



Sorbitol causes preferential selection of Müller glial precursors from late retinal progenitor cells in vitro

Tasneem Zahir,¹ Henry Klassen,² Minoru Tomita,¹ Michael J. Young¹

¹Schepens Eye Research Institute, Harvard Medical School, Boston, MA, ²Singapore Eye Research Institute, 168751, Singapore

Purpose: The replacement of glucose by sorbitol in growth medium causes selection of astroglial cells from heterogeneous primary cultures derived from the brains of newborn mice. The present study was undertaken to investigate the effects of sorbitol on in vitro selection of Müller glial precursors from expanded late retinal progenitor cells (RPCs).

Methods: RPCs used in these studies were isolated from the neural retina of postnatal day one green fluorescent protein (GFP) transgenic mice. The resulting GFP positive neurospheres were dissociated into a single cell suspension and grown on poly-D-lysine/laminin coated tissue culture flasks or slides to generate adherent RPCs. These adherent cells were treated with glucose free medium containing 25 mM sorbitol for 7 days and the expression of retinal-specific cell markers was determined by immunocytochemistry, reverse transcriptase polymerase chain reaction (RT-PCR) and immunoblot analysis

Results: In vitro studies showed that sorbitol treatment of late RPCs altered cellular morphology. Immunocytochemical studies showed an increase in the proportion of cells expressing glial cell markers, most of which co-expressed CRALBP, GFAP, and vimentin. An increase in the proportion of cells expressing PKC α , a bipolar cell marker, was also observed. RT-PCR analysis showed down-regulation of nestin transcripts with a concomitant increase in CRALBP, GFAP, vimentin and PKC α . These findings were confirmed by immunoblot analysis, where down-regulation of nestin expression with simultaneous up-regulation of CRALBP, GFAP and PKC α was observed.

Conclusions: Sorbitol treatment of multipotent late RPCs, in the absence of glucose, results in the preferential selection of Müller glial precursors and their subsequent differentiation into cells that morphologically resemble Müller cells and co-express multiple glial markers.

The vertebrate retina is comprised of seven distinct cell types that are derived from a common progenitor cell. These include six classes of neurons and one glial cell type, the Müller glial cell [1]. Müller cells are astrocyte-like glial cells that span the width of the retina from the inner to outer limiting membranes [2]. They are considered the principal glial cells of the retina because they perform many of the functions carried out by astrocytes, oligodendrocytes and ependymal cells in other regions of the central nervous system [3]. Functions provided by Müller cells include maintaining the complex retinal architecture, giving structural and metabolic support to retinal neurons. Müller cells are also involved in the visual cycle through the synthesis and renewal of cone visual pigments. As part of this process they bind all-trans retinol, convert it into 11-cis-retinol and then release it into the extracellular space for uptake by cone photoreceptors [4,5].

More recently, another quite different role has been attributed to Müller glia, namely that of generating new neuronal cells during retinal regeneration. Because they also generate glial cells, Müller cells may constitute a previously unidentified stem cell in the vertebrate retina that becomes activated upon injury. Evidence of this comes from studies in the post-hatch chick retina where the endogenous Müller cells

dedifferentiate, proliferate, express markers common to retinal progenitors, and produce new neurons in response to neurotoxic damage [6,7]. Furthermore, it has been demonstrated that neurotoxic damage is not essential for activation of retinal regenerative mechanisms since the endogenous glial cells become neurogenic following intravitreal injections of exogenous growth factors [8-10]. In a recent study, researchers showed that the capacity for retinal regeneration is not restricted to lower vertebrates, in that Müller cells generated new retinal neurons in the adult mammalian retina after toxic damage [11]. Furthermore, the fate of these regenerated neurons could be partially controlled via extrinsic factors or intrinsic genes.

The efforts of several groups have been focused on demonstrating that mature Müller glia have the potential to acquire the characteristics of retinal progenitors and can serve as a source of regeneration in the vertebrate retina. In contrast, our goal is to develop strategies for generating Müller glia from cultured late retinal progenitor cells. (RPCs) This work has implications for both retinal tissue engineering and the control of Müller cell activation and subsequent gliosis in a range of retinal diseases.

In this study, we determine the effect of sorbitol on the in vitro selection of Müller glial precursors from expanded late RPCs and their subsequent differentiation. The enzymes of the sorbitol pathway, aldose reductase and sorbitol dehydrogenase, along with the sorbitol uptake system, are present in

Correspondence to: Michael J. Young, Schepens Eye Research Institute, Department of Ophthalmology, Harvard Medical School, 20 Staniford Street Boston, MA 02114; Phone: (617) 912 7419; FAX: (617) 912 0101; email: mikey@vision.eri.harvard.edu

rat astroglia-rich primary cultures but have not been detected in rat neuron-rich cultures [12,13]. Because of the absence of the appropriate enzymes for the uptake and metabolism of sorbitol, mouse brain-derived oligodendroglia, microglia and ependymal cells cannot survive in media containing sorbitol instead of glucose. Hence, the replacement of glucose by sorbitol has been used for the selection of astroglia from heterogeneous primary cultures of newborn mouse brains [14].

The present study was undertaken to investigate the effects of sorbitol on *in vitro* differentiation of late RPCs. As the progeny of late retinogenesis include rod photoreceptors, bipolar neurons and Müller glia, we sought to determine if sorbitol treatment of late RPCs would preferentially promote Müller cell differentiation, either directly through the induction of late RPCs down the glial pathway or indirectly by selection of Müller glial precursors.

