Retinal neurons regulate proliferation of postnatal progenitors and Müller glia in the rat retina via TGFβ signaling

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Summary

The number of proliferating cells in the rodent retina declines dramatically after birth. To determine if extrinsic factors in the retinal micro-environment are responsible for this decline in proliferation, we established cultures of retinal progenitors or Müller glia, and added dissociated retinal neurons from older retinas. The older cells inhibited proliferation of progenitor cells and Müller glia. When these experiments were performed in the presence of TGFβRII-Fc fusion protein, an inhibitor of TGFβ signaling, proliferation was restored. This suggests a retina-derived TGFβ signal is responsible for the developmental decline in retinal proliferation. TGFβ receptors I and II are expressed in the retina and are located in nestin-positive progenitors early in development and glast-positive Müller glia later in development. RT-PCR and immunofluorescence data show TGFβ2 is the most highly expressed TGFβ ligand in the postnatal retina, and it is expressed by inner retinal neurons. Addition of either TGFβ1 or TGFβ2 to postnatal day 4 retinas significantly inhibited progenitor proliferation, while treatment of explanted postnatal day 6 retinas with TGFβ signaling inhibitors resulted in increased proliferation. Last, we tested the effects of TGFβ in vivo by injections of TGFβ signaling inhibitors: when TGFβ signaling is inhibited at postnatal day 5.5, proliferation is increased in the central retina; and when co-injected with EGF at postnatal day 10, TGFβ inhibitors stimulate Müller glial proliferation. In sum, these results show that retinal neurons produce a cytostatic TGFβ signal that maintains mitotic quiescence in the postnatal rat retina.

Key words: TGFβ, Müller glia, Proliferation, Progenitors, Retinogenesis, Cytostasis, Rat

Introduction

In the developing nervous system, progenitor proliferation is regulated by both extrinsic and intrinsic factors. Many extrinsic factors that are mitogenic for retinal progenitors have been identified. Sonic hedgehog (Shh), fibroblast growth factors (FGFs), epidermal growth factor (EGF) and transforming growth factor α (TGFα) stimulate proliferation of progenitors isolated from the developing retina (Anchan et al., 1991; Anchan and Reh, 1996; Jensen and Wallace, 1997; Lillien and Cepko, 1992), while Shh is required for normal levels of retinal progenitor proliferation in vivo (Wang et al., 2002).

However, little is known regarding the role of extrinsic factors in the decline of proliferation that occurs during late retinogenesis (Alexiades and Cepko, 1996; Young, 1985). Retinal progenitor proliferation peaks around the day of birth, and declines until approximately the end of the first postnatal week (Sidman, 1960; Young, 1985). After this time, there is little evidence for renewed proliferation of either progenitors or Müller glia in the mammalian retina, except under abnormal conditions (Fariss et al., 2000; Moshiri and Reh, 2004; Nork et al., 1986; Nork et al., 1987; Robison et al., 1990; Sueishi et al., 1996). This appears to be true of most of the CNS; after the period of embryonic and neonatal neurogenesis, only a few regions of the CNS retain neural progenitors (Gage, 2002).

The molecular basis for the establishment and maintenance of mitotic quiescence in the CNS is not well understood. Co-culture studies have demonstrated that neurons can inhibit glial proliferation (Gomes et al, 1999). For example, Hatten (Hatten, 1987) found that proliferation of postnatal mouse cerebellar glia was inhibited fivefold when cultured with cerebellar neurons. Recently, TGFβ family members have been implicated in the inhibition of proliferation in the nervous system. Constam et al. (Constam et al., 1994) demonstrated that the postnatal decline in cerebellar precursor proliferation is paralleled by an increase in neuronal TGFβ2 expression, and that TGFβ2 inhibits precursor proliferation in culture. Moreover, growth and differentiation factor 11 (GDF11), a member of the TGFβ superfamily expressed in the olfactory epithelium, was shown to inhibit proliferation of neuronal precursors in explant cultures of mouse olfactory epithelium (Wu et al., 2003).

In light of these studies, we sought to determine whether the postnatal reduction in progenitor and glial proliferation is regulated by signaling factors present in the developing retina. We found that progenitor and Müller glial proliferation was inhibited by co-culture with retinal cells, and further characterized the nature of the mitotic inhibitor using a combination of receptor blocking experiments, addition of TGFβs, intraocular injections and explant cultures of retinal

glia and progenitors. The results of our experiments support a model in which TGFβ2, primarily derived from retinal neurons, inhibits proliferation of retinal progenitors and glia at the end of retinogenesis.

Materials and methods

Animals and injections

All animals used in this study were treated according to guidelines of the University of Washington IACUC. Long Evans rats were purchased from Charles River Laboratories. For P5.5 intraocular injections, animals were anesthetized by hypothermia, and their eyelids opened with iridectomy scissors. Proparacaine topical anesthetic was applied, followed by intraocular injection nasal to the cornea using a 30.5 G needle and Hamilton syringe. For P10 intraocular injections, P10/P11 animals were anesthetized with ketamine/xylazine and injected as described for P5.5 animals. Factors used for intraocular injection experiments include 40 nmoles SB-431542 in dimethylsulfoxide (Sigma) mTGFβRII-hFc (R&D Systems), mouse-anti-TGFβ blocking antibody (MAB1835, R&D Systems) and rhEGF (R&D Systems). BrdU injections were performed intraperitoneally using a sterile 30.5 G needle and 1 ml syringe. For the birthdating study, pups were weighed and given three injections of BrdU (50 mg/kg) over 9 hours on postnatal day 4, 6, 8, 10 or 12, sacrificed at P15 by CO_2 overanesthesia and processed for BrdU immunohistochemistry.

Immunohistochemistry

Tissues were rinsed in PBS, fixed in 4% paraformaldehyde/4% sucrose in PBS for 1 hour, and cryoprotected in 30% sucrose prior to cryosectioning. Cryosections were mounted on Superfrost slides (VWR). Slides stained with anti-BrdU were incubated for 10 minutes in 4 N HCl, washed in PBS and blocked at room temperature for 1.5 hours in 0.3% TritonX-100/5% goat serum/PBS. All primary antibody staining procedures were performed overnight at room temperature in 0.3% TX-100/PBS, followed by four 15 minute PBS washes. Secondary antibody incubations were performed for 1 hour at room temperature in 0.3% TX-100/PBS, followed by four 15 minute PBS washes. For DAPI staining, 1 μg/ml DAPI (sigma) was used, followed by two PBS rinses. Sections/coverslips were rinsed in water, dried, and mounted in Flouromount (Southern Biotechnology) medium.

Antibodies used include: mouse anti-rat β3 tubulin (1:500, BabCo), mouse-anti-BrdU(1:150, G3G4 Developmental Studies HB), mouse anti-nestin (1:80, DSHB), rat anti-BrdU (1:100, Accurate), rabbit anti-bovine CRALBP (1:500, UW55, gift from Jack Saari, University of Washington), guinea pig anti-glast (1:3000, Chemicon), rabbit anti-human TGFβ2 (1:200, Santa Cruz), rabbit anti-human TGFβR1 (1:100, Santa Cruz), rabbit anti-human TGFβRII (1:100, Santa Cruz). Secondary antibodies used include: goat anti-rabbit Alexa 568 (1:500, Molecular Probes), goat anti-rabbit Alexa 488 (1:500, Molecular Probes), goat anti-mouse Alexa 488 (1:500, Molecular Probes), goat anti-mouse Alexa 568 (1:500, Molecular Probes), goat anti-rat 488 (1:500, Molecular Probes) and goat anti-guinea pig Cy3 (1:700, Chemicon).

