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Combinatorial regulation of optic cup progenitor cell fate by SOX2 and PAX6

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SUMMARY

In humans, haploinsufficiency of either SOX2 or PAX6 is associated with microphthalmia, anophthalmia or aniridia. In this study, through the genetic spatiotemporal specific ablation of SOX2 on both wild-type and *Pax6*-haploinsufficent backgrounds in the mouse, we have uncovered a transcriptionally distinct and developmentally transient stage of eye development. We show that genetic ablation of SOX2 in the optic cup results in complete loss of neural competence and eventual cell fate conversion to non-neurogenic ciliary epithelium. This cell fate conversion is associated with a striking increase in PAX6, and genetically ablating SOX2 on a *Pax6*-haploinsufficient background partially rescues the *Sox2*-mutant phenotype. Collectively, these results demonstrate that precise regulation of the ratio of SOX2 to PAX6 is necessary to ensure accurate progenitor cell specification, and place SOX2 as a decisive factor of neural competence in the retina.

KEY WORDS: Multipotential retinal progenitors, Ciliary epithelium, SOX2, PAX6, Gene dosage, Mouse

INTRODUCTION

The vertebrate eye, which is composed of neurogenic and non-neurogenic structures, arises from a single progenitor pool in the optic vesicle. The eye, therefore, provides a useful and accessible model for studying potential interactions of signaling pathways in the specification of distinct cell fates. At around embryonic day (E) 10.5 of mouse development, the optic vesicle receives signals from the surface ectoderm telling it to invaginate, forming the bilayered optic cup. The inner layer of the optic cup gives rise to the neural retina (NR) and the outer layer becomes the retinal pigment epithelium (RPE). The interface of these two domains is the peripheral optic cup margin, which gives rise to the non-pigmented ciliary body epithelium (CE) and the inner iris epithelium. Progenitor cells at the boundary between prospective NR and CE make a binary cell fate decision to become either neurogenic (NR) or non-neurogenic (CE) (Beebe, 1986). The molecular and cellular mechanisms regulating this cell fate decision are poorly understood, in part because cells fated to become CE exhibit pervasive expression of the retinal progenitor transcription factors *Rax*, *Chx10* (*Vsx2* – Mouse Genome Informatics) and *Pax6* (Cho and Cepko, 2006; Fuhrmann et al., 2000; Furukawa et al., 1997; Hodgkinson et al., 1993; Kubo and Nakagawa, 2008; Liu et al., 2006; Liu et al., 2003; Pittack et al., 1997; Rowan and Cepko, 2004).

A number of cell-extrinsic signaling pathways have been implicated in CE fate specification. One report showed that CE markers are found at the edges of tissue that ectopically expresses fibroblast growth factor (FGF), suggesting that the ciliary body is specified in the optic vesicle where bone morphogenetic protein (BMP) and FGF signals overlap (Dias da Silva et al., 2007). In addition, studies in multiple species have shown canonical Wnt signaling to be a potent regulator of peripheral eye structures (Cho and Cepko, 2006; Liu, H. et al., 2007; Tomlinson, 2003). A role for Wnt signaling in specifying CE fate in the mouse comes from the observation that constitutive activation of β-catenin in optic cup progenitor cells results in ectopic expression of CE-specific genes at the expense of NR-specific genes (Liu, H. et al., 2007). However, these ectopic CE-like cells fail to express *Pax6* and *Chx10*, both of which are normally maintained in the prospective CE of control eyes at early stages. In the adult, *Pax6* is maintained in the CE of the iris and ciliary body.

The reduction of *Pax6* expression upon activated Wnt signaling is surprising given that PAX6 is a positive regulator of peripheral eyecup development (Davis-Silberman et al., 2005). A member of the paired-box and homeobox-containing family of transcription factors, PAX6 has been shown to be required for iris specification, optic cup morphogenesis, lens formation and retinal neuronal differentiation (Baumer et al., 2002; Davis-Silberman and Ashery-Padan, 2008; Davis-Silberman et al., 2005; Grindley et al., 1997; Marquardt et al., 2001; Philips et al., 2005; Smith et al., 2009; Xu et al., 1999). These developmental processes require a critical threshold of PAX6 as demonstrated by the fact that heterozygous carriers of *PAX6* deletions (Davis-Silberman et al., 2005; Hill et al., 1991; Hogan et al., 1986; Ton et al., 1991) and transgenic mice with increased levels of PAX6 (Ericson et al., 1997; Schedl et al., 1996) display eye abnormalities (Favor et al., 2001; Hack et al., 2004; Heins et al., 2002; Kim and Lauderdale, 2008; Manuel et al., 2007). Humans with mutations in *PAX6* exhibit aniridia (no iris) and often have smaller ciliary bodies (reviewed by Hanson and Van Heyningen, 1995; Hayashi et al., 2004; Okamoto et al., 2004; Prosser and van Heyningen, 1998). Mice that are haploinsufficient for *Pax6* exhibit reduced size of the optic cup margin, implicating a shift in the boundary between NR and CE (Davis-Silberman et al., 2005).

Here, we test the hypothesis that there is an antagonistic relationship between transcription factors that are restricted to the prospective NR and those that, like PAX6, span the boundary

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between prospective NR and CE. One of these potential regulators of NR specification is the high mobility group (HMG)-containing transcription factor SOX2. Conditional deletion of *Sox2* in the developing mouse retina results in the loss of competence to undergo neuronal differentiation, and mice that are hypomorphic for *Sox2* exhibit reduced eye size (Taranova et al., 2006). Moreover, ~10% of human individuals with anophthalmia (lack of eye) or severe microphthalmia (small eye) carry a *SOX2* mutation (Fantes et al., 2003; Hagstrom et al., 2005; Hanson and Van Heyningen, 1995; Ragge et al., 2005a; Ragge et al., 2005b; Zenteno et al., 2005; Zenteno et al., 2006) (for a review, see Hever et al., 2006).

Although both SOX2 and PAX6 have been shown to be essential for the maintenance of multipotent retinal progenitor cells (RPCs) (Marquardt et al., 2001; Taranova et al., 2006; Xu et al., 1999) and studies in mouse illustrate that changes in SOX2 and PAX6 dosage result in developmental defects of the eye, no study has yet addressed their epistatic relationship in the developing optic cup. To examine the relationship between *Sox2* and *Pax6* in the optic cup, we performed genetic analysis in the mouse and uncovered a mechanism through which the eyecup is regionalized into NR and CE. We show that SOX2 and PAX6 are expressed in an inverse gradient in the developing optic cup and find that ablation of SOX2 in multipotent optic cup progenitor cells biases them towards a non-neurogenic CE fate. The immediate molecular readout of this cell fate conversion is the upregulation of PAX6. Accordingly, the deletion of *Sox2* on a *Pax6*-haploinsufficient background ($Pax6^{Sey/+}$) significantly rescues the *Sox2*-mutant phenotype. Therefore, in the absence of SOX2, multipotent RPCs cannot maintain neuronal differentiation capacity (i.e. NR identity) and undergo cell fate conversion to CE. These results identify SOX2 as a crucial factor defining multipotent neural retinal progenitor identity (Taranova et al., 2006) and suggest a model of dosage-dependent transcriptional regulation of cell fate in the optic cup.

MATERIALS AND METHODS

Mouse breeding

$Sox2^{cond/+}$ mice (Taranova et al., 2006) were crossbred to $\alpha P0^{CREiresGFP}$ (Dr P. Gruss, Max-Planck-Institute of Biophysical Chemistry, Germany) (Marquardt et al., 2001) or $Chx10^{CreGFP}$ (Jackson Laboratories, Bar Harbor, ME, USA) (Rowan and Cepko, 2004) to generate $Sox2^{cond/+}$; $\alpha P0^{CREiresGFP}$ and $Sox2^{cond/+}$; $Chx10^{CreGFP}$ mouse lines. These lines were then backcrossed to the $Sox2^{cond}$ line to generate homozygous mutant genotypes. Lineage tracing was carried out using *Rosa26Reporter* (*R26R*) mice (Jackson Laboratories) (Soriano et al., 1987). $Pax6^{Sey/+}$ mice (Dr A. LaMantia, The George Washington University, DC, USA) (Hill et al., 1991) were bred to $Sox2^{cond/+}$; $\alpha P0^{CREiresGFP}$ mice to obtain $Sox2^{cond/+}$; $\alpha P0^{CREiresGFP}$; $Pax6^{Sey/+}$ mice and then backcrossed to $Sox2^{cond/+}$ mice to yield the $Sox2^{cond/cond}$; $Pax6^{Sey/+}$; $\alpha P0^{CREiresGFP}$double mutant. β-$catenin^{activated}$ mice (Dr R. Wechsler-Reya, Duke University, Durham, NC, USA) (Harada et al., 1999) were crossed with the $\alpha P0^{CREiresGFP}$ to obtain the constitutively activated genotype β-$catenin^{activated}$; $\alpha P0^{CREiresGFP}$. Primer sequences for all of the alleles mentioned can be found in Table S1 in the supplementary material. It was necessary to genotype for the $Sox2^{\Delta cond}$ allele to eliminate animals in which germline recombination occurred. All animal work was carried out in accordance with University of North Carolina at Chapel Hill IACUC and DLAM approval.

