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Bone Morphogenetic Proteins and Secreted Frizzled Related Protein 2 Maintain the Quiescence of Adult Mammalian Retinal Stem Cells

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ABSTRACT

Rare retinal stem cells (RSCs) within the ciliary epithelium at the retinal margin of the adult mouse and human eyes can divide in vitro in the absence of growth factors to generate clonal, self-renewing spheres which can generate all the retinal cell types. Since no regenerative properties are seen in situ in the adult mammalian eye, we sought to determine the factors that are involved in the repression of endogenous RSCs. We discovered that factors secreted by the adult lens and cornea block the proliferation of adult RSCs in vitro. Bone morphogenetic protein (BMP)2, BMP4, and secreted frizzled related protein 2 were identified as principal effectors of the anti-proliferative effects on RSCs. As a similar induced quiescence was observed in vitro on both mouse and human RSCs, targeting these molecules in vivo may reactivate RSCs directly in situ in the eyes of the blind. STEM CELLS *2013;31:2218–2230*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Most retinal diseases involve the degeneration of photoreceptors, which leads to visual impairment and further blindness. Unfortunately, there is no current method to replace the lost retinal cells and repair sight. Fish and amphibians produce new retinal neurons throughout life from a germinal region at the peripheral margin of the retina called the ciliary marginal zone (CMZ), which regulates normal retinal development and contributes to retinal regeneration [1–5]. Furthermore, lineage tracing studies have demonstrated that some CMZ cells can give rise to clones containing all types of retinal neurons and Müller glial cells [4,6] emphasizing the existence of bona fide retinal stem cells (RSCs) within this specific area. Since no such extensive proliferative and regenerative capacities have been observed in mammals (beyond the postnatal day 10 [PND10] in mice), specifically at the margin of the retina within the ciliary epithelium (CE) [7,8], it was hypothesized that no CMZ per se or bona fide RSCs persist in the adult mammalian eye. Moreover, two laboratories recently have suggested that all adult mammalian CE cells remain as pigmented ciliary epithelial cells with at best a limited potential to transdifferentiate into photoreceptors [9,10]. However, recently it was revealed that more than 90% of the progeny of the putative adult RSCs from the adult mouse and human CE showed the potential to differentiate into mature non-pigmented rod photoreceptors [11].

In opposition to the hypothesis of random CE transdifferentiation, our studies have suggested that endogenous bona fide RSCs may exist within the adult CE in mammals. many recent studies have demonstrated that clonal RSC-derived spheres can be grown from the CE of distinct mammals [12–15]. Furthermore, a very rare population (1 in 500 cells, i.e., 0.2%) of pigmented cells within the CE adjacent to the edge of the retina of embryonic to adult mouse and human eyes was shown to clonally exhibit the self-renewal and multipotentiality properties of RSCs when grown in culture [16,17]. The rare frequency (0.2%) of sphere-forming CE cells supports a stem cell hypothesis, which one would not expect from an overall transdifferentiation of all the pigmented cells present within the CE [10]. Moreover, using cell-sorting methods we recently have enriched prospectively for a rare population of medium-sized, heavily pigmented, P-cadherin low-expressing cells within the adult CE that exhibit RSC properties in vitro. However, given that sorting using these markers does not lead to a pure adult sphere-forming RSC population, stochastic events also might underlie the number of cells that proliferates clonally to form spheres in these culture conditions. Indeed, in P-cadherin knockout mice, we recently have shown that more CE cells survive cell dissociation, thus leading to a significant increase in clonal primary RSC colonies (B.L.K. Coles and D. van der Kooy, unpublished data).

The fact that rare putative adult pigmented RSCs readily proliferate in vitro to form clonal, self-renewing pigmented spheres even in the absence of exogenous growth factors [17], while no proliferation has been observed in vivo, led us to hypothesize that RSC may be present in the mammalian eye but repressed within the adult CE RSC niche. Although little is known about how RSCs are controlled in the mammalian eye, several findings support the idea that stem cells are present within the perinatal and the adult CE and that this area retains a certain level of plasticity (i.e., some cells can re-

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enter the cell cycle). First, a study using adult Patched1$^{+/-}$ heterozygous mice revealed the persistence of a small number of mitotic cells within the second postnatal week CE, whereas no proliferative cells are found in wild-type retinas of the same age [18]. Second, it was shown that retinal precursors can be stimulated to proliferate following the injection of insulin growth factor (IGF) and fibroblast growth factor 2 (FGF2) in the perinatal mammalian eye [19]. Third and perhaps more convincing, we previously observed enlarged CE areas in two *Mitf* and *Chx10* null-mutant mouse strains impairing retinal pigmented epithelium (RPE) and neural retina progenitor proliferation, respectively [20]. Moreover, three to eight times more RSC-derived primary spheres were isolated in vitro from both retinal progenitor-deficient mutant mice compared to wild-type counterparts indicating increased RSC population [20]. Finally, using ephrinA3 null-mutant mice and in humans with retinal detachment or anterior vitreoretinopathy, two recent studies have demonstrated that proliferation may be reactivated within the adult CE [21] [22]. All together, these findings suggest that the adult mammalian CE cells may retain a proliferative reactivity like the CMZ cells of lower vertebrates, and that RSC quiescence is an induced rather than an intrinsic property of adult RSCs. Therefore, we sought to determine factors present in the mammalian CE/RSC niche that are responsible for inducing quiescence in adult RSCs and thus prevent the retinal regeneration that occurs in some cold-blooded vertebrates.

In this study, we discovered that the lens and cornea eye tissues from adult mouse and human eyes specifically release factors that inhibit adult RSC proliferation in vitro. Our findings further reveal that bone morphogenetic proteins (BMPs) are secreted into adult (but not into perinatal) mouse cornea and lens conditioned media, and that only BMP2 and BMP4 inhibit the proliferation of both adult RSCs and their progenitors. This result suggests that active BMP signaling may repress RSC proliferation within the adult mouse CE in vivo. These in vitro anti-proliferative effects were reversible, since upon BMP removal, RSCs and their progenitors resumed their proliferation. In addition, secreted Frizzled-related protein (sFRP) 2 also reduced the numbers of adult RSC primary spheres. Inhibiting BMP signaling through Noggin and functionally blocking sFRP2 both compensated for the effects of cornea- and lens-secreted inhibitory factors, with the primary effects of Noggin on cornea inhibition and anti-sFRP2 on lens inhibition. Factors from the human adult cornea and lens also blocked the proliferation of adult human RSCs in vitro, suggesting that similar factors may repress RSCs in the human eye. All together, these findings demonstrate that a combination of factors released by the cornea and lens inhibits adult mouse and human RSCs in vitro, which may explain the lack of RSC proliferative responses in vivo to damage or retinal degeneration.

Materials and Methods

Animals

Six- to 10-week-old adult wild-type C57/Bl6 (Charles River, Canada, http://www.criver.com/) or T-Cell Factor (TCF)-*LacZ* mice were housed and used in accordance with "The use and care of experimental animals" guidelines. Our animal protocols were reviewed and approved by the Animal Care Committee of the University of Toronto.

Clonal Sphere Assays

RSCs were isolated from the CE at the peripheral edge of the retina of adult mice. Culture conditions and the clonal sphere assay were performed as previously described [17,20]. Briefly, CE-derived cells were plated at a clonal cell density (10 cells per microliter) in standard culture conditions (SFM+FH), that is, serum free media (SFM) [17] supplemented with FGF2 (10 ng/mL) and heparin (2 μg/mL) (Sigma Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). Human eyes were obtained from the Eye Bank of Canada (Toronto, ON) within 24 hours post mortem. The eyes were dissected as previously described [16] in artificial cerebral spinal fluid [17]. Dissociated cells from the CE were counted and plated at 10 cells per microliter in SFM+FH. After 7 days in culture, mouse and human clonal primary spheres were counted. Exogenous factors were added just prior to plating and replaced every other day at specific concentrations as indicated.

Individual mouse clonal primary RSC spheres were harvested and placed in an enzyme solution (trypsin 1.33 mg/mL [Sigma], hyaluronidase 0.67 mg/mL [Sigma], kinurenic acid 0.2 mg/mL [Sigma], collagenase type 1 and type 2 (0.5 mg/mL each) [Worthington Biochemical Corp. http://www.worthington-biochem.com/], elastase (0.1%) [Worthington Biochemical Corp.]). RSC colonies were kept in this enzyme solution for 30 minutes at 37°C. Upon enzyme exposure, spheres were triturated 30 times and then spun down for 5 minutes. Partially dissociated colonies were then resuspended in SFM-containing 1 mg/mL trypsin inhibitor (Sigma) and triturated additional 30 times to single cells. Cells were then centrifuged again for 5 minutes and resuspended in SFM+FH. Passaged spheres were counted after additional 7 days in culture.

Lentiviruses and RSC Transduction

Self-inactivating lentiviral expression vector, pMF351-YFP [23] (kind gift from Dr. Fussenegger) was used to subclone BMP response elements (BRE) of Id1 protein upstream the luciferase reporter gene (generous gift from Dr. ten Dijke) in pGL3 expression vector (Promega, Madison, WI, http://www.promega.com). BRE-Luciferase was excised from pGL3 using MluI/XbaI enzymes and subcloned into pMF351-YFP backbone using AscI/SpeI.

