Tumorigenesis and Neoplastic Progression

Tumor-Associated Retinal Astrocytes Promote Retinoblastoma Cell Proliferation Through Production of IGFBP-5

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Retinoblastomas consist of cone-like neoplastic cells and diverse non-neoplastic cells whose roles in tumorigenesis have not been defined. Here, we investigated the glial cells that constitute 2% to 3% of the cells in retinoblastoma tumors, including their origin, their relationship to a potential retinoblastoma stem cell population, and their effects on tumor cell proliferation. Retinoblastoma glia consistently expressed the retinal astrocyte marker Pax2 but inconsistently expressed the Müller cell and occasional astrocyte marker CRALBP. Many of the glia expressed the stem cell-associated Sox2 but nevertheless were non-neoplastic as they coexpressed Rb and/or retained two *RB1* alleles. Conversely, the glia were distinct from the non-neoplastic cells that strongly expressed the stem cell-associated ABCG2. Adherent Pax2⁺, Sox2⁺, Rb⁺ glia readily grew from explanted retinoblastomas and produced soluble factors that enhanced the proliferation of cocultured retinoblastoma cells. This effect was emulated by normal retinal glia and appeared to be mediated by insulin-like growth factor binding protein-5 (IGFBP-5), as it was mimicked by recombinant IGFBP-5 and mitigated by neutralizing IGFBP-5 antibody. As glia-derived IGFBP-5 was earlier found to promote photoreceptor survival, our findings indicate that retinal astrocytes enhance the proliferation of cone-like retinoblastoma cells by deploying a factor that also provides trophic support to the tumor cells' non-neoplastic counterparts. These observations suggest that a tissue-specific microenvironmental

feature cooperates with oncogenic mutations in a cancer cell of origin to promote retinoblastoma tumorigenesis. (Am J Pathol 2010, 177:424–435; DOI: 10.2353/ajpath.2010.090512)

Retinoblastoma is a childhood retinal cancer that develops in response to the inactivation of the *RB1* gene, the loss of functional Rb protein, and subsequent genetic changes.^{1,2} As germline *RB1* mutations predispose to retinoblastoma with high penetrance, the tumors must arise from cells that are exceptionally sensitive to the loss of Rb function. A recent study showed that the tumors depend on cone-specific signaling circuitry and suggested that they arise from cone photoreceptor precursors.³ Thus, the intrinsic cone precursor circuitry may be one of the factors that sensitizes to *RB1* mutations and predisposes to retinoblastoma tumorigenesis. However, in addition to the circuitry of the cell of origin, the retinal microenvironment could also contribute to the high rate of tumorigenesis that follows the loss of Rb function. Nevertheless, there is currently little understanding of the interactions of the cone-like neoplastic cells and their non-neoplastic neighbors during retinoblastoma tumorigenesis.

One aspect of the retinoblastoma microenvironment that has been examined in some detail is the tumor vasculature. As in other cancers, retinoblastoma growth appears to be limited by the vascular supply, as the tumors often have perivascular cuffs in which cell proliferation and survival correlate with the proximity of the tumor cells to the blood vessel lumens.^{4,5} However, in addition to vascular cells, retinoblastomas contain other cell types whose roles in tumorigenesis have not been explored. These include microglia,⁶ which are macrophage-like cells of the central nervous system, macroglia

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(hereafter referred to simply as “glia”) characterized by their expression of glial fibrillary acidic protein (GFAP),^{7,8} and cells with stem cell–related features such as expression of neural stem cell markers or the ATP-binding cassette transporter G2 (ABCG2).^{9,10} Establishing the roles of the tumor microglia, glia, and putative stem cell populations could be crucial for understanding how the retinal microenvironment collaborates with Rb loss in the cells that give rise to retinoblastoma tumors.

The current study focuses on the properties of the retinoblastoma-associated glia, which constitute ~2% to 3% of the cells in retinoblastoma tumors.³ Until recently, it was unclear whether these cells derive from the normal retina or from the neoplastic, *RB1* mutant cells that give rise to the malignancy. A neoplastic origin was suggested by reports that cultured retinoblastoma cells can undergo glial differentiation¹¹ and that glia-like cells in the tumors lacked detectable Rb protein.¹² However, later studies refuted the evidence of glial differentiation¹³ and revealed that Rb is generally expressed, and both *RB1* alleles are retained, in the tumor associated GFAP⁺ cells.³ Retinoblastoma xenograft studies further suggested that the tumor glia are non-neoplastic, as subretinal grafts appeared to be infiltrated by GFAP⁺ host cells¹⁴ and were propagated by cone-like cells.³

Here, we examine whether retinoblastoma glia have properties of astrocytes, which normally line the retinal vasculature, or properties of Müller cells, which are produced within the neural retina where the tumors initially form. As glial cells can have stem cell–related properties, we also examined whether tumor cells that express stem cell–associated proteins^{9,10} represent non-neoplastic glial subpopulations. Finally, as retinal glia can promote cone photoreceptor survival,^{15,16} we examined whether the tumor glia have a similar effect on the cone-like retinoblastoma cells. The studies establish that retinoblastoma-associated glia have properties of retinal astrocytes and suggest that retina-specific microenvironmental features contribute to retinoblastoma tumorigenesis.

Materials and Methods

Sample Preparation

Human retinoblastomas and fetal eyes were obtained under protocols approved by the Weill Cornell Medical College and Memorial Sloan-Kettering Cancer Center Institutional Review Boards. Murine eyes were obtained under protocols approved by the Weill Cornell Institutional Animal Care and Use Committee. Cryosections of retinoblastomas and retinas were prepared as previously described.^{3,17}

Immunofluorescence Analysis of Frozen Sections and Cultured Cells

Staining was performed as described^{3,17} with minor modifications as follows.