Tissue preparation, immunohistochemistry and in situ hybridization

Mouse embryos were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS). Tissue was immersed sequentially in 10%, 20% and 30% sucrose in DEPC PBS overnight and then embedded and frozen in OCT (optimal cutting temperature) medium (Tissue-Tek). Horizontal cryostat sections (14 μm) were blocked in 1% goat serum and 0.1% Triton X-100 in PBS and incubated with primary antibodies at 4°C overnight and secondary antibodies for one hour at room temperature. The following antibodies were used in this study: rabbit anti-cleaved caspase 3 (1:300, Cell Signaling), chicken anti-GFP (1:2000, Abcam), rabbit anti-Ki67 (1:1000, Abcam), mouse anti-PAX6 (1:100, Hybridoma), rabbit anti-SOX2 (1:3000, Millipore), mouse anti-β-tubulin III (1:1000, Covance), Alexa Fluor 488-conjugated anti-chicken (1:2000, Molecular Probes), Alexa Fluor 546-conjugated anti-mouse (1:2000, Molecular Probes) and Alexa Fluor 546-conjugated anti-rabbit (1:2000, Molecular Probes). In situ hybridization was performed on cryostat sections (20 μm) using digoxygenin (DIG)-labeled antisense probes followed by enzymatic detection according to manufacturer's protocols (Roche). The following in situ probes were employed in this study: Axin2 [Dr F. Costantini (Jho et al., 2002)], Bmp4 [Dr A. LaMantia (Bhasin et al., 2003], Bmp7 [Dr B. Hogan (Lyons et al., 1995)], Chx10 [Dr R. McInnes (Horsford et al., 2005)], Hes5 [Dr E. Anton (Chenn and Walsh, 2002)], Lef1 [Dr R. Grosschedl (Galceran et al., 1999)], Msx1 [Dr Y. Liu (Liu et al., 2006)], NeuroD1 [Dr J. Lee (Lee et al., 1995)], Notch1 [Dr U. Lendahl (Lardelli and Lendahl, 1993)], Rax [Dr C. Cepko (Furukawa et al., 1997)], Sfrp2 [Dr J. Nathans (Rattner et al., 1997)], Otx1 [Dr J. P. Martinez (Simeone et al., 1992)], Pax6 [Dr A. LaMantia (Anchan et al., 1997)] and Zic1 [Dr K. Millen (Aruga et al., 1994)]. For β-galactosidase (β-gal) staining, slides were washed with PBS and immersed in β-gal staining solution [final concentrations: 5 mM $K_3Fe(CN)_6$ (Sigma), 5 mM $K_4Fe(CN)_6.3H_2O$ (Sigma), 2 mM $MgCl_2$ (Mallinckrodt), 0.02% Igepal CA-630 (Sigma), 0.01% sodium-deoxycholate (Sigma) and X-gal (1:50, Promega)] overnight at 37°C. Fluorescent and light microscopy images were taken on a Leica inverted microscope (Leica DMIRB) using a Q-imaging Retiga-4000RV digital CCD camera (Vashaw Scientific, Raleigh, NC, USA).

BrdU labeling

Pregnant mothers were weighed and injected with 6 μl/g of 15 mg/ml BrdU (Sigma B5002) in PBS 2 hours before dissection. Embryos were sectioned and prepared for immunohistochemistry as stated above and stained overnight with mouse anti-BrdU (1:500, BD Biosciences).

PAX6 immunofluorescence intensity analysis

PAX6 immunofluorescence intensity was measured in tissue sections taken from the central-most portion of E14.5 eyes. To exclude intensity variations caused by the appearance of different cell sections (and correspondingly, different volumes) at the focus of the objective, optical sectioning was performed to generate the maximum image projection of the resulting slices. To capture the whole cell volume, initial optical sectioning was conducted to determine the objective position at which the fluorescence intensity in the selected region of interest (ROI) would be maximal. The ROI (at least 20 cells per ROI) was selected at similar locations in each sample. The maximum intensity plane served as a reference point. For the final imaging for intensity calculations, a stack of 13 μm (6.5 μm above and below the reference point) was collected. Twenty cells from each region of the eyecup (the lens epithelium, prospective CE, retinal ganglion cells, SOX2-positive neural progenitors and SOX2-ablated neural progenitors) were selected from the final 8-bit image for fluorescence intensity calculations and the intensity was plotted using the Olympus Fluoview 2.1c. All intensity measurements were conducted on an Olympus Fluoview FV1000 confocal microscope with a 40× NA0.6 objective (Olympus Corp.). Statistics calculations were performed using Excel software (Microsoft, Redmond, WA, USA).

RESULTS

The central NR and the peripheral optic cup margin are defined by an inverse gradient of SOX2 and PAX6

To establish the functional interaction between SOX2 and PAX6 in the specification of optic cup progenitor cells, we first compared their expression patterns using immunohistochemistry. SOX2 and PAX6 are co-expressed in the anterior neural plate and throughout the optic vesicle prior to optic cup formation (Rex et al., 1997;

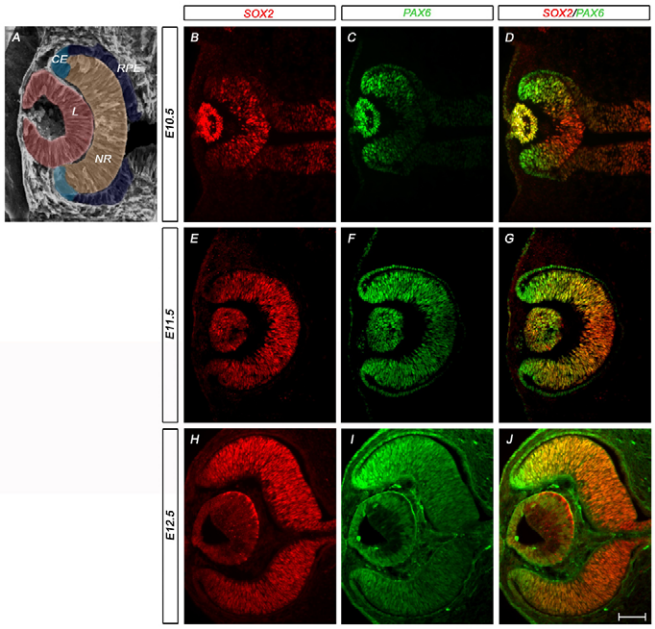

Fig. 1. The neural retina and optic cup margin are defined by an inverse gradient of SOX2 and PAX6. (**A**) Schematic of an E12.5 eye indicating the boundaries of the mouse neural retina (NR), lens (L), prospective ciliary body epithelium (CE) and retinal pigment epithelium (RPE). (**B-D**) Immunohistochemistry on horizontal sections of wild-type embryos shows high SOX2 expression (red) in the central optic cup and high PAX6 expression (green) in the peripheral optic cup margin. (**E-J**) As the eye develops, the inverse gradients of SOX2 and PAX6 expression are maintained. Scale bar: 100 μm.

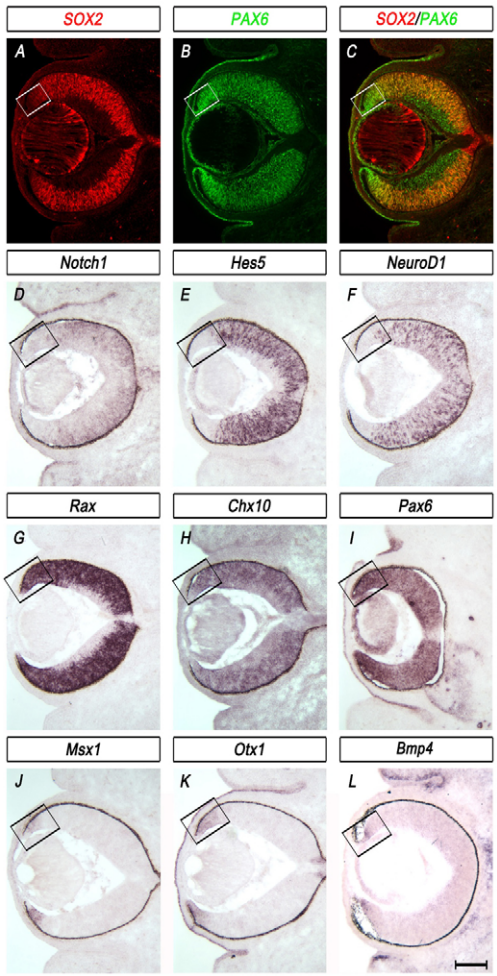

Fig. 2. Expression profiles of the central neural retina and peripheral optic cup margin at E13.5 reflect the inverse expression patterns of SOX2 and PAX6. (**A-C**) Immunohistochemistry for SOX2 (red) and PAX6 (green) on horizontal sections of wild-type mouse eye illustrates inverse gradients of expression, with SOX2 highly expressed in the central neural retina (NR) and PAX6 highly expressed in the peripheral optic cup margin. (**D-F**) In situ hybridization (ISH) of NR-specific genes shows that *Notch1* (D), *Hes5* (E) and *NeuroD1* (F) are co-expressed with SOX2 in the central NR. (**G-I**) ISH of the optic cup progenitor transcription factors *Rax* (G), *Chx10* (H) and *Pax6* (I) shows expression in both the prospective NR and CE. (**J-L**) ISH of *Msx1* (J), *Otx1* (K) and *Bmp4* (L) shows expression in the optic cup margin. Boxes delineate the optic cup margin. Scale bar: 200 μm.

