Sox9*, 5′-CTCGATATCTAACCCTTCCCCGCCCCTCAG-3′ and 5′-CGCTCTAGATTAAGGCCGGGTGAGCTGCGTG-3′. The amplified cDNAs were ligated into the pCRII-TOPO vector (Invitrogen, CA, USA), sequenced and excised with *Eco*RV and *Xba*I. All the above fragments were inserted into *Sma*I-*Xba*I sites of pCXIZ. The vectors used for constitutive expression of *Fgf4* and a repressor form of SoxB1 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 μg/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 by 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 (Genepaddles, 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/μl 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-3 Prime, CO, USA), anti-N-cadherin (1:1000; GC-4; 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-HuC/D (1:500; 16A11; Invitrogen, CA, USA), anti-Isl1 (1:200; 39.4D5, concentrate; Developmental Studies Hybridoma Bank, IA, USA), anti-visinin (1:800; 7G4, 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 phalloidin (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).

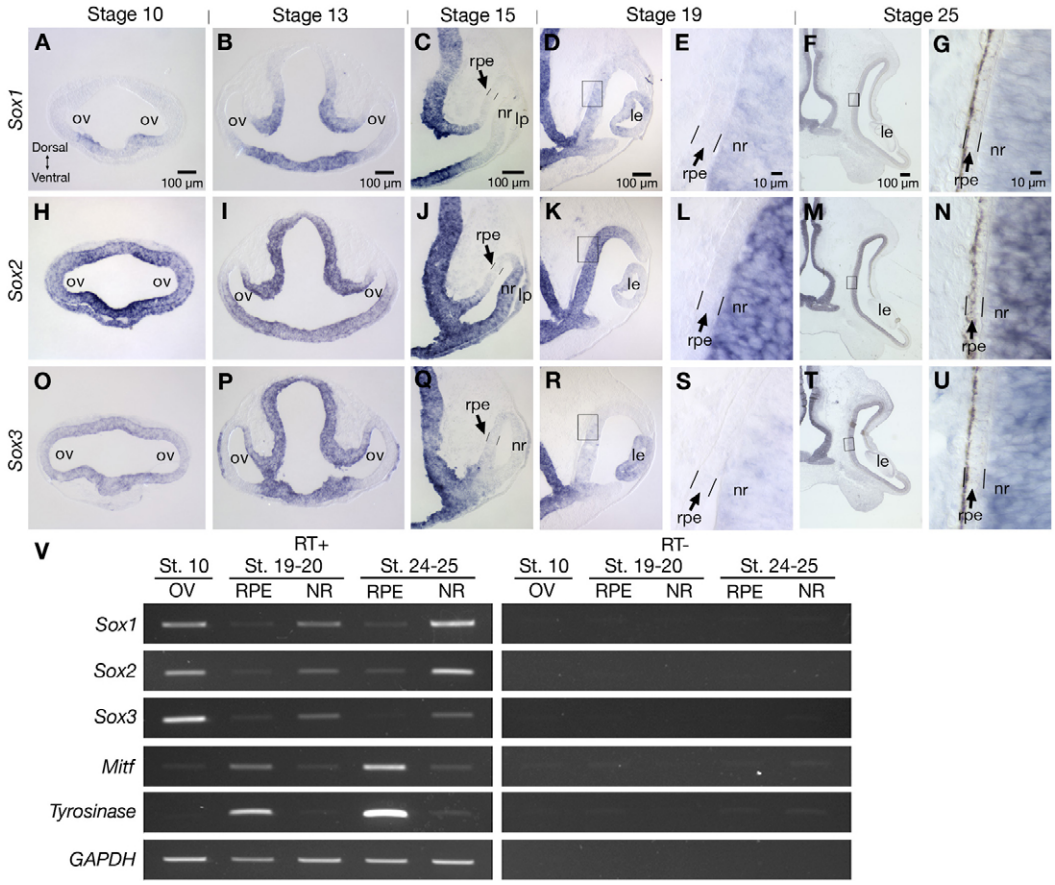

Fig. 1. SoxB1 downregulation in the presumptive RPE in the developing chick eye. (**A-U**) Transverse sections through the eye primordium at various developmental stages stained by in situ hybridization for *Sox1*, *Sox2* and *Sox3* transcripts, as indicated. Dorsal, up; ventral, down. E,G,L,N,S,U are high magnification images of boxed regions in D,F,K,M,R,T, respectively. At stage 10 (A,H,O), broad expression of *Sox2* and *Sox3* is detectable in the optic vesicle. After the formation of the double-layered optic cup (C-G,J-N,Q-U), however, none of three SoxB1 genes shows strong expression in the presumptive RPE. le, lens vesicle; lp, lens placode; nr, presumptive neural retina (NR); ov, optic vesicle; rpe, presumptive retinal pigmented epithelium (RPE). Scale bars: 100 µm in A-C,D,F; 10 µm in E,G. (**V**) RT-PCR analysis for *Sox1*, *Sox2*, *Sox3*, *Mitf* and tyrosinase. *Gapdh* was used as a control. RT reaction mixtures without reverse transcriptase (RT–) were used as negative controls, and gave no detectable signal in our conditions.

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,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 at stages 19-20 and became even stronger by stages 24-25. Coinciding with RPE marker gene expression, expression of *Sox1*, *Sox2* and *Sox3* dramatically declined in the 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