Pretreatments

Frozen sections were postfixed in 4% paraformaldehyde (PFA) in 0.15 M NaCl and 20 mmol/L Tris (pH 8.0) (TBS) for 5 minutes, washed in TBS, dried, heated in 250 ml 1 mmol/L EDTA for 20 minutes at 95–100°C, treated with 1% sodium borohydride in deionized water for 5 minutes, and washed with TBS. Retinal sections were treated similarly except that heating was replaced by incubation in 1 mmol/L EDTA-TBS for 5 minutes at room temperature. Cultured cells were fixed on coverslips in 4% PFA for 5 minutes without post fixation.

Immunostaining

Sections were treated with ABC kit (Vector Laboratories, Burlingame, CA) reagent A and reagent B in TBS for 15 minutes each, washed in TBS, blocked and permeabilized in 2.5% horse serum, 2.5% donkey serum, 2.5% human serum, 1% nonfat milk, 1% BSA, 0.1% Triton-X-100, and 0.05% Tween-20 in TBS (block 1, cleared by centrifugation and filtration with 0.2 μ m cellulose acetate filter) for 20 minutes, incubated in primary antibody in block 1 overnight (or for some Rb staining for 36 hours) at 4°C, and then washed three times in TBS. For Rb, GFAP, and Ki-67 (mouse antibody) staining, samples were incubated with biotin-conjugated horse anti-mouse (Vector BA-2000, 1:135) for 30 minutes, washed in TBS, followed by FITC-conjugated streptavidin (Vector SA-5001, 1:200) for 30 minutes in 0.1 M sodium bicarbonate and 0.15 M NaCl, pH 8.2. For costaining, sections stained as above were incubated with an additional primary antibody and appropriate secondary antibody as described.³ Images were analyzed by epifluorescence microscopy using a Zeiss Axiovert 35 or by confocal microscopy using a Leica DMIRE2. The specificity of all immunostaining was confirmed with “no primary antibody” or “isotype IgG antibody” controls.

Primary Antibodies

Mouse antibodies were to Rb (G3-245, BD Biosciences, San Jose, CA 1:200; and Rb-Ab5, Calbiochem, San Diego, CA, 1:500), Ki-67 (B56, BD Biosciences, 1:50), GFAP (G-A-5, Sigma, St. Louis, MO 1:50), ABCG2 (BXP-21, Santa Cruz Biotechnology, Santa Cruz, CA 1:200), and CD31 (FITC-conjugated, IM1431 Beckman Coulter, Brea, CA 1:5). Rabbit antibodies were to CRX¹⁸ (gift of C. Gregory-Evans, Imperial College London, UK; 1:50); Pax2 (Covance, Princeton, NJ 1:100), Ki-67 (NLC-Ki67P Novocastra, Newcastle Upon Tyne, UK 1:100), CRALBP (UW55,¹⁹ gift of J. C. Saari, University of Washington, Seattle, WA; 1:500), and nestin (AB5922 Chemicon, Billerica, MA 1:200). A goat antibody was to Sox2 (Y17, Santa Cruz Biotechnology, 1:300).

Rb + Sox2 Immunostaining Followed by RB1 Fluorescence in Situ Hybridization

5-micron cryosections were stained with Rb-Ab5 and then with Sox2 antibodies as above. Ten confocal images

Table 1. Oligonucleotide Primers for qRT-PCR-Analyses

Gene	Sense primer	Anti-Sense primer
<i>BDNF</i>	5'-TTTACCCAATAAGATACATCACAGTCAC-3'	5'-ACAGAACAAGAACGAACAACAG-3'
<i>CNTF</i>	5'-GTTAGATGTCTCAGAAAGTCAGTTG-3'	5'-CCTCCTCACCGCCAAGTATG-3'
<i>CTGF</i>	5'-TTAAGACTTGACAGTGGAACTACATTAG-3'	5'-ATAGCAGGCATATTACTCGTATAAGATG-3'
<i>FGF2</i>	5'-CTCCACCTCCTCTCCACCTG-3'	5'-TGCCTGTTTCATTGCTCCACTC-3'
<i>GDNF</i>	5'-GCTATGAAACCAAGGAGGAAGT-3'	5'-TGCCTGCCCTACTTTGTGCAC-3'
<i>IGF1</i>	5'-TCTGAATCTTGGCTGCTGGAG-3'	5'-AGGGAGGTGGTGGGTATAGAC-3'
<i>IGF2</i>	5'-CCAGCCAGAGTGAGGAAGGAG-3'	5'-CAGGAGCACACCAGGGAGTC-3'
<i>IGF1R</i>	5'-CTACCTCCACATCCTGCTCATC-3'	5'-GCCAGCCACTCGGAACAG-3'
<i>IGFBP5</i>	5'-CCTTCAGATTCCGAGTTGCCTAC-3'	5'-AATATGTACTGTGGTTATTGCTGTCTTC-3'
<i>NT3</i>	5'-CTCCTCTCCCTTCCGAACAG-3'	5'-CGCCGACGCACTACC-3'
<i>VEGFA</i>	5'-CCTTGCCTTGCTGCTCTACC-3'	5'-GATGATTCTGCCCTCCTCTCTC-3'

of each section were obtained and the positions of Rb⁻, Sox2⁺ cells, Rb⁻, Sox2⁻ cells, and Rb⁺, Sox2⁺ cells recorded. Sections were probed by fluorescence *in situ* hybridization (FISH) for *RB1* using Vysis LSI 13q14.3 D13S319 SpectrumOrange for *RB1*, LSI 13q34 Spectrum-Aqua, and CEP 12 (12p11.1-q11) SpectrumGreen Multi-color Probe kit (Vysis, Des Plaines, IL). Slides were reimaged with a Zeiss immunofluorescence microscope equipped with Metasystems Isis software. Cells with two 13q34 FISH signals were identified and the number of *RB1* FISH signals recorded. The aqua 13q34 probe signal was redesignated as green for display purposes.