METHODS

Isolation and culture of late retinal progenitor cells: RPCs were isolated from the neural retina of postnatal day one GFP transgenic mice [15]. Briefly, retinas from newborn GFP transgenic mice (gift from Dr. Masaru Okabe, University of Osaka, Japan) [16] were surgically removed and the ciliary marginal zone and optic nerve head dissected. The tissue was finely chopped with sterile forceps and digested with 0.1% type I collagenase (Sigma-Aldrich, St. Louis, MO) for 20 min. The supernatant containing dissociated cells was then passed through a 100 mm mesh strainer, centrifuged and seeded in complete medium (CM) containing Neurobasal media (with 25 mM D-glucose; Invitrogen, Carlsbad, CA), B27 neural supplement (Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Sigma-Aldrich), 100 mg/ml penicillin-streptomycin (Sigma-Aldrich) and 20 ng/ml epidermal growth factor (recombinant human EGF; Invitrogen). This cycle was repeated until all retinal tissue was digested. Cells were refed every 2 days. GFP+ neurospheres appeared within 2 to 3 weeks and were passaged at regular intervals. To assess the capacity for self-renewal, spheres were broken up and plated as single cells. Individual cells formed new spheres over a period of 5-7 days. These spheres labeled positively for nestin (a marker for neural progenitor cells; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA) and Ki67 (a marker for cell proliferation; Novocastra Laboratories, Newcastle, UK) [15].

***In vitro* differentiation of late retinal progenitor cells:** GFP+ neurospheres were dissociated into a single cell suspension and grown on poly-D-lysine and laminin coated tissue culture plates to generate adherent RPCs that were subsequently exposed to sorbitol treatment. Briefly, passage 20 cells from confluent T75 flasks were trypsinized and dissociated into a single cell suspension (200,000 cells/ml). These cells were then seeded onto Biocoat poly-D-lysine/mouse laminin coated eight well culture slides or flasks (BD Biosciences, Bedford, MA) and allowed to grow for 12-16 h at 37 °C in CM. The CM containing EGF was then removed and the cells washed with Ca²⁺ and Mg²⁺ free HBSS (Invitrogen, Carlsbad, CA). Selection/Differentiation medium containing DMEM F12 modified (with 2 mM L-glutamine, with 25 mM D-sorbitol,

without glucose, custom ordered from SAFC BioSciences, Lenexa, KS), B27 neural supplement (Invitrogen, Carlsbad, CA), and 100 mg/ml penicillin-streptomycin (Sigma-Aldrich), but no EGF, was then added to the experimental wells or flasks. Controls were treated with CM containing glucose but no sorbitol. The medium was changed every 3 days for up to 7 days. The cells grown in flasks were used for RT-PCR and immunoblot analysis whereas the cells grown on culture slides were fixed at 7 days post-sorbitol treatment for immunocytochemical studies.

Immunocytochemistry: RPCs cultured on Biocoat poly-D-lysine/mouse laminin culture slides were processed according to standard protocols. Briefly, cells were fixed in 4% paraformaldehyde (pH 7.2) in 0.1 M phosphate buffered saline (PBS) and blocked in PBS containing 2% BSA and 0.5% Triton X-100. They were then incubated with appropriate primary antibodies (Table 1) for 2 h at room temperature. Antibody incubations were conducted in PBS containing 2% BSA. Cells were washed three times for 10 min each with PBS and incubated with species specific secondary antibodies conjugated with Cy2 or Cy3 (Jackson ImmunoResearch Laboratory, Bar Harbor, ME) for 1 h at room temperature. Subsequently, cells were washed three times with PBS, coverslipped in 2.5% PVA (polyvinyl alcohol)/DABCO (1,4 diazabicyclo/2,2,2 octane) and examined under an epifluorescence microscope (Nikon Eclipse, E800). For double-labeling studies, cells were treated with 100% methanol for 30 min at -20 °C, prior to fixing with 4% paraformaldehyde. This was followed by several washes of 20 min each with PBS to eliminate GFP fluorescence. Note that for double labeling studies the cells were not incubated with a mixture of primary and secondary antibodies. Instead they were incubated with one set of primary and secondary antibodies followed by the second set of primary (rabbit polyclonal GFAP and goat polyclonal vimentin, Table 1) and secondary antibodies. Semi-quantitative immunocytochemistry was performed by counting a total cell number of at least 200 cells per well in randomly selected fields using 4',6-Diamidino-2-phenylindole (DAPI) labeling. Each determination was performed in quadruplicate (n=4), and each experiment was repeated at least three times.

Reverse transcriptase polymerase chain reaction analysis: Total RNA was isolated from RPCs (10⁶ cells per sample), grown in the absence or presence of sorbitol, using an RNAqueous-4PCR kit (Ambion, Austin, TX). Reverse transcription reactions were set up using the RETROscript kit (Ambion). A 2-step RT-PCR approach was used, involving an initial step of heat denaturation of RNA to obtain cDNA followed by a step of PCR amplification using gene specific primers (Table 2). The thermocycler program used for PCR amplification included a hot start (95 °C for 1 min) followed by denaturation at 94 °C for 30 s, annealing at specific temperatures (Table 2) for 30 s for 30 cycles, extension at 72 °C for 30 s for 30 cycles and final extension at 72 °C for 5 min. RT-PCR reactions (cDNA) were run on 2% agarose gels (containing ethidium bromide at a final concentration of 0.5 mg/ml) against a 100 bp ladder and products visualized under UV light. The amount of cDNA was normalized based on the signal from

constitutively expressed S15 mRNA, which encodes a small ribosomal subunit protein.