The lentiviral vectors were produced by cotransfecting 293T cells with the lentiviral expression vector, pLP/VSVG (encoding the VSV-G envelope protein), and the packaging constructs pLP1 and pLP2 (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Lentiviruses were concentrated by ultracentrifugation to reach 1.0×10^7 transduction units per milliliter. Lentiviruses were added to adult RSC cultures 6 hours after plating with a multiplicity of infection = 10 and left overnight. The next morning, virus-containing media was removed and replaced by regular SFM+FH. On day 7, YFP expression was observed in 90%–95% on average of all the adult clonal spheres generated. Clonal spheres were then harvested, and the luciferase activity was quantified using the Dual-Glo Luciferase assay system (Promega).

Co-culture and Tissue Conditioned Media

Perinatal (PND2) and adult eye tissues including the sclera, the RPE, the neural retina (NR), the iris, the lens, the cornea, and the CE were microdissected and isolated from a minimum of three C57/Bl6 mice (six eyes) per individual experiment. Since the presumptive CE of PND2 animals is not separated from the iris yet, both tissues were processed together. The limbal region of the peripheral adult cornea and the external capsule of both PND2 and adult lens were removed, respectively. Prior to culture, the NR tissue was pretreated with Dispase (BD Biosciences, San Diego, CA, http://www.bdbiosciences.com) for 2 minutes at 37°C to eliminate RPE cell contamination.

Co-culture experiments were performed only with adult tissues. Individual eye pieces were placed on the upper compartment of 24-well culture plates prepared with special well culture inserts (Nunc). Primary cells from the adult or presumptive perinatal CE were plated at the bottom. The 8-μm-pore size enabled

the diffusion of secreted factors while preventing any cell–cell or tissue–cell contacts.

Conditioned media from perinatal and adult tissue pieces were prepared in pre-determined volumes of SFM in respect to tissue sizes in order to obtain consistent total protein concentrations in tissue supernatant (~50 μg/mL per tissue). On day 2, tissue supernatants were harvested, filtered (0.22 μm), and mixed at 10%, 25%, and 50% to SFM accordingly. Basic FGF (bFGF2, 10 ng/mL) and heparin (2 μg/mL) were last to be added into the different conditioned media to maintain their concentration constant across all conditions. Perinatal or adult RSCs were then plated at clonal cell density (10 cells per microliter) and grown for a week.

Reverse Transcription Polymerase Chain Reaction

RNA from the adult CE of three animals per experiment or primary pigmented adult RSC spheres (~20 colonies) was extracted using RNAeasy mini kit (Qiagen, Hilden, Germany, http://www1.qiagen.com) and complemented with DNase treatment. RNAs were then reverse-transcribed using one-step reverse transcription kit (Qiagen). Equivalent cDNA amounts were used to analyze the expression of BMP receptors. The different sets of primers used in this study are listed in Supplemental or Supporting information Table 1.

Western Blot

Filtered conditioned media from individual adult eye tissues were collected and boiled in Laemmli sample buffer (BioRad, Hercules, CA, http://www.bio-rad.com). Approximately 20 μg of protein extracts was loaded per lane and ran on 12% SDS-PAGE precast gels (BioRad), electrotransferred onto polyvinylidene di-fluoride (PVDF) membranes and blotted with the monoclonal anti-BMP2/4 antibody (R&D Systems, Minneapolis, MN, http://www.rndsystems.com) and the monoclonal anti-β-actin antibody (Sigma).

Immunohistochemistry

Adult animals were anesthetized and fixed through transcardial perfusion of 4% paraformaldehyde (Sigma-Aldrich). Eye balls were removed from adult mouse skulls, postfixed in 4% paraformaldehyde (Sigma) overnight before being transferred to a cryoprotectant 30% sucrose solution for a minimum of 24 hours. Eyes were embedded in Tissue Tek and sectioned at 16 μm using a cryostat. Eye sections were then stained with anti-phospho Smad1/5/8 (Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com) antibodies, followed by appropriate secondary Alexa 488 goat anti-rabbit (Invitrogen) antibodies. Nuclei were counterstained with Hoescht 33258 (Sigma-Aldrich). Images were acquired with a Zeiss (Axiovert) microscope.

Evaluation of Toxicity

Toxicity of an anti-sFRP2 blocking antibody (R&D Systems) and Noggin were assessed by culturing adult RSCs in the presence of increasing doses (0–1,000 ng/mL). Their specificity was tested by combining various concentrations of sFRP2 or BMPs in presence of their respective inhibitors.

RSC Quiescence

Adult CE-derived cells were grown in standard culture conditions or subjected to 50 ng/mL BMP2, BMP4, BMP7, or sFRP2 (R&D systems), or to 25% conditioned media from the adult cornea, iris, CE, lens, NR, RPE, or sclera. On day 7, the numbers of spheres were determined. Quiescence-induced effects were further evaluated in individual wells by harvesting the spheres and cells present in the different conditions described above. Cells and forming spheres were washed twice with SFM, then replated into standard culture conditions. The conditioned media- or drug-free cultures were allowed to grow for additional 7 days and colonies were counted again. Conditions in which the total numbers of spheres were equivalent to control (i.e., 2 weeks in standard culture conditions), although reduced after the first week of treatment, revealed an induced quiescence.

Quiescence was further assessed by dissociating individual spheres grown the first week in control or drug conditions to single cells and before plating them either in control or drug conditions and grown for an additional week. If equivalent numbers of passaged spheres between control and drug-removed conditions were observed, although reduced the first week in the presence of drugs, this indicates that the given drug blocks temporarily cell divisions suggesting induced quiescence.

Statistics

Data are expressed as mean ± SEM. GraphPad Prism 4.0 *t* tests or ANOVA were used for statistical comparisons where appropriate, with Dunnett's and Bonferroni's post hoc tests as required. Minimal statistical significance was defined at $*p < .05$.

Results

Secreted Factors from the Adult Lens and Cornea Block the In Vitro Proliferation of Adult RSCs

In the absence of specific markers for RSCs in vivo, the clonal sphere assay is the only way to prospectively identify the RSC population within the CE of the mammalian eye. In clonal sphere assays, the number of spheres produced reflects the number of endogenous stem cells [17,24]. Since more than 99% of the cells in the spheres are progenitors [17,25], the average sphere diameter of clonally expanded spheres represents a good estimate of retinal progenitor proliferation. Additionally, RSC self-renewal capacities were evaluated by the passage of single spheres [16,17,20].

To model how RSCs are regulated in the adult eye, dissociated adult CE cells were co-cultured in the presence of various intact adult eye pieces. Results show that the majority of the tested tissues reduced the numbers of primary spheres compared to control, thus indicating alterations in adult RSC proliferation and/or survival (Fig. 1A). However, several culture parameters such as the size of individual eye tissue pieces, the competition between adult RSCs and adult eye tissues for nutrients and growth factors, or the release of toxic factors by adult tissues may lead to a non-specific decrease in primary clonal sphere formation.

To overcome the nutrient and growth factor depletions as well as any size difference between adult eye tissues, conditioned media from these distinct eye pieces were produced. This approach was designed to ensure that the volume of media in which the adult eye tissues were cultured was proportional to their respective sizes in order to obtain the same amount of total proteins per tissue supernatants (~50 μg/mL per tissue). Adult RSCs then were cultured in the presence of 10%, 25% and 50% conditioned media, supplemented with the same concentrations of FGF2 and heparin across all tested conditions. These experiments reveal that the conditioned media derived from the adult lens, the cornea, and to a lesser extent the iris tissues reduced the numbers of primary spheres compared to control (Fig. 1B). NR and RPE conditioned media at high concentrations (50%) also demonstrated a significant decrease in the numbers of clonal adult primary RSC spheres compared to control suggesting that these tissues may release inhibitory or toxic factors. To address this toxicity issue, primary dissociated adult CE-derived cells were grown in presence of 25% adult conditioned media for 7 days before removing cells and growing clonal spheres isolated from this potentially deleterious environment and culturing them in control culture conditions for additional 7 days. The removal of