Primary Retinoblastoma Cultures

Coculture of Retinoblastoma Cells with Tumor-Derived Glia

Primary retinoblastomas were explanted to RB culture medium prepared as described.²⁰ After 7 days, tumor cells in suspension were removed, and the tumor-associated glia trypsinized and either adhered to coverslips for staining or replated for passaging in the presence of the suspension retinoblastoma cells. RB174-derived glia were cocultured with suspension cells for an additional 7 days, plated at 1.8×10^3 cells per well of a 96-well dish, and cocultured either with RB174 tumor cells from the same cocultures (at 1.8×10^4 cells per well) or with freshly explanted RB177 tumor cells (at 0.8×10^4 cells per well) in RB culture medium without insulin and with media changed every 3 days. Tumor cells were removed from the attached glia by gentle pipetting, stained with trypan blue, and viable cells counted. In some experiments, RB174 and RB177 tumor cells were plated in 24-well dishes, either directly contacting GL177 glia or separated from the glia by a 0.4 μ m polycarbonate Transwell membrane (Corning Life Sciences, Lowell, MA).

Coculture of Retinoblastoma Cells with Mouse Retinal Glia

Mouse retinal glia were isolated according to a standard protocol.²¹ Briefly, retinas were isolated at postnatal day 7, digested using the Papain Dissociation System (Worthington Biochemical Corporation, Lakewood, NJ), dissociated by gentle pipetting, and cultured in DMEM

with 10% fetal bovine serum, 0.1% fungizone, and 2% penicillin/streptomycin solution. After 7 days, the retinal glia were trypsinized and either attached to coverslips for immunostaining or replated at 1.8×10^3 cells per well of a 96-well plate and cocultured with 3×10^4 RB170 tumor cells.

Retinoblastoma Cultures with Exogenous Factors or Antibodies

RB191 and RB192 were explanted and the suspension and adherent cells cocultured for 30 or 20 days, respectively. The suspension cells were then removed and replated at 3.5×10^3 or 1.3×10^4 cells per well of a 96-well dish, and incubated with 50 ng/ml connective tissue growth factor (CTGF), 50 ng/ml vascular endothelial growth factor (VEGF; Shenandoah Biotechnology, Warwick, PA), 50 ng/ml fibroblast growth factor 2 (FGF2; Chemicon), 50 ng/ml recombinant porcine insulin-like growth factor binding protein-5 (IGFBP-5; generously provided by M. White, University of Minnesota),²² 50 ng/ml IGF-1 (Sigma), or PBS (for the control), with replenishment of the media and factors every two days. Alternatively, the same RB191 and RB192 cells were cocultured with 3×10^3 RB192 glia (GL192), in 300 μ l media with 200 μ g/ml chicken anti-porcine IGFBP-5 (M. White, University of Minnesota),²² or 200 μ g/ml of control chicken IgG (Gallus Immunotech, Cary, NC). Differences in cell number were evaluated by using analysis of variance least significant difference, with SPSS software.

Gene Expression Analyses

RNAs from primary retinoblastoma and tumor-derived glial cells that had been in culture for 20 days (RB185, RB186, GL185, GL186), for 40 days (RB192 and GL192), for 50 days (RB191), for ~300 days (RB177), or for ~1.5 years (GL55T) were subjected to qRT-PCR as described,³ with gene-specific oligonucleotide primers (Table 1).

Analysis of Cocultures

For BrdU, Ki-67, and terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining,

cells were collected and dissociated by pipetting, plated on poly-L-lysine coated 1.2 cm coverslips, and incubated at 37°C for 2 to 3 hours. After incubation, the medium was removed and the cells fixed with 4% PFA/PBS for 5 minutes, rinsed three times with TBS, dried, and stored at -20°C.

Attached cells were costained for Ki-67 and CRX as described above. For BrdU staining, BrdU (10 µg/ml) was added to the media for 3 hours. Attached cells were permeabilized in 0.2% Triton X-100 in TBS for 10 minutes, treated with 2 N HCl for 30 minutes at 37°C, neutralized in 0.1M sodium borate, pH 8.5, blocked in block 1 (see above), stained with rat anti-BrdU antibody (Bu1/75(ICR1), Accurate Chemical & Scientific, Westbury, NY; 1:100) for 2 hours, followed by FITC-conjugated goat anti-rat second antibody (Jackson Laboratories, Bar Harbor, ME; 1:100) for 30 minutes, and then stained for CRX.

TUNEL was performed using the TumorTACS *In Situ* Apoptosis Detection Kit (Trevigen, Gaithersburg, MD), modified as follows. Slides were rinsed with TBS, digested with 25 µg/ml proteinase K for 5 minutes, washed in deionized water, and fixed, labeled with Bio-16-dUTP, and FITC-conjugated streptavidin as described.³

The proportion of CRX⁺ tumor cells that were Ki-67⁺, BrdU⁺, or TUNEL⁺ was determined in triplicate under fluorescence microscopy, by counting at least 600 CRX⁺ cells per sample. Statistical analyses were determined using the Student two-sample *t* test.