Immunoblot analysis: RPCs, grown in the absence or presence of sorbitol, and B6 mouse retinas (6 weeks), were homogenized separately in lysis buffer (1% Triton-X 100, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 50 mM NaCl, 50 mM NaF) containing protease inhibitor cocktail (1:100 dilution; Sigma-Aldrich) and phosphatase inhibitor (1:100 dilution; Sigma-Aldrich). Protein levels in total cell lysates were quantified using a protein assay kit (Bio-Rad, Hercules, CA). The protein samples (20 mg) were separated on poly-acrylamide gels (NuPage; Invitrogen) for 40 min at 160 V and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen; Invitrogen) for 60 min at 30 V. After transfer, the membranes were blocked in 5% nonfat dry milk in TBS-T (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) for 30 min. The blots were incubated with the following primary antibodies: nestin (1:20 dilution; DSHB), CRALBP (1:10,000 dilution; ABR), GFAP (1:1000 dilution; Chemicon), PKC α (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and recoverin (1:1000 dilution; Chemicon, Temecula, CA). Subsequently, blots were incubated with horseradish peroxidase (HRP) conjugated species-specific secondary antibodies and the signals visualized with the ECL Western blotting detection system (GE Healthcare Bio-Sciences, Piscataway, NJ).

RESULTS

Sorbitol-induced changes in morphology of late retinal progenitor cells: Adherent RPCs generated from dissociated RPC neurospheres continued to proliferate and formed a monolayer. Most of these cells remained morphologically undifferentiated, although a small number extended short processes (Figure 1A). After sorbitol treatment the proliferation of adherent RPCs decreased dramatically. Many of these cells displayed a bipolar, spindle shaped morphology, often giving rise at one pole to relatively wide cytoplasmic projections resembling endfeet and at the opposite pole to a long smooth process terminating in a growth cone-like structure (Figure 1B).

Semi-quantitative evaluation of effect of sorbitol on protein expression using immunocytochemistry: The effect of sor-

bitol on the expression of proliferative, progenitor, glial and bipolar cell markers was determined by immunocytochemistry (Table 3). In the adherent RPC control population, 30.2% of cells expressed Ki67 (Table 3; Figure 2A) compared to 4.5% of adherent RPCs after sorbitol treatment (Table 3; Figure 2B). After 7 days of treatment, the percentage of RPCs expressing CRALBP, GFAP and vimentin had increased from 0 to 32.2%, 7.2% to 52.2%, and 0 to 49.3%, respectively (Table 3; Figure 2E-J). Sorbitol also had an inductive effect on PKC α expression. The percentage of cells expressing PKC α increased from 9% to 22.3% (Table 3; Figure 2K-L). In contrast to the increase observed in the percentage of cells expressing bipolar and glial markers, sorbitol treatment decreased the percentage of cells that stained positive for nestin from 92.5% to 46.7% (Table 3; Figure 2C-D).

Double-labeling with glial markers for cell identification: The expression of different glial markers (CRALBP, GFAP, vimentin) by RPCs grown in the presence of sorbitol led us to test whether these RPCs co-labeled with different combinations of glial markers (CRALBP and GFAP, CRALBP, and vimentin, vimentin, and GFAP). After 7 days of treatment 100% of the cells that expressed CRALBP (32.2% of total cell population) also expressed GFAP and vimentin (Figure 3G-I, Figure 3J-L). However, not all the GFAP-positive cells were CRALBP-positive (32.2% \pm 1.9 versus 52.2% \pm 2.3 of total cell population, Figure 3G-I). In contrast, all the GFAP-positive cells were vimentin-positive (52.2% \pm 2.3 versus 49.4% \pm 3.4 of total cell population, Figure 3M-O). In addition, the sorbitol treated cells were also examined for co-expression of Ki67 and GFAP, nestin and GFAP, as well as PKC α and GFAP. These cells did not co-express Ki67 and GFAP or PKC α and GFAP (Figure 3A-C, Figure 3P-R). In contrast, co-localization of nestin and GFAP immunoreactivity was observed although the overlap was not 100% (Figure 3D-F).

Effect of sorbitol on transcription of glial, bipolar, and progenitor cell markers: Semi-quantitative RT-PCR analysis was carried out to determine the effects of sorbitol on the preferential selection of Müller glial precursors from late RPC and their subsequent differentiation (Figure 4A). RPCs grown in the absence of sorbitol (referred to as RPCs in Figure 4A)

TABLE 1. LIST OF PRIMARY ANTIBODIES USED FOR IMMUNOCYTOCHEMISTRY

Antibodies	Type	Specificity	Source	Dilution
CRALBP	Mouse monoclonal	RPE and Muller glia	ABR	1:1000
GFAP	Mouse monoclonal Rabbit polyclonal	Astrocytes and Muller glia	Chemicon Dako	1:400 1:3000
Ki67	Mouse monoclonal	Proliferating cells	Novocastra	1:100
nestin (Rat-401)	Mouse monoclonal	Neural progenitors	DSHB	1:1
PKC α	Mouse monoclonal	Bipolar neurons	Santa Cruz	1:200
recoverin	Rabbit polyclonal	Rod photoreceptors and midget cone bipolar neurons	Chemicon	1:1000
vimentin	Mouse polyclonal Goat polyclonal	Astrocytes and Muller glia	Sigma Sigma	1:200 1:40

The following primary antibodies were utilized for immunocytochemical analysis of late RPCs grown in the absence or presence of sorbitol.