Retinal cell cultures

Rats were sacrificed by CO_2 overanesthesia and cervical dislocation. Eyes were removed and retinas were dissected in Hank's buffered Salt Solution (HBSS, Gibco-BRL). Retinal explants were cultured in DMEM/F-12 and B27 (Invitrogen). Following culture, explants were either fixed and processed for immunohistochemistry, or dissociated and plated on poly-ornithine-coated glass coverslips for 2 hours, then fixed and processed for immunohistochemistry. For dissociated cell cultures, retinas were rinsed in sterile Ca^{2+} and Mg^{2+}-free HBSS (CMF) and dissociated at 37°C for 5-10 minutes in a 0.5% trypsin/CMF solution. Trypsin was inactivated by one-fifth the volume of fetal bovine serum and the cells were spun at 1500 rpm for 5 minutes and resuspended in DMEM/F12 media supplemented with 0.6% glucose, 0.1125% $NaHCO_3$, 5 mM HEPES, 1% fetal bovine serum (Gibco-BRL), penicillin (1 unit/ml) and streptomycin(1 μg/ml), and hormone supplement including putrescine (9.66 μg/ml), progesterone (0.02 μM), selenium (30 μM), apo-Transferrin (0.1 mg/ml) and insulin (0.025 mg/ml). Dissociated retinas were plated on poly-D-Lysine coated glass coverslips, overlaid with Matrigel basement membrane (Collaborative Research) and maintained at 37°C. 5-Bromo-2′Deoxyuridine (BrdU; Sigma) was used at 10 μg/ml. Growth factors/blocking factors (all from R&D Systems) used include recombinant human TGFβ2, mTGFβRII-hFc (0.5 μg/ml), mActivinRIIB-hFc (0.5 μg/ml) and mBMPRIB-hFc (2 μg/ml).

Cell counting and statistics

Cells were counted using a Zeiss Axiophot fluorescent microscope and Spot II camera. For P4 and P6 explants, three or four individuals from three litters were used. For each dissociated retinal explant, five or six random fields were chosen, and the percentage of $BrdU^+$ cells (out of a total of 150-200 cells) was calculated for each field. For the P5.5 intraocular injections, sections were selected in which the optic nerve was visible, and the number of $BrdU^+$ cells/mm^2 was determined for eight fields. Six animals were analyzed for the control (DMSO) group and five animals were analyzed in the treated group (SB-431542). For the P10 intraocular injections, the following numbers of animals were analyzed: four in the control group, two with the anti-TGFβ cocktail alone, four in the 250 ng EGF-treated group, and four in the group with 250 ng EGF + anti-TGFβ cocktail. Student's *t*-test and ANOVA were used to compare the groups for significant differences.

Quantitative PCR

Quantitative RT-PCR was performed as previously described (Kubota et al., 2004) using an Opticon monitor from MJ Research and Sybr Green PCR Master Mix (Applied Biosystems). RNA samples were taken from three different individuals at the age indicated. Total RNA was collected using Trizol (Invitrogen), DNAse treated using Rneasy mini-kit (Qiagen) and quantified by spectrophotometry. RNA (1 μg) was used for the reverse transcription reaction, using Oligo-dTs and Superscript II RT. cDNA samples were run in triplicate for each primer set. The cycle at which a given sample/primer combination reached log-phase was noted and normalized to GAPDH levels. Primers were obtained from Invitrogen and designed using Primer3 (MIT) to amplify 200 bp of each gene. Primer sequences, 5′ to 3′: Gapdh 5′, AAGGTCATCCCAGAGCTGAA; GAPDH 3′, GTCCTCAGTGTAGCCCAGGA; TGFβ1 5′, ATGACATGAACCGACCCTTC; TGFβ1 3′, ACTTCCAACCCAGGTCCTTC; TGFβ2 5′, CAACACCATAAACCCCGAAG; TGFβ2 3′, GGCTTTCCCGAGGACTTTAG; TGFβ3 5′, CTTACCTCCGCAGCTCAGAC; TGFβ3 3′, CCTCAGCTGCACTTACACGA; TGFβRI 5′, ACCTTCTGATCCATCCGTTG; TGFβRI 3′, CTTCCTGTTGGCTGAGCTGT; TGFβRII 5′, CCTGTGTGGAGAGCATCAAA; TGFβRII 3′, ATCTGGGTGCTCCAGTTCAC.

Efficiency curves were performed by diluting template DNA 8-, 16-, 32- and 64-fold. The average difference for all primers between each twofold dilution was one.

Western blotting

Retinas were dissected in PBS, and the central and peripheral retinas were separated. Protein was extracted using M-PER buffer (Pierce) and quantified by Coomassie Reagent (BioRad) as per manufacturer's instructions. Protein (30 μg) was loaded in each well. Membranes were incubated with primary antibodies rabbit anti-human TGFβ2 (1:200, Santa Cruz), rabbit anti-human TGFβRI (1:100, Santa Cruz) and rabbit anti-human TGFβRII (1:100, Santa Cruz), rabbit anti-Foxo1 (1:200, CeMines), rabbit anti-Smad2/3 (1:1000, BD Biosciences). Secondary antibodies were goat anti-mouse alkaline

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phosphatase and goat anti-rabbit alkaline phosphatase from the BioRad Immunostar Chemilluminescence kit.

Results

Retinal progenitor proliferation is complete by postnatal day 10

Although previous birthdating studies have documented the timing of neurogenesis and proliferation in the rodent retina (Alexiades and Cepko, 1996; Young, 1985), no study has specifically addressed the pattern of BrdU incorporation at the end of rat retinogenesis. To determine the pattern of proliferation in the postnatal rat retina, we performed BrdU injections on postnatal days 4-12 (P4-12). Animals were injected three times daily, allowed to survive to P15, then sacrificed and processed for immunohistochemistry (Fig. 1A-E). Retinas from animals injected on P4 showed robust BrdU incorporation throughout the retina in the inner and outer nuclear layers (Fig. 1A, inset). By postnatal day 6, BrdU incorporation is nearly absent in the central retina (Fig. 1B, inset), though some progenitors are still mitotically active in the peripheral retina (asterisks). Animals injected at P8 showed no BrdU incorporation centrally, though BrdU-labeled cells are present peripherally (arrow, Fig. 1C). BrdU-labeled cells in the P10 injected animals were confined to the far periphery (arrow, Fig. 1D), and by P12 no BrdU incorporation was detected in the retina (arrows indicate the retinal edge, Fig. 1E). Therefore, proliferation in the postnatal rat retina undergoes a dramatic decline between postnatal day 4 and 12, and progenitors in the central retina become mitotically quiescent between P4 and P6. These data demonstrate that neurogenesis is essentially complete in most of the rat retina by P6, in agreement with previous studies (Alexiades and Cepko, 1996).

A TGFβ signal from the retina inhibits proliferation in the retina

The termination of proliferation in the postnatal retina might be explained by either intrinsic changes in the progenitor cells or by extrinsic factors in the progenitor micro-environment. We hypothesized that a signal from mature retinal cells might be responsible for the termination of proliferation in the retina. To test this hypothesis, we used the experimental protocol shown in Fig. 2A. P4 rat retinas were dissociated and cultured for 24 hours. Surviving cells were re-dissociated and plated onto Matrigel-coated, glass coverslips. Following the first passaging, many of the surviving, proliferating cells are progenitors, as indicated by immunoreactivity for the progenitor marker nestin (Fig. 2B,D). On the third day of culture, retinas from P13 animals were harvested, dissociated and plated on 4 μm filter culture plate inserts, which were inserted into the wells that contain the P4-derived retinal cells. Thus, any soluble factors from the newly added retinal cells that could mediate an effect on proliferation should have access to the underlying progenitors. The cells were co-cultured in this manner for 12 hours, with BrdU added during the last 2 hours.

Fig. 2B shows a typical field of nestin-positive progenitor cells under control conditions, with no additional retinal cells added to the culture. Under control conditions, 25% (±2.4) of nestin-expressing cells enter S-phase during the BrdU pulse (Fig. 2F). However, when 5 million P13 retinal cells are added