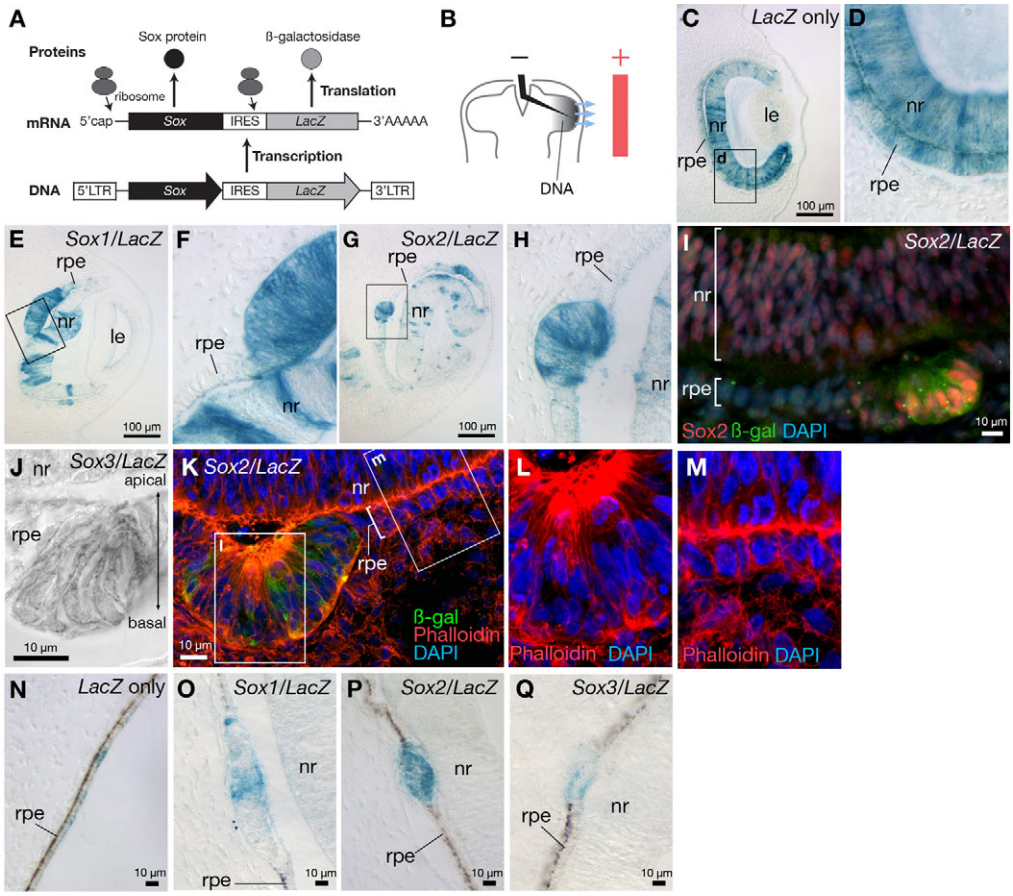

Fig. 2. Maintained expression of SoxB1 inhibits RPE morphogenesis and cytodifferentiation. (**A**) A dicistronic expression vector, pCXIZ. Sox protein and β-Gal are cotranslated through 5′ cap-dependent and -independent mechanisms. (**B**) Electroporation of stage 10-11 optic vesicle. A tungsten needle electrode (cathode; black) and a gold-plated wire electrode (anode; red) were used to achieve region-specific gene transduction. (**C-J**) Eyes electroporated with control *LacZ* (C,D), *Sox1/LacZ* (E,F) and *Sox2/LacZ* (G,H) expression vectors. The eyes were fixed at stage 19-20, stained with X-Gal (blue) and paraffin-sectioned transversely. D,F,H are high magnification images of boxed regions in C,E,G, respectively. (I) Coexpression of exogenous Sox and β-Gal proteins in a thickened epithelial area in the presumptive RPE. A frozen section of a *Sox2/LacZ*-electroporated eye stained with Sox2 (red) and β-Gal (green) antibodies and DAPI (blue, nuclei). Endogenous Sox2 signal is also visible in the presumptive NR (nr). (J) A differential interference contrast image of SoxB1-transfected RPE cells. This paraffin section image was taken in grayscale with a green filter for better visualization of the morphology of *Sox3*-transfected cells (stained with X-Gal). (**K**) A confocal image of SoxB1-transfected RPE cells. A frozen section of a *Sox2/LacZ*-electroporated eye stained for β-Gal (green), actin filament (phalloidin, red) and nuclei (DAPI, blue). (**L**,**M**) High magnification images of boxed areas in K, as indicated by lower case letters, showing only phalloidin (red) and DAPI (blue) staining. (**N-Q**) Lack of pigmentation in SoxB1-transfected presumptive RPE cells. Paraffin sections of eyes at stage 21-22 electroporated with *LacZ* (N), *Sox1/LacZ* (O), *Sox2/LacZ* (P) and *Sox3/LacZ* (Q) expression vectors. le, lens vesicle; nr, presumptive NR; rpe, presumptive RPE. Scale bars: 100 µm in C,E,G; 10 µm in I-K,N-Q.

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,El 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 ∂-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; Yamagata et al., 1990)]. In untransfected eyes, strong N-Cad staining was detected in the central NR, lens and brain, but not in the RPE (Fig. 3A-C). By contrast, *Sox2*-transfected RPE cells exhibited intense apical staining for N-Cad (*n*=12; Fig. 3D-F). Furthermore, transfected regions contained TuJ1 (*n*=8; Fig. 3G,H)-, 3A10 (*n*=4; Fig. 3I,J)-, HuC/D (*n*=11; Fig. 3K,L)-, Isl1 (*n*=6; Fig. 3M,N)- and visinin (*n*=6; Fig. 3O,P)-positive cells. Many of these cells were found on the basal side of the epithelium, consistent with their neuronal characteristics. Similar results were obtained with *Sox1* (*n*=5; Fig. 3Q,R) and *Sox3* (*n*=5; Fig. 3S,T) vectors, but not with a control *LacZ* vector (*n*=4; data not shown).