TABLE 2. LIST OF PRIMERS USED FOR REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION ANALYSIS

Genes	Primer sequences	Product size (bp)	Genbank accession number	Temp ($^{\circ}$ C)
nestin	F: AACTGGCACACCTCAAGATGT R: TCAAGGGTATTAGGCAAGGGG	235	NM_016701	60
CRALBP	F: TACCCTGGTGTCTTCCAG R: GGTTCCTCATTTCACAGC	150	NM_020599	63
GFAP	F: CACGAACGAGTCCCTAGAGC R: ATGTTGATGCGGTTTCTTC	234	NM_010277	60
vimentin	F: ATGCTTCTCTGGCAGCTCT R: AGCCACGCTTTCATACTGCT	206	NM_011701	64
PKC α	F: CCCATTCCAGAAGGAGATGA R: TTCCTGTCAGCAAGCATCAC	212	NM_011101	60
recoverin	F: ATGGGGAATAGCAGAGCGG R: GAGTCCGGAAAACTTGGGAATA	179	NM_009038	60
S15	F: TTCGCAAGTTCACCTACC R: CGGGCCGGCCATGCTTACG	361	NM_001018	60

The following primers were utilized for RT-PCR analysis of late RPCs grown in the absence or presence of sorbitol.

showed high levels of nestin transcripts. After 7 days of sorbitol treatment there was down-regulation of nestin transcripts and a simultaneous up-regulation of CRALBP, GFAP and vimentin transcripts. The down-regulation of nestin transcripts, along with the concomitant increase in CRALBP, GFAP, vimentin, and PKC α transcripts, suggested that sorbitol increased the number of RPCs expressing markers of glial and bipolar neurons. In addition, the complete absence of recoverin confirmed the lack of induction of photoreceptor or cone bipolar differentiation by sorbitol. The RT-PCR results were corroborated by immunoblot analysis of late RPCs grown in the absence or presence of sorbitol (Figure 4B). Late RPCs grown in the absence of sorbitol (referred to as RPCs in Figure 4B) expressed high levels of nestin protein. In addition, low levels of GFAP and PKC α protein expression were also detected in these cells at baseline levels. Treatment with sorbitol for 7 days resulted in an induction of CRALBP and GFAP expression along with a dramatic decrease in nestin expression. Furthermore, RPCs treated with sorbitol showed an increase in PKC α expression (Figure 4B). In this study retinas from B6 mice (4 weeks) were used as negative control for nestin expression and positive control for CRALBP, GFAP, PKC α and recoverin expression. The data from immunoblot analysis confirmed that sorbitol treatment induced the late RPCs to up-regulate glial and bipolar markers. As with the RT-PCR analysis, the absence of recoverin expression excluded the possibility of differentiation into photoreceptors or cone bipolar cells.

DISCUSSION

Primary cultures derived from the brains of newborn rodents contain neurons, oligodendrocytes, ependymal and other cell

types, but are dominated by astroglial cells. Sorbitol, a long-chained polyol, is taken up in glial cells by diffusion through a proteinaceous channel-like structure that does not involve a carrier or direct penetration of the lipid bilayer [12]. Since the enzymes of the sorbitol pathway and sorbitol uptake system, are present in astroglia but not neurons [12,13], the replacement of glucose by sorbitol has been used for astroglial selection from cultured mouse brain [12,14]. The effect of sorbitol on *in vitro* selection and differentiation of Müller glial precursors from expanded late RPCs has not been studied previously.

The RPCs studied here are multipotent and have the capacity to differentiate into the cell types generated during late retinogenesis [1,17,18]. Results from our immunocytochemical, RT-PCR and immunoblot analyses indicated that sorbitol induced a significant number of late RPCs to differentiate, either directly through the induction of late RPCs down the glial pathway or indirectly by selection of Müller glial precursors, into cells that morphologically resembled Müller cells and also expressed the glial markers CRALBP, GFAP and vimentin. In

TABLE 3. QUANTITATIVE EVALUATION OF EFFECT OF SORBITOL ON THE EXPRESSION OF DIFFERENT CELL MARKERS

Antibodies	Specificity	-Sorbitol in vitro ^a	+Sorbitol in vitro ^b
Ki67	Proliferating cells	30.2 \pm 2.1	4.5 \pm 1.0
nestin (Rat-401)	Neural progenitors	92.5 \pm 3.0	46.7 \pm 3.6
CRALBP	Muller glia	0	32.2 \pm 1.9
GFAP	Astrocytes and Muller glia	7.2 \pm 0.8	52.2 \pm 2.3
vimentin	Astrocytes and Muller glia	0	49.3 \pm 3.4
PKC α	Bipolar neurons	9.0 \pm 2.2	22.3 \pm 3.0

^{a,b} represents retinal progenitor cells grown in the absence or presence of sorbitol were subjected to quantitative immunocytochemical analysis using the listed markers. Values are expressed as percentage \pm SD, and n=6.