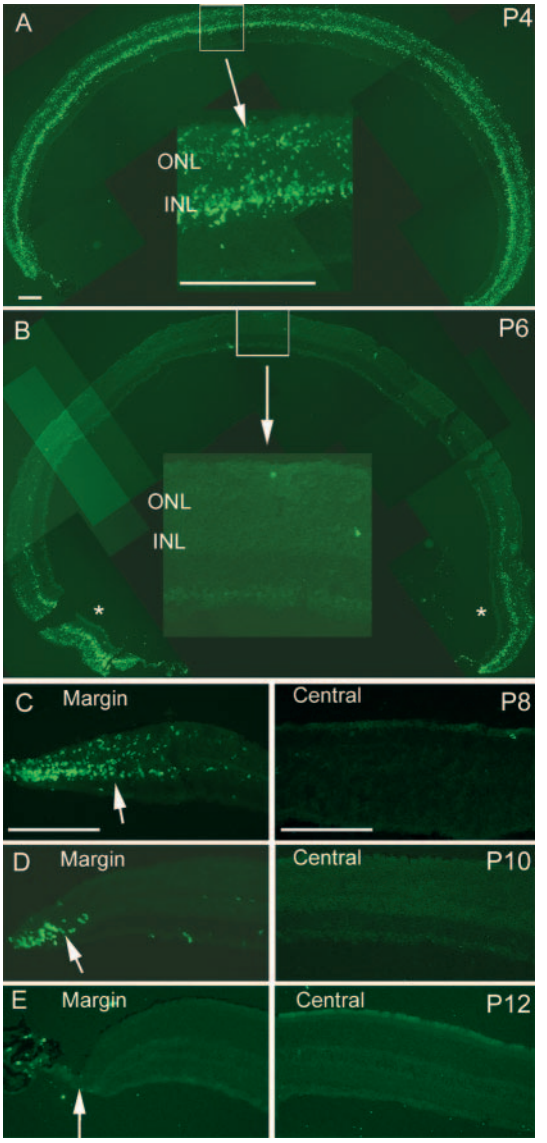

Fig. 1. Retinal progenitor proliferation declines between P4 and P12. Rats were injected every 3 hours for 9 hours with BrdU on the postnatal day indicated. At P4 (A), BrdU-positive cells can be found centrally and peripherally in both the INL and ONL (inset). By P6 (B), few or no cells incorporate BrdU in the central retina; (B, inset), however, BrdU-positive cells can be found in the peripheral regions (asterisks). At P8 (C), BrdU incorporation is restricted to the periphery. By P10 (D), some BrdU labeling can be found at the margin (arrow). At P12 (E), there is no BrdU labeling within the retina (arrow marks the retinal margin). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar: 200 μm.

(Fig. 2D), the percentage of progenitor cells entering S-phase drops to 15% (±2.2), consistent with the hypothesis that a soluble cytostatic factor is produced by retinal cells.

A similar experimental paradigm was used to test for effects of retinal cells on Müller glial proliferation. Cultures enriched for Müller glia were co-cultured with retinal cells from older animals in a similar manner to that described above, except the length of the BrdU pulse was 6 hours. Fig. 2C shows a typical field of CRALBP-positive Müller glial cells cultured with no

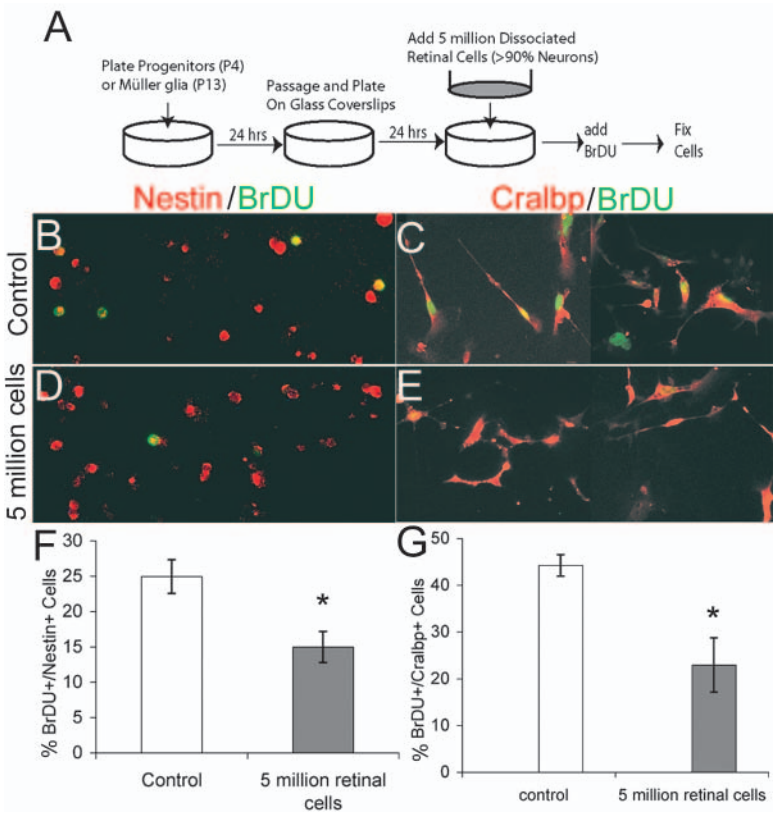

Fig. 2. Soluble signal from retinal cells inhibits postnatal proliferation. (A) Experimental protocol (see text). Cells were cultured in the presence of 5 million retinal cells for 12 hours, BrdU was added for the final 2 hours of progenitor cell culture (B,D) and the final 6 hours of glial cell culture (C,E). (B-E) Example fields of nestin$^+$ (red) BrdU-labeled (green) progenitor cells (B,D), and CRALBP$^+$ (red) BrdU-labeled (green) Müller glia (C,E), under control conditions (no added cells: B,C) or with the addition of 5 million dissociated retinal cells (D,E). (F) Quantification of BrdU$^+$/nestin$^+$ cells shows a significant reduction in proliferation with 5 million added cells (15±2.2%) compared with control (24.9±2.4%). (G) Quantification of BrdU$^+$/CRALBP$^+$ cells shows significant inhibition of glial proliferation with addition of 5 million cells (23.0±2.8%) compared with control treated cells (44.3±2.3%). *P<0.02, pairwise comparison (Student's t-test).

additional retinal cells. Quantification shows that ~44% (±2.3) of CRALBP-positive Müller glia are BrdU-positive in the control condition (Fig. 2C,G) However, when 5 million dissociated retinal cells are added to these glia, the percentage of CRALBP-positive cells incorporating BrdU shrinks to 23% (±2.8) (Fig. 2E,G). These data therefore indicate that a soluble factor released by retinal cells inhibits Müller glial proliferation.

To determine the identity of this soluble factor, we performed these experiments in the presence of TGFβ superfamily receptor-Fc fusion proteins (Tsang et al., 1995). These receptor bodies act to bind TGFβ ligands in solution and sequester them, hence inhibiting the signaling cascade. As quantified in Fig. 3, TGFβ receptor II-Fc (0.5 μg/ml) virtually restored progenitor (Fig. 3A) and Müller glial (Fig. 3B) proliferation to control levels in the presence of 5 million retinal cells. Neither Activin receptor II-Fc nor BMP receptor I-Fc was capable of restoring progenitor or Müller glial proliferation to control levels in these assays (Fig. 3A,B). These data suggest that retinal cells produce factors which signal through the TGFβ receptor, and which can act to inhibit proliferation in the postnatal retina. As the changes in the number of BrdU-positive cells could be secondary to changes in cell death of a specific population in these cultures, we examined DAPI-stained nuclei in each of the conditions and found no difference in the percentage of pyknotic nuclei present (data not shown). Therefore, TGFβ signaling is probably inhibiting cell cycle progression without affecting cell death in these cultures.

TGFβ ligands and receptors are expressed in the postnatal retina

TGFβ signaling has been implicated in controlling the proliferation of a variety of cell types (Anchan and Reh, 1995; Hunter et al., 1993; Pillaire et al., 1999). To determine whether TGFβ signaling components are expressed in the first postnatal weeks, RT-PCR and immunohistochemistry were performed on retinas from rats aged P4 to adult. Levels of transcription of TGFβ ligands and receptors were investigated via quantitative RT-PCR.

At P4, mRNA encoding TGFβ ligands and receptors was present in the retina (Fig. 4K). Quantitative RT-PCR results suggest the most highly expressed TGFβ ligand was TGFβ2, as TGFβ2 transcripts are 80-fold more abundant than TGFβ3 and eightfold more abundant than TGFβ1 (Fig. 4G). Thus, the mRNA for TGFβs and their receptors are present at P4, and TGFβ2 is the predominant ligand expressed. Immunolocalization of receptor protein expression reveals that TGFβRI and RII are expressed in the nestin-positive processes of P4 retinal progenitors (arrowheads, Fig. 4A-F). In addition, at higher magnification, we observe nestin-positive cell bodies that co-label with both TGFβ receptor proteins (see Fig. S1 in the supplementary material). Furthermore, TGFβ2 is most highly expressed in β3 tubulin-positive cells in the ganglion cell layer and the inner part of the INL (presumably amacrine cells) (Fig. 4H-J). All sections shown are taken from central retina, and we did not observe significant gradients of expression in either the ligands or receptors when examined by immunostaining or western blot (data not shown). However, we did observe an increase in retinal Smad2/3 expression between P4 and P6 by western blot (see Fig. S2 in the supplementary material). This increase might enhance the effectiveness of TGFβ signaling that occurs at this time. Furthermore, at P6, when proliferation is absent from the central retina, we observed higher levels of forkhead box family member Foxo1 expression in the central retina compared with peripheral retina by western blot (see Fig. S2 in the supplementary material). As Foxo1 has been shown to enhance TGFβ signaling in neuroepithelial cells, its presence in the central retina may indicate higher levels of TGFβ signaling (Seoane et al., 2004).