In chick embryos, the presumptive RPE retains the potential to differentiate into the NR until E4.5 (Guillemot and Cepko, 1992; Park and Hollenberg, 1989; Pittack et al., 1991). We investigated

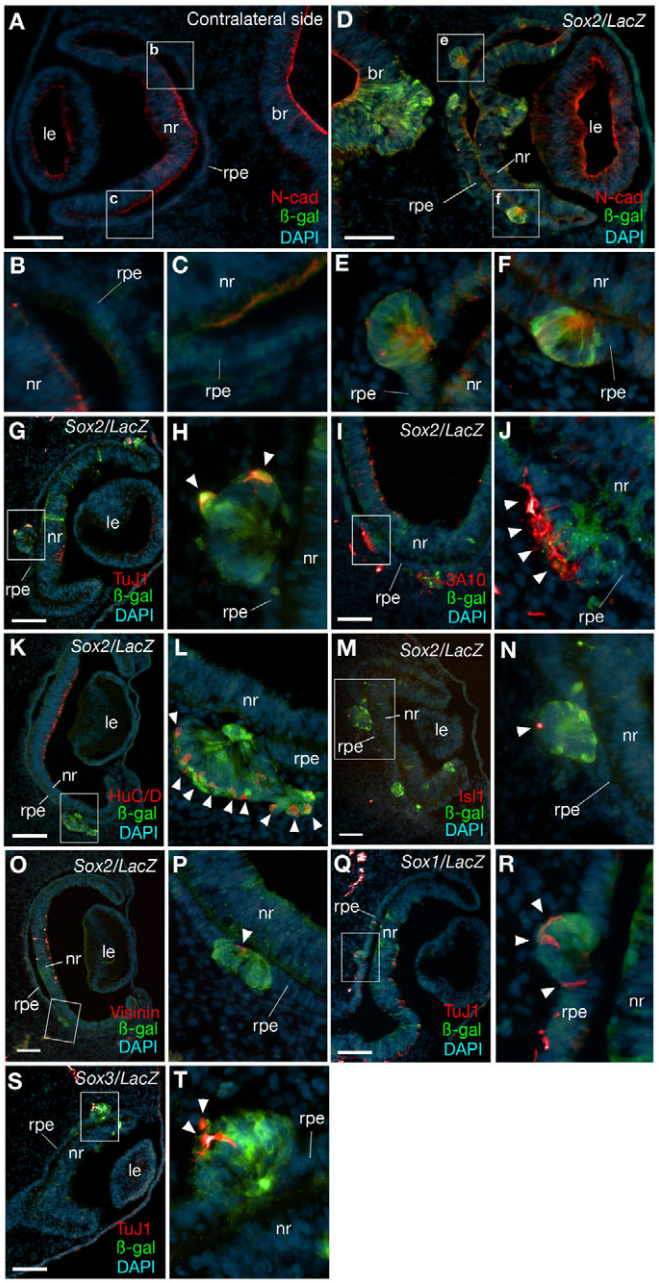

Fig. 3. Maintained expression of SoxB1 confers neural characteristics. (**A-F**) Ectopic expression of N-Cad in *Sox2/LacZ*-transfected cells in the presumptive RPE. In untransfected eyes (A-C), an intense signal for N-Cad (red) was seen in the presumptive NR (nr) and the brain (br), but not in the presumptive RPE (rpe). In transfected eyes (D-F), intense N-Cad staining was seen ectopically in the presumptive RPE. B,C and E,F are high magnification images of boxed regions in A and D, as indicated. (**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 (O,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 *Sox3/LacZ* (S,T) expression vectors. H,J,L,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; rpe, presumptive RPE.