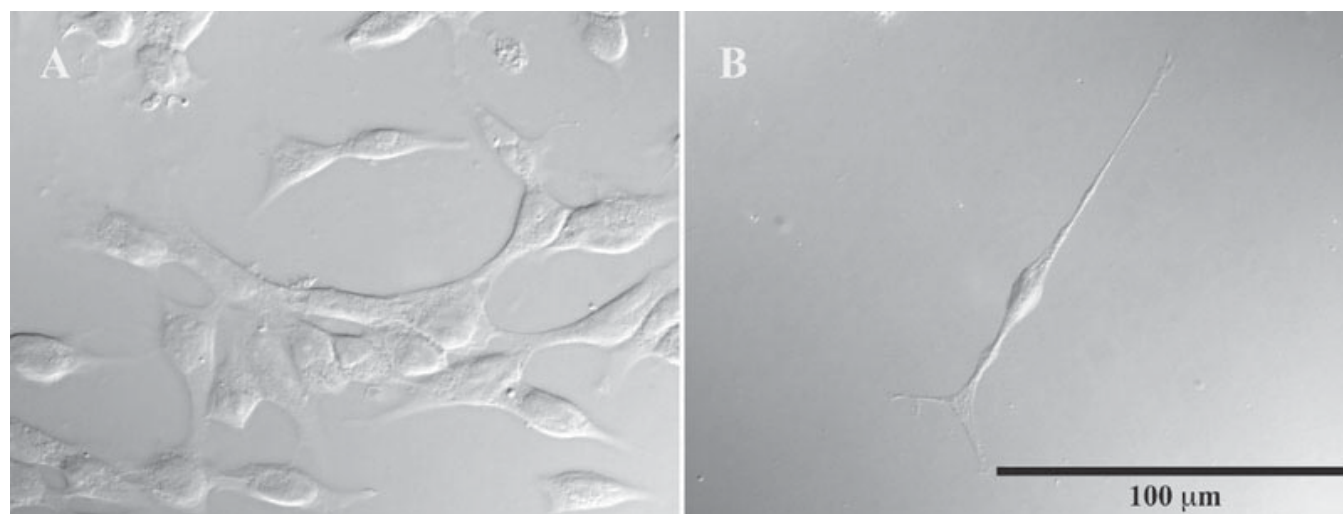


Figure 1. Sorbitol induced changes in the morphology of late RPCs. Panels **A** and **B** show phase contrast images of control RPCs and sorbitol treated RPCs. Control RPC cultures grown in CM media for 12-16 h grew as an undifferentiated monolayer with a few cells giving out short processes (**A**, 600x). However, in the sorbitol treated cultures where CM was replaced with differentiation media after 12-16 h and the cells were grown in this media for 7 days, most of the cells adopted a bipolar, spindle shaped morphology. They became asymmetrical with elongated cell bodies often giving rise at one pole to relatively wide cytoplasmic expansions resembling endfeet, and at the opposite pole to a long smooth process terminating in a growth cone (**B**, 600x).