These data suggest that: (1) retinal progenitors in the postnatal retina possess the TGFβ receptor complement necessary for signaling; and (2) the predominant TGFβ ligand expressed at this stage, TGFβ2, is expressed by retinal neurons. Furthermore, at P4 and P6, downstream TGFβ signaling

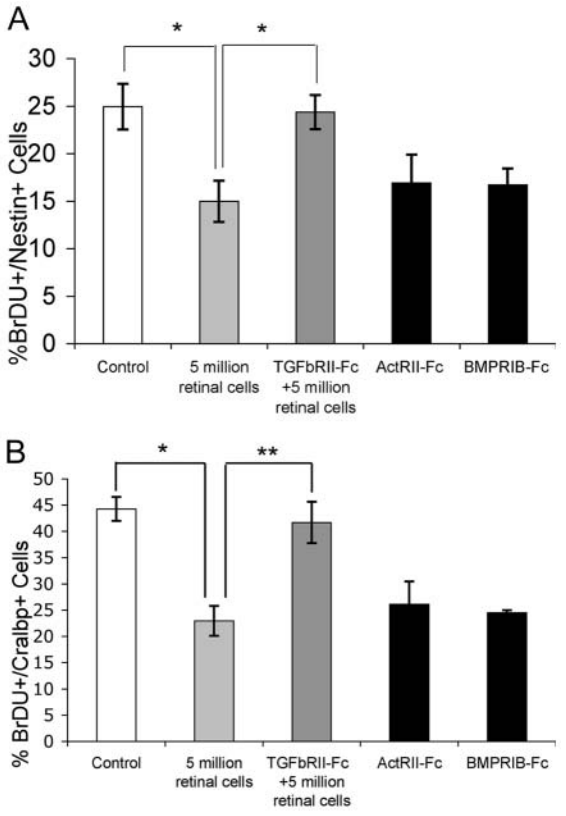

Fig. 3. Inhibition of TGFβ signaling restores proliferation in addback conditions. (A) Quantification of nestin$^+$/BrdU$^+$ cells shows restoration of proliferation to near control levels (white bar; 24.9±2.4%) in the presence of TGFβRII-Fc and 5 million retinal cells (dark gray bar; 24.4±1.8%) compared with 5 million cells alone (light gray bar; 15±2.2%). Neither ActivinRII-Fc nor BMPRIB-Fc had a significant effect on proliferation (black bars). (B) Quantification of CRALBP$^+$/BrdU$^+$ cells shows TGFβRII-Fc restores proliferation of CRALBP$^+$ Müller glial cells in the presence of 5 million retinal cells (dark gray bar; 41.7±3.9%), compared with 5 million cells alone (light gray bar; 23±2.8%), neither ActivinRII-Fc, nor BMPRIB-Fc affected glial proliferation in this assay (black bars). Error bars=s.e.m. *P<0.02, **P<.005 pairwise comparisons (Student's t-test).

components, such as Smad2/3 and Foxo1 are present and may play a role in the regulation of retinal proliferation.

At postnatal day 10, RT-PCR results indicate that TGFβ1 and TGFβ2 are expressed in the retina, as well as TGFβRI and II (Fig. 5J). Quantitative RT-PCR shows that TGFβ2 is the most highly expressed ligand in the P10 retina, at ~12-fold higher expression than TGFβ1 (Fig. 5K). Again, immunostaining results show TGFβ2 expression primarily in β3 tubulin-positive neurons in the inner nuclear layer (arrowheads, Fig. 5G-I). At this stage, TGFβ receptor I and II expression can be found in Glast-positive Müller cell bodies of the inner nuclear layer (arrowheads) and Müller glial processes (arrows) in the outer nuclear layer (Fig. 5A-F), indicating that Müller glia are competent to respond to TGFβ signals. Again, all sections shown are taken from the central retina, and we did not observe a significant central-peripheral gradient in either receptor or ligand expression at any developmental timepoint. At both P4 and P10, the TGFβ receptor is also expressed in ganglion cells and it is possible that some of the effects of TGFβ on progenitors and glia are not direct.

Taken together, these data indicate that: (1) the primary TGFβ receptor ligand in the postnatal retina is TGFβ2; (2) TGFβ2 is primarily expressed by inner retinal neurons; and (3) both postnatal progenitors and Müller glia express TGFβ receptors. The data are consistent with the hypothesis that production of TGFβ2 by retinal neurons during development acts to limit progenitor and Müller glial proliferation.

TGFβ ligands inhibit proliferation in postnatal rat explants

To determine if TGFβ ligands are capable of inhibiting retinal

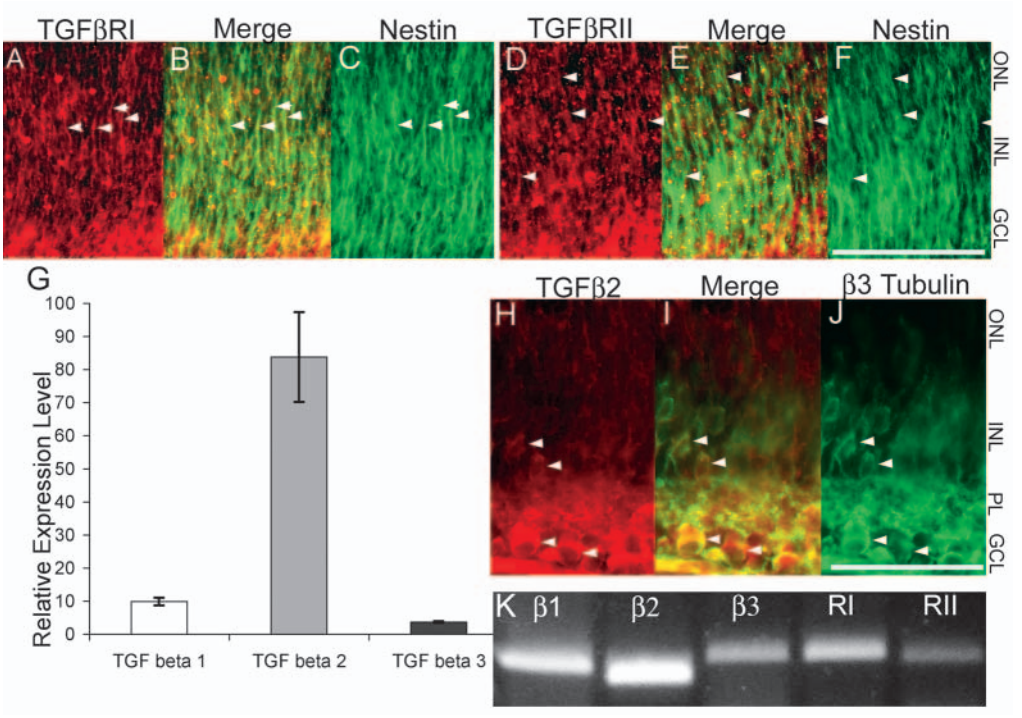

Fig. 4. TGFβ ligands and receptors are expressed in P4 rat retina. (A-F) Immunolocalization of TGFβRI (red, A) and II (red, B) in nestin-positive (green, C,F) processes of progenitor cells in the central retina. Arrowheads indicate double-labeled cells. (G) Quantitative RT-PCR results show TGFβ2 is expressed 80-fold higher than TGFβ3 and eightfold higher than TGFβ1 in P4 retinal tissue. (H-J) TGFβ2 (red) is expressed by β3 tubulin-positive (green) ganglion and amacrine cells (arrowheads). (K) RT-PCR for TGFβs and receptors P4 retinal mRNA. Scale bar: 50 μm.