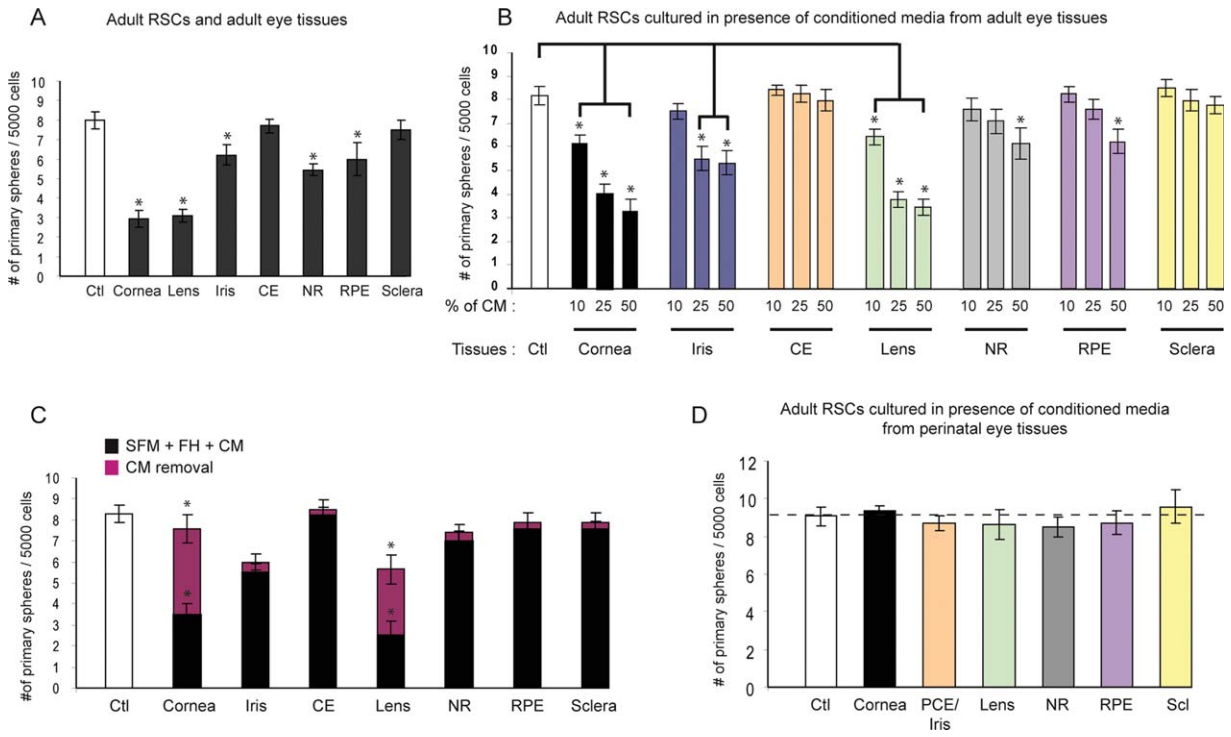

Figure 1. Secreted factors from the adult lens and cornea significantly reduce the proliferation of adult RSCs. **(A):** Adult CE-derived cells were grown in standard culture conditions (Ctl) or co-cultured with different adult eye pieces such as the cornea, lens, iris, CE, NR, RPE, and sclera. Tissues were isolated from a single eye within each mouse with the exception of the adult CE (which was isolated from both eyes in each mouse) and placed in the upper compartments of the culture wells. Separated by well inserts, adult CE-derived cells were plated at a clonal cell density of 10 cells per microliter (cultured in 500 μL media) in the lower compartments. A one-way ANOVA indicated a significant main effect of the treatment [$F(7,184) = 37.05$, $p < .05$, $n = 6$]. Dunnett's post hoc tests revealed that all the adult eye tissues tested, except the adult CE and sclera (Ctl vs. CE and Ctl vs. sclera, $p > .05$), led to significantly reduced numbers of clonal primary adult RSC spheres (* $p < .05$). **(B):** Adult CE-derived cells were cultured in standard conditions (Ctl) or in control media supplemented with 10%, 25%, and 50% conditioned media (CM) made from various adult eye tissues grown in SFM for 2 days. A two-way ANOVA revealed a significant interaction between conditioned media treatment and dose [$F(18,252) = 4.36$, $p < .05$, $n = 10$). Bonferroni post tests indicated that 10% CM from the adult cornea and lens, and 25% CM from the lens, cornea, and iris, as well as 50% CM from the lens, cornea, iris, NR, and RPE induced a significant decrease in the numbers of clonal primary adult RSC spheres compared to control (* $p < .05$). **(C):** Adult RSCs were grown initially in standard culture conditions or in presence of different adult eye tissue-derived CM for 7 days (SFM+FH+CM) as indicated. The black bars represent the numbers of clonal primary adult RSC spheres. Each group was then collected, washed, and plated again in standard culture conditions and grown for additional 7 days (CM removal). The red bars display the total numbers of clonal RSC spheres formed during the "rescue" period. A repeated-measures two-way ANOVA showed significant main effects of treatment and rescue and a significant interaction [$F(7,112) = 4.88$, $p < .05$, $n = 6$]. Bonferroni post hoc tests showed a significant rescue upon removal of cornea and lens CM (cornea CM vs. cornea CM removal and lens CM vs. lens CM removal; * $p < .05$), since there were as many clonal primary adult RSC spheres formed as in control conditions. **(D):** Adult CE-derived cells were cultured in control conditions (Ctl) or in presence of 25% CM from the perinatal (PND2) cornea, PCE/iris, lens, NR, RPE, and sclera as indicated. A one-way ANOVA showed no significant main effect of PND2 tissue CM [$F(6,105) = 0.89$, $p > .05$, $n = 6$]. Abbreviations: CM, conditioned media; CE, ciliary epithelium; Ctl, control; NR, neural retina; PCE, presumptive CE; RPE, retinal pigmented epithelium; RSC, retinal stem cell; SFM, serum-free media.

adult cornea and lens conditioned media enabled the formation of equivalent numbers of primary RSC spheres compared to control, suggesting that secreted factors from the cornea and lens induced RSC simple quiescence (Fig. 1C). However, no significant recovery in RSC sphere numbers was observed after withdrawal of the adult iris conditioned media suggesting a partial toxicity and emphasizing the reversible specificity of the inhibition released by the adult lens and cornea (Fig. 1C).

To investigate further whether these inhibitory effects on RSC proliferation are specific to the adult eye tissues, we generated conditioned media from early PND2 eye tissues, a developmental stage where retina still is forming [6,26] and RSCs still are proliferating in vivo [17]. None of the perinatal conditioned media (representing 25% of the total media volume) showed any effects on adult RSC proliferation (Fig. 1D) or on perinatal RSCs (Supporting Information Fig. 2), suggesting that the proliferation of RSCs is repressed specifically by factors from the adult eye.

BMP2 and BMP4 Are Secreted by the Adult Cornea and Lens

To identify the nature of these inhibitory factors, conditioned media from the different adult eye tissues were boiled to denature proteins but spare ions and metabolites. Once boiled, none of the conditioned media had effects on the formation of primary spheres indicating that the inhibition is most likely mediated by proteins (Supporting Information Fig. 3). We next sought to identify the factors that are released from the adult lens and cornea. The BMP signaling pathway has been shown to play important roles in the formation of the ciliary body during eye development [27] and to be expressed in the adult lens and cornea [28,29]. To investigate the presence of BMPs in the supernatants of adult eye tissues, single adult RSCs were transduced with a lentivirus containing BMP response element (BRE) upstream of the luciferase reporter gene and cultured in the presence of the different adult eye conditioned media. The