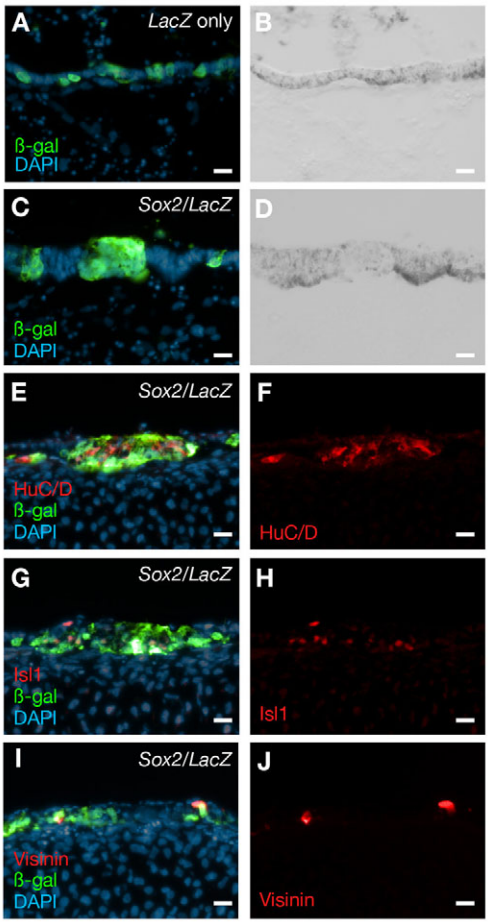

Fig. 4. SoxB1 misexpression converts pigmented RPE into a neural-like tissue. Explants consisting of E6 RPE and the underlying mesenchyme were electroporated, cultured in vitro, cryosectioned and immunostained. Upon electroporation, the explants were precisely oriented so that only RPE layer was transfected. (**A**,**B**) An explant electroporated with the control *LacZ* vector. β-Gal immunostaining (A, green) and bright field (B) images. (**C**,**D**) An explant electroporated with a *Sox2/LacZ* vector. β-Gal immunostaining (C, green) and bright field (D) images, demonstrating diminished pigmentation in highly transfected cells. (**E-J**) Expression of HuC/D (E,F), Isl1 (G,H) and visinin (I,J) (red) in cultured RPE electroporated with a *Sox2/LacZ* vector. β-Gal, green. F,H,J show only signals for these neuronal markers. Scale bars: 10 μm.

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; Fig. 4A,B). Conversely, RPEs electroporated with a *Sox2/LacZ* vector exhibited an overall increase in epithelial thickness and reduced pigmentation (n=4; 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; 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; Martinez-Morales et al., 2001; Martinez-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; Furhmann 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. 6J-L,P-R). *Fgf4* similarly downregulated *Mitf* and *Otx2* in transfected presumptive RPE (Fig. 6S,T; i-nr), but upregulated all NR marker genes (i.e. *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.