Uchikawa et al., 2003; Uwanogho et al., 1995). However, in the early optic cup (E10.5), SOX2 and PAX6 began to exhibit inverse expression gradients. Whereas SOX2 was highly expressed in the prospective NR (Fig. 1A,B) in a graded central^{high}-to-distal^{low} pattern, PAX6 was highly expressed in prospective CE and RPE (Fig. 1A,C) in a gradient from central^{low}-to-distal^{high} (Baumer et al., 2002; Grindley et al., 1997; Kamachi et al., 2001; Walther et al., 1991). These inverse expression patterns were maintained throughout early optic cup development (Fig. 1D,G,J). Consequently, in the adult, SOX2 appeared to be excluded from the non-neural CE and RPE (see Fig. S1A,C in the supplementary material), whereas PAX6 was maintained in the CE (see Fig. S1B,C in the supplementary material).

To determine whether the SOX2-PAX6 gradient is concurrent with the early divergence of cell fate into central prospective NR and peripheral prospective CE, we examined a repertoire of established optic cup markers at E13.5, before the NR and CE become morphologically distinguishable. We found that in the prospective NR, SOX2 and PAX6 (Fig. 2A,B) were co-expressed with the NR-specific genes *Notch1* (Lardelli and Lendahl, 1993), *Hes5* (Chenn and Walsh, 2002) and *NeuroD1* (*Neurod1* – Mouse Genome Informatics) (Lee et al., 1995) (Fig. 2D,E,F) and with the multipotent optic cup progenitor genes *Rax* and *Chx10* (Fig. 2G,H). By contrast, SOX2 was downregulated in the prospective CE (Fig. 2A,C, box), where *Msx1* (Liu et al., 2006), *Otx1* (Simeone et al., 1992), *Bmp4* (Zhao et al., 2002) (Fig. 2J,K,L) and *Zic1* (data not shown) (Trimarchi et al., 2009) were preferentially expressed. PAX6 was highly maintained in the prospective CE (Fig. 2B,I,box).

Ablation of SOX2 results in neurogenic to non-neurogenic cell fate conversion

We previously generated mice carrying a conditional floxed allele of *Sox2* (*Sox2$^{cond/+}$*) (Taranova et al., 2006). To conditionally ablate SOX2 from multipotent cells throughout the peripheral optic cup, we used α*P0^{CREiresGFP}* transgenic mice, in which CRE expression is driven by the *Pax6* retina-specific enhancer α and minimal promoter P0 beginning at E10.0 (Kammandel et al., 1999; Marquardt et al., 2001). This mouse line can be used as a transgenic reporter of α enhancer-driven *Pax6* expression in the optic cup (Baumer et al., 2002). We previously crossed *Sox2$^{cond/+}$* mice with *Sox2$^{cond/+}$*; α*P0^{CREiresGFP}* mice and showed that ablation of SOX2 results in

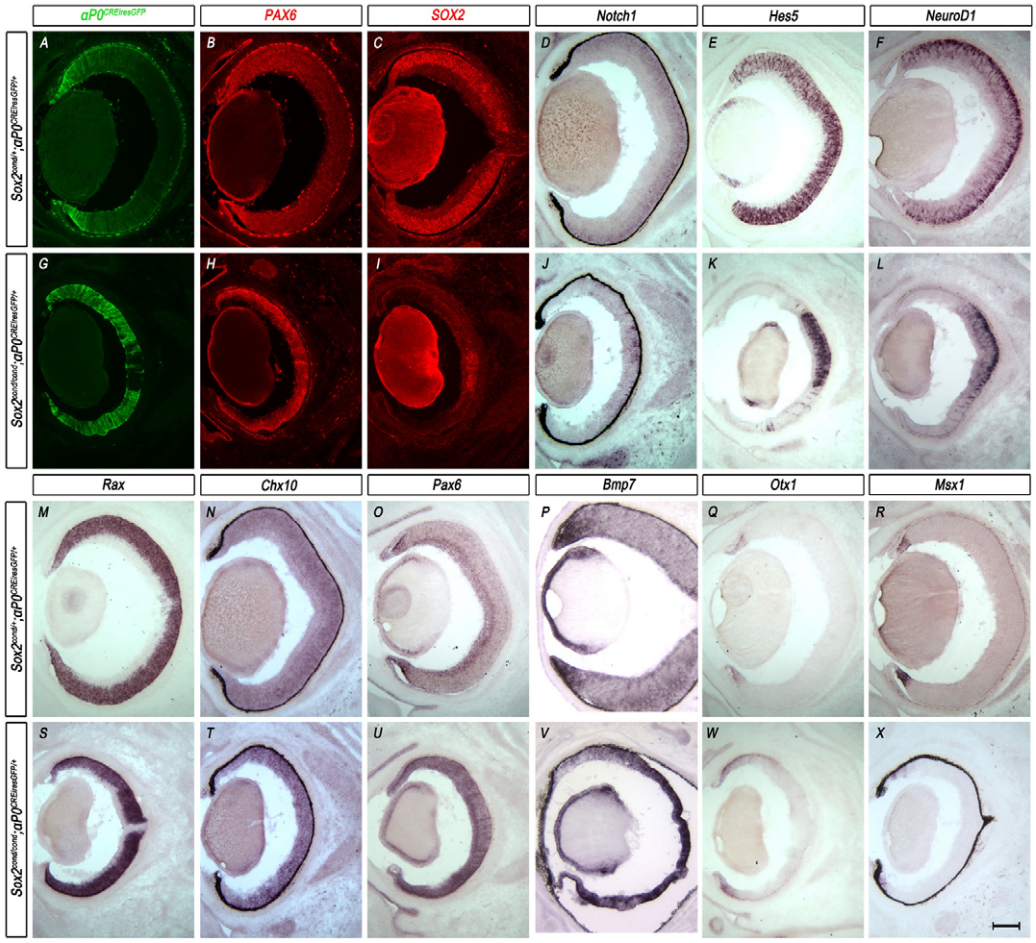

Fig. 3. Ablation of SOX2 in peripheral optic cup progenitor cells results in loss of neural characteristics, maintenance of optic cup progenitor transcription factors and central expansion of the optic cup margin. (**A-X**) Horizontal sections through the eyes of E16.5 mouse embryos. (A,G) $\alpha P0^{CREiresGFP}$ reporter expression in control (*Sox2*$^{cond/+}$; $\alpha P0^{CREiresGFP}$) and mutant (*Sox2*$^{cond/cond}$; $\alpha P0^{CREiresGFP}$) eyes as indicated by CRE-GFP expression (green). $\alpha P0^{CREiresGFP}$ is centrally expanded in mutant embryos (G) compared with controls (A). (B,C,H,I) Immunohistochemistry of SOX2 and PAX6 indicates upregulation of PAX6 (H) throughout the central eyecup in which SOX2 has been ablated (I) when compared with PAX6 expression (B) in SOX2-positive cells (C) of wild-type controls. (D-F,J-L) In situ hybridization (ISH) of the NR-specific genes *Notch1* (N,J), *Hes5* (E,K) and *NeuroD1* (F,L) indicates loss of expression of members of the Notch1 signaling pathway, *Notch1* (J), *Hes5* (K) and *NeuroD1* (L), in regions where SOX2 has been ablated (J-L) when compared with wild-type controls (D-F). (M-O,S-U) ISH of *Rax* (M,S), *Chx10* (N,T) and *Pax6* (O,U) shows no change in expression of *Rax* (S) or *Chx10* (T) but increased expression of *Pax6* (U) in SOX2-ablated eyes (S-U) when compared with wild-type controls (M-O). (P,Q,V,W) ISH shows the expansion of the CE markers *Bmp7* (P,V) and *Otx1* (Q,W) into the prospective NR of *Sox2*-mutant eyes. (R,X) ISH of the CE marker *Msx1* shows little change in expression between the control (R) and mutant eye (X). Scale bar: 200 μm.

severe ocular deformities, including extremely reduced eye size (Taranova et al., 2006). Here, we determine the fate of *Sox2*-mutant optic cup progenitor cells by comparing gene expression, proliferation and cell death in mutant (*Sox2*$^{cond/cond}$; $\alpha P0^{CREiresGFP}$) eyes with that of control (*Sox2*$^{cond/+}$; $\alpha P0^{CREiresGFP}$) eyes.

We first examined *Sox2* and *Pax6* expression at E16.5, when the prospective NR and CE initially become morphologically distinguishable. In control eyes, the inverse SOX2-PAX6 gradient was maintained throughout the optic cup, with PAX6 expression (Fig. 3B) highest in the distal tips and SOX2 expression (Fig. 3C) highest in the central optic cup. By contrast, SOX2-ablated cells (Fig. 3I) throughout the prospective NR exhibited an increase in $\alpha P0^{CREiresGFP}$ reporter (Fig. 3G), PAX6 protein (Fig. 3H) and *Pax6* mRNA expression (Fig. 3U). To confirm this increase in PAX6 protein, we quantified PAX6 immunofluorescence intensity in different regions of the eyecup of control and mutant embryos (see Fig. S2 in the supplementary material). PAX6 was significantly upregulated exclusively in CRE-positive, SOX2-ablated progenitor cells of the central optic cup when compared with wild-type SOX2-positive central progenitor cells (P<0.0001).

To determine the identity of these *Sox2*-mutant cells, we examined the expression of NR-specific genes. *Sox2*-mutant cells failed to express the NR markers *Notch1* (Fig. 3D,J) and *Hes5* (Fig. 3E,K) and markers of postmitotic neurons, including *NeuroD1* (Fig. 3F,L) and β-*tubulin III* (*Tubb3* – Mouse Genome Informatics) (data not shown).

