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Reprogramming RPE to differentiate towards retinal neurons with *Sox2*

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Abstract

Guiding non-neural, retinal pigment epithelium (RPE) to produce retinal neurons may offer a source of developing neurons for cell-replacement. *Sox2* plays important roles in maintaining neural progenitor/stem cell properties and in converting fibroblasts into pluripotent stem cells. This study tests the possibility of using *Sox2* to reprogram RPE to differentiate towards retinal neurons in vivo and in vitro. Expression of *Sox2* in the chick retina was detected in progenitor cells, in cells at a discrete location in the layers of amacrine and ganglion cells, and in Műller glia. Overexpression of *Sox2* in the developing eye resulted in hypo-pigmentation of the RPE. In the affected regions, expression of retinal ganglion cell markers became apparent in the RPE layer. In RPE cell culture, *Sox2* promoted the expression of retinal ganglion and amacrine markers and suppressed the expression of genes associate with RPE properties. Mechanistic investigation using the developing retina revealed a co-expression of *Sox2* and *bFGF*, a growth factor commonly used in stem cell culture and capable of inducing RPE-to-retina transdifferentiation (or reprogramming) during early development. Similar patterns of changes in *Sox2* expression and in *bFGF* expression were observed in atrophic retina and in injured retina. In RPE cell culture, *Sox2* and *bFGF* mutually enhanced one another's expression. Up-regulation of *bFGF* expression by *Sox2* also occurred in the retina. These results suggest that *Sox2* can initiate a reprogramming of RPE cells to differentiate towards retinal neurons and may engage *bFGF* during the process.

Keywords

differentiation; fibroblast growth factor; regeneration; retinal ganglion cells; transcription factor

Introduction

In the vertebrate eye, the multilayered neural retina lies anatomically beneath a homogenous-looking monolayer of non-neural, darkly pigmented cells, the retinal pigment epithelium (RPE). Developmentally, the non-neural RPE and the neural retina share a common developmental origin – the optic vesicle, which originates from the lateral aspects of the forebrain. Invagination of the optic vesicle forms a double-layered optic cup. Cells in the outer layer of the optic cup are destined to become the RPE, and cells in the inner layer will constitute the neural retina. The RPE maintains its simple, monolayer structure throughout life. The neural retina, on the other hand, consists of highly ordered structure with several major neuron types,

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including ganglion, amacrine, bipolar, horizontal and photoreceptor cells, and Műller glia. The diverse cell types in the retina are generated from a pool of multipotent progenitors through states of developmental competence by coordinated cell cycle exit and cell fate specification [1-4].

Degeneration of retinal neurons results in vision loss, because retinal neurons are unable to regenerate. RPE cells, on the other hand, can re-enter the cell cycle to proliferate. Further, progeny cells from RPE proliferation can differentiate into other cell types [5]. Classic studies demonstrated that embryonic chick RPE can be induced by basic fibroblast growth factor (bFGF), either administered exogenously or released from the retina, or by aFGF, to transdifferentiate into a neural retina [6-10]. This capability of bFGF-induced RPE-to-retina transdifferentiation/reprogramming is lost in chick embryos older than embryonic day 4.5 [E4.5; 6,8]. Additionally, under in vitro conditions, the transdifferentiation/reprogramming occurs only when RPE is cultured as an explant or sheet, and does not occur when RPE is cultured as dissociated cells [8]. In the mature eye, RPE cells normally remain quiescent. However, when stimulated, such as by physical triggers that occur during surgery, RPE cells can proliferate. This proliferative response is presently regarded as an undesirable side effect, because the amplified RPE cells often differentiate into cells that generate tractional force that causes retinal detachment and leads to visual impairment. On the other hand, the proliferative response raises an intriguing possibility of exploring the RPE as a convenient source of retinal neurons for replacement in situ, provided that the RPE progeny cells can be guided to differentiate into retinal neurons.

Sox2 is one of four genes used to convert adult fibroblasts into induced pluripotent stem cells in mouse and in human [11-14]. Its protein belongs to the SoxB1 subfamily of transcription factors characterized by a high mobility group (HMG) DNA-binding domain [15,16]. Gene targeting studies show a cell-autonomous requirement for *Sox2* in both epiblast and extraembryonic ectoderm, and mice homozygous for *Sox2* mutants die shortly after implantation [17]. During embryonic neurogenesis, *Sox2* is expressed in the neural plate and in neural stem cells and progenitors. Expression of *Sox2* in neural stem cells and progenitors also occurs in neurogenesis of adulthood [17-20]. Constitutive expression of *Sox2* inhibits neuronal differentiation. Conversely, inhibition of *Sox2* signaling results in the loss of progenitor markers, premature cell cycle exit, and initiation of neuronal differentiation [19, 21]. *Sox2* could therefore play an important role in maintaining neural progenitor/stem cell properties. In mouse retina, the lack of *Sox2* expression causes retinal progenitor cells to lose competence to both proliferation and differentiation, and there is a dose-dependent regulatory effect of *Sox2* on retinal neurogenesis [22]. In human, *Sox2* mutations cause anophthalmia with variable extraocular defects [18]. In the chick and the mouse retina, *Sox2* is expressed in proliferating cells and a subpopulation of amacrine cells [22-24].

To investigate whether *Sox2* can reprogram RPE cells to differentiate towards retinal neurons, we used the Replication Competent Avian Splice (RCAS) retrovirus [25] to ectopically express *Sox2* in RPE cells in the developing chick eye and in cultured chick RPE cells. We then analyzed the expression of retinal neuronal markers in the otherwise RPE cells. We found that *Sox2* induced the expression of genes active in retinal neurons, inhibited the expression of genes associated with RPE properties, and caused hypo-pigmentation of the RPE cells. Furthermore, we found a positive relationship between *Sox2* and *bFGF* expression and a negative relationship between *Sox2* and the expression of pigment epithelium-derived factor (*PEDF*).

Materials and Methods

Chick embryos

Fertilized, pathogen-free White Leghorn chicken eggs were purchased from Spafas and incubated in a Petersime incubator. All use of animals adhered to the procedures and policies set by the Institutional Animal Use and Care Committee at the University of Alabama at Birmingham.

Construction of retroviruses expressing *Sox2*, *Sox2*ΔB, *Sox2*ΔC, and *En-Sox2*

To generate RCAS expressing *Sox2*, the coding region of chick *Sox2* was amplified by RT-PCR based on the published sequence [26] and cloned into pGEMT (Promega, Madison, WI). The cloned sequence was verified. To generate deletion constructs, the DNA-binding domain was removed by restriction digestion with Tth111 I and Psi I, followed by Klenow filling-in and inframe, self-ligation to create *Sox2*ΔB, and the transactivation domain [27] was removed by restriction digestion with Psi I and Spe I (in the multiple cloning site) followed by self-ligation to create *Sox2*ΔC. To generate an active repression construct, the repressor domain of Drosophila Engrailed [28-30] was fused (in frame) to the N-terminus of Sox2, creating En-Sox2. After sequence verification, the DNA sequences were subcloned into shuttle vector Cla12Nco and then inserted into RCAS [25]. Recombinant viral particles were produced as previously described [31]. The titers of the virus stocks ranged from 5×10^7 to 2×10^8 pfu/ml. Different batches of independently produced viruses were used when repeating experiments to rule out the possibility that experimental observations were attributable to retroviral recombination.