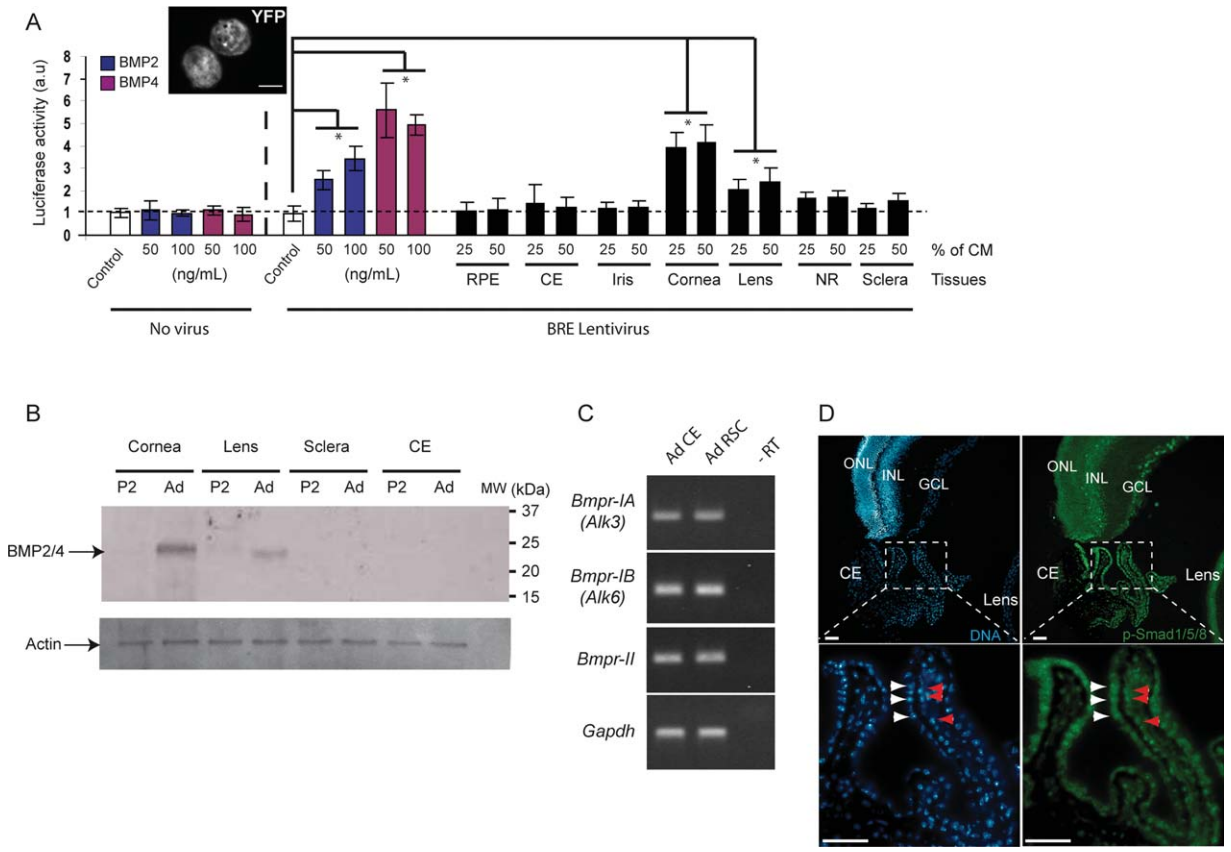

Figure 2. BMP2 and BMP4 are secreted specifically by the adult lens and cornea. **(A):** Luciferase activity was measured in clonal primary adult RSC spheres non-infected (no virus) or infected with a lentivirus containing BMP-response element upstream of a luciferase reporter gene and cultured in standard culture conditions (Ctl), in the presence of increasing doses of BMP2 or BMP4 or in 25% or 50% adult CM from the eye tissue indicated. BMP additions were used as positive controls. Luciferase activity of infected primary adult RSC spheres cultured in control conditions were used as the baseline standard. The inset represents two typical YFP^+ infected adult RSC spheres. The pigmentation within clonal adult RSC spheres obscures the YFP fluorescence in some cells. Scale bar = 50 μm. A two-way ANOVA showed a significant interaction between dose and treatment [$F(16,243) = 3.02$, * $p < .05$, $n = 10$]. Multiple comparison post hoc tests indicated a significant increase in luciferase activity between control versus BMP2/BMP4 treatments and between control versus cornea and lens CM (* $p < .05$). **(B):** Western blot analyses of conditioned media prepared from postnatal day 2 (P2) or Adult (Ad) cornea, lens, sclera, and CE tissues. Approximately 40 μg of the proteins was loaded per lane, and BMP2/4 and β-actin antibodies were used to blot the membranes. The lane on the extreme right (MW) indicates the protein ladder. β-Actin was used as a loading control. **(C):** Reverse transcription polymerase chain reaction (RT-PCR) analyses of type I (*BmprI*, *Alk*) and type II (*BmprII*) BMP receptors in Ad CE and in clonal primary adult RSC spheres (Ad RSC). *Gapdh* was used as an internal control. No amplification was observed in the absence of RT (−RT). **(D):** Adult eye section immunostained for BMP downstream targets phospho-Smad1/5/8 (green) and counterstained with Hoescht (DNA) (blue), with labeling of the distinct eye structures to indicate the eye section orientation (the ONL, the INL, and the GCL, the lens, and the CE RSC niche). High magnification pictures (bottom panels) of the adult CE show the nuclear expression of phosphorylated Smad 1/5/8 in the pigmented stem (internal cell layer, red arrowheads) and non-pigmented progenitor (external cell layer, white arrowheads) cells. Scale bar = 50 μm. Abbreviations: Ad, adult; BMP, bone morphogenetic protein; CE, ciliary epithelium; CM, conditioned media; GCL, ganglion cell layer; INL, inner nuclear layer; NR, neural retina; ONL, outer nuclear layer; P2, postnatal day 2; RPE, retinal pigmented epithelium; RSC, retinal stem cell.

presence of BMPs in the media was assessed by measuring the luciferase activity. Only spheres cultured in presence of the lens- or cornea-derived conditioned media demonstrated a two-fold and four-fold increase in luciferase activity, respectively, compared to infected spheres cultured in standard culture conditions (Fig. 2A). We further demonstrated the specific presence of BMP2 and/or BMP4 in the adult, but not in the perinatal (PND2) lens and cornea supernatants by Western blot. The non-inhibitory perinatal and adult sclera and CE tissues also did not display detectable BMP expression (Fig. 2B).

To verify whether adult RSCs are able to respond to BMP signals, the expression of BMP receptors in the adult CE and in adult primary RSC colonies was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR). As shown in Figure 2C, BMP type II receptor, as well as BMP type IA and IB receptors, were expressed in the adult CE and in the adult primary RSC spheres (Fig. 2C; Supporting Information Fig. 4), thus highlighting the fact that RSCs can respond to BMPs signals in vitro. Furthermore, active BMP signaling was observed in vivo in the adult RSC niche as shown by the specific nuclear expression of the phospho-Smad 1/5/8 BMP effectors in the pigmented stem (internal cell layer) and non-pigmented progenitor (external cell layer) cells within the adult CE (Fig. 2D).

BMP2 and BMP4 Inhibit the Proliferation of Adult RSCs and Retinal Progenitor Cells

After identifying the presence of BMP family molecules in the adult lens and cornea conditioned media, the effects of BMP2, BMP4, and BMP7 (three factors involved in CE development) were assessed directly on adult RSC cultures.

Treatment with increasing doses of BMP2 decreased the numbers and the sizes of clonal primary RSC-derived spheres in a dose-dependent manner compared to controls (Fig. 3A, 3B). BMP4 showed a more dramatic effect on the numbers of clonal primary RSC spheres formed, as a 50% decrease was observed at low concentrations (10 ng/mL) (Fig. 3C). The

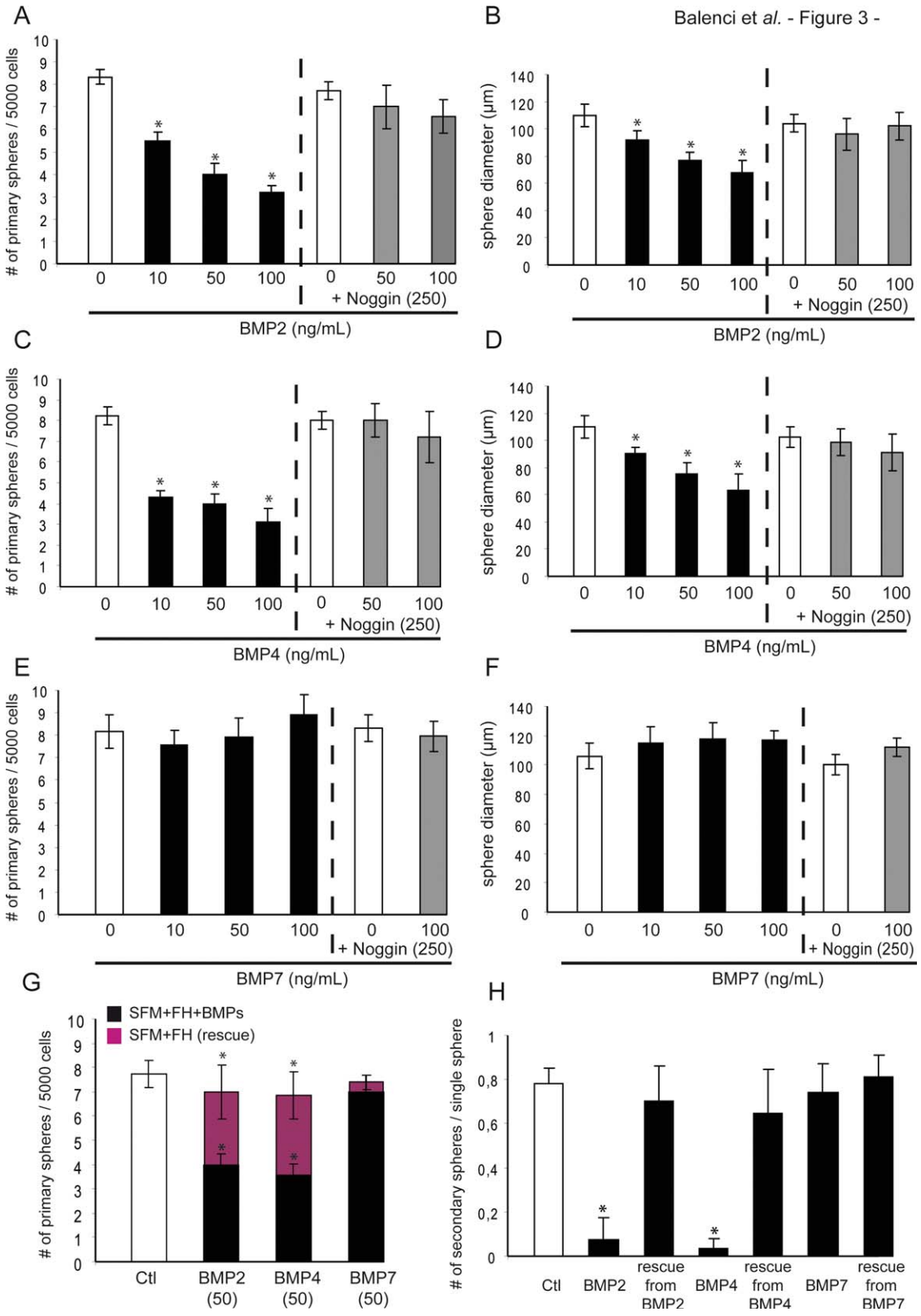

Figure 3.