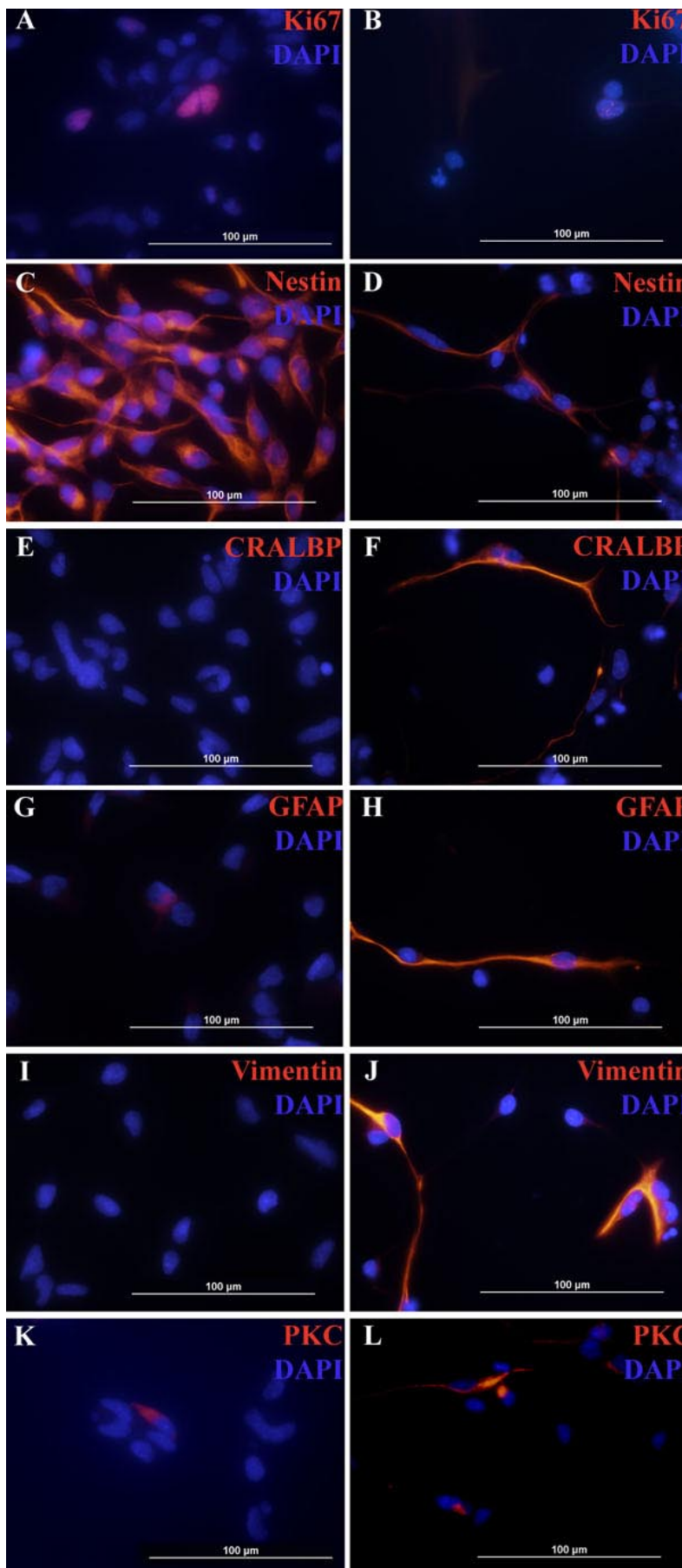


Figure 2. Expression of proliferation, progenitor, glial, and bipolar cell markers by late RPCs before and after sorbitol treatment. Panels A through L show fluorescent images of Ki67, nestin, CRALBP, vimentin, GFAP and PKC α immunoreactivity in combination with DAPI (4',6-Diamidino-2-phenylindole) staining before (A, C, E, G, I, K, 600x) and after (B, D, F, H, J, L, 600x) sorbitol treatment. Sorbitol induced RPCs to up-regulate CRALBP, GFAP, vimentin, and PKC α expression and adopt glial morphology. In contrast, sorbitol treatment resulted in a down regulation of Ki67 and nestin expression.