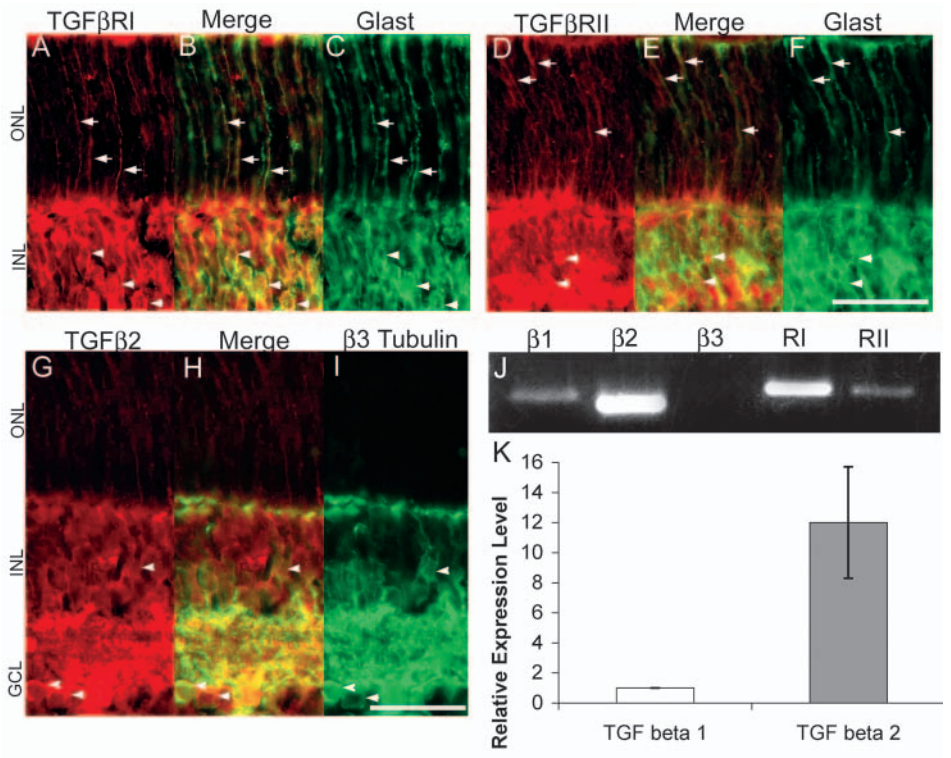

Fig. 5. TGFβ ligands and receptors are expressed in P10 rat retina. (A-F) Immunolocalization of TGFβRI (red, A) and II (red, D) glast-positive (C,F green) cell bodies (arrowheads) and processes (arrows) in central retina. (G-I) TGFβ2 (red) is expressed by β3 tubulin-positive (green) neurons of the inner retina (arrowheads). (J) RT-PCR analysis shows that transcripts for TGFβ ligands 1 and 3, as well as for receptors I and II, are expressed in P10 rat retinas. (K) Quantitative RT-PCR showing TGFβ2 is expressed 12-fold higher than TGFβ1 at P10. Scale bar: 50 μm.

progenitor proliferation, we cultured intact, postnatal day 4 rat retinas for a period of 24 hours in the presence of 5 ng/ml TGFβ1, TGFβ2 or TGFβ3, and 10 μg/ml BrdU. Following explant culture, the retinas were either fixed, sectioned and processed for BrdU immunohistochemistry, or dissociated and plated onto poly-ornithine coated coverslips for quantification of BrdU-positive cell numbers. In these experiments, nearly all of the BrdU-positive cells were co-labeled with the neural progenitor marker nestin (93±3% for control, 97±1% for TGFβ2 treated). The percentage of cells positive for BrdU was determined for at least three different retinas in each condition, and then normalized to control. Fig. 6A,B shows examples of intact retinal explant sections from control and TGFβ2-treated explants, respectively. BrdU staining predominates in the progenitor zone in both treated and control explants. Treatment of the explants with either TGFβ1 or TGFβ2 reduced the percentage of cells that incorporated BrdU to 51.3% (±6.3) and 52.4% (±1.2) of control levels, respectively (Fig. 6C). TGFβ3 treatment did not consistently reduce proliferation in these explants. In explants treated with TGFβ1 or TGFβ2, we did not observe any region-specific reduction in proliferation.

As mentioned, TGFβ2 is the most highly expressed TGFβ ligand at postnatal day 4 (Fig. 4G). To determine the dose response characteristic of postnatal day 4 retinas, a dose-response curve was generated at various TGFβ2 concentrations. Postnatal day 4 retinas were cultured as intact explants in 0, 0.5, 5 or 50 ng/ml TGFβ2 for 24 hours in the presence of BrdU. Following this explant culture period, the explants were dissociated and the percentage of BrdU-positive cells determined for each condition. In control retinas, 13.4% (±1.7) of cells were BrdU-positive after the 24 hour culture period (Fig. 6D). This percentage dropped to 8.4% (±1.6) in the presence of 0.5 ng/ml TGFβ2, 7.3% (±0.7) in the 5 ng/ml condition, and 6.4% (±0.4) in the 50 ng/ml condition (Fig. 6D). This dose-response data indicate that TGFβ2 is an effective cytostatic signal at a wide range of concentrations.

In addition to their role in the regulation of proliferation, TGFβs have been shown to promote cell death in embryonic mouse retina (Dunker and Krieglstein, 2003; Dunker et al., 2001). To determine whether the changes we observed in the number of BrdU-positive cells in these explant cultures were due to a TGFβ-mediated increase in apoptosis, we performed TUNEL analysis on sectioned, TGFβ-treated explants. No consistent or significant changes in the numbers or locations of apoptotic cells could be observed between control and TGFβ treated explants (data not shown).

Inhibition of TGFβ activity restores proliferation to the P6 retina in vitro and in vivo

As noted above, postnatal day 6 retinas show a markedly reduced level of proliferation when compared with P4 retinas (see Fig. 1). To determine if proliferation in P6 retinas could be restored by inhibiting TGFβ signaling, we used a TGFβ neutralizing monoclonal antibody, which binds TGFβ ligands 1, 2 and 3 of multiple species, including rat (Dasch et al., 1989). When explants were treated with the anti-TGFβ antibody for 24 hours in the presence of BrdU, there was a 170% (±19) increase in the percentage of cells incorporating BrdU during the culture period, compared with controls (mouse IGG alone, Fig. 7A-C). At this stage of development, the proliferating cells could be either Müller glia or retinal progenitors. To determine this, we labeled the dividing cells with anti-BrdU and CRALBP. The anti-TGFβ treated explants showed an increase in BrdU labeling for both CRALBP$^+$ Müller glia and progenitor cells (data not shown). Qualitatively, the increase in proliferation in these explants occurred most often as an expansion of the peripheral zone into more central areas of the retina (data not shown).

These explant experiments show that TGFβ signaling has an anti-proliferative effect on cells of the postnatal rat retina, and that inhibiting this endogenous signal maintains proliferation of the progenitors past the developmental period in which the retina would normally become mitotically quiescent.

Inhibition of TGFβ signaling in vivo at postnatal day 6 also

Development

extends the period of proliferation. For these experiments, we used SB-431542, a small molecule inhibitor of TGFβRI/Alk5 (Callahan et al., 2002; Inman et al., 2002). Postnatal day 5.5 pups were given intraocular injections of either DMSO (Fig. 8A) or 40 nanomoles SB431542 dissolved in DMSO (Fig. 8B), followed by a single BrdU injection 12 hours later, at P6. There was an increase in the number of BrdU$^+$ cells in SB431542-treated animals (Fig. 8B) compared with DMSO-treated animals (Fig. 8A); control retinas contained an average of 105 (±27) BrdU-labeled cells/mm^2 of central retina, compared with 240 (±40) in animals treated with SB-431542. These data further support the possibility that TGFβ is an important inhibitor of progenitor proliferation in the postnatal retina.