We next examined whether this loss of neural characteristics is specific to the deletion of *Sox2* or is associated with decreased expression of other optic cup progenitor transcription factors, *Rax* and *Chx10*. In contrast to the upregulation of *Pax6* upon SOX2 ablation (Fig. 3H,U) *Rax* (Fig. 3M,S) and *Chx10* (Fig. 3N,T) remained unchanged between controls and mutants. Given that *Rax* and *Chx10* are expressed in CE progenitors, we hypothesized that

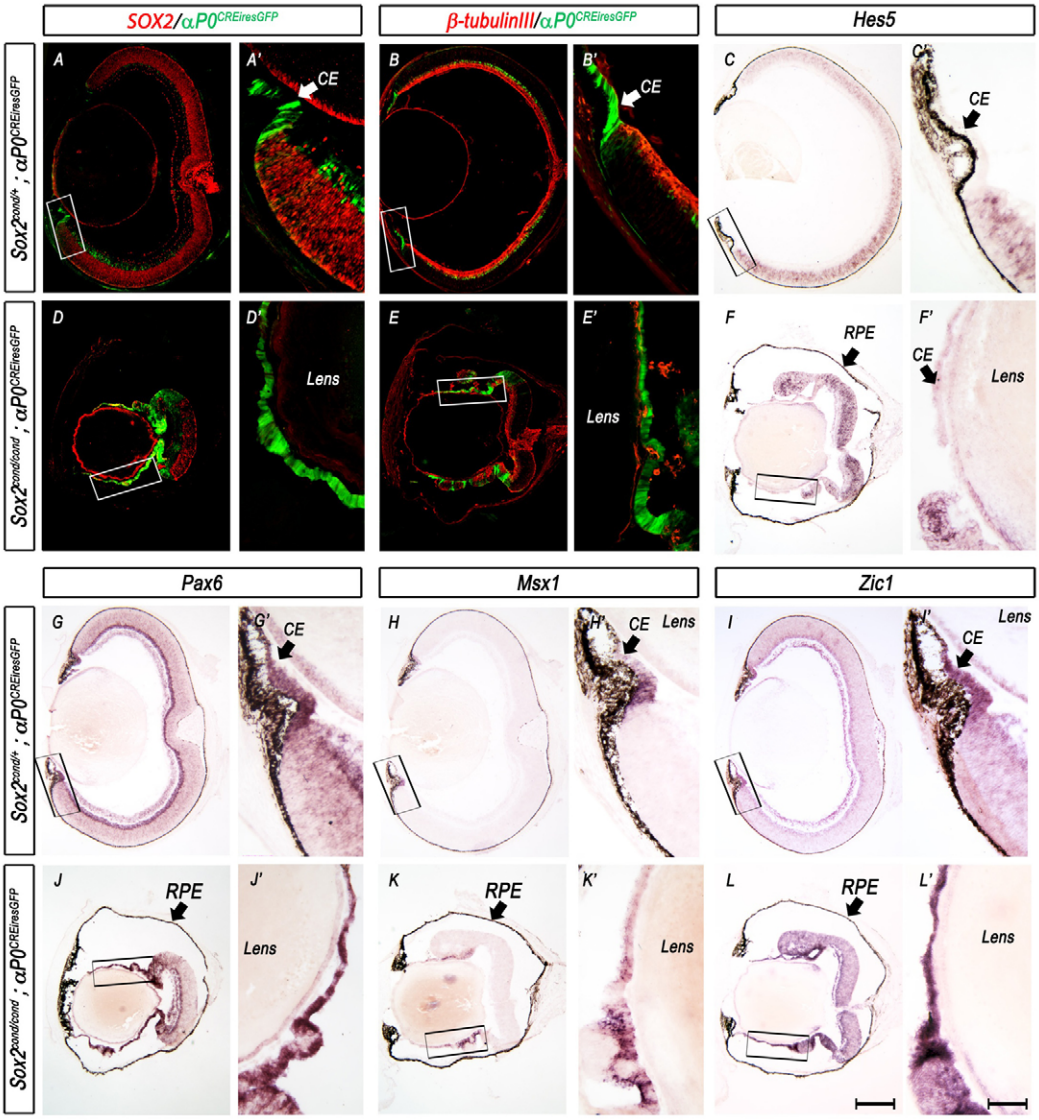

Fig. 4. Ablation of SOX2 by αP0^{CREiresGFP} results in cell fate conversion of the neurogenic retina to non-neurogenic CE. (**A-L'**) Low (A-L) and high magnification (A′-L′) images of horizontal sections through the eyes of P0 control (*Sox2*$^{cond/+}$; αP0^{CREiresGFP}) and mutant (*Sox2*$^{cond/cond}$; αP0^{CREiresGFP}) mouse pups. Boxes indicate the magnified regions. αP0^{CREiresGFP} expression is in the distal tips as well as in the inner nuclear layer and retinal ganglion cell layer of control eyes (A,A′). By contrast, αP0^{CREiresGFP} expression expands throughout the prospective NR of *Sox2*-mutant eyes (D,D′). β-tubulin III antibody staining (B,B′,E,E′) and in situ hybridization (ISH) for *Hes5* (C,C′,F,F′) are present only throughout the SOX2-positive neural retina (NR) of mutant eyes and not in SOX2-ablated regions when compared with controls. ISH of CE-specific genes, including *Pax6* (G,G′,J,J′), *Msx1* (H,H′,K,K′) and *Zic1* (I,I′,L,L′), indicates upregulation in mutant eyes when compared with controls. CE, ciliary epithelium; RPE, retinal pigment epithelium. Scale bars: in L, 400 μm for A-L; in L′, 100 μm for A′-L′.

Sox2-mutant cells might gain CE characteristics, so we examined the expression of established CE markers. Our data indicate that some *Sox2*-mutant cells ectopically express a subset of genes normally restricted to the prospective CE, including *Bmp7* (Fig. 3P,V) and *Raldh2* (*Aldh1a2* – Mouse Genome Informatics; data not shown). *Otx1* (Fig. 3Q,W), which is normally restricted to the distal tips of the optic cup, exhibited slight central expansion in *Sox2*-mutant eyes. However, most *Sox2*-mutant cells failed to express other prospective CE genes, including *Msx1* (Fig. 3R,X) and *Mitf* (data not shown) at E16.5.

The Wnt/β-catenin signaling pathway has been implicated in the specification of CE fate (Cho and Cepko, 2006; Liu, F. et al., 2007; Liu et al., 2003). We examined components of this pathway to test the hypothesis that the Wnt signaling domain is expanded upon *Sox2* deletion. *Axin2*, an endogenous readout of Wnt activity (Fuhrmann et al., 2009; Jho et al., 2002), was increased in *Sox2*-mutant cells compared with wild-type controls (see Fig. S3F,M in the supplementary material), but *Lef1* expression appeared to be unchanged (see Fig. S3E,L in the supplementary material). *Sfrp2*, a Wnt signaling antagonist expressed in NR progenitors (Liu et al., 2003), was centrally shifted (see Fig. S3D,K in the supplementary material). Taken together, these data suggest that the active Wnt signaling domain is centrally expanded upon SOX2 ablation.

Forced expression of a stabilized form of β-catenin in optic cup progenitors has been used previously to model the role of Wnt signaling in CE induction (Liu, H. et al., 2007). We therefore compared expression of prospective NR and CE genes between *Sox2*-mutant embryos and embryos with constitutive activation of Wnt signaling. Consistent with the previous results of Liu et al. (Liu, H. et al., 2007), regions with stabilized β-catenin (β*catenin*^{activated}; αP0^{CREiresGFP}) exhibited upregulation of *Lef1* and *Axin2* (see Fig. S3S,T in the supplementary material) and failed to express the NR markers *Sox2*, *Hes5* and *Sfrp2* (see Fig. S3O-R in the supplementary material). However, in contrast to *Sox2*-mutant eyes, which exhibited central expansion of CRE and increased *Pax6* expression, regions that constitutively express β-catenin did not show expansion of αP0^{CREiresGFP} and did not express *Pax6* (see Fig. S3I,N,P,U in the supplementary material). Thus, the loss of SOX2 parallels activation of β-catenin at E16.5 in the expansion of the *Axin2*-positive domain and the loss of NR characteristics.