BMP4 treatment demonstrated comparable effects on retinal stem and progenitor cells to those shown with BMP2, as similar dose-dependent reductions in the sphere diameters and sizes of clonal adult RSC spheres were observed compared to non-treated spheres (Fig. 3B, 3D). By contrast, BMP7 did not have effects on primary sphere formation or on the sphere diameters of clonal adult RSC spheres (Fig. 3E, 3F). These BMP2 and BMP4 effects could be due to either a decreased cell proliferation or increased cell death of retinal stem and/or retinal progenitors.

To test for any potential non-specific toxic effects of BMP factors, primary RSC cultures were grown in presence of BMPs and Noggin, a potent inhibitor that binds and sequesters BMPs. The addition of Noggin counterbalanced the BMP effects and restored the sphere numbers and sizes, pointing out the pharmacologically specific effects of BMP2 and BMP4 on retinal stem and retinal progenitor proliferation (Fig. 3A–3F).

To address whether BMPs may induce retinal differentiation, adult CE-derived cells were cultured in the presence of 50 ng/mL BMP2, BMP4, and BMP7 for a week, before clearing these drugs from the cell culture media and allowing the respective groups of cells/spheres to grow in control conditions for an additional "rescue" period of 7 days (Fig. 3G). As previously shown, BMP2 and BMP4, but not BMP7, reduced the numbers of primary RSC spheres during the first week, but new primary RSC spheres were formed during the second week in drug-free conditions to reach equivalent sphere numbers to control conditions. These results suggest that the BMPs blocked adult RSC proliferation, which resumed once the inhibitor was no longer present in the culture dishes, with no influence on cell fate (Fig. 3G).

To confirm these data, primary spheres were grown in standard culture conditions or BMP conditions. Individual spheres cultured in standard conditions were then dissociated to single cells and grown for a second week in the same standard control conditions. Other single spheres grown in BMP conditions were dissociated and then plated in BMP conditions (BMP) or in standard control culture conditions (rescue from BMP). Spheres maintained in BMP2 or BMP4, but not BMP7 conditions led to an almost complete quiescence of adult RSCs (Fig. 3H). This quiescence was removed when spheres grown in BMP conditions for the first week were passaged into control conditions (rescue from BMP), as the numbers of passaged spheres became comparable to spheres grown and passaged into control conditions (Fig. 3H). These findings suggest that BMP2 and BMP4 inhibit RSC self-renewal, which is consistent with the previous data (Fig. 3A–3D) demonstrating a BMP-mediated blocking of cell divisions regardless of the retinal precursor cell type. These BMP effects could not be explained by a lack of cell survival since spheres grown in BMP conditions could be passaged at equivalent ratios of sphere forming to non-sphere forming cells, compared to spheres grown in control conditions (data not shown).

sFRP2 Modulates RSC Proliferation

Recently, the Wnt signaling pathway has been implicated in the development of the lens and cornea [30], and exogenous Wnt-3a was shown to increase adult RSC proliferation [31]. Furthermore, tissues surrounding the CE express sFRP1 and sFRP2, which antagonize Wnt signaling by sequestering Wnt ligands [32,33]. Consequently, we examined the Wnt signaling pathway as an additional inhibition of adult RSC proliferation.

To address whether canonical Wnt signaling is active within primary adult RSC spheres, adult CE-derived cells from a reporter mouse expressing β-galactosidase driven by a minimal promoter containing six Tcf binding sites (*Tcf-LacZ*) [34] were cultured in standard culture conditions. X-gal labeling of these spheres after 7 days in culture showed that a subset of cells within the spheres was LacZ-positive with no consistent localization within the center versus periphery of clonal adult RSC spheres, suggesting that endogenous Wnt signaling is present within at least a subpopulation of adult RSCs and/or their progeny (Fig. 4A). This finding suggests that some Wnt factors may be endogenously secreted in standard culture conditions. Adult primary RSC spheres expressed a specific set of Frizzled (Fzd) Wnt receptors such as Fzd 4, Fzd 6, and Fzd 7, but not Fzd 3 (Fig. 4B), as shown previously [35].

To investigate the roles of Wnt signaling on adult RSCs in vitro, primary CE-derived cells were treated with increasing concentrations of sFRP2. The presence of sFRP2 resulted in a dose-dependent decrease in the numbers of primary spheres, similar to that of BMP2 and BMP4 (Fig. 4C). In

Figure 3. BMP2 and BMP4, but not BMP7, reduce the proliferation of both adult RSCs and their retinal progenitors. **(A, B):** Numbers (A) and sphere diameters (B) of primary RSC spheres arising from adult ciliary epithelium (CE)-derived cells cultured at a clonal cell density of 10 cells per microliter (in 500 μL total 500 μL total media / well) and grown in standard conditions (0) or in presence of increasing doses (from 10 to 100 ng/mL) of BMP2 without and with Noggin (250 ng/mL) as indicated. Two-way ANOVAs revealed a significant interaction between dose and treatment on the numbers of clonal primary RSC spheres [$F(3,72) = 4.47$, * $p < .05$, $n = 10$], and a significant effect of Noggin treatment [$F(1,72) = 6.20$], a significant effect of dose [$F(3,72) = 2.74$] but no significant interaction on the sphere diameters [$F(3,72) = 1.76$, $p = .16$]. **(C, D):** Numbers (C) and sizes (D) of clonal primary RSC spheres from adult CE-derived cells submitted to increasing concentrations of BMP4 with or without Noggin. Two-way ANOVAs indicated a significant interaction between dose and treatment on the total numbers of clonal primary RSC spheres [$F(3,72) = 4.59$, * $p < .05$, $n = 10$], and a significant effect of dose [$F(3,72) = 3.62$], a significant effect of Noggin treatment [$F(1,72) = 4.26$, $p < .05$], but no significant interaction between dose and treatment on the sphere diameters [$F(3,72) = 1.49$, $p = .22$]. **(E, F):** Numbers (E) and sphere diameters (F) of clonal primary RSC spheres obtained upon treatments with increasing doses of BMP7 with or without Noggin. Two-way ANOVAs indicated no significant interaction between dose and treatment on the total sphere numbers [$F(3,40) = 0.32$, $p = .81$, $n = 10$] or on the sphere diameters [$F(3,40) = 0.15$, $p = .93$]. **(G):** Adult RSCs were cultured initially in standard culture conditions (Ctl) or in 50 ng/mL BMP2, BMP4, or BMP7 (SFM+FH+BMPs) for 7 days. The black bars represent the numbers of clonal adult primary RSC spheres. Cells and growing spheres from individual wells were then harvested and plated in standard culture conditions for an additional week (rescue). The red diagrams display the total numbers of clonal RSC spheres formed during the "rescue" period. A repeated-measures two-way ANOVA showed significant effects of treatment and rescue and a significant interaction [$F(3,40) = 2.85$, * $p < .05$, $n = 6$]. BMP2, BMP4 (* $p < .05$) but not BMP7 ($p > .05$), significantly blocked the formation of clonal adult primary RSC spheres. Removal of BMP2 and BMP4 led to a complete rescue in the numbers of clonal adult RSC primary spheres (* $p < .05$). **(H):** Individual clonal adult primary RSC spheres were grown and passaged into control conditions (Ctl) or grown in BMP conditions for a week and then passaged into either control (rescue from BMP) or in BMP (BMP) conditions. A one-way ANOVA indicated a significant main effect of treatment [$F(6,161) = 8.386$, * $p < .05$, $n = 6$]. Bonferroni post hoc tests demonstrated that BMP2 and BMP4 (* $p < .05$), but not BMP7 ($p > .05$) significantly blocked the formation of clonal adult secondary RSC spheres compared to control. This inhibition was suppressed once BMP2 and BMP4 were removed from adult RSC cultures, as equivalent numbers of adult secondary RSC spheres were observed compared to spheres kept in control conditions. Abbreviations: Ctl, control; BMP2, bone morphogenetic protein 2; SFM, serum free media.