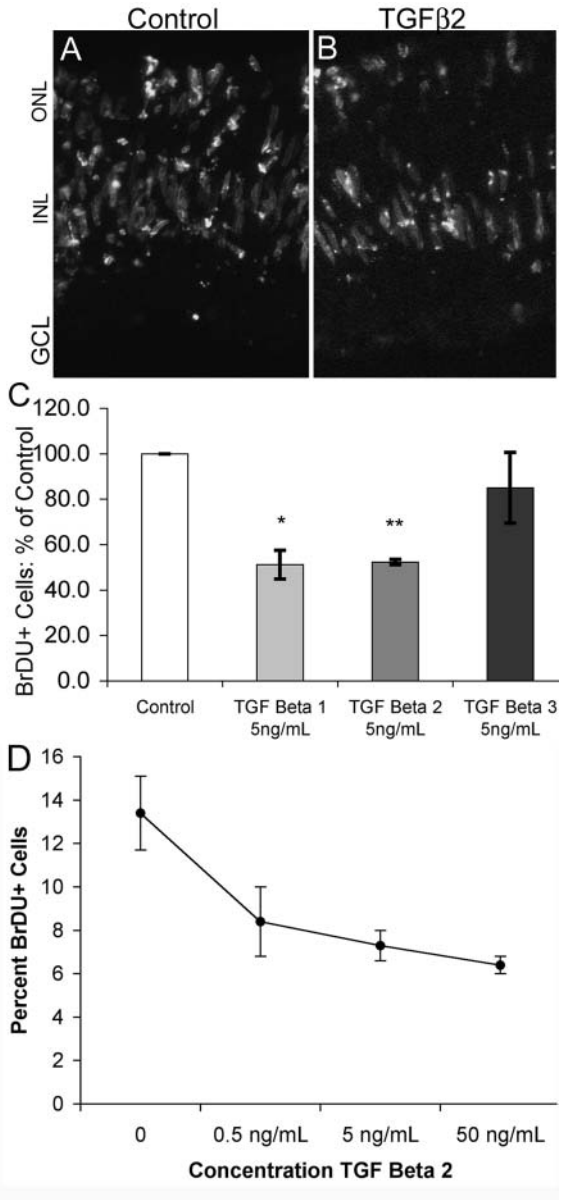

Fig. 6. TGFβ treatment reduces proliferation in P4 retina. P4 retinas were cultured as whole explants for 24 hours with BrdU. (A,B) BrdU labeling of control (A) and TGFβ2-treated (B) explants. Fewer BrdU-positive cells are present in TGFβ2-treated P4 explants. (C) P4 explants were dissociated after 24 hours of culture and processed for immunohistochemistry. TGFβ1 (gray bar) and 2 (dark gray bar) reduced proliferation to 51.3% (±6.3) and 52.4% (±1.2) of control (white bar), respectively. No significant difference in BrdU$^+$ cell numbers was observed in TGFβ3 treated explants (black bar). (D) Dose-response curve indicates 13.4% (±1.7) of cells are BrdU-positive in control treated explants, compared to 8.4% (±1.6) at 0.5 ng/ml TGFβ2, 7.4% (±0.7) at 5 ng/ml, and 6.4% (±0.4) at 50 ng/ml. **P*<0.01, ***P*<0.005, pairwise comparison (Student's *t*-test).

Inhibition of TGFβ signaling potentiates EGF stimulated Müller glial proliferation in vivo

As noted above, little or no proliferation occurs in the retina after P10. To determine if Müller glia might re-enter the cell cycle when TGFβ signaling is inhibited in vivo, we used a combination of the TGFβ receptor II-Fc protein previously mentioned and a TGFβ neutralizing monoclonal antibody that binds to and inhibits signaling via TGFβ ligands 1, 2 and 3 of multiple species, including rat (Dasch et al., 1989). Rat pups were injected intraocularly on P10 and P11 with either PBS/BSA (control), a cocktail of TGFβ signaling inhibitors (5 μg mouse-anti-TGFβ and 1.25 μg TGFβRII-fc), 250 ng EGF, or a combination of 250 ng EGF and TGFβ signaling inhibitors (mouse-anti-TGFβ and TGFβRII-fc combined). These intraocular injections were followed by systemic BrdU injections every 8 hours for 24 hours. Control or anti-TGFβ/TGFβRII-fc injections were quantitatively identical, and resulted in little or no BrdU labeling in Müller glial cells (Fig. 9A-C,G,J), although a few endothelial cells were labeled. EGF injection resulted in BrdU incorporation, predominately in the INL, with a few cells labeled in the ONL (Fig. 9H). However, when 250 ng of EGF was co-injected with the anti-TGFβ

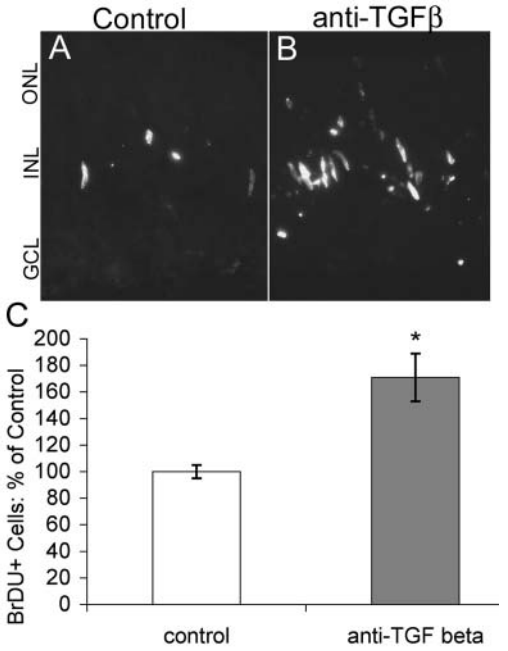

Fig. 7. Inhibition of TGFβ signaling increases proliferation in P6 retinal explants. (A,B) BrdU labeling of P6 explants cultured as whole, floating retinas for 24 hours with BrdU, in control media, including mouse IGG (A) or in the presence of monoclonal antibody against TGFβ ligands 1, 2 and 3 (B). Treatment with anti-TGFβ antibody resulted in a 170.8% (±18.9) increase in the percentage of cells that were BrdU$^+$, compared with control. **P*<0.01, pairwise comparison (Student's *t*-test).

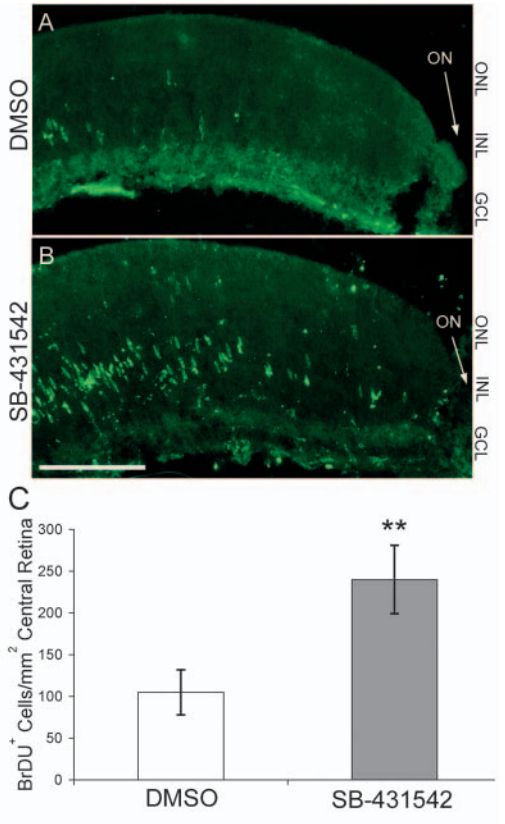

Fig. 8. Inhibition of TGFβRI signaling increases proliferation in the central P6 retina. Postnatal day 5.5 rat pups were given intraocular injections of either DMSO (control) or 40 nanomoles SB431542, small molecule inhibitor of the TGFβRI kinase domain. Pups were injected with BrdU 12 hours later, at P6. (A) A section located at the level of the optic nerve (ON) shows few cells are $BrdU^+$ (green) after intraocular injection with DMSO. (B) SB431542-treated retinas contain numerous BrdU-labeled (green) cells in the region of the optic nerve (ON). (C) Quantification of the number of $BrdU^+$ cells in sections of the retina adjacent to the optic nerve show DMSO-treated control animals (n=6) average 105 (±27) $BrdU^+$ cells/mm^2 central retina, whereas SB-431542 treated animals (n=5) average 240 (±40) $BrdU^+$ cells/mm^2 central retina. $^{**}P<0.01$. Scale bar: 100 μm.

cocktail (Fig. 9D-F,I) there was a large number of BrdU-labeled cells. Co-labeling with CRALBP and BrdU indicated that most of the BrdU+ cells in both the INL and ONL of EGF and EGF/anti-TGFβ injected animals are CRALBP-positive Müller glia (see arrowheads). Although EGF treatment alone stimulated Müller glial proliferation (Fig. 8J), we found nearly twice as many BrdU-positive Müller glia in EGF/anti-TGFβ cocktail treated retinas (633±81) as in EGF treated retinas (330±91). Thus, TGFβ signaling appears to inhibit Müller proliferation in vivo as well as in vitro.