Results

Retinoblastoma Glia Coexpress GFAP and Nestin, but Inconsistently Express the Müller Cell and Occasional Astrocyte Marker, CRALBP

To characterize the retinoblastoma-associated glia, we first examined their expression of established glial cell markers, including GFAP, nestin, and CRALBP. GFAP is thought to be expressed in all retinal astrocytes and activated Müller cells;^{23,24} nestin in retinal progenitor cells, astrocytes, and Müller cells;^{25–27} and CRALBP in all Müller cells and a subset of astrocytes during retinal vasculogenesis.^{19,28}

We initially costained 10 retinoblastomas for GFAP and nestin and six tumors for GFAP and CRALBP. Nestin was detected in virtually all GFAP⁺ cells, regardless of whether they were in perivascular clusters, in glial hyperplasias, in Flexner-Wintersteiner rosettes, or isolated in the midst of neoplastic cells (Figure 1A). Nestin was also coexpressed with CD31 in a subset of Rb⁺ endothelial cells, in keeping with its previously reported expression during tumor angiogenesis,²⁹ but was not detected in other GFAP⁺ cell types. In contrast, CRALBP was detected in only three of six tumors, and several tumors had CRALBP⁺ as well as CRALBP[−] regions (Figure 1, B–C), as previously observed.⁸ We conclude that GFAP is a specific marker of retinoblastoma glia, that nestin is not specific to glia, and that CRALBP is inconsistently expressed in GFAP⁺ glia in retinoblastoma tumors.

Retinoblastoma Glia Express the Retinal Astrocyte Marker Pax2

As CRALBP is expressed in all Müller cells and in astrocytes during retinal vasculogenesis,^{19,28} its inconsistent expression in retinoblastomas could reflect that a subset of the glia are CRALBP⁺ Müller cells or that the glia are generally astrocytes of which only a subset express CRALBP. To distinguish between these possibilities, we stained retinoblastomas for Pax2, which is expressed in optic nerve pre-astrocytes and immature retinal astrocytes but not in retinal progenitor or Müller cells.^{23,30}

We first confirmed that Pax2 is expressed in GFAP⁺ retinal astrocytes but not in Müller or progenitor cells of the developing retina,²³ using a gestational week-16 sample (data not shown). We then examined Pax2 expression in a tumor-associated retina from an 8-month-old retinoblastoma patient to determine whether its expression changes in the presence of a retinoblastoma tumor. In this sample, Pax2 was again detected in GFAP⁺ astrocytes but not in Müller cells, whereas astrocytes as well as Müller cells stained for Sox2 (Figure 2A). Notably, the Müller cells in this retina expressed GFAP and thus were “activated,” most likely due to the tumor’s disruption of the normal retinal architecture.³¹ Thus, Pax2 was restricted to retinal astrocytes and was not induced along with GFAP during Müller cell activation.

Importantly, Pax2 was also detected in the majority of GFAP⁺ cells in each of eight retinoblastomas, regardless of the tumor differentiation status, including cells in perivascular positions, in glial hyperplasias, interspersed throughout the tumors, and in Flexner-Wintersteiner rosettes (Figure 2B). Moreover, the majority (97% ± 4%) of the Pax2⁺ cells expressed Rb, confirming that they were non-neoplastic, and expressed Sox2, as expected for proliferating astrocytes³² (Figure 2, B–D). The consistent presence of Pax2 indicated that most of the retinoblastoma glia have properties of retinal astrocytes, rather than Müller glia or retinal progenitor cells.

Non-Neoplastic Retinoblastoma Glia Proliferate and Express the Stem Cell-Associated Sox2

The widespread presence of Sox2 in retinoblastoma glia implied that the glial cells might proliferate together with neoplastic retinoblastoma cells. Concordantly, Ki-67 was detected in a subset of the Sox2⁺, GFAP⁺ cells in each of six retinoblastoma samples (Figure 3A).

Whereas the immunophenotype of Sox2⁺, GFAP⁺, Pax2⁺ cells was consistent with that of retinal astrocytes, we also considered whether a subset of the Sox2⁺ cells might comprise a distinct population. Specifically, as Sox2 is generally expressed in neural stem cells,³³ and as cells with neural stem cell features were proposed to function as retinoblastoma cancer stem cells,¹⁰ we explored whether a subset of the Sox2⁺ cells might have cancer stem cell properties. To do so, we examined whether Sox2 was restricted to non-neoplastic cells that expressed GFAP and Rb, and that retained wild-type *RB1*, or was also expressed in *RB1*-

Figure 1. Retinoblastoma glia coexpress GFAP and nestin, but only a subset express CRALBP. **A:** Immunofluorescent staining of GFAP (green) and nestin (red), showing representative coexpression of nestin in GFAP⁺ cells (arrows). **B and C:** Costaining of GFAP (green) with CRALBP (red). Arrows indicate GFAP⁺ cells that coexpressed CRALBP in some tumors (**B**) but not in others (**C**). All panels show nuclear staining with DAPI (blue). Scale bars, 20 μ m.

mutated cells as expected of retinoblastoma cancer stem cells.

In these studies, Sox2 was detected in nearly all GFAP⁺ cells but not in GFAP[−] cells, both in undifferentiated regions (Figure 3A) and in highly differentiated zones (Figure 3B). As in our previous analysis of GFAP⁺ cells,³ Rb was detected in the vast majority (95% $\pm$ 3.0%) of the Sox2⁺ cells, including those in differentiated Flexner-Wintersteiner rosettes and in undifferentiated tumors (Figure 3B and data not shown). Importantly, Rb was also detected in the Sox2⁺ cells in a tumor (RB172) that had a biallelic *RB1* deletion and could not produce mutant Rb protein. This implies that the majority of Sox2⁺ cells expresses Rb and does not derive from the *RB1* mutant cells that initiate retinoblastoma tumorigenesis.