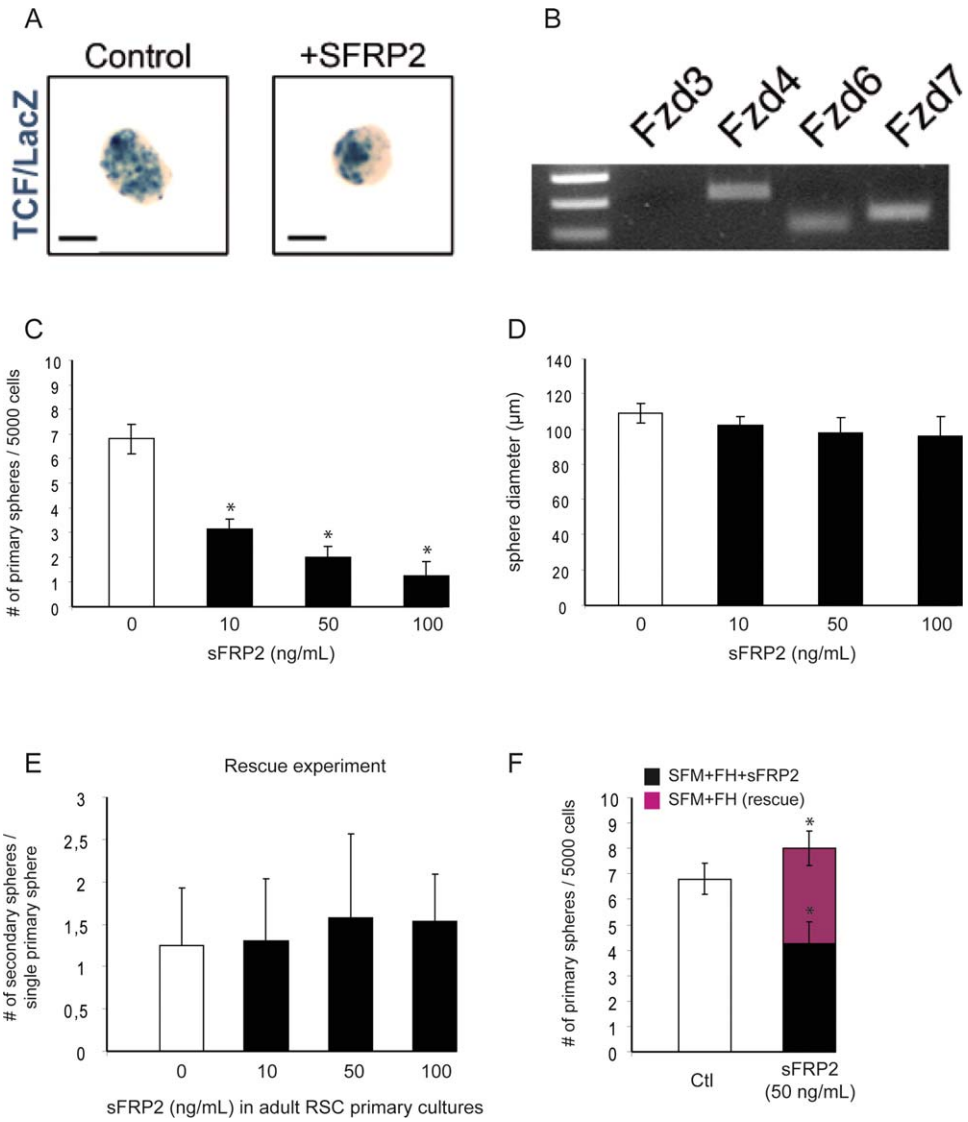

Figure 4. sFRP2 blocks the formation of clonal adult primary RSC spheres. **(A):** Analysis of Wnt signaling activity within clonal primary RSC spheres isolated from adult *Tcf-LacZ* mice and grown in control conditions or in presence of the Wnt inhibitor sFRP2 (50 ng/mL). *Tcf-LacZ* activity was measured by X-gal staining shown in blue. **(B):** Reverse transcription polymerase chain reaction analysis of Fzd 3, 4, 6, and 7 Wnt receptors in clonal adult primary RSC spheres. **(C, D):** Numbers (C) and sphere diameters (D) of clonal primary adult RSC spheres grown in control conditions or in presence of increasing doses of sFRP2 (10–100 ng/mL) were measured. One-way ANOVAs indicated a significant dose-dependent decrease in numbers of clonal adult primary RSC sphere formed [$F(3,8) = 19.20$, * $p < .05$, $n = 5$] but revealed no main effect on sphere diameter [$F(3,8) = 0.38$, $p > .05$]. **(E):** Numbers of secondary spheres grown in control conditions were assessed upon the passaging of single clonal primary adult RSC spheres grown either in control conditions or in presence of various concentrations of sFPR2. A one-way ANOVA showed no main effect of sFRP2 exposure on the numbers of clonal adult secondary RSC spheres [$F(3,16) = 0.18$, $p > .05$, $n = 5$]. **(F):** Adult RSCs were cultured initially in standard culture conditions (Ctl) or in 50 ng/mL sFRP2 for 7 days. The black bar represents the numbers of clonal adult primary RSC spheres. Cells and growing spheres from individual wells were then harvested and plated in standard culture conditions for an additional week (rescue). The red diagram displays the total numbers of clonal RSC spheres formed during the "rescue" period. A repeated-measures two-way ANOVA showed significant effects of treatment and rescue and a significant interaction [$F(1,20) = 5.87$, * $p < .05$, $n = 6$]. sFRP2 significantly blocked the formation of clonal adult primary RSC spheres and its removal led to a complete rescue in the numbers of clonal adult primary RSC spheres (* $p < .05$). Abbreviations: Fzd, Frizzled; RSC, retinal stem cell; sFRP2, secreted Frizzled-related protein; SFM, serum-free media; TCF, T-Cell Factor.

striking contrast to the BMP results, however, the spheres that did appear under sFRP2 conditions were the same size as the ones grown under control conditions (Fig. 4D). Thus, we conclude that sFRP2 acts primarily on RSCs rather than on retinal progenitors.

In addition, when sFRP2-treated primary spheres were passaged into control conditions, they produced clonal secondary spheres at a similar frequency to control spheres grown in and passaged into standard control conditions (Fig. 4E). This suggests first, that sFRP2 is not toxic and second, that the decrease in primary spheres produced by sFRP2 was not due to a global reduction in proliferation, as the average size of primary spheres was similar across all concentrations of sFRP2. The treatment of *Tcf-LacZ* adult CE-derived forming spheres with sFRP2 led to a decrease, but not a complete abolishment of X-gal activity, suggesting that sFRP2 did

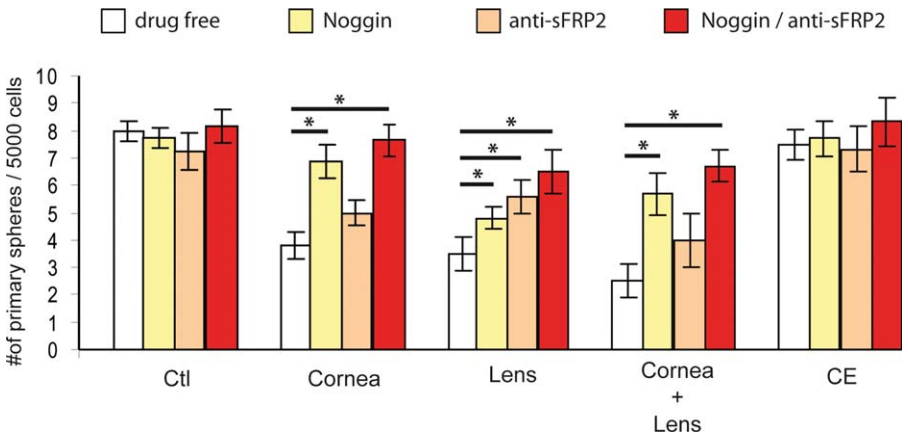

Figure 5. Bone morphogenetic protein (BMP)2, BMP4, and adult cornea conditioned media (CM) induce a quiescent state in adult RSCs (RSCs) in vitro. Adult RSCs were grown in presence of 25% adult CE-, cornea-, lens-, and cornea+lens-derived conditioned media in inhibitor-free conditions or in presence of Noggin (250 ng/mL, yellow bars), or the anti-sFRP2 blocking antibody (100 μg/mL, orange bars), or both inhibitors (red bars). A two-way ANOVA showed a significant interaction between drug and treatment [$F(12,140) = 1.94$, * $p < .05$, $n = 8$]. Bonferroni post hoc tests revealed that the addition of Noggin reversed significantly the inhibitory effects of both cornea and lens conditioned media (with a bigger effect on the cornea conditioned media), whereas the anti-sFRP2 blocked significantly only the lens conditioned media effects. The combination of both Noggin and anti-sFRP2 in the adult RSC cultures grown in lens and/or cornea conditioned media restored adult RSC sphere formation to control levels (* $p < .05$). Abbreviations: Ctl, control; CE, ciliary epithelium; sFRP, secreted Frizzled-related protein.

not inhibit all Wnt signaling in adult RSC spheres (Fig. 4A, right panel). Based on these findings, we conclude that sFRP2 induced adult RSCs to become non-proliferative but did not appear to affect any subsequent divisions once a RSC had begun to divide.

To confirm this statement, adult CE-derived cells were cultured in presence of 50 ng/mL sFRP2 for a week, before washing off sFRP2 from the cell culture media and allowing cells and forming spheres to grow in control conditions for an additional rescue period of 7 days. After withdrawal of sFRP2, equivalent numbers of clonal primary RSC spheres were formed compared to CE cells grown in control conditions for 2 weeks, indicating that sFRP2 induced RSC quiescence (Fig. 4F).