$P27^{kip}$ expression is disrupted in Müller glia following inhibition of TGFβ signaling

One mechanism by which TGFβs inhibit cell proliferation is by activating transcription of cyclin-dependent kinase inhibitors (CDKIs) of the INK4 and Cip/Kip families, such as $p15^{INK4b}$, $p21^{Cip}$ and $p27^{Kip1}$(Pillaire et al., 1999; Polyak et al., 1994; Reynisdottir et al., 1995). These proteins inhibit cell cycle progression at the G_1 to S phase transition by preventing cyclin-dependent kinase (cdk) phosphorylation of the retinoblastoma (Rb) protein (Sherr and Roberts, 1999). TGFβ might inhibit proliferation by upregulating $p27^{Kip1}$ in retinal progenitors and Müller glia, as $p27^{Kip1}$ is crucial for cell cycle arrest of both cell types (Dyer and Cepko, 2000; Dyer and Cepko, 2001; Levine et al., 2000).

To test whether TGFβ acts through $p27^{kip1}$ to inhibit proliferation, we added TGFβ2 to cultures of Müller glia. We found that TGFβ treatment can upregulate $p27^{kip1}$ expression in dissociated cultures of Müller glia to 130% of control levels (data not shown). We also examined $p27^{kip1}$ expression in the retina of rats that had received injections of EGF and EGF combined with TGFβ inhibitors. In the control condition, $p27^{kip1}$ expression was expressed by CRALBP-positive, Müller glial cells located in the inner nuclear layer (arrows, Fig. 10A-C). In animals injected with 250 ng EGF alone, $p27^{kip1}$ expression appeared slightly downregulated in the inner nuclear layer (Fig. 10D-F); however, in animals treated with EGF and the anti-TGFβ cocktail, $p27^{kip}$ expression was substantially reduced (Fig. 10G-I).

Discussion

In this study, we show that: (1) retinal neurons secrete a cytostatic factor; (2) this factor can be blocked by TGFβ receptor II-Fc fusion protein; (3) addition of exogenous TGFβ inhibits postnatal day 4 retinal progenitor proliferation; (4) inhibiting TGFβ signaling through the use of a TGFβ blocking antibody in vitro, or the small molecule inhibitor of TGFβRI, SB-431542, resulted in an increase in retinal proliferation at P6, a timepoint when a decline in proliferation is normally observed in the retina; and (5) inhibition of TGFβ signaling through a combination of the TGFβRII-Fc fusion protein and the pan-TGFβ blocking antibody enhances the ability of Müller glia to re-enter the cell cycle in response to EGF, in part through the loss of $P27^{kip1}$ expression. These experiments demonstrate that TGFβ negatively regulates the proliferation of retinal progenitors and Müller glia in the developing retina.

The immunostaining pattern we observed for TGFβ2, the most abundantly expressed TGFβ ligand, and its receptors suggests a paracrine signaling mechanism is responsible for the inhibition of proliferation; TGFβ2 expression was found in the amacrine and ganglion cells at P4, and later in the β3-tubulin-positive, inner-retinal neurons. The presence of the paracrine signaling pathway could ensure that progenitors provide the necessary numbers of late-born cell types needed to make connections with the already existing early-born cells. As noted in the Introduction, mitogenic Shh is produced in the retinal ganglion cells during development (Jensen and Wallace, 1997; Levine et al, 1997; Dakubo et al., 2003; Wang et al., 2002). In light of our results, retinal neurons appear to provide both mitogenic and cytostatic factors. The co-expression of mitogenic and anti-mitogenic signals within the same tissue has also been observed in the cerebellum; postmitotic neurons produce the mitogen, Shh and the mitotic inhibitory signal, TGFβ (Constam et al., 1994; Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). In addition, in the olfactory epithelium, where GDF11 has been shown to inhibit neurogenesis, mitogenic Fgf8 and Follistatin, an inhibitor of GDF11, are expressed (Calof et al., 1998; Shou

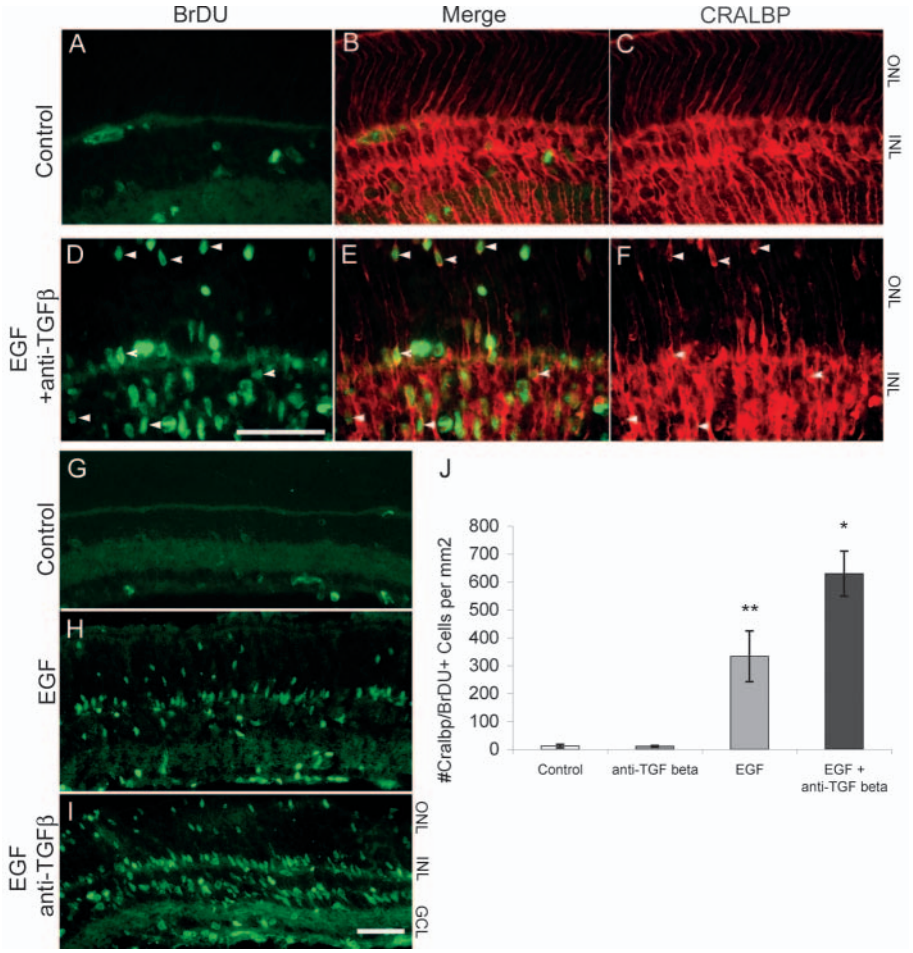

Fig. 9. Inhibition of TGFβ signaling in vivo enhances Müller glial proliferation in response to EGF at P10. Postnatal day 10 rat pups received intraocular injections of either PBS/BSA (A-C,G), anti-TGFβ cocktail (5 μg mouse-anti-TGFβ and 1.25 μg TGFβRII-fc), 250 ngEGF(H), or a combination of EGF and anti-TGFβ cocktail (D-F,I). Intraocular injections were followed by BrdU injections. (A-C) In PBS/BSA-injected animals, only an occasional BrdU (green) labeled cell was found. (D-F) In the anti-TGFβ/EGF-treated animals, BrdU (green) and CRALBP (red) staining shows multiple double-positive cells (arrowheads) in both the inner and outer nuclear layers of the retina. (G-I) Low magnification views of control injected retina (G), EGF injected retina (H) or EGF + anti-TGFβ injected retina (I). (J), Quantification of in vivo results, from counts of $CRALBP^+/BrdU^+$ cells present in central retinal sections: control, 12 (±6) cells/mm^2 retina; anti-TGFβ, 11.1 (±4) cells/mm^2; EGF, 333.5 (±92) cells/mm^2; EGF + anti-TGFβ, 630 (±81) cells/mm^2; *P<0.05, **P<0.005, pairwise comparison (Student's t-test). Scale bar: 50 μm.

et al., 2000; Wu et al., 2003). A common source of mitogen and growth-inhibitory signals might finely tune the numbers and ratios of cells as they are born or as neurons die, resulting in properly functioning circuits.