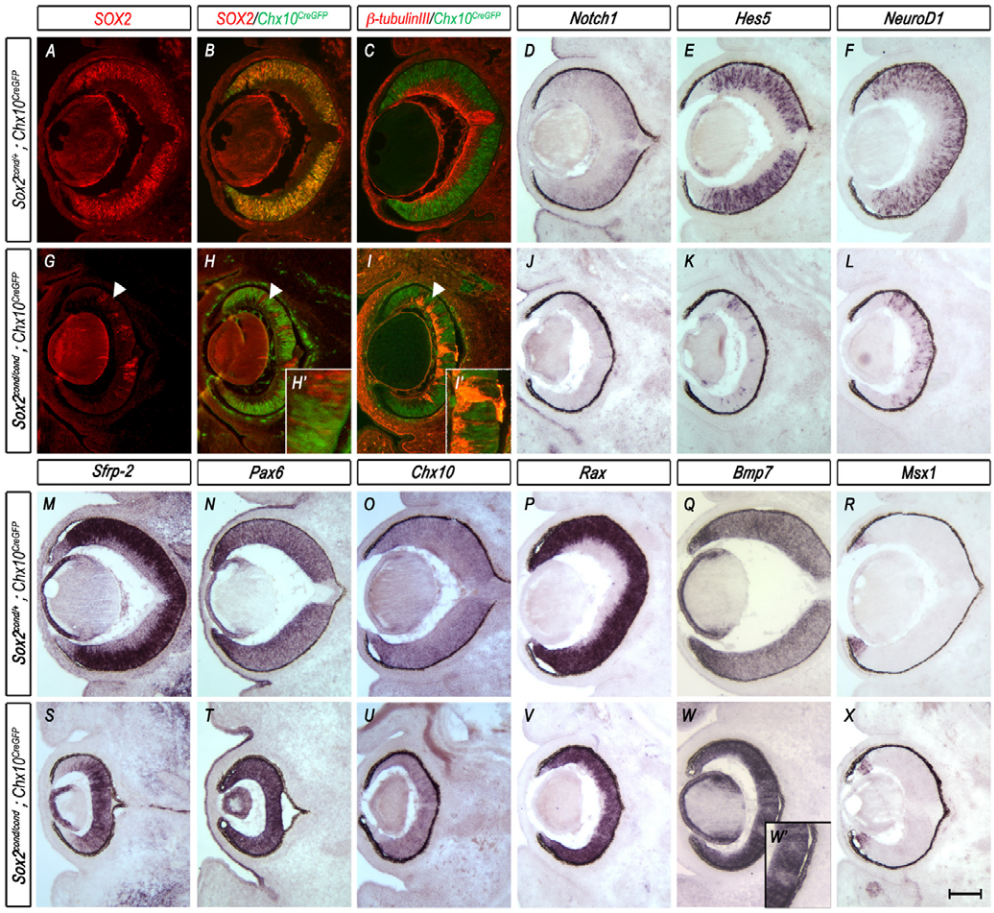

Fig. 5. Mosaic ablation of SOX2 in neural progenitor cells using *Chx10*^{CreGFP} results in loss of neuronal differentiation, maintenance of optic cup progenitor genes and central expansion of the optic cup margin. (**A-X**) Horizontal sections through the eyes of E14.5 control (*Sox2*$^{cond/+}$; *Chx10*^{CreGFP}) and mutant (*Sox2*$^{cond/cond}$; *Chx10*^{CreGFP}) mouse embryos. (A-C,G-I′) *Chx10*^{CreGFP} reporter (B,C,H-I′; green) double labeled with SOX2 (A,B,G-H′; red) or β-*tubulin III* (C,I,I′; red) shows expression throughout the whole optic cup in control eyes. In mutant eyes, CRE-GFP is mutually exclusive of SOX2 (H) and β-*tubulin III* (I). Arrowheads indicate regions in which SOX2 has been ablated. (D-F,J-X) In situ hybridization (ISH) of *Notch1* (D,J), *Hes5* (E,K), *NeuroD1* (F,L), *Sfrp2* (M,S) and the optic cup progenitor genes *Pax6* (N,T), *Chx10* (O,U) and *Rax* (P,V) shows loss of expression of members of the Notch1 signaling pathway (J-L), central restriction of the Wnt antagonist *Sfrp2* (S), upregulation of *Pax6* (T) and maintenance of the progenitor markers *Chx10* (U) and *Rax* (V) in regions where SOX2 has been ablated (G) when compared with wild-type controls (D-F,M-P). ISH of the CE marker *Bmp7* (Q,W,W′) shows expansion into the prospective NR of mutant embryos, whereas *Msx1* (R,X) shows little difference in expression between the control and mutant eye. Scale bar: 200 μm.

Based on the central expansion of some genes known to be involved in specifying CE fate, we hypothesized that the loss of SOX2 induces a transient liminal or 'in-between' state in which a maturation period is required for cells to fully adopt CE identity. To test this hypothesis, we examined the expression of NR- and CE-specific genes in control and *Sox2*-mutant eyes at postnatal day (P) 0. In control eyes, the NR exhibited laminar morphology and α*P0*^{CREiresGFP} expression in the inner nuclear layer and retinal ganglion cell layer (Fig. 4A,A′). The CE contained a single layer of cuboidal cells with high α*P0*^{CREiresGFP} expression (Fig. 4A, box and 4A′, arrow). The NR-specific gene *Hes5* was expressed throughout the laminar NR, marking an abrupt boundary between the neurogenic retina and non-neurogenic CE (Fig. 4C,C′). The neurogenic and non-neurogenic regions were also distinguishable by the edge of β-*tubulin III* expression (Fig. 4B,B′). In stark contrast to control eyes, *Sox2*-mutant eyes showed expanded α*P0*^{CREiresGFP} expression throughout the central eyecup (Fig. 4D,D′). Similar to what was observed in the eyes of mutant embryos, postnatal *Sox2*-mutant cells failed to express genes specific to the NR, including *Hes5* (Fig. 4C,C′,F,F′), and lost neuronal differentiation capacity as demonstrated by the mutual exclusivity of β-*tubulin III* and α*P0*^{CREiresGFP} expression (Fig. 4B,B′,E,E′). In addition to the upregulation of α*P0*^{CREiresGFP}, *Sox2*-mutant cells gained expression of genes that were preferentially expressed in the CE at P0, including *Pax6*, *Msx1* and *Zic1* (Fig. 4G-I′). These ectopic CE-like regions exhibited the thin single-layered morphology characteristic of wild-type CE. By contrast, the SOX2-positive NR regions, which presumably developed from cells that did not undergo CRE-mediated recombination, exhibited proper thickness and laminar morphology when compared with the NR of control eyes. These results suggest that *Sox2*-mutant progenitors autonomously undergo cell fate conversion from neurogenic retina to non-neurogenic CE.

Previous studies have shown that the development of the CE monolayer results from a decrease in cell division. Indeed, the optic cup margin exhibits a lower proliferation rate than does the prospective NR (Beebe, 1986; Cho and Cepko, 2006; Kubota et al.,