However, as ~5% of the Sox2⁺ cells did not have detectable Rb, we also assessed whether these rare Sox2⁺,Rb[−] cells derive from *RB1* mutant tumor cells. To do so, we identified a tumor (RB64) in which the neoplastic cells had one deleted and one retained *RB1* allele. We then immunostained for Rb and Sox2, and defined the *RB1* status of the Sox2⁺,Rb[−] cells, as well as that of Sox2⁺,Rb⁺ glia and Sox2[−],Rb[−] tumor cells, using two-color FISH for *RB1* (at 13q14) and for a 13q34 locus that served as a FISH positive control. As expected, most (~85%) of the Sox2⁺,Rb⁺ glial cells had two *RB1* signals and most (~83%) of the Sox2[−],Rb[−] tumor cells had only one *RB1* signal (Figure 3, C and D). The ~15% of Sox2⁺,Rb⁺ cells with only one *RB1* signal and the Sox2[−],Rb[−] cells with no *RB1* signal are thought to reflect the limited sensitivity of the FISH assay. Importantly, most (~87%) of the Sox2⁺,Rb[−] cells also had two *RB1* hybrid-

Figure 2. Retinoblastoma glia express the retinal astrocyte marker Pax2. **A:** Immunofluorescent staining of Pax2 in a tumor-associated retina from an 8-month-old child, showing coexpression of Pax2 (white) with Sox2 (red) and GFAP (green) in astrocytes in the nerve fiber layer (NFL; arrow), but not in the Sox2⁺,GFAP⁺ Müller glia in the inner nuclear layer (INL; asterisk). **B:** Immunofluorescent staining of Pax2 (white) in Sox2⁺ (red) and GFAP⁺ (green) cells in an undifferentiated retinoblastoma. **C:** Expression of Pax2 (white) in glia that coexpress Rb (blue-green) and nestin (red, the merged image is yellow). **D:** Coexpression of Pax2 (blue-green) and Sox2 (red) in a differentiated retinoblastoma-like region. All panels show nuclear staining with DAPI (blue). Arrows in **B–D** denote Pax2⁺ cells that coexpress the indicated markers. Scale bars, 20 μ m.

ization signals (Figure 3, C and D). The presence of two *RB1* signals in a similar proportion of Sox2⁺,Rb⁺ and Sox2⁺,Rb[−] cells implied that most and potentially all of the Sox2⁺,Rb[−] cells retained two *RB1* alleles despite their lack of Rb expression. These studies indicate that Sox2 is restricted to non-neoplastic GFAP⁺ cells in retinoblastoma tumors.

Retinoblastoma Glia Are Distinct from the Endothelial Cells that Prominently Express the Stem Cell-Associated ABCG2

We further examined whether retinoblastoma glia express ABCG2, which was reported to be highly expressed in a subset of retinoblastoma cells that have cancer stem cell properties.^{9,10} We found that the GFAP⁺ cells were distinct from the strongly ABCG2⁺ cells, and that the latter had the

Figure 3. Sox2⁺ and ABCG2⁺ cells in retinoblastoma tumors. **A:** Costaining of Ki-67 (red) in a glial cell (arrow) that costains for Sox2 (green) and GFAP (white). Ki-67 specifically localized to DAPI-stained nuclear material (blue). **B:** Costaining of Rb (green), Sox2 (white), and GFAP (red) in glial cells in a highly differentiated retinoma-like region. Note Rb expression in a Sox2⁺,GFAP⁺ cell (arrow) within a Flexner-Wintersteiner rosette (dashed circle). **C:** Rb + Sox2 immunostaining plus RB1 FISH analysis of Sox2⁺,Rb⁻ cells, Rb (green) and Sox2 (red) were stained and imaged (left and center), followed by FISH (right) for RB1 (red) and 13q34 (green). Note Sox2⁺,Rb⁻ cells (white arrows) and Sox2⁺,Rb⁺ cell (green arrow) have two 13q34 and two RB1 signals. **D:** Percentage of Sox2⁻,Rb⁻ neoplastic cells, Sox2⁺,Rb⁺ non-neoplastic cells, and the Sox2⁺,Rb⁻ cells that have 0, 1, or 2 RB1 signals. Error bars indicate SD. **E:** Costaining of ABCG2 + GFAP (left), ABCG2 + Rb (center), and ABCG2 + CD31 (right). White arrows indicate strongly ABCG2⁺ endothelial cells; yellow arrow, weak-staining ABCG2⁺ tumor cells. Scale bars, 20 μ m.

appearance and Rb and CD31 expression pattern of endothelial cells (Figure 3E). Moreover, the ABCG2 signal in the Rb⁺,CD31⁺ endothelial cells was far greater than that of the surrounding Rb⁻ tumor cells, and we did not detect Rb⁻

Figure 4. Retinoblastoma-derived and mouse retina-derived glial cells. **A:** Retinoblastoma-derived adherent cells stained for Sox2, nestin, Pax2, and Rb, consistent with a homogeneous retinal astrocyte population. **B:** P7 mouse retina-derived adherent cells uniformly stained for Sox2 and nestin, but with only a subset stained for Pax2 or CRALBP, consistent with a mixed astrocyte and Müller cell population.

tumor cells with prominent ABCG2 staining. We conclude that GFAP⁺ retinoblastoma glia are distinct from the strongly ABCG2⁺ tumor endothelium.

Tumor-Derived as well as Normal Retinal Glia Promote Retinoblastoma Cell Proliferation and Survival

We noted that GFAP⁺ glial processes were present in nearly all retinoblastoma regions and often extended from perivascular glial cell bodies into the surrounding tumors (e.g., Figure 2B). Accordingly, we examined whether tumor-associated glial cells could affect retinoblastoma cell growth or survival. To do so, we used primary retinoblastoma cultures, in which the cone-like tumor cells remained in suspension and in which rare cells adhered to the culture dish and expanded either in the presence or the absence of the suspension cells. Like the tumor glia that were characterized *in situ*, the adherent cells had properties of retinal astrocytes, as they expressed Sox2, nestin, Pax2, and Rb but generally did not have detectable CRALBP (Figure 4A and data not shown).