Infection of embryonic chick eye with RCAS-Sox2, RCAS-NSCL2, and RCAS-GFP

Retroviruses expressing *Sox2*, *NSCL2* [32], a neural-tissue specific basic helix-loop-helix gene belonging to the stem cell leukemia (SCL) family, or *GFP* [31] as a control, were microinjected into the subretinal space between E2.5 and E3, as previously described [33]. The eyes were enucleated between E5 to E18 and fixed with ice-cold 4% paraformaldehyde. The fixed samples were cryoprotected with OCT: 20% sucrose (2:1), frozen with liquid nitrogen, and kept at −80°C. Cross sections of 10 μm were used in immunocytochemistry and in situ hybridization.

RPE cell culture

Chick E6 RPE was dissected free from the neural retina as described [31]. Pooled RPE tissues were incubated with typsin-EDTA, and the dissociated cells were cultured with knock-out DMEM plus 20% serum replacement (Invitrogen, San Diego, CA). For bFGF treatment, RPE cells were cultured in the presence of 10 μg/ml bFGF. When the culture became ~50% confluent, 10-20 μl of concentrated retrovirus expressing *Sox2*, *Sox2*ΔB, *Sox2*ΔC, *En-Sox2* or GFP was added to a 35 mm dish. Cultures were maintained for an additional 8-15 days, and cells in the culture were then harvested for reverse transcription-polymerase chain reaction (RT-PCR) or fixed for immunocytochemistry.

Glial cell culture

E18 chick eyes were enucleated. The anterior segment along the middle between limbus and equator was surgically removed. After an incubation of the eye cup in 2% dispase at 37°C for 10-20 minutes, the retina was peeled off the RPE. Retinal cells were dissociated with typsin-EDTA and seeded in 35 mm dishes with Medium 199 supplemented with 10% fetal calf serum for 4 hours. At this point, cells not attached to the dish were removed, and new medium was added. When the culture reached about 40-50% confluency, RCAS-Sox2 or RCAS-GFP (30

μl) was added to the dishes. Cells in the culture were harvested for RT-PCR 3 days after the administration of the virus.

Immunocytochemistry

Monoclonal antibody RA4 (1:1000 dilution) was a gift from Dr. Steven McLoon (University of Minnesota, Minneapolis, MN). Monoclonal antibody (HM-2; 1:200) against microtubule-associated proteins (MAP2) was purchased from Sigma. Monoclonal antibody against Brn3a (1:100) was purchased from Chemicon (Temecula, CA). The following monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA): anti-bromodeoxyuridine (anti-BrdU, G3G4, 1:100; developed by Dr. Stephen J. Kaufman), 3A10 (1:100; developed by Dr. Thomas Jessell), anti-islet-1 (39.4D5, 1:100; developed by Dr. Thomas Jessell), anti-Ap2a (3B5, 1:200, developed by Dr. Trevor Williams), anti-vimentin (H5, developed by Dr. Joshua Sanes), and anti-visinin (7G4, 1:500; developed by Dr. Constance Cepko). Standard immunocytochemistry was performed with horse radish peroxidase (HRP)-conjugated, alkaline phosphatase-conjugated (Vector Laboratories, Burlingame, CA), or fluorophore-conjugated (Molecular Probes, Eugene, OR) secondary antibodies.

In situ hybridization and double labeling

Digoxigenin (Dig)-labeled antisense RNA probes against the coding sequences of *Sox2* (555 nucleotides) and *bFGF* (476 nucleotides) [34] were synthesized using the Genius kit (Roche Molecular Biochemicals, Indianapolis, IN) following the manufacturer's instructions. FITC-labeled antisense RNA probes against *bFGF* mRNA was also synthesized for double-in situ hybridization along with dig-labeled antisense RNA probes against *Sox2* mRNA, following procedures previously described [35]. In situ hybridization was performed with 8-10 μm frozen sections on glass slides with final staining with alkaline phosphatase substrate NBT [35] or with fluorescein-tyramide or rhodamine-tyramide [35]. For double labeling, in situ hybridization was performed first followed by immunocytochemistry.

Double labeling for BrdU incorporation and *Sox2* expression

Chick embryos at E8, E10, or E14 infected with RCAS-Sox2, or RCAS-GFP as a control, were pulsed with BrdU (50 μg in 50 μl of HBSS) dropped through an opening window in the shell through the vitelline membrane. The embryos were incubated for 4 hours before the eyes were harvested and fixed with 4% paraformaldehyde. Frozen sections on glass slides were first subjected to in situ hybridization with dig-labeled anti-*Sox2* RNA probes and then to BrdU detection using a specific antibody as previously described [36]. E15 embryos infected with RCAS-Sox2 or RCAS-GFP were subjected to anti-BrdU immunostaining only.

Chemically damaging the retina

N-methyl-D-aspartate (NMDA, 2-3 μl of 34 mM solution in 0.9% saline) was injected into the vitreous of E12 chick eyes to induce retinal injuries [37-39]. Injection of saline solution alone served as the control. At E15, retinas from 3 experimental eyes or control eyes were collected for analysis.

RT-PCR

Total RNAs were isolated from the RPE and the retina dissected from chick embryos and from cells in RPE and glial cell cultures, using the RNeasy protect mini kit (QIAGEN). First-strand cDNA was synthesized with a cDNA synthesis kit (Ambion) using oligo-dT as the primer. After a 5-10 fold dilution, 1 μl of the first-strand cDNA was used as the template in each 30 μl PCR reaction. The small ribosomal protein 17 (S17) was used as an internal control to normalize the amount of cDNA in each sample [40] with 20 cycles of amplification using

primers gtgatcatcgagaag and agcaacataacgagc annealed at 44°C. *Mitf*, *mp115*, *Otx2*, *Npy*-1, *Pax6*, and *PEDF* were amplified by 30 cycles and *bFGF* was amplified by 25 cycles, at an annealing temperature of 56°C derived empirically using temperature gradients. The specific primer set for each of the genes spans intron of 689 to 7396 bp: *Mitf*, ccttggcttgatggaccca and gcatgatcagtgtcctccat (GenBank accession # D88363); *Mmp115*, cacagcagtggagggaagc and ggctgtgggcagcagcg (GenBank accession # NM_205112); *Otx2*, gcatgatgtcttatcttaagcaa and ggaacttccatgaggatgtc (GenBank accession # AY600957); *Npy*-1, cctctcccagagccgctcc and ggaatgcacaaataatttatttaaat (GenBank accession # M87294); *Pax6*, caacactcccagccacatc and ccaacacagatcaaacatcc (GenBank accession # NM_205066); *bFGF*, atggcggcgggggcgg and tcagcttttagcagacattgg (GenBank accession # NM_205433); and *PEDF*, gaagatgtgatttctcgtgc and gaggctttcctcgatcag (NCBI's Annotation Process, XM_001234864). For quantificational and statistical analyses, the integrated optical density (IOD) of the PCR product on agarose gels was measured using LabWorks™ (version 4.0, UVP Inc.). The means and SDs of IODS from 3 independent RT-PCR reactions were calculated using Origin 7.0 (OriginLab Corp.) One-way ANOVA of Origin 7.0 was used for statistical significance analysis at 0.05 (*) and 0.01 (**) levels.