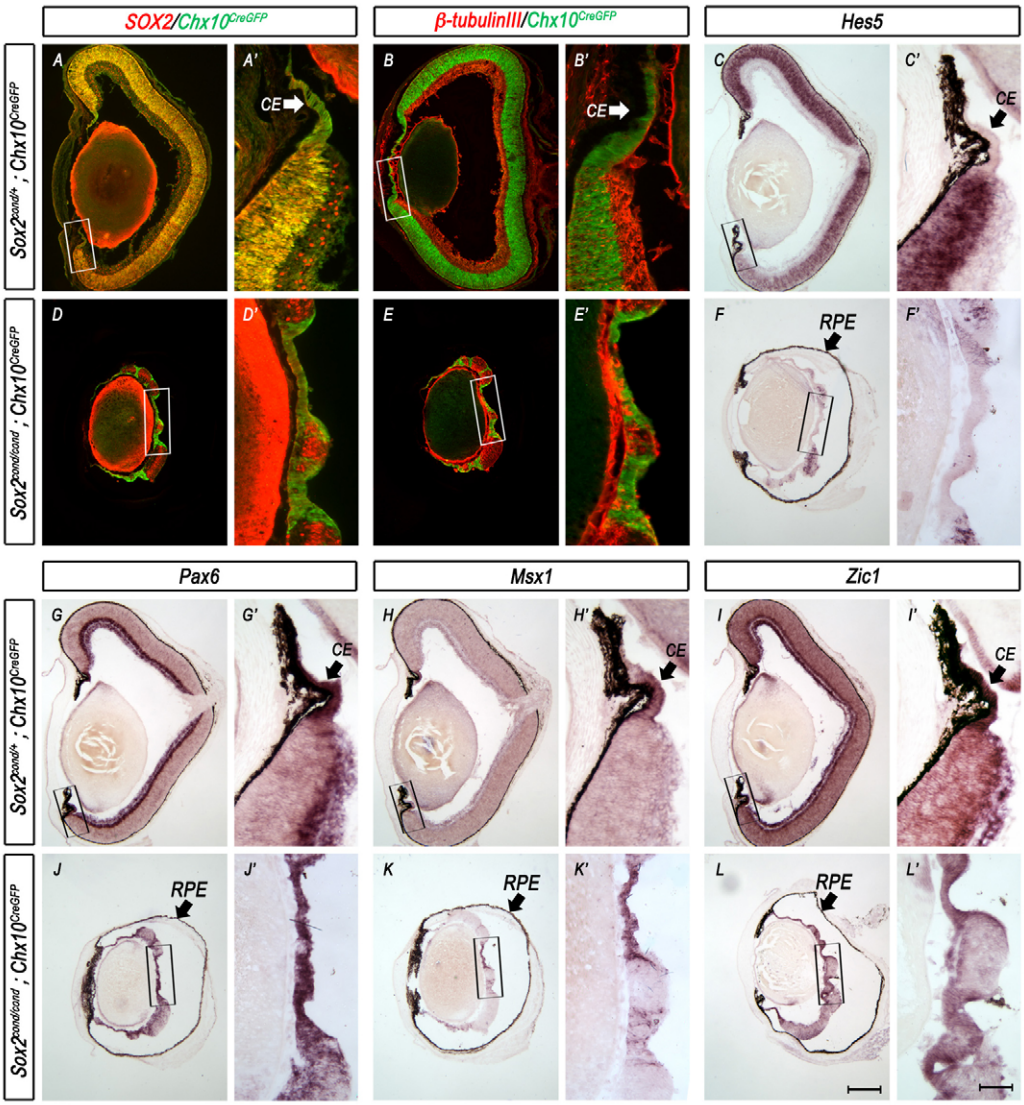

Fig. 6. At P0 mosaic regions of SOX2 ablation by *Chx10^{CreGFP}* in the central eyecup indicate cell fate conversion to CE. (**A-L′**) Low (A-L) and high magnification (A′-L′) images of horizontal sections through the eyes of P0 control (*Sox2$^{cond/+}$; Chx10^{CreGFP}*) and mutant (*Sox2$^{cond/cond}$; Chx10^{CreGFP}*) mouse pups. Boxes indicate magnified regions. Immunohistochemistry for SOX2 (A,A′,D,D′; red), β-tubulin III (B,B′,E,E′; red) and GFP (A-E′; green) in control and mutant eyes shows thinning of the retinal neuroepithelium to ciliary epithelial (CE)-like morphology (D-E′) and loss of neuronal differentiation capacity (E,E′) in mosaic regions of SOX2 ablation (D,D′) throughout the mutant eyecup (D-E′) when compared with wild-type controls (A-B′). In situ hybridization shows the loss of expression of the NR marker *Hes5* (F,F′), upregulation of *Pax6* (J,J′), and gain of expression of the CE markers *Msx1* and *Zic1* (K-L′) in SOX2-ablated regions (D,D′) when compared with wild-type controls (C,C′,G-I′). CE, ciliary epithelium; RPE, retinal pigment epithelium. Scale bars: in L, 400 μm for A-L; in A′, 100 μm for A′-L′.

2004). Examination of proliferation markers revealed that many SOX2-ablated cells, particularly in the peripheral region of the eyecup, did not incorporate BrdU (see Fig. S4B,F in the supplementary material) or express Ki67 (see Fig. S4C,G in the supplementary material). However, many SOX2-ablated cells in the central eyecup did. These results suggest that upon the deletion of *Sox2* by *αP0^{CREiresGFP}*, there is decrease in the number of proliferating cells, particularly throughout the peripheral eyecup. By contrast, there was no significant change in apoptosis as indicated by cleaved caspase-3 expression (see Fig. S4D,H in the supplementary material).

Optic cup progenitor transcription factors PAX6, CHX10 and RAX are not sufficient to maintain neuronal differentiation capacity in the absence of SOX2

The previous data suggest that ablation of SOX2 in multipotent peripheral progenitor cells that can give rise to both NR and CE results in their eventual restriction to CE fate. To test further the hypothesis that cells that are specified to become NR will convert to CE upon loss of SOX2, we used the *Chx10^{CreGFP}* mouse line to ablate SOX2 in a mosaic pattern of progenitor cells throughout the whole optic cup beginning at E11.0. Thus, SOX2 was removed from alternating patches of cells that had been specified to become NR, and neighboring wild-type cells could serve as internal controls (Fig. 5B,C,H-I′) (Donovan and Dyer, 2004; Jadhav et al., 2006; Oron-Karni et al., 2008; Rowan et al., 2004; Zhang et al., 2004). In *Sox2$^{cond/cond}$; Chx10^{CreGFP}* mutant eyes, SOX2 was specifically ablated in CRE-expressing cells marked by GFP (Fig. 5B,G-H′). As a consequence of SOX2 loss, mutant cells did not undergo neuronal differentiation, as shown by the mosaic expression of β-*tubulin III* (Fig. 5C,I,I′) and *NeuroD1* (Fig. 5F,L) in a pattern mutually exclusive of *Chx10^{CreGFP}* expression (Fig. 5I, arrowhead). Moreover, SOX2-ablated cells failed to express the NR-specific genes *Notch1*, *Hes5 and Sfrp2* (Fig. 5D,E,J,K,M,S).

To determine if these *Sox2*-mutant progenitor cells undergo cell fate conversion to CE, we examined the localization of genes normally expressed in the prospective CE at E14.5. In regions of SOX2 ablation, *Pax6* was upregulated (Fig. 5N,T) and *Chx10* (Fig. 5O,U) and *Rax* (Fig. 5P,V) were maintained. Moreover, *Sox2*-mutant cells expressed some prospective CE markers, including *Bmp7* (Fig. 5Q,W,W′), but not others, including *Msx1* (Fig. 5R,X). This expression profile recapitulates that of the early *Sox2$^{cond/cond}$; aP0^{CREiresGFP}* mutant cells described above.

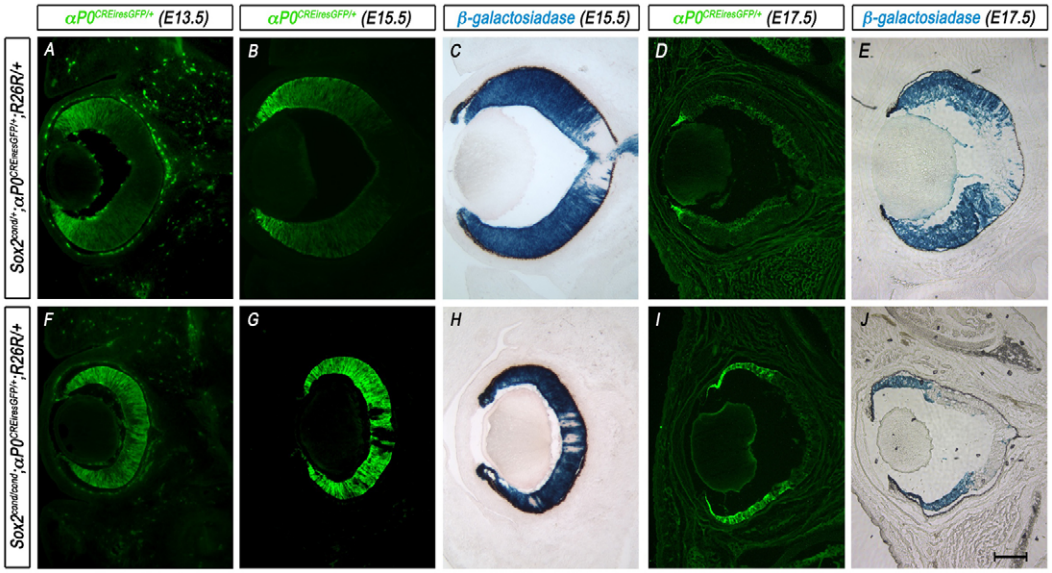

Fig. 7. Fate mapping *Sox2*-mutant progenitor cells using $\alpha P0^{CREiresGFP}$ and R26R. (**A**,**F**) $\alpha P0^{CREiresGFP}$ (green) is expressed in peripheral progenitor cells in control (*Sox2*$^{cond/+}$; $\alpha P0^{CREiresGFP}$; *R26R*) eyes at E13.5, whereas $\alpha P0^{CREiresGFP}$ reporter expression in mutant (*Sox2*$^{cond/cond}$; $\alpha P0^{CREiresGFP}$; *R26R*) eyes is expanded into the central prospective neural retina (NR). (**B**,**D**,**G**,**I**) As the wild-type eye develops, $\alpha P0^{CREiresGFP}$ reporter expression (green) is further restricted to the peripheral eyecup at E15.5 (B) and serves as a marker of CE by E17.5 (D). In the mutant eye, $\alpha P0^{CREiresGFP}$ reporter expression remains an indicator for all recombined cells throughout the NR and prospective ciliary epithelium (CE; G,I). (**C**,**E**,**H**,**J**) β-gal reporter assay on E15.5 and E17.5 eyes indicates that $\alpha P0^{CREiresGFP}$-positive progenitor cells are capable of giving rise to both prospective NR and CE in control eyes but appear to be restricted in *Sox2*-mutant eyes, in which β-gal colocalizes with $\alpha P0^{CREiresGFP}$. Scale bar: 200 μm.

The upregulation of *Pax6* and *Bmp7* suggests that ablation of SOX2 by *Chx10*^{CreGFP} induces a liminal state similar to that observed in *Sox2*$^{cond/cond}$; $\alpha P0^{CREiresGFP}$ mutants. At P0, mosaic regions of *Chx10*^{CreGFP} expression/SOX2-ablation were clearly distinguishable from neighboring *Chx10*^{CreGFP}-negative/SOX2-postive regions (Fig. 6A,A′,D,D′). In mutant eyes, SOX2-ablated regions lacked expression of the NR gene *Hes5* (Fig. 6C,C′,F,F′) and the postmitotic neuronal marker β-*tubulin III* (Fig. 6B,B′,E,E′). Conversely, *Sox2*-mutant regions upregulated *Pax6* (Fig. 6G,G′,J,J′) and cell-autonomously gained expression of the CE markers *Msx1* and *Zic1* (Fig. 6H-I′,K-L′). These data support our finding that loss of SOX2 in multipotent progenitor cells results in a temporary liminal state typifying their maturation to CE. In addition, at P0, *Sox2*-mutant regions exhibited thin, single-layered morphology that starkly contrasts with neighboring control regions, which exhibited proper NR laminar morphology. Therefore, *Sox2*-mutant progenitors fated to become NR lose neuronal differentiation capacity and undergo cell fate conversion to CE (Cho and Cepko, 2006; Liu, H. et al., 2007).