We then cocultured the tumor-associated glia with the neoplastic retinoblastoma cells that remained in suspension. The tumor-associated glia consistently enhanced the proliferation of cocultured primary tumor cells (Figure 5, A and B), increased the proportion of cells that incorporated BrdU and expressed Ki-67 ($P < 0.01$), and decreased the proportion that underwent apoptosis as measured by TUNEL staining ($P < 0.01$) (Figure 5C). However, the stimulatory effect depended on the ratio of the glial and tumor cells, as the effect was most clear when there was a >5-fold excess of retinoblastoma cells, and confluent glial monolayers inhibited tumor cell proliferation (data not shown). Moreover, in some cultures the dependence on the glia was transient, and tumor cells expanded in a glia-independent manner after 1 to 2 months, as previously reported.³⁴

Figure 5. Tumor- and normal retina-derived glia enhance retinoblastoma cell proliferation and survival. **A** and **B**: Effect of retinoblastoma-derived glia on proliferation of the homologous Rb174 (**A**) and Rb177 (**B**) tumor cells. **C**: Effect of Rb174-derived glia on BrdU incorporation, Ki-67 expression, and TUNEL staining of Rb174 and Rb177 tumor cells. **D**: Effect of Rb174 or Rb177 glia on the homologous Rb174 or Rb177 tumor cells, either separated by a 0.4 μ m membrane or in direct contact. **E**: Effect of mouse retinal glia on Rb170 cell proliferation, Ki-67 expression, and TUNEL staining. Error bars indicate SD. * $P < 0.01$; ** $P < 0.05$.

To determine whether the glial trophic effect depended on direct contact with the tumor cells, we cocultured tumor cells with their homologous glia, either permitting direct contact or separating the two with a 0.4- μ m pore membrane. As shown in Figure 5D, glia that were separated from the tumor cells stimulated tumor cell proliferation ($P < 0.05$), implying that diffusible factors at least partially mediate the glial effects.

We next addressed whether the tumor-associated glia differ from normal retinal glia in their ability to support retinoblastoma cell proliferation, using a retinal glia preparation²¹ from 7-day-old mice. When examined 7 days after plating, the adherent retinal glia expressed Sox2 and nestin, similar to the tumor-associated glia. However, only ~30% of the glia expressed Pax2, and ~40% expressed CRALBP (Figure 4B), suggesting that the cultures were mixtures of retinal astrocytes and Müller cells. This idea was further supported by our finding that the proportion of Pax2⁺ cells was fairly constant, despite that GFAP expression in the Pax2⁺ cells progressively declined to undetectable levels over two weeks in culture (data not shown). Nevertheless, as with the tumor-associated glia, the cocultured retinal glia increased tumor cell growth and the proportion of cycling Ki-67⁺ tumor cells and decreased the proportion of TUNEL⁺ cells (Figure 5E). Thus, as for the tumor-associated glia, normal retinal glia enhanced retinoblastoma cell proliferation and survival.

IGFBP-5 Contributes to the Glial Trophic Effect

As retinoblastoma cells have properties of neoplastic cone photoreceptors,³ we explored whether factors that are known to promote cone photoreceptor survival mediate the trophic effect of the glia on cocultured retinoblastoma cells. Cone photoreceptor survival was previously shown to be enhanced by retinal glia-conditioned medium,^{35,36} and CTGF, IGFBP-5, and FGF2 were identified as retinal glia-derived factors that promote photoreceptor survival.^{16,37} Other factors that promote photoreceptor survival, but which have not as yet been identified in retinal glia-conditioned medium, include brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), glia-derived neurotrophic factor (GDNF), neurotrophin-3 (NT3), and VEGF.^{38–40} Thus, we determined whether any of these factors were selectively expressed in retinoblastoma-derived glial preparations.

In qRT-PCR assays, IGFBP5, CTGF, and VEGFA RNAs were detected in each of three retinoblastoma glial preparations, but were not detected, or were present at far lower levels, in retinoblastoma cells (Figure 6A). In contrast, FGF2, BDNF, CNTF, NT3, and GDNF were inconsistently expressed in retinoblastoma glia and also detected in some early passage retinoblastoma cell preparations. Notably, IGFBP5 and CTGF RNAs were far more abundant than the others, when compared with β -actin RNA (see x axis scales in Figure 6A). Furthermore, IGFBP5 was expressed at higher levels in glia that were cocultured with abundant as opposed to rare retinoblastoma cells (Figure 6B, compare GL185 and

Figure 6. Expression and function of IGFBP-5 in retinoblastoma cultures. **A:** qRT-PCR analyses of the indicated RNAs in retinoblastoma glia preparations (GL185, GL186, and GL55T) and in primary RB185, early passage RB177, and established Y79 retinoblastoma cells. All expression is normalized to β -actin mRNA. P values for the mean of the three glial versus retinoblastoma preparations were determined by the Student t test and are shown within each graph. NS indicates not significant, with $P > 0.05$. **B:** qRT-PCR analysis of IGFBP5 RNA in GL185 (cultured with abundant RB185 cells), GL186 (cultured with rare RB186 cells), GL194 (cultured either with or without RB194 cells), and the indicated retinoblastoma preparations. Cocultured glial and retinoblastoma cells were separated before RNA preparation. P value determined by Student t test. **C:** Effect of the indicated factors or tumor-derived glia in the presence of IGFBP-5 antibody (Ab) or an isotype control (IgY) on proliferation of RB191 (left) and RB192 (right) retinoblastoma cells. Values indicate cell number at eight days after plating, relative to the cell number at day 0. The RB191a and RB191b panels represent separate experiments. Cell number differences were evaluated using the analysis of variance least significant difference method. $**P = 0.05$, $*P < 0.05$ relative to the control. **D:** qRT-PCR analyses of IGFBP5 (left) and IGFBP5R (right) expression in the indicated tumor-derived glia (GL) or retinoblastoma (RB) cells. P values relate to the mean expression for the three glial versus the six retinoblastoma samples, determined using the Student two-sample t test.