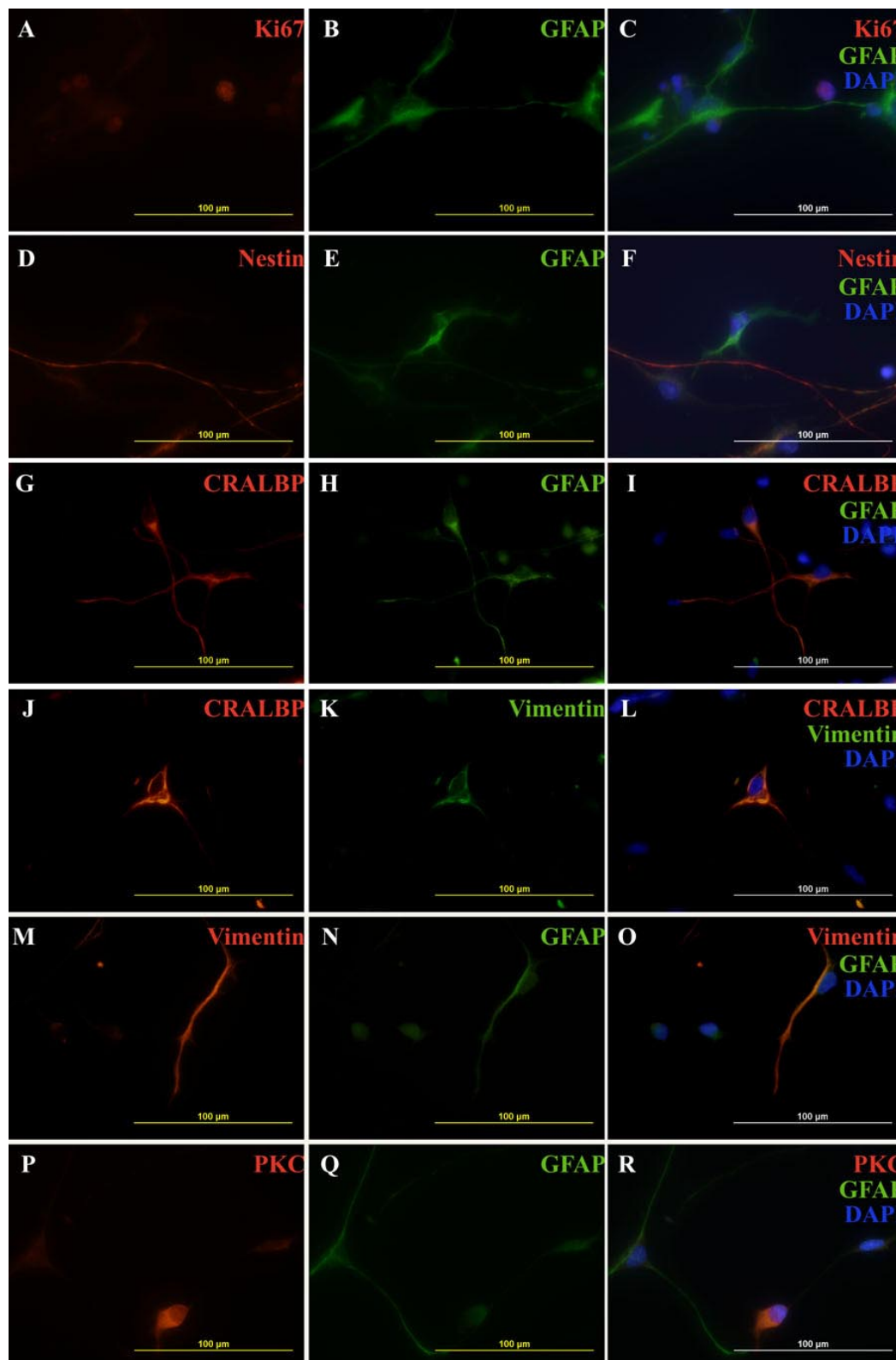


Figure 3. Double-labeling of late RPCs after sorbitol treatment with different cell specific markers. Panels **A, D, G, J, M, and P** (600x) show fluorescent images of Ki67, nestin, CRALBP vimentin, and PKC α immunoreactivity while (**B, E, H, K, N, Q**, 600x) show that the same RPCs express GFAP or vimentin. (**C, F, I, L, O, R**, 600x) represent merged images of (**B, E, H, K, N, Q**) and (**C, F, I, L, O, R**). After 7 days of treatment 100% of the cells that expressed CRALBP also expressed GFAP and vimentin (**G-I, J-L**). However, not all the GFAP-positive cells were CRALBP-positive (**G-I**). In contrast, all the GFAP-positive cells were vimentin-positive (**M-O**). In addition, nestin and GFAP co-expression was observed, although the overlap was not 100% (Figure 3**D-F**), while Ki67 or PKC α were never seen to co-localize with GFAP (**A-C, P-R**).

addition to up-regulation of glial markers, an increase in cells expressing the bipolar cell marker PKC α was also observed. To our knowledge, this is the first study demonstrating that sorbitol has an inductive effect on bipolar cell differentiation. In contrast to its effects on glial and bipolar markers, sorbitol did not induce RPCs to express rod photoreceptor markers.

In the case of nestin and Ki67, sorbitol treatment decreased the percentage of RPCs that stained positive for both markers. Nestin [19], an established marker for CNS progenitor cells, is expressed by these cells in their proliferating, undifferentiated state [15,20,21]. When these cells undergo their final mitotic division and begin to differentiate into mature neurons, nestin expression is down-regulated. Similarly, Ki67 is a marker for cell proliferation (like BrdU pulse labeling) and its expression is used to determine the proportion of cells that are mitotically active. It is a prototypic cell cycle related nuclear protein, expressed by proliferating cells in all phases of the active cell cycle (G1, S, G2, and M phase). The expression of Ki67 is down-regulated in RPCs upon differentiation.

In addition to the first set of immunoreactivity studies, where cells were stained with a single marker, double-labeling studies were also carried out. After 7 days of sorbitol treat-

ment, many of the cells that expressed CRALBP also expressed GFAP and vimentin. The co-expression of CRALBP (cellular retinaldehyde-binding protein) and vimentin (intermediate filament protein) strongly supports Müller cell differentiation, since both these proteins have been extensively used to identify Müller cells. The co-localization of GFAP, an intermediate filament protein that is primarily expressed in retinal astrocytes, with CRALBP, a marker for Müller cells in the adult retina, was not altogether unexpected. While resting Müller cells in the mammalian retina express little or no GFAP *in vivo*, this marker is strongly up-regulated in reactive Müller cells following retinal detachment, retinal injury and photoreceptor degeneration [22-24]. The up-regulation of GFAP expression observed in this study may be a result of the *in vitro* culture system. This possibility is supported by previous reports of increased GFAP expression by cultured Müller glia [25-27]. Thus our finding that Müller cells in culture express GFAP suggests that they are more similar to reactive than resting Müller cells.

Although all the CRALBP positive cells were GFAP-positive, the converse was not the case. However, all GFAP-positive cells were vimentin-positive. We can only speculate as to