Fate mapping of *Sox2* mutant cells depicts loss of neural progenitor capacity in the retina

To establish directly whether ablation of SOX2 results in an autonomous cell fate change from NR to CE, we genetically fate mapped *Sox2*-mutant cells using the $\alpha P0^{CREiresGFP}$ mouse line. *Sox2*$^{cond/+}$; $\alpha P0^{CREiresGFP}$ mice were crossed with mice carrying the Rosa26R CRE reporter allele (*Sox2*$^{cond/+}$; *R26R*/+), which expresses β-galactosidase (β-gal) following CRE-mediated excision of a translational stop cassette, permanently marking the progeny of CRE-expressing cells (Soriano et al., 1987). Therefore, cells that express CRE at the time of analysis can be detected using GFP fluorescence, whereas all the progeny of CRE-positive cells express β-gal. In control *Sox2*$^{cond/+}$; $\alpha P0^{CREiresGFP}$; *R26R*/+ embryos, $\alpha P0^{CREiresGFP}$ was initially expressed throughout the peripheral optic cup (Fig. 7A). By E15.5, it became restricted to the distal tips (Fig. 7B), maintaining high expression in the CE by E17.5 (Fig. 7D). However, at E15.5 and E17.5, β-gal was detected throughout both the laminar NR ($\alpha P0^{CREiresGFP}$-negative) and the distal CE (Fig. 7C,E), leaving only a portion of central NR cells unmarked. This β-gal expression pattern confirms that $\alpha P0^{CREiresGFP}$-expressing progenitor cells can give rise to both NR and CE. By contrast, as previously shown, *Sox2*$^{cond/cond}$; $\alpha P0^{CREiresGFP}$; *R26R*/+ mutant eyes exhibited expanded $\alpha P0^{CREiresGFP}$ expression into the central optic cup (Fig. 7F,G,I), and all $\alpha P0^{CREiresGFP}$-positive cells appeared to express β-gal (Fig. 7H,J). By E17.5, these β-gal-positive regions exhibited thin morphology when compared with the SOX2-postive β-gal-negative regions in the same eye (Fig. 7J).

Sox2 and *Pax6* genes interact to coordinate eye development

Based on the increase in PAX6 expression upon SOX2 ablation, we hypothesized that proper regionalization of the optic cup depends on a fine balance of SOX2 and PAX6 dosage. To test this hypothesis directly, we performed genetic epistasis analysis of SOX2 and PAX6 in the developing optic cup. To modulate PAX6 dosage, we used the *Pax6*$^{Sey/+}$ mouse line in which a spontaneous mutation in the *Pax6* gene produces a truncated protein that lacks a DNA-binding homeodomain and the C-terminal transactivation domain. This truncated PAX6 is considered to be functionally inactive and is widely used as a *Pax6*-null allele (Hill et al., 1991; Hogan et al., 1986; Osumi et al., 2008). Compared with *Sox2*$^{cond/+}$ or *Sox2*$^{cond/cond}$ mice, which display normal eye development (Taranova et al., 2006), *Pax6*$^{Sey/+}$ mice exhibit reduced external eye size, iris hypoplasia and small lens (Hill et al., 1991).

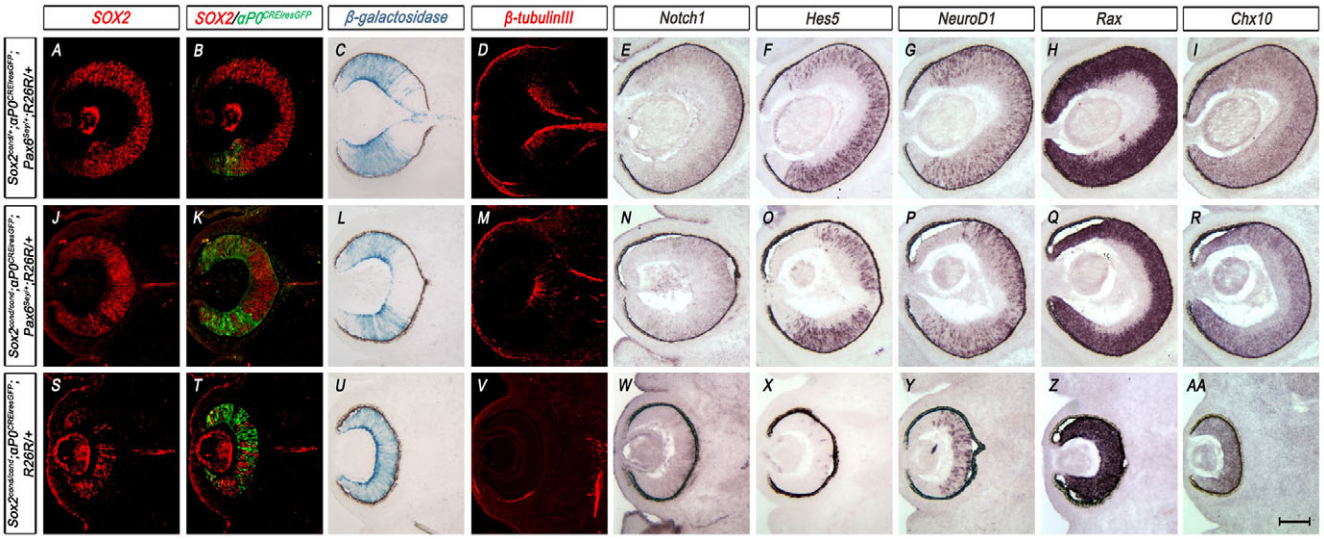

Fig. 8. *Pax6*-haploinsufficiency significantly rescues the *Sox2*-mutant NR. (**A**,**B**,**J**,**K**,**S**,**T**) Comparison of SOX2 immunohistochemistry (red) with $\alpha P0^{CREiresGFP}$ expression (green) in *Pax6* single mutant ($Sox2^{cond/+}$; $\alpha P0^{CREiresGFP}$; $Pax6^{Sey/+}$), *Sox2 Pax6* double mutant ($Sox2^{cond/cond}$; $\alpha P0^{CREiresGFP}$; $Pax6^{Sey/+}$) and *Sox2* single mutant ($Sox2^{cond/cond}$; $\alpha P0^{CREiresGFP}$) eyes indicating little to no SOX2 expression in the *Sox2* single mutant compared with the other two genotypes. (**C**,**L**,**U**) β-gal activity illustrating the progeny of all $\alpha P0^{CREiresGFP}$-expressing cells indicates rescue of $\alpha P0^{CREiresGFP}$ expression in *Sox2 Pax6* double mutants. (**D**,**M**,**V**) β-*tubulin III* (red) shows maintenance of neuronal differentiation capacity in *Pax6* single mutants and *Sox2 Pax6* double mutants but not in *Sox2* single mutants. (**E-I**,**N-R**,**W-AA**) In situ hybridization of *Notch1* (E,N,W), *Hes5* (F,O,X), *NeuroD1* (G,P,Y), *Rax* (H,Q,Z) and *Chx10* (I,R,AA) shows maintenance of prospective NR markers in the *Sox2 Pax6* double mutants. Scale bar: 200 μm.

To ablate specifically SOX2 in peripheral progenitors on this *Pax6*-haploinsufficient background, we crossed $Sox2^{cond/+}$; $\alpha P0^{CREiresGFP}$ mice with $Sox2^{cond/+}$; $Pax6^{Sey/+}$ mice. We then compared the resulting $Sox2^{cond/cond}$; $\alpha P0^{CREiresGFP}$; $Pax6^{Sey/+}$ (*Sox2 Pax6* double mutant) embryos with $Sox2^{cond/cond}$; $\alpha P0^{CREiresGFP}$ (*Sox2* single mutant) embryos and $Sox2^{cond/+}$; $\alpha P0^{CREiresGFP}$; $Pax6^{Sey/+}$ (*Pax6* single mutant) control embryos. *Sox2 Pax6* double mutant eyes, which have reduced levels of both SOX2 and PAX6, were significantly normalized compared with *Sox2* single mutant eyes, which are wild type for *Pax6* but lose SOX2 upon CRE-mediated ablation (Fig. 8J-AA). The central expansion of $\alpha P0^{CREiresGFP}$ seen in *Sox2* single mutant eyes was significantly rescued in *Sox2 Pax6* double mutant eyes (Fig. 8J-AA). Lineage tracing analysis using the Rosa26R CRE reporter showed that the domain of $\alpha P0^{CREiresGFP}$ activity, as marked by β-gal expression, is peripherally restricted in *Sox2 Pax6* double mutants compared with *Sox2* single mutants. These data indicate that $\alpha P0^{CREiresGFP}$ expression in *Sox2 Pax6* double mutants is restored to an expression pattern that more closely resembles that of controls (Fig. 8B,K). Nevertheless, $\alpha P0^{CREiresGFP}$ expression co-localized with β-gal activity in *Sox2 Pax6* double mutants (Fig. 8B,C,K,L), which is consistent with the maintenance of $\alpha P0^{CREiresGFP}$ in SOX2-ablated cells as described above (Fig. 7). The ablation of SOX2 in the *Pax6*-haploinsufficient background significantly rescued neuronal differentiation capacity, as illustrated by normalization of β-*tubulin III* (Fig. 8D,M,V), *Notch1* (Fig. 8E,N,W), *Hes5* (Fig. 8F,O,X) and *NeuroD1* (Fig. 8G,P,Y). Modulating SOX2 or PAX6 levels did not affect *Rax* (Fig. 8H,Q,Z) or *Chx10* (Fig. 8I,R,AA) expression.