GL186), and dramatically declined when retinoblastoma cells were removed (Figure 6B, compare the two GL194 samples).

We next examined whether factors that were selectively expressed in glia (CTGF, IGFBP-5, and VEGF) or that were identified as trophic components of glia-conditioned medium (CTGF, IGFBP-5, and FGF2)^{16,37} could

mimic the trophic effects of the glia on primary RB191 and RB192 retinoblastoma cells. In the absence of glia or exogenous factors, RB191 cells proliferated slowly and RB192 cell numbers declined (Figure 6C). However, IGFBP-5 significantly increased the proliferation of both preparations, whereas FGF2, VEGF, and CTGF had smaller or insignificant effects. Moreover, a neutralizing

IGFBP-5 antibody but not an IgY isotype control suppressed the trophic effect of cocultured glial cells (Figure 6C). This antibody was likely to have acted by interfering with IGFBP-5, as it did not recognize other IGF binding proteins.²² The glial cells' prominent expression of IGFBP-5, the trophic effect of recombinant IGFBP-5, and the suppression of the glial trophic effect with neutralizing IGFBP-5 antibody all support the conclusion that glia-derived IGFBP-5 promoted retinoblastoma cell proliferation and survival.

As IGFBP-5 can act through IGF-dependent as well as IGF-independent mechanisms,⁴¹ we addressed whether IGFBP-5 might collaborate with IGFs that are produced either by the retinoblastoma glia or by the primary retinoblastoma cells. As shown in Figure 6D, RNAs encoding IGF1, IGF2, and the major IGF1 receptor (IGF1R) were detected in four primary, one early passage, and one established retinoblastoma cell preparation, but were present at significantly lower levels in retinoblastoma-associated glia. Moreover, exogenous IGF1 promoted retinoblastoma cell proliferation to a similar extent as coculture with retinoblastoma glia, and IGFBP-5 provided little or no benefit when provided in combination with exogenous IGF1 (Figure 6C). Thus, IGFBP-5 augmented the proliferation of IGF-expressing retinoblastoma cells, but had minimal benefit in the presence of supraphysiologic IGF levels.

Discussion

It is increasingly recognized that non-neoplastic cells in the tumor microenvironment influence tumor behavior and ultimately the disease course of the host.⁴² As such, an understanding of cancer pathogenesis requires that all cell types in a tumor are identified and their contributions to tumorigenesis defined. Characterizing the collaborating cell types in retinoblastoma could be particularly informative, as it could establish how non-neoplastic cells cooperate with a specific oncogenic mutation—the inactivation of *RB1*—in the cells that give rise to this malignancy. To gain insight into the role of the microenvironment, this study focused on the glial cells that are a consistent component of retinoblastoma tumors.

GFAP⁺ retinoblastoma glia were previously found to express Rb and/or to retain wild-type *RB1* alleles,³ indicating that most and potentially all of the glia are non-neoplastic. This study further establishes that the majority of the glia have properties of retinal astrocytes, proliferate along with the neoplastic retinoblastoma cells, and comprise a subset of the stem cell-related cells within the tumors. In addition, our results indicate that retinoblastoma glia could contribute to tumorigenesis by providing IGFBP-5-mediated trophic support to the cone-like Rb-deficient tumor cells.

Retinoblastoma Glia Have Properties of Non-Neoplastic Retinal Astrocytes

Several lines of evidence suggest that retinoblastoma glia originate from the astrocytes that enter the eye via the

optic nerve during retinal vasculogenesis. Most importantly, the majority of the glia coexpressed GFAP and the retinal astrocyte lineage marker Pax2, regardless of whether the cells were coalesced around the tumor vasculature, as is typical of astrocytes in the normal retina, or were interspersed among the neoplastic cells. Notably, Pax2 was detected in astrocytes but not Müller cells in a retinoblastoma-associated retina, despite that the Müller cells became GFAP⁺ in the presence of the tumor, indicating that Pax2 is not induced as a consequence of Müller cell activation. The Pax2⁺ cells also expressed Sox2 and proliferated, in keeping with the proliferation of Sox2⁺ astrocytes in other settings.³² Thus, the behavior of the Pax2⁺, GFAP⁺ cells is consistent with that of retinal astrocytes and an origin from the retinal astrocyte lineage.

An astrocytic origin of the glia is also supported by studies of early eye development. In particular, prior studies showed that Pax2 is an important determinant of the retinal astrocyte lineage,⁴³ and that cells that give rise to retinal astrocytes are differentiated from those that generate the neural retina through the reciprocal repression of Pax2 and Pax6.⁴⁴ This reciprocal repression could underlie the failure of retinal progenitor cells (which are Pax6⁺) to produce astrocytes during normal retinal development,⁴⁵ and could similarly require that Pax2⁺ tumor-associated astrocytes derive from Pax2⁺ retinal astrocytes, rather than from Pax6⁺ retinal progenitors, during retinoblastoma tumorigenesis.

Importantly, Pax2 was expressed by the adherent cells that grew from explanted retinoblastomas, suggesting that these cells likewise derived from retinal astrocytes. It is noted that the adherent cells lacked the astrocyte marker GFAP, yet this was consistent with the progressive loss of GFAP in freshly explanted Pax2⁺ retinal astrocytes under the same tissue culture conditions (data not shown). As these cells grew quite robustly, it is plausible that adherent cells that were previously cultured from retinoblastomas³⁴ or from mouse retinal tumors⁴⁶ constituted a similar retinal astrocyte population.