Results

The spatial and temporal expression pattern of *Sox2* in the chick retina

To facilitate an understanding of how *Sox2* participates in retinal neurogenesis, we examined its expression pattern at different developmental stages, from E5 to post-hatching day 30 (P30). In situ hybridization showed heavy staining of *Sox2* expression between E5 and E7 (Fig. 1), the time when retinal cell proliferation is most active [41,42]. During this period, cells expressing *Sox2* localized across the entire thickness of the neuroepithelium, as shown in E5 central retina (Fig. 1A) or in E7 peripheral retina (Fig. 1B). In E7 central retina, where development is more advanced than at the periphery, cell proliferation activity subsides, and postmitotic cells accumulate on the vitreous side as differentiating ganglion cells and amacrine cells and on the RPE side as developing photoreceptor cells. At this stage, fewer cells remained positive for *Sox2* mRNA (Fig. 1C), and most of the positive cells were confined to the zone of progenitor cells (Fig. 1D). *Sox2* expression continued to decrease From E7 through E15, with the majority of the expression confined to a narrowing zone of cells in the middle of the developing retina (Fig. 1E, F). This expression pattern parallels that of retinal neurogenesis in general. The lens, the RPE, and the surrounding mesenchymal cells appeared to lack *Sox2* expression (Fig. 1A-D)

Starting from E7, *Sox2* expression was also apparent in a small number of cells spatially separated from those of the proliferating zone (arrows, Fig. 1D). These cells localized to the inner plexiform layers at E9 (Fig. 1E), to the amacrine layer at E9 and thereafter (Fig. 1E-H), and to the ganglion cell layer in E15 and thereafter (Fig. 1F-H).

In E15 retina, where there is little cell proliferation activity [41,42], *Sox2* expression persisted in cells at the middle of the inner nuclear layer (INL; Fig. 1F). Similar expression was detected in retinas of P0 (Fig. 1G) and P30 (Fig. 1H). Double-labeling showed that these cells were positive for vimentin (arrows, Fig. 1I-K), indicating *Sox2* expression in Műller glia.

Changes in RPE appearance induced by *Sox2*

The expression of *Sox2* in the retinal neuroepithelium and its known role in maintaining neural stem cell properties prompted us to ask whether *Sox2* could induce de novo retinal neurogenesis in the context of RPE cells. RCAS, a replication-competent retrovirus, was used to drive *Sox2* overexpression (or misexpression) in the developing chick eye by microinjecting RCAS-Sox2 into the space between the two layers of the optic cup between E2.5 and E3. Before E8,

the eye and the retina from embryos infected with RCAS-Sox2 were indistinguishable from those of the control. BrdU incorporation analysis showed no significant changes in retinal cell proliferation activities between retinas overexpressing *Sox2* and the controls at E8 and E10 (data not shown). Anatomically, the majority of the retina infected with RCAS-Sox2 appeared normal, with the exception of discreet places between the central and peripheral retina, as described below.

From E8 and thereon, the experimental eyes appeared "spotty," with regions markedly lacking the dark pigmentation (Fig. 2A). Cross sections showed that at the "spotty" places, the RPE cells partially or completely lacked dark pigments (Fig. 2A). At these affected places, the retina often detached from the de-pigmented RPE (Fig. 2C). The de-pigmentation of the RPE and the retinal detachment suggest that *Sox2* might have interfered with RPE development and function. Neither de-pigmentation nor retinal detachment was observed with control eyes infected with RCAS-GFP (Fig. 2A, B).

To examine whether cells in the de-pigmented regions could re-enter the cell cycle, we subjected the embryos to BrdU at E14, when cell proliferation has long ceased in the RPE, and analyzed BrdU incorporation at E15. While absent in the control (data not shown), $BrdU^+$ cells were detected in the de-pigmented regions of the RPE infected with RCAS-Sox2 (Fig. 2E, F).

Induction of neuron markers in the RPE by *Sox2*

To investigate whether *Sox2* had initiated the development of neural properties in the context of RPE, we examined the eyes infected with RCAS-Sox2 for the expression of genes associated with retinal neurons. One of the earliest neural markers in the developing chick retina is RA4 immunoreactivity in newborn ganglion cells [43]. In regions with significant de-pigmentation, a large portion of the cells (estimated to be >50%) in the RPE were $RA4^+$ (Fig. 3A-C). Some of the $RA4^+$ cells exhibited neural morphologies (arrows in Fig. 3A-C). Cells in the de-pigmented regions were also immunopositive for a neurofilament-associated protein recognized by monoclonal antibody 3A10 (Fig. 3D), and a 160-kDa neurofilament recognized by monoclonal antibody 4H6 (Fig. 3G). Neither protein was detected in the RPE cell layer of the control retina infected with RCAS-GFP (Fig. 3E-I). The RPE de-pigmentation and the ectopic expression of RA4, 3A10, and 4H6 were detected from E8 through E18, the last developmental stage analyzed. Notably, cells in the de-pigmented region of the RPE infected with RCAS-Sox2 did not express other ganglion markers, such as Brn3a or Islet-1, and they remained negative for general neural maker Map2, photoreceptor marker visinin, and amacrine markers Pax6 and AP2α (data not shown).

Reprogramming cultured RPE cells by *Sox2*

The possibility of *Sox2* reprogramming RPE to differentiate towards retinal neurons was also investigated in cultures of dissociated RPE cells. When E6 chick RPE cells are dissociated and seeded at low density in culture dishes, they enter the cell cycle and proliferate. During this proliferating phase of culture, RPE cells lose their pigment granules [44]. Later, as the culture becomes confluent, the cells become re-pigmented. When infected with RCAS-Sox2, however, cells in the culture failed to become re-pigmented after confluence and a week thereafter, in contrast to those in the control culture infected with RCAS-GFP (Fig. 4B). Thus, as in the eye, *Sox2* inhibited the pigmentation of RPE progeny cells in culture.

Immunocytochemistry analysis showed a large number of $RA4^+$ cells (accounting for >50% of the total cells) in RPE cultures infected with RCAS-Sox2 (Fig. 4D) and $3A10^+$ (accounting for >30% of the total cells; Fig. 4G). Expression of AP2α was also detected in RPE cell cultures infected with RCAS-Sox2 (Fig. 4J). Thus, unlike in vivo in the eye, *Sox2* was able to induce AP2α expression in vitro in RPE cell culture. The control cultures lacked the expression of

these markers (Fig. 4C, F, I). Morphologically, some of the $RA4^+$ or $3A10^+$ cells exhibited long processes, which markedly deviated from the hexagonal or fibroblast-like morphologies of RPE cells in culture (Fig. 4D, F).

For additional controls and to determine whether transcription activation by Sox2 is required for the observed changes, we generated RCAS expressing mutation constructs of *Sox2* (Fig. 4A), including omission of the DNA-binding domain (*Sox2ΔB*), omission of the activation domain (*Sox2ΔC*), and the addition of the repressor domain of Drosophila Engrailed (*En-Sox2*). Drosophila Engrailed actively and specifically represses activated transcription [28, 28]. Its repressor domain can confer repression activity to heterologous DNA-binding domains when linked to them [28,29]. RPE cell cultures infected with RCAS viruses expressing these mutation constructs showed no differences from that of the GFP control in re-pigmentation (Fig. 4B), in lacking of $RA4^+$ cells (Fig. 4E), and in lacking of $3A10^+$ cells (Fig. 4H).