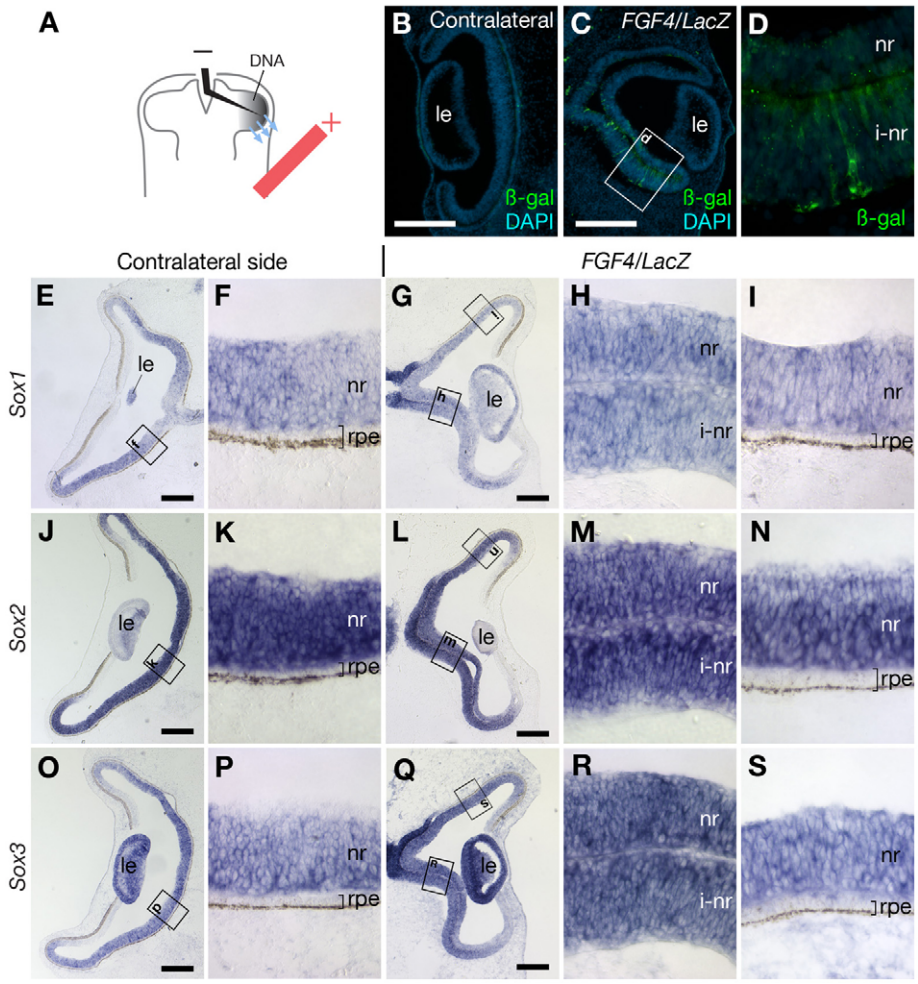

Fig. 5. FGF induces SoxB1 expression ectopically in the presumptive RPE. (**A**) Electroporation. Electrodes were arranged to introduce DNA into the posterior wall of the OV. (**B-D**) Distribution of transfected cells in a stage 19 optic cup, as detected by the β-Gal immunostaining (green). Anterior, top; posterior, bottom. No signal is detectable on the contralateral side (B). On the electroporated side (C,D), transfected cells are found only in the posterior portion of the optic cup. (**E-S**) Expression of *Sox1* (E-I), *Sox2* (J-N) and *Sox3* (O-S) in stage 22 eyes as detected by in situ hybridization. Contralateral side (E,F,J,K,O,P) and electroporated side (G-I, L-N, Q-S). Ectopic expression of all three SoxB1 genes is detectable on the electroporated side in a thickened area within the presumptive RPE (induced NR; i-nr) (G,H,L,M,Q,R). No ectopic expression of SoxB1 is detectable on the contralateral side (E,F,J,K,O,P) or the anterior portion of the optic cup on the electroporated side (G,I,L,N,Q,S), where the RPE is thin and pigmented. D,F,H,I,K,M,N,P,R,S are high magnification images of boxed regions in C,E,G,J,L,O,Q, respectively, as indicated by lower case letters. i-nr, induced neural retina; le, lens vesicle; nr, presumptive NR; rpe, presumptive RPE. Scale bars: 100 μm.

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-animal/germline 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.