Noggin and Anti-sFRP2 Prevent Adult RSC Quiescence

Since secreted factors from the adult cornea and lens, and more specifically BMP2, BMP4, and sFRP2, induced adult RSC quiescence in vitro, an anti-sFRP2 blocking antibody and Noggin were used to assess whether these tissue conditioned media inhibitions could be pharmacologically attenuated or abolished. We first characterized the ability of a commercially available function blocking polyclonal antibody to attenuate the inhibition of sphere formation caused by sFRP2. Adult CE-derived cells were grown in 50 ng/mL of sFRP2, a concentration that resulted approximately in a threefold decrease of the numbers of primary RSC spheres (Fig. 4C, 4F; Supporting Information Fig. 5). Increasing concentrations of sFRP2 antibodies were tested to determine whether sphere numbers could be rescued. A concentration of 100 ng/mL of anti-sFRP2 antibody was able to restore significantly the numbers of spheres to levels observed in untreated cultures (Supporting Information Fig. 5). In parallel, increasing doses of Noggin were applied to adult primary RSC cultures to verify that no endogenous BMPs were released in control RSC culture conditions and to assess the toxicity of the BMP inhibitor. No reduction in primary sphere formation was observed across the different doses of Noggin that were tested alone (Supporting Information Fig. 1B). Moreover, we demonstrated previously that 250 ng/mL of Noggin was sufficient to reverse the BMP2 and BMP4-induced quiescent effects (Fig. 3).

Adult CE-derived cells were grown in control media, in 25% inhibitory conditioned media from the lens and/or cornea, or in 25% non-inhibitory conditioned media from the CE in the presence or absence of Noggin and/or the anti-sFRP2 antibody. The combination of both the lens- and cornea-derived conditioned media led to a slight additive effect as the inhibition was stronger than with the conditioned media from the cornea or the lens alone (Fig. 5). This result suggests that a combination of factors or increased doses of common factors secreted by both lens and cornea media are responsible for the increased inhibition of adult RSC proliferation. Furthermore, the addition of Noggin to 25% cornea- or 25% lens-derived conditioned media led to a significant rescue in the numbers of adult primary RSC spheres. Similarly, blocking sFRP2 activity in 25% lens-derived conditioned media gave rise to a significant attenuation of the inhibitory effect on primary sphere formation. However, the anti-sFRP2 had no significant blocking effects on the inhibition of 25% cornea conditioned media on the numbers of adult RSC spheres. Additionally, the blocking effects of anti-sFRP2 tended to be bigger than those of Noggin on 25% lens conditioned media. Finally, the addition of both Noggin and anti-sFRP2 restored the numbers of adult primary RSC spheres in either 25% lens, 25% cornea or 25% lens/25% cornea conditioned media to levels similar to those grown in control media (Fig. 5). Therefore, the combination of inhibiting BMP signaling and blocking sFPR2 completely abolished the cornea- and lens-induced quiescence of adult RSCs in vitro.

Adult Human RSCs Are Maintained in a Quiescent State by Surrounding Cornea and Lens Tissues

Adult human RSCs, like adult mouse RSCs, are non-proliferative in situ and do not react following degeneration or injury but can be isolated and grown in vitro as clonal RSC spheres even in the absence of growth factors [16,17]. Working from the hypothesis that adult human RSCs also are inhibited by tissues adjacent to the CE, we performed similar experiments to determine the effects of conditioned media from various adult eye tissues on adult human RSCs. Identical to mouse adult RSCs, human adult cornea- and lens-derived conditioned media exhibited inhibitory effects on adult human RSC proliferation, as shown by the reduction in the numbers of

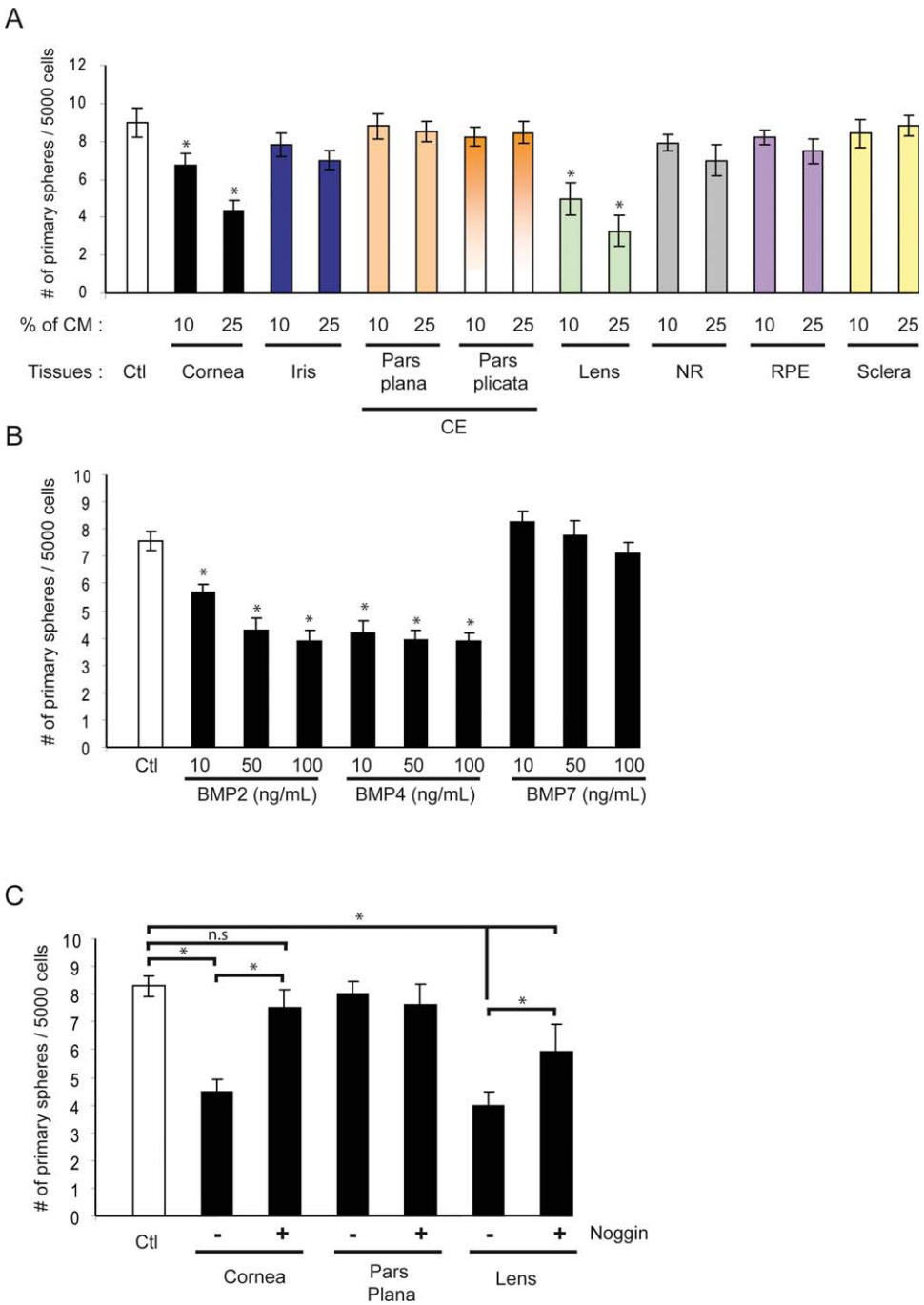

Figure 6. Human lens and cornea inhibit the proliferation of human RSCs. **(A):** Human adult RSCs were grown in standard culture conditions (Ctl) or in presence of 10% or 25% CM generated from adult eye tissues as indicated. The human adult CE was dissected into two parts: the pars plana and pars plicata. A two-way ANOVA revealed a significant interaction between dose and treatment [$F(14,72) = 2.97$, * $p < .05$, $n = 4$]. Multiple comparison post hoc tests showed that secreted factors from the adult lens and the cornea reduce significantly the numbers of clonal human primary RSC spheres (* $p < .05$). **(B):** Human adult RSC cultures were cultured (10 cells per microliter) in standard conditions (Ctl) or in increasing doses of BMP2, BMP4, or BMP7 (10–100 ng/mL). Clonal adult primary RSC spheres formed were counted on day 7 in vitro. A one-way ANOVA revealed a significant main effect of treatment [$F(9,170) = 21.81$, $p < .05$, $n = 4$]. Multiple comparison post hoc tests showed that BMP2 and BMP4 (* $p < .05$) but not BMP7 ($p > .05$) decrease significantly the numbers of clonal human primary RSC spheres compared to control. **(C):** Human adult RSCs were cultured in control conditions (Ctl) or in presence of 25% conditioned media from the human adult cornea, lens, or Pars plana. The Pars plana was used as a non-inhibitory negative control. Numbers of clonal primary adult human RSCs were determined in each condition media supplemented (+) or not (−) with Noggin (250 ng/mL). A two-way ANOVA revealed significant main effects of conditioned media and treatment and a significant interaction [$F(3,24) = 4.09$, * $p < .05$, $n = 4$]. Multiple comparison post hoc tests showed a partial and almost a complete rescue by Noggin on primary sphere formation for human adult RSCs cultured in presence of lens and cornea conditioned media, respectively (n.s = non-significant). Abbreviations: BMP, bone morphogenetic protein; CE, ciliary epithelium, CM, conditioned media; Ctl, control; NR, neural retina; RPE, retinal pigmented epithelium.