RT-PCR was used to examine whether *Sox2* suppressed the expression of genes key to RPE development and specification and/or promoted the expression of genes associated with neural properties. Genes important for RPE development include *Mitf* [45,46], *Otx2* [47-50] and melanosomal matrix protein *Mmp115* [51]. Mutation of *Mitf* caused RPE-to-retina transformation [52,53]. In cultures infected with RCAS-Sox2, we found reduced expression of *Mitf*, *Otx2*, and *Mmp115* (Fig. 4K), genes which are important in development and maintaining of RPE properties. On the other hand, *Sox2* increased the expression of *Pax6*, a homeodomain gene expressed in retinal progenitor cells, amacrine cells, and ganglion cells [54] and regulating neural identity and differentiation [55]. *Sox2* also increased the expression of *Npy-1* (Fig. 4K), a gene that encodes neuropeptide Y [56] and is expressed in a discrete set of amacrine cells [57]. The sizes of the RT-PCR products were as expected from the corresponding mRNA, not the genomic sequence with intron sequence.

Co-expression of *Sox2* with *bFGF* in the developing retina

The observed changes in RPE properties induced by *Sox2* share similarities with bFGF-induced RPE transdifferentiation/reprogramming [5,7,8,58]. In reprogramming embryonic chick RPE at early developmental stages to develop into a neural retina, aFGF (or bFGF) is thought to potentiate the fate of the first born neurons, the retinal ganglion cells, thereby setting the stage for subsequent retinal neurogenesis. Population analysis of dissociated cells several days after exposing RPE tissue to aFGF (or bFGF) indicated that as much as 80% of the cells in the transdifferentiated RPE cells express antigens normally present in retinal ganglion cells [9]. In RPE cell culture, bFGF, like *Sox2*, prevents the cells from becoming re-pigmented and induces the expression of early, but not later markers of retinal ganglion cells [59]. These similarities prompted us to examine the inductive relationship between *Sox2* and *bFGF* in our experimental systems.

In situ hybridization was used to examine the *bFGF* expression pattern in the developing chick retina. At E6, *bFGF* mRNA was present in cells across the neuroepithelium, while the developing ganglion cells accumulating on the vitreal side lacked obvious expression (Fig. 5A). By E8, cells expressing *bFGF* were concentrated into a zone at the middle of the INL where residual proliferating cells and/or developing Műller glia reside (Fig. 5B). Expression of *bFGF* in cells localized to where Műller glia reside became apparent in retinas of E10 (Fig. 5C), E12 (Fig. 5D), and E16 (data not shown), albeit the levels of expression were barely detectable with our in situ hybridization method in E12 and older retinas.

The similarity in the spatial patterns of *bFGF* expression and *Sox2* expression prompted an inquiry into whether the two were co-expressed. Double-in situ hybridization was carried out using thin (8 μm) E8 retinal sections and with prolonged proteinase K treatment, two measures incorporated to reduce cell density to minimize ambiguity in identifying double-labeled cells.

This method detected double-labeled cells in the middle region of the neuroepithelial layer of the E8 retina (Fig. 5E-H).

We then examined atrophic retinas and injured retinas to address the question of whether *Sox2* and *bFGF* were co-regulated in the retina. For atrophic retina, we used those partially infected with RCAS-NSCL2. Previous study has shown than retinas infected with RCAS-NSCL2 undergo severe Műller glial atrophy [32]. We found that in the retina partially infected with RCAS-NSCL2, the infected regions (Fig. 6A) lacked vimentin$^+$ (Műller) cells (Fig. 6B). These regions also lacked *bFGF*-expressing cells, which were present in the adjacent, uninfected regions (Fig. 6C). Similarly, in infected regions (Fig. 6D) with fewer vimentin$^+$ cells (Fig. 6E), fewer *Sox2*-expressing cells were present than in the adjacent, uninfected regions (Fig. 6F). Thus, in Műller glia-deficient atrophic retina, the expression of *Sox2* and *bFGF* were down-regulated.

Vitreal injection of NMDA is known to cause degeneration of retinal neurons, particularly amacrine and bipolar cells [37-39] and to induce glial activation [60]. One of the reactive responses of Műller glia to NMDA-induced retinal injury is to re-enter the cell cycle, producing progeny cells that migrate into all layers of the neural retina, leading to the hypothesis that Műller glia may serve as “stem cells” of the retina [60-62]. RT-PCR was used to analyze the effect of NMDA damage on the expression of Sox2 and bFGF, with primers spanning intron sequence to verify amplification from mRNA/cDNA and not from genomic DNA contamination. In retinas subjected to NMDA damage, the level of expression of *Sox2* was increased, and the level of *bFGF* expression was also increased (Fig. 6G). Computer assisted calculation of the integrated optical density (IOD) ratio to “housekeeping” gene S17 showed a more than 2-fold increase ($p<0.01$) in *Sox2* expression, from 0.50 ± 0.05 in the control to 1.29 ± 0.09 in the injured retina. The expression of *bFGF* showed a 58% increase ($p<0.01$), from 0.62 ± 0.01 in the control to 0.98 ± 0.11 in the experimental retina (Fig. 6H). Thus, in NMDA-treated retina, the expression of *Sox2* and *bFGF* was up-regulated.

Inductive relationship between *Sox2* and bFGF

The coincidental expression and the similar patterns of changes in expression between *Sox2* and *bFGF* prompted an examination of the inductive relationship between *Sox2* and *bFGF*. Semi-quantitative RT-PCR was carried out to examine the effect of *Sox2* overexpression in retinas infected with RCAS-Sox2 on *bFGF* expression. In these retinas, the level of *bFGF* mRNA was increased in comparison to the control infected with RCAS-GFP (Fig. 7A). The IOD ratio of *bFGF* to “housekeeping” gene S17 was increased over 3-fold ($p<0.01$), from 0.60 ± 0.22 in the control to 2.02 ± 0.24 in the experimental retina (Fig. 7B). No significant change was observed in the mRNA level of *PEDF* ($p>0.05$; Fig. 7A, B), a growth factor produced by Műller glia and the RPE [63-65]. The RPE from these embryos was also analyzed for alterations in *bFGF* expression, but no changes were found (data not shown). However, the level of *PEDF* expression in the RPE was significantly decreased by at least 30% ($p<0.01$) in embryos infected with RCAS-Sox2 (Fig. 7C, D). Similar results were obtained with RPE isolated from E15 embryos (Fig. 7C, D).