To determine whether this rescue phenotype is maintained postnatally, we examined *Sox2 Pax6* double mutants at P0 and found a slightly expanded region of $\alpha P0^{CREiresGFP}$-positive thin CE-like cells compared with *Pax6* single mutant controls (see Fig. S5A-H in the supplementary material, brackets). This result contrasts with the single-layered morphology and CE gene expression present throughout the center of the eyecup in *Sox2* single mutants (see Fig. S5I-L in the supplementary material). Moreover, the NR of *Sox2 Pax6* double mutants exhibited proper laminar morphology and neuronal differentiation capacity as indicated by β-*tubulin III* and *Hes5* expression, and *Msx1* expression was restricted to the distal tips (see Fig. S5F-H in the supplementary material). Therefore, *Sox2 Pax6* double mutants more closely resembled *Pax6* single mutants than *Sox2* single mutants.

DISCUSSION

SOX2 maintains neurogenic fate of proliferating neuroepithelial progenitor cells

Here, we have demonstrated for the first time that the genetic ablation of SOX2 causes a neurogenic-to-non-neurogenic cell fate conversion. These results place SOX2 as a crucial factor defining neurogenic identity in retinal neuroepithelium. In early stage mouse embryos, SOX2 expression marks the region fated to become neural ectoderm and its appearance in chicken embryos coincides with the onset of neural fate specification (Wood and Episkopou, 1999). Inhibition of SOX2 function in *Xenopus* embryos blocks neural differentiation, and SOX2 signaling in chick has been shown to promote proliferation and inhibit neuronal differentiation (Bylund et al., 2003; Graham et al., 2003; Kishi et al., 2000). In the retina, unlike in other regions of the CNS, SOX2 is expressed exclusively of the highly related SOXB1 factors, SOX1 and SOX3. Thus, the optic cup, which retains the unique capacity to generate non-neurogenic structures, provides an excellent model for studying the transcriptional role of SOX2 in maintaining neurogenic identity.

SOX2 ablation initiates gradual cell fate conversion from NR to CE

Secreted molecules known to affect neurogenic versus non-neurogenic optic cup cell fate include FGFs, BMPs and Wnts. A previous report showed that converging FGF and BMP signals

Table 1. Expression profiles of the prospective neural retina (NR) and ciliary body epithelium (CE)

	E13.5 wild type		P0 wild type	
Marker	NR	OCM	NR	CE
Neural retina				
Sox2	+	–	+	–
Sfrp-2	+	–	+	–
Notch1	+	–	+	–
Hes5	+	–	+	–
NeuroD1	+	–	+	–
Math5	+	–	+	–
OCPTF				
Chx10	+	+	+	–
Six3	+	+	+	–
Rax	+	+	+	–
Pax6	+	+	+	+
Ciliary epithelium				
Axin2	–	+	–	+
Bmp7	–	+	–	+
Lef1	–	+	–	+
Raldh2	–	+	–	+
Otx1	–	+	–	+
Msx1	–	+	–	+
Mitf	–	+	–	+
Zic1	–	+	–	+

At E13.5 and P0, NR markers are present throughout the central eyecup. The optic cup progenitor transcription factors are present in both the prospective NR and CE (OCM) at E13.5 but lose expression in the CE by P0. CE markers are restricted to the peripheral eyecup at both stages.
OCM, optic cup margin; OCPTF, optic cup progenitor transcription factors.

might define the prospective NR-CE boundary at optic vesicle stages of the chick (Dias da Silva et al., 2007). BMP signaling appears to be required for *Otx1* and *Msx1* expression in the prospective CE (Zhao et al., 2002), and *Msx1* expression has been shown to be induced by activated Wnt signaling (Willert et al., 2002). Thus, overlapping FGF, BMP and Wnt signals might coordinate the cell-intrinsic gene expression necessary for CE development. However, at least one study has demonstrated induced ectopic CE marker expression without the onset of Wnt2b expression (Dias da Silva et al., 2007). Interestingly, it has been proposed that extrinsic signals that direct neurogenic versus non-neurogenic fate converge at the regulation of SOX proteins (Wilson et al., 2001). Here, we have investigated the cell-intrinsic role of SOX2 and PAX6 in setting up the NR-CE boundary in the optic cup.

Using genetic fate mapping, we have demonstrated that removal of a transcription factor, SOX2, converts prospective NR to CE. Our results are consistent with previous data showing that neural RPCs maintain multipotent differentiation capacity (i.e. the capacity to form CE) at a developmental time point (E11.0) subsequent to the division of NR and CE fate (Fekete et al., 1994; Turner and Cepko, 1987; Turner et al., 1990). The gradual cell fate conversion upon *Sox2* deletion is characterized by: (1) loss of the NR markers *Notch1*, *Hes5*, *NeuroD1* and *Sfrp2* (see Table 1, neural retina markers); (2) maintenance of the multipotent progenitor genes *Chx10*, *Rax* and *Pax6* (see Table 1, optic cup progenitor transcription factors); (3) expansion of Wnt and BMP signals; and (4) decreased proliferation with progressive thinning of the neuroepithelium. These characteristics define a liminal or in-between state that culminates in the expression of CE markers, including *Otx1*, *Zic1* and *Msx1* (see Table 1, ciliary epithelium markers). A similar delay in the consolidation of CE identity in response to ectopic Wnt signaling has been previously demonstrated (Cho and Cepko, 2006). In both instances, the delay could be due to the presence of competing signals in the eyecup: Wnt signals promote non-neurogenic fate but multipotent progenitor genes and Wnt antagonists promote neurogenic fate. Intriguingly, activation of β-catenin in the eyecup was shown to produce a stronger and more rapid onset of CE characteristics than did Wnt2b overexpression, suggesting that stabilized β-catenin bypasses Wnt antagonists in the prospective NR to cause a more immediate increase in CE gene expression (Cho and Cepko, 2006). Thus, the presence of Wnt antagonists might explain why consolidation of CE fate is gradual upon SOX2 ablation but rapid upon β-catenin activation (Liu, F. et al., 2007).

Sox2 and *Pax6* interact to regionalize the optic cup

A major difference between SOX2 loss-of-function and β-catenin gain-of-function in the prospective retina is the effect on *Pax6*. Ablation of SOX2 leads to an immediate increase in *Pax6* expression, whereas activation of β-catenin diminishes *Pax6* expression. In humans, haploinsufficiency of *PAX6* is associated with anterior eye formations, including defects of the iris and ciliary body (for reviews, see Hever et al., 2006; Hill et al., 1991). In the mouse, deletion of one copy of *Pax6* in the distal optic cup resulted in the loss of CE precursors and a distal shift in the boundary between prospective NR and CE (Davis-Silberman et al., 2005). Moreover, transgenic overexpression of *Pax6* resulted in abnormalities of the ciliary body, the iris and the cornea (Schedl et al., 1996). These studies demonstrate the importance of regulating appropriate levels of PAX6 for the proper development of peripheral eye structures. Here, we have described increased *Pax6* expression in the optic cup upon the removal of a potential repressor.

Our genetic epistasis analyses provide evidence for a mechanism of PAX6 regulation in the developing eyecup. The dramatic increase in PAX6 upon *Sox2* deletion and the subsequent NR-to-CE cell fate conversion suggest that SOX2 normally antagonizes PAX6 signaling to maintain NR identity. Moreover, the genetic rescue of eye development by lowering PAX6 levels while deleting *Sox2* indicates a functional antagonism between the two genes. These data raise the possibility that neurogenic versus non-neurogenic fate in the optic cup is extremely sensitive to the ratio of PAX6 to SOX2.

We propose a model (see Fig. S6 in the supplementary material) in which SOX2 levels are high enough to antagonize *Pax6* expression in central optic cup progenitor cells, thereby maintaining neurogenic capacity. Conversely, in the peripheral optic cup, SOX2 levels are low, perhaps allowing PAX6 to activate its own expression via the α enhancer. In fact, previous studies have shown that PAX6 can directly bind a conserved site in α, and that lowering PAX6 levels decreases α-driven CRE-GFP expression (Baumer et al., 2002; Schwarz et al., 2000). Further studies are needed to address the question of whether SOX2 and PAX6 coordinate *Pax6* expression through the α enhancer. Indeed, SOX2 and PAX6 have been shown to co-regulate expression of the δ-*crystallin* gene in the lens, where they form a complex at the DC5 enhancer that is stabilized by both protein-protein and protein-DNA interactions (Kamachi et al., 2001). A similar co-regulation of α enhancer activity might control *Pax6* expression in the optic cup given that the α enhancer has been suggested to mediate the proximal^{low}-to-distal^{high} PAX6 gradient (Baumer et al., 2002; Davis-Silberman et al., 2005).

Although the role of secreted signaling molecules in positioning the boundary between prospective NR and CE has been studied extensively, little is known about the cell-intrinsic mechanisms responsible for setting up or maintaining this boundary. Our results suggest a tightly coordinated dosage-dependent transcriptional mechanism directing NR versus CE cell fate. We have shown that the functional antagonism between SOX2 and PAX6 is necessary for proper patterning of the eyecup, such that in the absence of SOX2, PAX6 is not sufficient to maintain NR identity, and cells originally fated to become retinal neurons instead take on a peripheral non-neurogenic cell fate.

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Competing interests statement
The authors declare no competing financial interests.

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