Our data also suggest a role for a neuronal source of TGFβ in maintaining Müller glial quiescence in the postnatal retina. Normally, mammalian Müller glia do not proliferate after retinal development is complete. Even in disease states, such as diabetic retinopathy, few Müller glia enter mitosis (Fariss et al.,

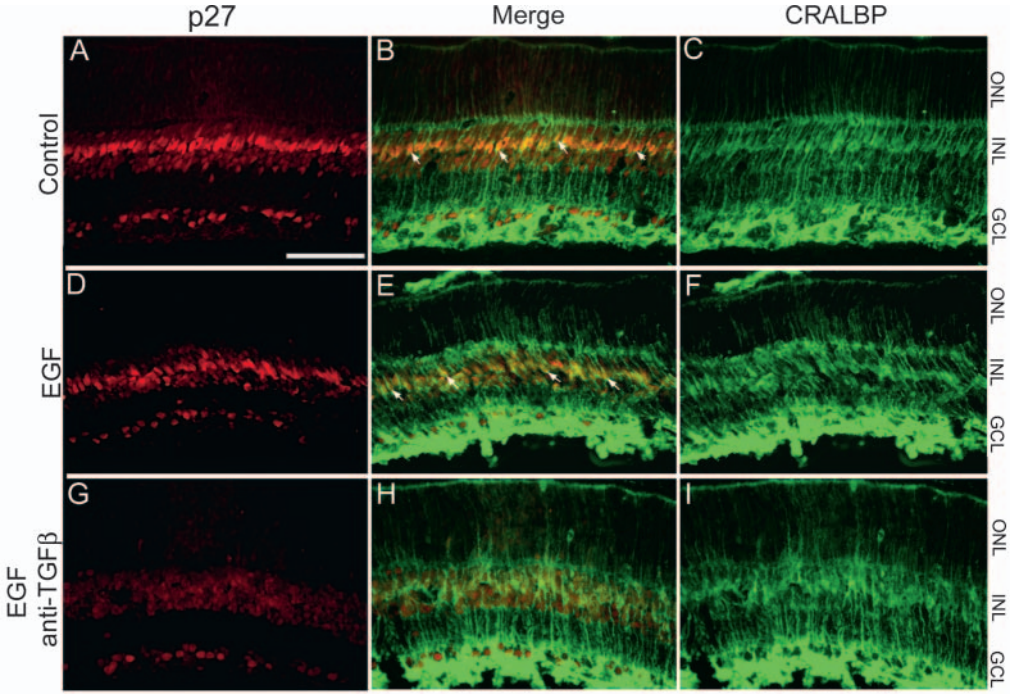

Fig. 10. $p27^{kip1}$ expression is disrupted in vivo with inhibition of TGFβ signaling. Sections shown represent typical sections from the central-most region of the retina from animals receiving intraocular injections of either PBS/BSA, 250 ng EGF or 250 ng EGF + anti-TGFβ cocktail. (A-C) PBS/BSA injected animals show distinct $p27^{kip1}$ (red, A) labeling of CRALBP-positive (green, C) cell bodies in the inner nuclear layer (arrows). (D-F) In EGF-treated animals, $p27^{kip1}$ staining (red, D) is still present in $CRALBP^+$ (green, F) Müller glia, although slightly reduced, compared with control-treated animals. (G-I) $p27^{kip1}$ (red, G) labeling is largely absent from the $CRALBP^+$ (green, I) Müller glia after treatment with EGF and anti-TGFβ cocktail combined. Scale bar: 50 μm.

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2000; Nork et al., 1986; Nork et al., 1987; Robison et al., 1990; Sueishi et al., 1996). Furthermore, attempts to stimulate rodent Müller glial proliferation with neurotoxins or growth factors does not increase their mitotic activity (J.L.C. and B.G., unpublished) (Dyer and Cepko, 2000) to the level of proliferation seen in the Müller glia of the chicken (Fischer et al., 2002; Fischer and Reh, 2001).

Our results are consistent with data from previous in vitro studies of Müller glia, demonstrating that EGF is a mitogen for Müller glial cells (Ikeda and Puro, 1995; Mascarelli et al., 1991; Milenkovic et al., 2003; Milenkovic et al., 2004; Roque et al., 1992; Scherer and Schnitzer, 1994). In addition, TGFβ has been implicated as an inhibitor of Müller glial proliferation in vitro (Ikeda and Puro, 1995). Furthermore Ikeda et al found that cultured Müller cells express type I and type II TGFβ receptors (Ikeda et al., 1998). Moreover, the antagonism between mitogenic factors like EGF and cytostatic factors like TGFβ may be present in astrocytes as well. For example, many studies have reported the mitogenic effects of EGF and related ligands on astrocyte proliferation (Bachoo et al., 2002; Doetsch et al., 2002; Huff et al., 1990; Leutz and Schachner, 1981; Rabchevsky et al., 1998). TGFβ can antagonize the EGF response in astrocytes (Hunter et al., 1993; Sousa Vde et al., 2004). In fact, de Sampaio e Spohr et. al. have proposed a paracrine interaction between neurons and astrocytes, also mediated by TGFβ1, similar to what we have proposed for Müller glia (de Sampaio e Spohr et al., 2002).

Together with these previous studies, our results support a model of neurogenesis in which the balance of mitogenic factors and mitotic inhibitors determine the level of proliferation in both the developing and postmitotic retina. The antagonistic interaction between the EGF and TGFβ pathways may be due to intracellular intersection of these signaling pathways (ten Dijke et al., 2000). This counteractive effect might allow EGF to play a mitogenic role in the presence of anti-mitogenic TGFβ signals in the postnatal retina. For example, the interferon γ (Jak/Stat) and EGF (Erk kinase) signaling pathways can upregulate inhibitory Smad7, which prevents nuclear translocation and transcriptional activation of Smad target genes (Ulloa et al., 1999). EGF also inhibits the TGFβ pathway through phosphorylation of Smad2/3 in the linker region, preventing nuclear translocation (Kretzschmar et al., 1999). EGF can also counteract the cytostatic effect of TGFβ by interfering with its ability to activate CDKI $p15^{INK4b}$ (Dunfield and Nachtigal, 2003).

One remaining question, however, is how does TGFβ inhibit proliferation in the central to peripheral pattern observed, when we saw no gradient in expression of signaling components? This might be accomplished through intracellular read-outs of EGF and TGFβ signaling. Indeed, retinal progenitor cells are known to change their responsiveness to EGF during development (Lillien and Cepko, 1992). This enhanced responsiveness to EGF in the late retinal progenitor cells is due, in part, to an increase in the level of the EGF receptor expression; however, it is likely that intracellular interaction with TGFβ signaling is also important.

Recent studies provide insight into how the intracellular readout of mitogenic versus cyctostatic TGFβ signals occurs molecularly. Seoane et. al. (Seoane et al., 2004) found that the forkhead box (Fox) family of transcription factors act as both positive and negative regulators of Smad-mediated transcription in neuroepithelial cells (Seoane et al., 2004). The authors found that Foxo proteins, which associate with Smads and facilitate transcriptional activation at the $p21^{cip}$ promoter, were expelled from the nucleus in the presence of PI3 kinase signaling. Foxg1, which promotes the differentiation of cortical progenitors, blocked the ability of the Foxo/Smad complex to activate transcription of $p21^{cip}$ in this same study (Hanashima et al., 2004; Hanashima et al., 2002). Therefore, it is possible that the response to the EGF and TGFβ signals received by a given cell are determined by the expression pattern or levels of factors such as the Fox proteins. Our data suggests Foxo1 might facilitate TGFβ signaling in the central retina at P6, as Foxo1 protein is more abundant centrally than peripherally. It is notable that in our in vivo P5.5 injections and P6 explant cultures, inhibition of TGFβRI alone was sufficient to stimulate proliferation. Yet at P10, the addition of EGF was required to promote Müller glial proliferation in vivo. Thus, both the response to mitotic inhibitors, as well as the availability of mitogens, shifts from conditions favoring proliferation to conditions that maintain quiescence at the termination of neurogenesis.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/13/3015/DC1

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