To confirm the induction of *bFGF* in the retina and the suppression of *PEDF* in the RPE by *Sox2*, we analyzed cultures of Műller glial cells and dissociated RPE cells. In Műller glial cell culture, the level of *bFGF* mRNA was increased (Fig. 7E), with its IOD ratio to S17 increased 2-fold ($p<0.01$), from 0.71 ± 0.33 in the control culture infected with RCAS-GFP to 1.98 ± 0.28 in the culture infected with RCAS-Sox2 (Fig. 7F). The level of *PEDF* expression (Fig. 7E), on the other hand, showed a 3 fold decrease ($p<0.01$), from 3.07 ± 0.27 in the control to 0.98 ± 0.04 in the experimental culture (Fig. 7F). In dissociated RPE cell culture, levels of *bFGF* expression was more than doubled ($p<0.01$) in a culture infected with RCAS-Sox2, in comparison with the control infected with RCAS-GFP (Fig. G, H). In RPE cell cultures infected

with RCAS expressing *Sox2ΔB*, *Sox2ΔC*, or *En-Sox2*, the levels of bFGF expression was similar ($p>0.05$) to that of the GFP control (Fig. 7G, H). In RPE cell cultures infected with RCAS-Sox2, *PEDF* expression was decreased (Fig. 7I, J).

We cultured dissociated RPE cells in the presence of bFGF, either alone or in combination with RCAS-Sox2, in order to examine bFGF's effect on the expression of *PEDF* and *Sox*. We found that bFGF, like *Sox2*, significantly ($p<0.01$) reduced *PEDF* expression (Fig. 7I, J). A further reduction was observed with a combination of bFGF and *Sox2* (from 0.22 to 0.01) than with either bFGF (to 0.05) or *Sox2* (to 0.15) alone (Fig. 7I, J). We also found that addition of bFGF to RPE cell culture induced *Sox2* expression (Fig. 7I).

Discussion

In situ hybridization detected *Sox2* mRNA in different retinal cell populations. The most prominent expression of *Sox2* was detected in cells of the neuroepithelium, suggesting a primary expression of *Sox2* in retinal progenitor cells. Although the number of cells expressing *Sox2* significantly decreased starting at E7 in the central retina, as cell proliferation diminished, Műller cells and a small number of cells in the amacrine and ganglion cell layers maintained detectable levels of *Sox2* mRNA. These results are in good agreement with previous reports of *Sox2* expression in the proliferating cells and a subpopulation of amacrine cells in the chick [24] and mouse retina [22]. The peak of *Sox2* expression during active cell proliferation in the chick retina is consistent with its established role in maintaining neural stem cell properties. Expression of *Sox2* in Műller glia could be related to its role in maintaining stem cell properties, as Műller glia have been shown to exhibit some properties of retinal stem cells [60-62]. The significance of *Sox2* expression in a small number of cells in the amacrine and ganglion cell layers is unclear, and it remains to be examined whether they were quiescent progenitor cells, reminiscent of rod progenitors in the inner nuclear layer of the goldfish retina [66]. In the mouse retina, this particular subgroup of *Sox2*-expressing cells coexpress neural markers Islet-1 and calretinin [22].

A large number of studies show *Sox2's* importance in maintaining neural progenitor/stem cell properties, and recent studies have established its role in reprogramming adult fibroblasts into pluripotent stem cells. Yet, our overexpression experiments showed no increase in the size of the retina or the eye, suggesting that *Sox2* alone is insufficient to prolong cell proliferation activity in retinal progenitor cells. It remains to be investigated whether prolonging retinal cell proliferation activity by *Sox2* will require the other 3 genes in the group of 4 used to reprogram fibroblasts into pluripotent stem cells [11-14]. The most profound effect of overexpression of *Sox2* in the developing chick eyes was to change certain RPE cell properties. In the eye, *Sox2* induced de-pigmentation and the expression of ganglion cell markers recognized by RA4, 3A10, and 4H6 in cells within the RPE layer. This suggests that *Sox2* is able to redirect the differentiation of RPE cells towards that of retinal neurons. Reprogramming by *Sox2* also occurred in RPE cell culture, in which genes key to RPE properties were suppressed, and genes associated with neurons were induced. These suggest that a reprogramming of RPE cells toward retinal neurons was initiated by *Sox2* both in vivo and in vitro. Phenotypically, *Sox2*-induced reprogramming of RPE towards retinal neuron bears certain similarities to bFGF-induced RPE reprogramming.

The importance of *Sox2* and bFGF signaling in maintaining the undifferentiated state of stem cells is widely known; the underlying mechanisms, however, remain unclear. In osteoblasts, activating bFGF signaling induces *Sox2* expression [67]. During neural induction in Xenopus embryos, FGF signaling is not required for the induction of *Sox2* expression, but is required for the maintenance of *Sox2* expression [68]. Results from the current study suggest an inductive relationship between *Sox2* and *bFGF*. Their spatial patterns of expression overlapped

in the developing retina, and their co-expression was detected in some retinal cells. Expression of both *Sox2* and *bFGF* was reduced with Műller glia atrophy and was enhanced by NMDA damage. Furthermore, *Sox2* was induced in RPE cell cultures treated with bFGF, while *bFGF* expression was enhanced by *Sox2* in the retina and in Műller glia culture.

Conclusion

The importance of *Sox2* and bFGF for stem cells is widely accepted. Our results imply a close relationship between *Sox2* expression and *bFGF* expression. *Sox2* can reprogram RPE cells towards retinal neurons both in vivo and in vitro. In future studies, *Sox2* may be used in combination with neural differentiation factors to reprogram RPE cells to produce retinal neurons.

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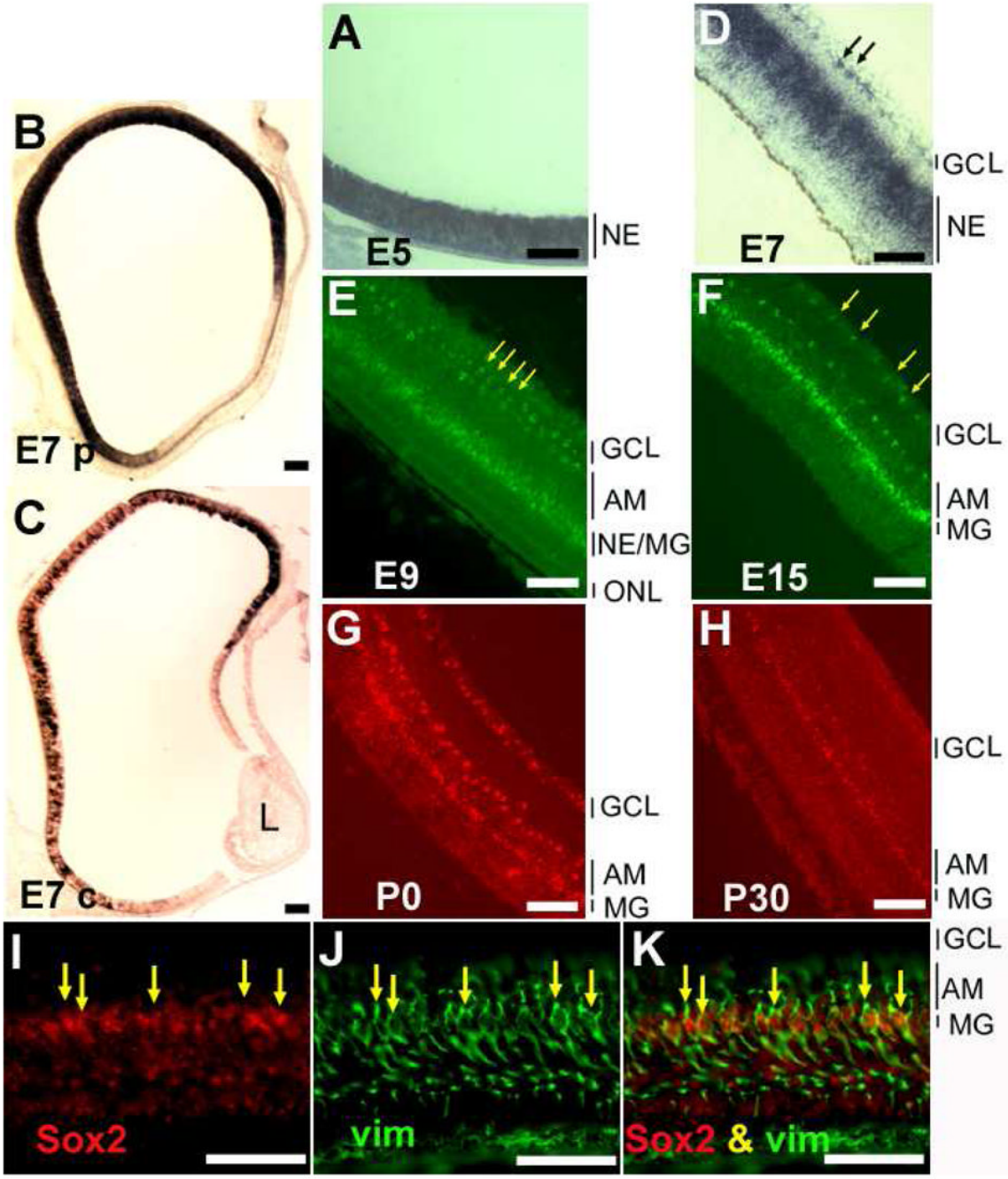