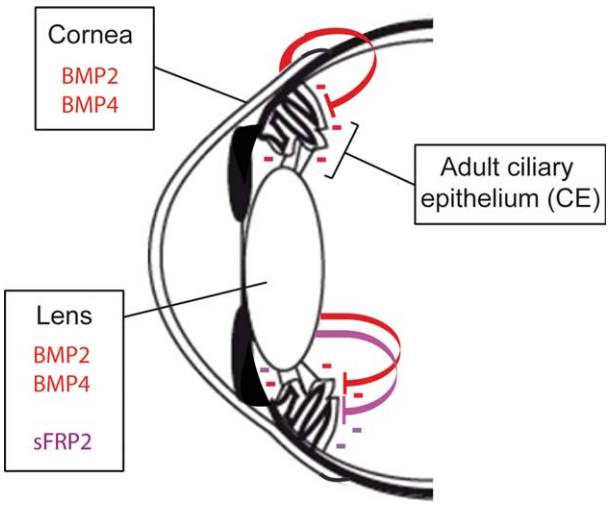

Figure 7. Summary. In the adult mammalian eye, the adult cornea and the lens secrete BMP2 and BMP4 which both inhibit the retinal stem and retinal progenitor cells present within the adult CE. The adult lens releases the sFRP2 protein that also contributes to adult RSC quiescence in the adult eye. Abbreviations: BMP, bone morphogenetic protein; CE, ciliary epithelium; sFRP2, secreted Frizzled-related protein 2.

clonal primary RSC spheres formed compared to human RSCs grown in the standard control conditions (Fig. 6A). We also showed that BMP2 and BMP4, but not BMP7 reduced by nearly 50% the numbers of primary RSC spheres compared to control (Fig. 6B). Finally, the addition of Noggin partially rescued the proliferation of human adult RSCs grown in the presence of 25% cornea- or 25% lens-derived conditioned media (Fig. 6C). These results suggest that, similar to the adult mouse eye, BMPs might be secreted by the cornea and the lens in the adult human eye, and thus prevent human RSCs present within the CE from being reactivated.

Discussion

The CMZ of some cold-blooded vertebrates continuously produces new retinal cells throughout life [36], but these active neurogenic properties are not seen in mammals including humans under normal conditions [8]. In this study, we asked which factors present in the adult mammalian eye might prevent the rare pigmented RSC population within the CE from proliferating to actively replace retinal neurons that are lost to retina degeneration or disease. Interestingly, a few recent studies have shown that neurogenic activities can persist in the postnatal or adult CE in mice with induced or inherited retinal degeneration [37–39].

The present findings demonstrate that factors released within adult CE/RSC niche are not inhibitory, but the ones secreted by surrounding tissues (especially the cornea and lens) block the in vitro proliferation of adult RSCs, as shown by the reduced numbers of clonal adult primary RSC spheres. Inhibition from both lens and cornea were shown in the developing chick eye to prevent the trigeminal innervation of embryonic corneal tissues [40]. Several other adult murine eye tissues also appeared to exert some inhibition of adult RSC proliferation. Thus, the inhibitory molecules from tissues other than the lens and cornea may explain the absence of complete blocking of adult RSC proliferation in vitro, as is normally the case in the adult mammalian eye in vivo.

In addition, the inhibitory effects of both adult lens and cornea are observed only with adult eye tissues, since none of the perinatal eye tissue conditioned media tested had any effects on adult or perinatal RSC proliferation. Indeed, at perinatal developmental stages, RSCs and retinal progenitors still are proliferating to form the maturing retina [17,20,26], and thus it was anticipated that no factors would be present in perinatal eye tissues to prevent RSCs from proliferating. All together, these results point out the importance of the temporal regulation of RSCs in the mammalian eye, where RSCs are active during retinal development and completely quiescent in the adult eye, even following most injuries.

BMP7, along with BMP2 and BMP4, were shown to be expressed in the developing CE [41–44], where active BMP signaling is required for normal development of the ciliary body [27]. However, both BMP2 and BMP4, by reducing the numbers and the sizes of adult primary RSC spheres, demonstrated their capacity to block RSC and retinal progenitor proliferation. By contrast, BMP7 showed no such effects. Moreover, we identified the presence of BMP2 and BMP4 in the lens and the cornea supernatants, which could potentially target the adult CE/RSC niche where we observed the activation of BMP target genes (Fig. 7).

The fact that adult RSCs resumed their proliferation when BMPs were removed from the culture media led us to conclude that BMP2 and BMP4 induced a quiescent state in RSCs. Similar BMP-induced quiescence effects have also been demonstrated in the adult hippocampus to maintain the proliferative potential of subgranular zone neural progenitors [45]. These BMP2 and BMP4 blocking effects on adult RSC proliferation are specific, since the addition of Noggin abolished this inhibition. Conversely, Noggin was shown to block the formation of the ciliary body during embryonic eye development [27]. In the adult hippocampus, the overexpression of Noggin increases the upstream precursor cell population, while endogenous levels of Noggin attenuate BMP signaling in proliferating cells within the dentate gyrus [46]. These studies demonstrate that regulating BMP signaling within a specific niche may drive opposite effects according to the temporal or spatial contexts.

Other recent studies have demonstrated that the modulation of Wnt signaling can affect the numbers of RSCs in developing and adult mouse eyes [31,47], as well as promoting retinal regeneration in adult mammals [48]. In this study, we observed that sFRP2, which binds to Wnt ligands and prevents them from activating their Fzd receptors, specifically blocked the proliferation of RSCs as the numbers but not the sizes of clonal adult RSC spheres were decreased compared to control. Modulating Wnt signaling activity recently was shown to be important for promoting the expansion of the neural stem cell pool in the adult brain in response to stroke [49]. sFRP2 could not abolish all Wnt activity in this study, since treatment with sFRP2 only decreased partially β-gal activity in clonal adult RSC spheres from TCF-LacZ mice. This could be explained either by Wnts being produced continuously by RSCs or retinal progenitors or by sFRP2 partially working through other signaling pathways. All the clonal primary RSC colonies treated or not treated with sFRP2 contained some degree of Tcf activity with no consistent localization within individual spheres, suggesting that if Wnts are secreted, they can activate Wnt signaling in both retinal stem and retinal progenitor cells. However, sFRP2 appears to function specifically on RSCs as the numbers of primary RSC colonies were reduced to almost zero at the highest dose tested, and sFRP2 has no functional effects on retinal progenitors. In addition, sFRPs can regulate retinal neurogenesis in the embryonic mouse eye by downregulating Notch activity [50], and decreased Notch activity has been

found to impair RSC proliferation (L. Balenci and D. van der Kooy, unpublished data).

Mixing conditioned media from the lens and the cornea together produced a stronger decrease in clonal adult RSC sphere formation than RSCs cultured in either the cornea or the lens conditioned medium alone. The current findings suggest that distinct factors from each adult eye tissue may participate preferentially in inducing the hypothesized quiescence of adult RSCs. Indeed, larger numbers of clonal adult primary RSC spheres were observed when Noggin was added to cornea conditioned media and the blocking sFRP2 antibody was added to lens conditioned media. We suggest that BMPs are secreted preferentially from the adult cornea, whereas sFRP2 originates primarily from the adult lens (Fig. 7). However, these combination experiments never gave rise to the complete inhibition of RSC proliferation observed in vivo in the adult mammalian eye. One possibility could be that additional non-characterized factors might be present in vivo and completely repress adult RSC proliferation. An alternative explanation might be that, in vivo, RSCs are part of an epithelium harboring numerous cell–cell contacts, which are important in regulating the proliferation of stem cells in several systems and may participate in the induction of adult RSC quiescence [51–53].

Secreted factors from the cornea and the lens clearly decreased the proliferation of both human and mouse RSCs. As observed with adult mouse RSCs, BMP2 and BMP4 also decreased the proliferation of adult human RSCs. This inhibition was reversed by the addition of Noggin. In vivo infusions within the CE of pharmacological compounds targeting the specific inhibitors released by the adult eye tissues may facilitate the activation of endogenous RSCs in case of disease or injury if the presence of reactive RSCs in vivo could be demonstrated definitely in mammals.

Conclusions

Adult mouse and human RSCs exhibit the same quiescence properties in vivo [21,22], and this study shows that mouse and human RSCs may be regulated by similar cellular and molecular mechanisms to maintain that quiescence. The adult cornea and lens release BMPs and sFRP2 that block the proliferation of adult mouse and human retinal precursor cells. Inhibiting these factors may pave a new regenerative avenue using the in situ proliferation and appropriate differentiation of the progeny of putative endogenous adult RSCs to help eyes self-repair and thus eliminate the invasive surgery and immunological rejection of transplants.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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