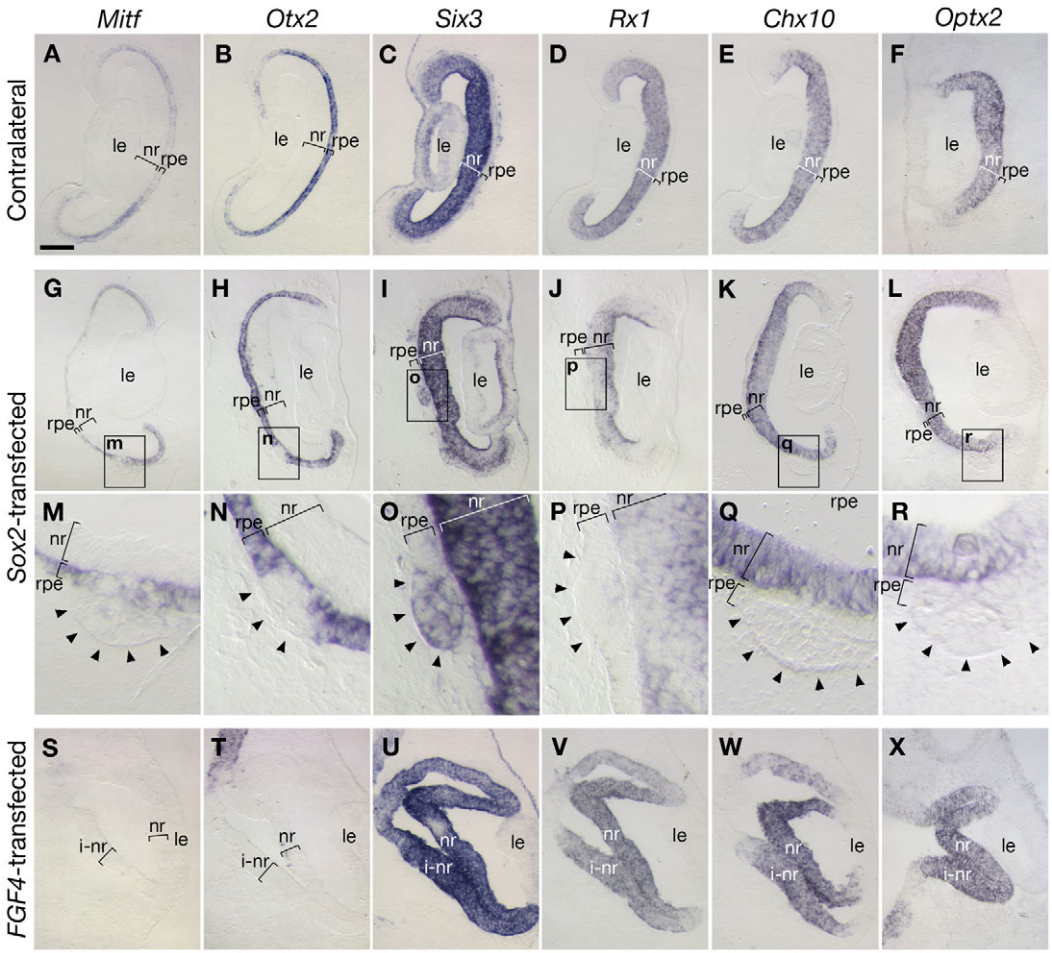

Fig. 6. SoxB1 mediates part of the FGF effect. (**A-X**) Expression of *Mitf* (A,G,M,S), *Otx2* (B,H,N,T), *Six3* (C,I,O,U), *Rx1* (D,J,P,V), *Chx10* (E,K,Q,W) and *Optx2* (F,L,R,X) in untransfected eyes on the contralateral side (A-F) and in eyes transfected with *Sox2* (G-R) and *Fgf4* (S-X), as detected by in situ hybridization. Anterior, top; posterior, bottom. M,N,O,P,Q,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.

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.

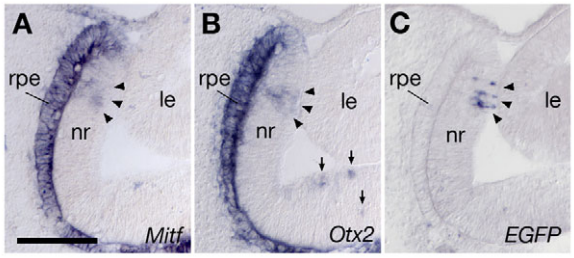

Fig. 7. SoxB1-dependent transactivation plays a role in suppressing RPE marker genes. Ectopic expression of *Mitf* (**A**) and *Otx2* (**B**) (arrowheads) in the NR at stage 18 after coelectroporation with HMG-EnR and EGFP vectors. Transfected regions were visualized by in situ hybridization for *EGFP* 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.

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. Mutations in *Mitf* and *Otx2* lead to ectopic NR-like development in the presumptive RPE (Bumsted and Barnstable, 2000; Martinez-Morales et al., 2001; Mochii et al., 1998b; Nakayama et al., 1998; Nguyen and Arnheiter, 2000). The absence of detectable expression

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, ∂-crystallin is expressed only in the lens and not in the optic cup, even though Sox2 and Pax6 directly regulate the ∂-crystallin promoter (Kamachi et al., 2001). Nevertheless, the synergistic action of Sox2 and Brn2 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 proteins on different downstream genes.

Mitf 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 *Rx1*, *Chx10* or *Optx2*. 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 dissection 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.

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Supplementary material

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

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