Fig. 1.

Spatial and temporal pattern of *Sox2* expression in chick retina. The in situ hybridization signals were visualized with nitroblue tetrazolium (A-D), fluorescein-tyramide (E,F), or rhodamine-tyramide (G,H). A: E5 retina. B: E7 peripheral retina. C: E7 central retina. D: E7 central retina at higher magnification. E: E9 retina; F: E15 retina. G: P0 retina. H: P30 retina. I-K: Double-labeling for *Sox2* expression and for Muller glia protein vimentin (recognized by Mab H5) in E15 retina. I: In situ hybridization detection of *Sox2* mRNA with rhodamine-tyramide. J: Immunostaining for vimentin with fluorescein. K: Simultaneous view of both stainings. Arrows in D: positive cells in the inner plexiform layer. Arrows in E and F: positive cells that appear spatially paired. Arrows in I-K point to double-labeled cells. AM: amacrine cells. GCL:

ganglion cell layer. L: lens. MG: Muller glia. NE: neuroepithelium. ONL: outer nuclear layer. Scale bars: 50 μm.

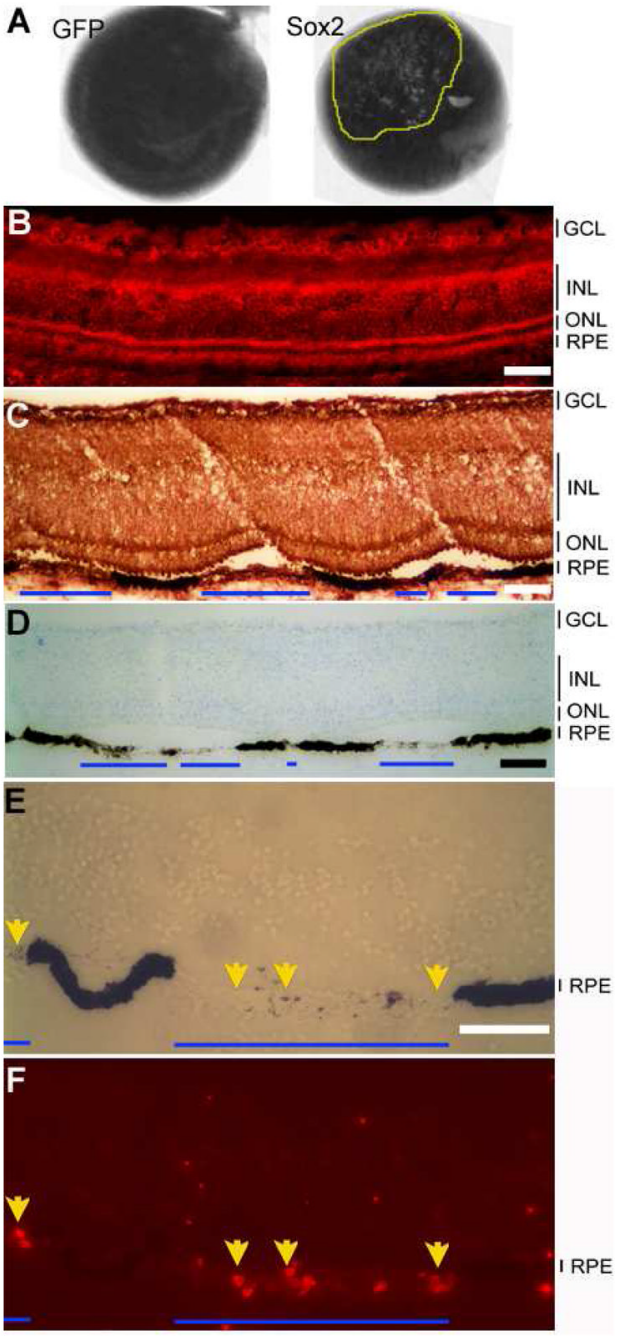

Fig.2.

RPE de-pigmentation from *Sox2* overexpression. A: E15 eyes from an embryo infected with RCAS-Sox2 (left) or RCAS-GFP (right). The “patchy” area is encircled by a yellow line. B: Immunostaining with anti-viral protein P27 to detect viral infection in E15 retina infected with RCAS-GFP. C: Immuno-detection of viral infection (anti-viral protein P27 staining) in E15 retina infected with RCAS-Sox2. D: Immuno-detection of viral infection (with anti-viral protein P27) in E15 retina infected with RCAS-GFP. Blue lines mark regions with de-pigmentation of the RPE and retinal detachment. E-F: BrdU incorporation in de-pigmented regions of E15 retina infected with RCAS-Sox2. E: A bright-field views. F: BrdU

immunostaining for BrdU. The arrows show $BrdU^+$ cells in the RPE layer. GCL: ganglion cell layer. INL: inner nuclear layer. ONL: outer nuclear layer. Scale bars: 50 μm.