Stem Cell-Like Features of Retinal Astrocytes and Other Non-Neoplastic Cells in Retinoblastoma Tumors

This study also addressed the relationship of the retinoblastoma glia to recently described stem cell-like cells in retinoblastoma tumors. The stem cell-like cells were reported to express neural stem cell and other stem cell-associated proteins and were suggested to act as cancer stem cells, although their neoplastic status was not established.^{9,10} We found that Pax2⁺ retinoblastoma glia coexpressed Sox2 and that the Sox2⁺ cells were non-neoplastic, as they expressed Rb and/or retained two *RB1* alleles. As Sox2 is a consistent neural stem cell marker,³³ these findings raise the possibility that some of the previously described neural stem cell-like cells in retinoblastomas consisted of non-neoplastic retinal astrocytes. On the other hand, the retinoblastoma glia were distinct from cells that prominently expressed the stem cell-

associated ABCG2. Nevertheless, the strongly ABCG2⁺ cells expressed Rb and had the appearance and CD31 staining that is characteristic of endothelial cells, in keeping with the presence of ABCG2⁺ endothelial cells in the normal retina and other neuronal tumors.^{47,48}

We conclude that non-neoplastic retinal astrocytes could largely account for the neural stem cell like-cells in retinoblastomas, whereas non-neoplastic endothelial cells could largely account for the ABCG2⁺ cells. Thus, our data do not support the view that stem cell-like cells in retinoblastomas generally comprise a cancer stem cell population.

Proliferative Effects of the Retinoblastoma Glia and IGFBP-5

This study also established that tumor-derived as well as normal retinal glia enhance the proliferation and survival of cultured retinoblastoma cells, and that the trophic effect is mediated at least in part by diffusible factors. IGFBP-5 contributed to this trophic effect, as the glia expressed IGFBP-5 and their effect was mimicked by recombinant IGFBP-5 and abrogated by neutralizing IGFBP-5 antibody. Interestingly, neoplastic retinoblastoma cells greatly enhanced *IGFBP5* expression in the cocultured glia (Figure 6B), implying that the two cell types partake in bidirectional paracrine signaling. The involvement of IGFBP-5 is of particular interest, as IGFBP-5 is produced by normal retinal glia and can promote cone photoreceptor survival.³⁷ Thus, IGFBP-5 is a potent glia-derived trophic factor for normal cone photoreceptors as well as for their neoplastic retinoblastoma counterparts.

It is currently unclear how IGFBP-5 exerts its trophic effect on retinoblastoma cells, including whether it primarily increases cell division or cell survival, and whether it exerts its effects via IGF signaling. However, it may be relevant that IGF1 largely supplanted the trophic effect of IGFBP-5, and that retinoblastoma cells expressed IGF1, IGF2, and IGF1R (Figure 6, C and D). These observations are consistent with a scenario in which glia-derived IGFBP-5 augments the autocrine effect of retinoblastoma-derived IGFs, similar to the ability of IGFBP-5 to augment IGF-mediated tumor cell proliferation and survival in other settings.^{49–51} However, additional studies are needed to evaluate this possibility.

Implications for Retinoblastoma Tumorigenesis

An important yet unresolved issue is whether the trophic effects of the retinoblastoma astrocytes and IGFBP-5 that were evident *in vitro* also contribute to retinoblastoma tumorigenesis *in vivo*. In support of this possibility, *IGFBP5* RNA is expressed in the normal retina and is up-regulated in angiomatous tufts during retinal neovascularization.⁵² Moreover, an oncogenic role of IGFBP-5 is not without precedent, as IGFBP-5 is overexpressed in diverse cancers,^{53,54} is required for neuroblastoma anchorage-independent growth,⁵¹ and enhances the *in vivo* tumorigenic potential of androgen-dependent prostate cancer cells.^{49,50}

If retinal astrocytes were indeed to contribute to retinoblastoma tumorigenesis, then this could have several important ramifications. For example, it might then be necessary for early *RB1*-mutated retinoblastoma cells to gain access to retinal astrocytes before developing into clinically detectable tumor foci. As retinal astrocytes extend processes only to the ganglion cell or inner plexiform layer, such a requirement could favor the development of retinoblastoma foci in the inner retina, regardless of whether the tumor cell of origin derives from outer nuclear layer cone precursors as we have suggested,³ or from inner nuclear layer cells as proposed by others.⁵⁵ A requirement that retinoblastoma cells gain access to astrocytes could also delay the development of retinoblastomas that originate in the outer nuclear layer, such that their initial appearance (which usually occurs from ~8 months gestation through the second postnatal year) substantially follows retinal cell cycle exit and differentiation in the mid-gestation fetal retina.⁵⁶ Moreover, astrocytic trophic support could influence the metastatic spread of retinoblastoma along the astrocyte-rich optic nerve²³ and might be therapeutically targeted, such as by interfering with IGFBP-5.

In summary, the current studies reveal that non-neoplastic retinal astrocytes populate retinoblastoma tumors and produce factors that support retinoblastoma cell proliferation and survival. The retinal astrocyte trophic effect appears to be mediated, at least in part, by IGFBP-5, which is a factor that also provides trophic support to normal cone photoreceptors. Thus, the findings suggest that the beneficial effects of retinal astrocytes on normal photoreceptors could be co-opted during tumorigenesis to promote the growth and survival of the cone-like retinoblastoma cells. In this way, retinal astrocytes could contribute to a microenvironment that is exceptionally conducive to retinoblastoma tumorigenesis. More generally, the studies suggest that tissue-specific non-neoplastic cells may cooperate with oncogenic mutations in a tumor cell of origin to promote the development of human cancers.

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