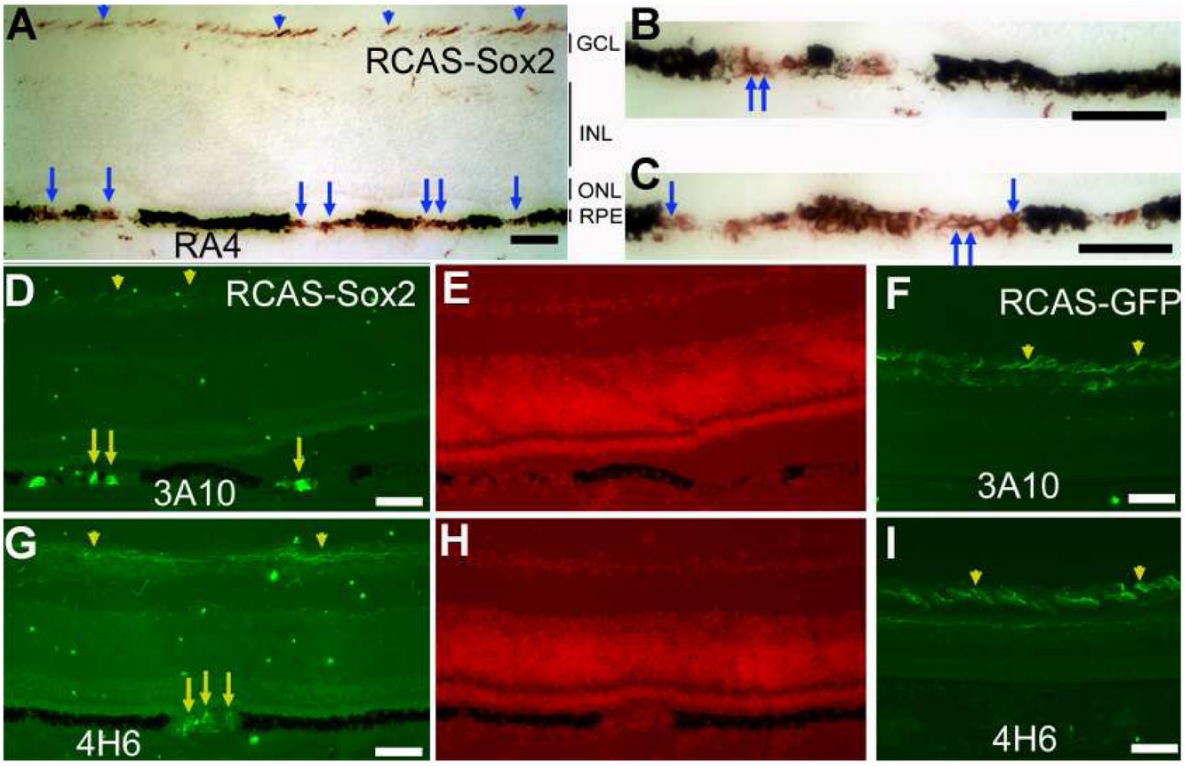

Fig. 3.

Ectopic expression of retinal ganglion cell markers in the RPE of E18 embryos infected with RCAS-Sox2. A: Immunostaining (in red) with RA4. B: Higher magnification of the left side of A. C: Higher magnification of the right side of A. D: Immunostaining (FITC) with 3A10 of retina infected with RCAS-Sox2. E: Nuclear staining of C. F: Immunostaining (FITC) with 3A10 of retina infected with RCAS-GFP. G: Immunostaining (FITC) with 4H6 of retina infected with RCAS-Sox2. H: Nuclear staining of C. I: Immunostaining (FITC) with 4H6 of retina infected with RCAS-GFP. Arrows point to cells with neuron-like morphologies. Arrowheads point to immunostaining of the neural fibers of retinal ganglion cells. GCL: ganglion cell layer. INL: inner nuclear layer. ONL: outer nuclear layer. Scale bars: 50 μm.

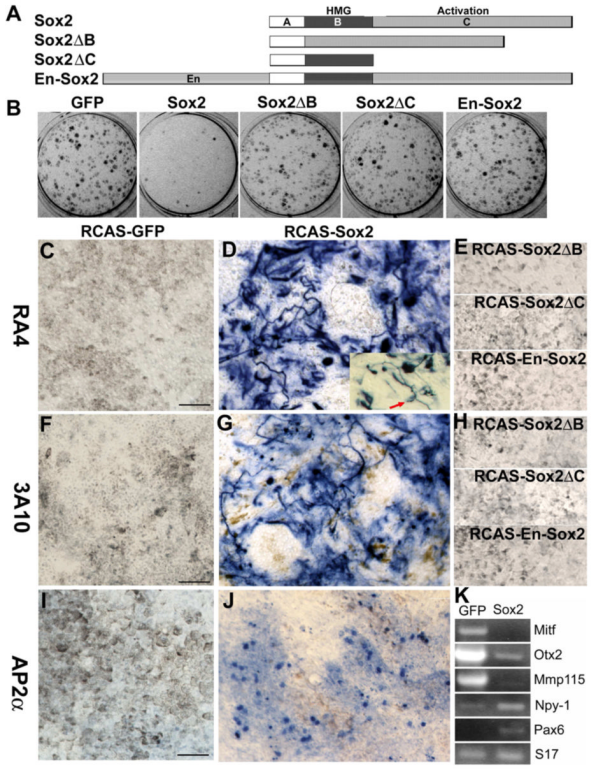

Fig. 4.

Suppression of RPE properties and induction of neural genes by *Sox2* in RPE cell cultures. A: A schematic diagram showing the structural regions included in each recombinant DNA construct. B: Appearance of RPE cell cultures, showing hypopigmentation of the infected with RCAS-Sox2 as compared with the control infected with RCAS-GFP. Also shown are parallel cultures infected with RCAS expressing *Sox2ΔB*, *Sox2ΔC*, or *En-Sox2*. C-J: Expression of retinal neural markers in RPE cell cultures infected with RCAS-Sox2. C-E: Immunocytochemistry with RA4 of cultures infected with RCAS-GFP, RCAS-Sox2, and RCAS expressing 3 mutations constructs of Sox2: *Sox2ΔB*, *Sox2ΔC*, and *En-Sox2*. F-H: Immunocytochemistry with 3A10. I, J: Immunocytochemistry for AP2α. The arrow in the inset of D: a cell displaying morphology typical of a retinal ganglion cell. K: RT-PCR analysis of the expression of RPE genes (*Mitf*, *Otx2*, and *Mmp115*) and of neural genes (*Npy*-1 and *Pax6*) in RPE cell cultures infected with RCAS-Sox2 or RCAS-GFP. Small ribosomal protein 17 (S17) was used as an internal control for the amount of first-strand cDNA present in the samples. Scale bars: 50 μm.

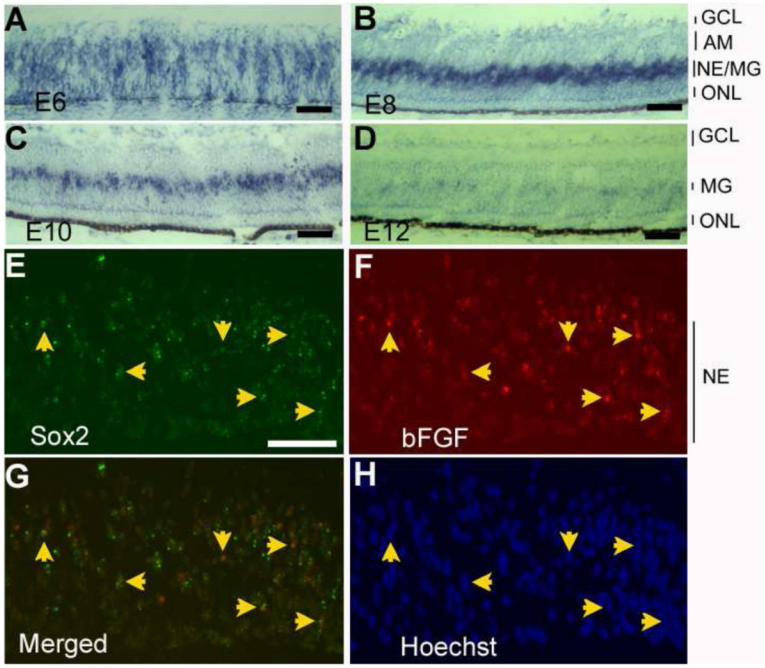

Fig.5.

The spatial pattern of *bFGF* expression in the developing chick retina and its co-expression with *Sox2*. A-D: In situ hybridization for bFGF expression in E6 (A), E8 (B), E10 (C), and E12 (D) chick retina. E-F: Double in situ hybridization for *Sox2* mRNA (E, fluorescein-tyramide, in green) and *bFGF* mRNA (F, rhodamine-tyramide, in red) in E8 chick retina. G: A merged view of E and F. H: Hoechst nuclear staining. Arrows point to double-labeled cells in the neuroepithelial layer (NE). AM: amacrine cells. GCL: ganglion cell layer. MG: Muller glia. NE: neuroepithelium. ONL: outer nuclear layer. Scale bars: 50 um.

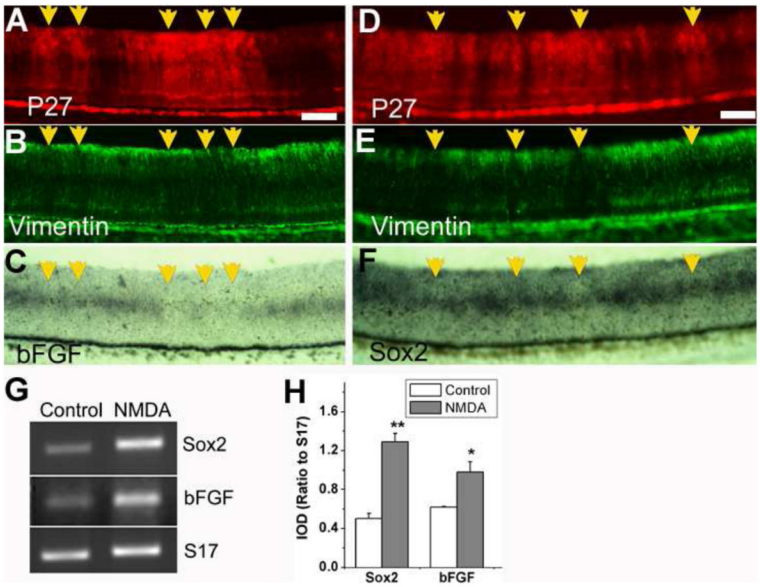

Fig. 6.

Similar patterns of changes in expression of *Sox2* and *bFGF* in Muller glia atrophic retina and after chemically induced retinal injury. A-F: E10 retinas partially infected with RCAS-NSCL2, which causes Muller glia atrophy [ref]. A, D: Immunostaining for antiviral protein p27 to identify regions infected by RCAS-NSCL2. B, E: Immunostaining for Muller glial protein vimentin. C, F: In situ hybridization for bFGF expression (C) and Sox2 expression (F). Arrows point to RCAS-NSCL2 infected regions with fewer vimentin$^+$ cells and fewer bFGF$^+$ cells or with fewer Sox2$^+$ cells than the adjacent, uninfected regions. G: RT-PCR analysis of Sox2 and bFGF expression in control and in NMDA-damaged retina. H: A plot of IOD ratios to S17 of the DNA band intensities of Sox2 and bFGF PCR products. Shown are the means and S.D.s from 3 independent RT-PCR reactions. "*" indicates statistically significant at $p<0.05$ level, and "**" at $p<0.01$ level. Scale bars: 50 um.

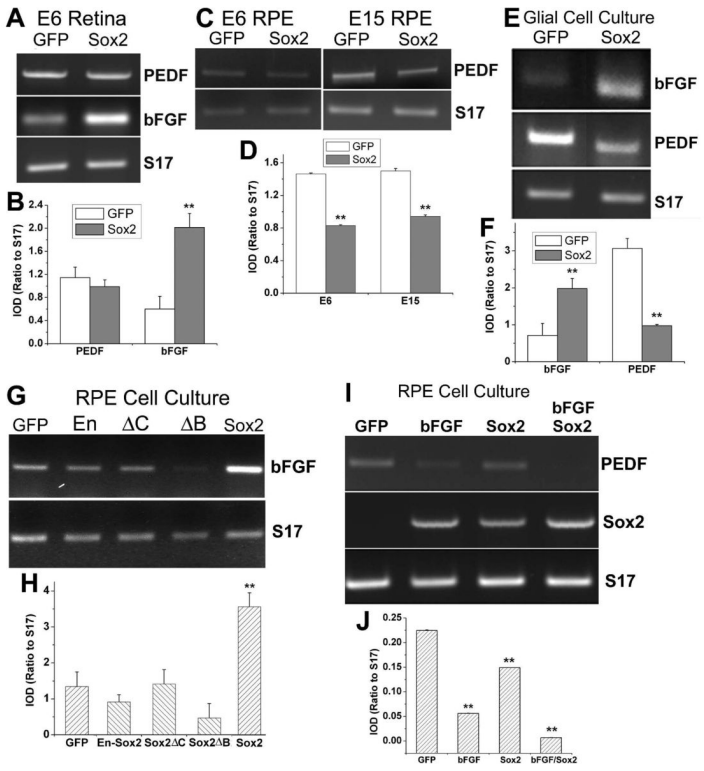

Fig. 7.

Increasing *bFGF* expression and decreasing *PEDF* expression by *Sox2*. A: RT-PCR analysis of *bFGF* and *PEDF* expression in E6 retina infected with RCAS-GFP (GFP) or RCAS-Sox2 (Sox2). Housekeeping gene *S17* was used as an internal control of the relative amount of cDNA present in each sample. B: A plot of IOD ratios (to *S17*) of the DNA band intensities of *PEDF* and *bFGF* PCR products. C, D: RT-PCR and a plot of IOD ratios (to *S17*) of *PEDF* expression in E6 and E15 RPE infected RCAS-GFP (GFP) or RCAS-Sox2 (Sox2). E, F: RT-PCR and a plot of IOD ratios (to *S17*) of *bFGF* expression and *PEDF* expression in glial cell culture infected with RCAS-GFP (GFP) or RCAS-Sox2. G, H: RT-PCR and a plot of IOD ratios (to *S17*) of *bFGF* expression E6 RPE cell culture infected with RCAS-GFP (GFP), RCAS-Sox2ΔB, RCAS-Sox2ΔC, RCAS-En-Sox2, or RCAS-Sox2. I, J: RT-PCR and a plot of IOD ratios (to *S17*) of the expression of *PEDF* and *Sox2* in RPE cell cultures infected with RCAS-GFP (GFP), treated with bFGF (bFGF), infected with RCAS-Sox2 (Sox2), or treated with bFGF as well as infected with RCAS-Sox2 (bFGF Sox2). Bars shown in all plots are the means and S.D.s from 3 independent RT-PCR reactions. "*" indicates statistically significant at $p<0.05$ level, and "**" at $p<0.01$ level.