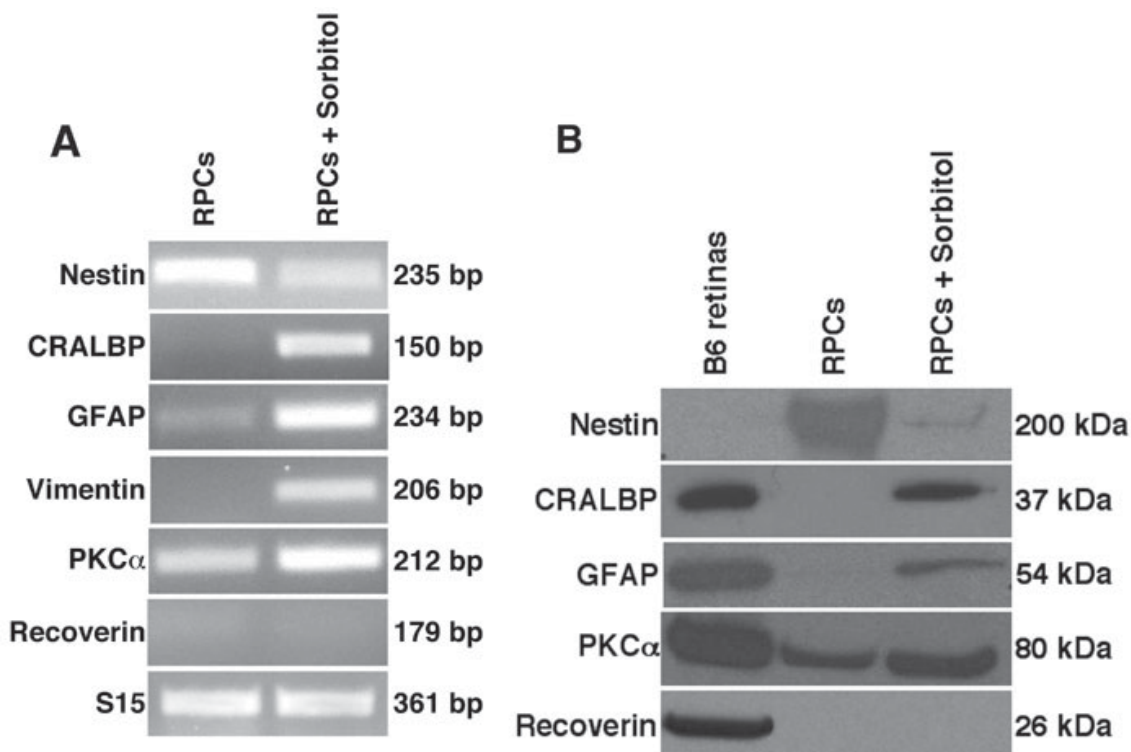


Figure 4. RT-PCR and immunoblot analysis of late RPCs grown in the absence or presence of sorbitol. RT-PCR (A) and Immunoblot (B) analysis of late RPCs grown in the absence or presence of sorbitol. Late RPCs grown in the absence of sorbitol expressed high levels of nestin (neuronal progenitor cell marker, A). After 7 days of sorbitol treatment there was down-regulation in nestin transcripts, along with a concomitant increase in CRALBP, GFAP, vimentin (glial cell markers), and PKC α (bipolar cell marker, A). Transcripts for recoverin (photoreceptor/cone bipolar cell marker) were not detected in untreated or treated RPCs (A). Immunoblot analysis of late RPCs, grown in the absence of sorbitol showed high levels of nestin expression (B). In addition, GFAP and PKC α protein expression was also detected in these cells at baseline levels (B). Treatment with sorbitol for 7 days resulted in an up-regulation of CRALBP, GFAP, vimentin and PKC α expression and a simultaneous down-regulation of nestin expression (B). However, recoverin expression was not detected in untreated or treated RPCs (B). Retinas from B6 mice (4 weeks) were used as negative or positive controls (B).

the identity of the subset of GFAP and vimentin co-expressing cells that did not express CRALBP (approximately 20% of total number of cells). It is tempting to simply call them retinal astrocytes, since both GFAP and vimentin are well-established astrocyte markers [26-29]. However, the lineage of neuroretinal cells does not include retinal astrocytes, which instead are derived from the brain, and migrate into the retina along the optic nerve during development. We instead favor a description of these GFAP/vimentin positive cells as immature Müller cells that do not express all of the markers of mature Müller glia.

In addition, sorbitol treated RPCs did not co-express GFAP and either Ki67 or PKC α . Co-localization of nestin and GFAP was observed, although the overlap was not complete. From these findings we conclude that the cells generated by treatment of RPCs with sorbitol are similar to reactive Müller cells because of up-regulation of GFAP and co-expression of nestin by the same cells [30,31].

Semi-quantitative RT-PCR analysis was carried out to further investigate the effects of sorbitol on late RPC differentiation. The findings of down-regulation of nestin transcripts with concomitant increases in CRALBP, GFAP, vimentin, and PKC α suggested that sorbitol increased the number of RPCs expressing markers of glial and bipolar neurons. Additionally, the complete absence of recoverin indicated the lack of induction of photoreceptor or cone bipolar differentiation by sorbitol.

The RT-PCR results were corroborated by immunoblot analysis. These data confirmed that sorbitol treatment induced the late RPCs to up-regulate glial and bipolar markers. The absence of recoverin expression again argued against the possibility of induction of differentiation of RPCs by sorbitol into photoreceptors or cone bipolar cells.

In the sorbitol treated cultures where CM was replaced with differentiation media after 12-16 h and the cells were grown for 7 days, 30% of the total cells survived compared to the cells in control cultures that were grown in CM media for 12-16 h. Although sorbitol may in fact favor the survival of Müller and bipolar cell precursors over other cell types that exist within the total RPC population (i.e rod photoreceptors), the results of the subsequent differentiation studies offer clues as to the relative importance of this mechanism. As the bipolar cell marker PKC α increased in expression from 9% to 22.3% of the surviving cells upon treatment with sorbitol and subsequent differentiation, this may be wholly attributed to the survival or increased proliferation of bipolar cell progenitors over other cell types. In contrast, Müller cell markers are increased from undetectable levels to 33-50% of the surviving cells after treatment with sorbitol. Even a profound increase in proliferation of glial precursors could not fully explain such an increase in Müller cell phenotypes. Furthermore, sorbitol tended to decrease, rather than increase, markers of cell division. Taken together these data point to an inductive role for sorbitol in Müller cell differentiation from RPCs.

Thus it remains unclear as to whether the pro-differentiation effects of sorbitol on PKC α expressing cells arise primarily from selectively enhancing the survival of bipolar precu-

ror cells or by more directly influencing terminal differentiation, our results suggest that the induction of Müller cell differentiation of RPCs in part underlies the effect of sorbitol on vimentin and CRALBP expression. Single cell or clonal analyses of lineage experiments are needed to make this determination, and unfortunately, such studies have proven difficult with cultured RPCs.

In summary, our results show that sorbitol can promote expression of glial and bipolar cell markers along with simultaneous down-regulation of progenitor and proliferative markers. Up-regulation of cell-specific markers is accompanied by morphological differentiation consistent with glial phenotypes. Although much is known about sorbitol metabolism in the retina as it relates to diabetic retinopathy, this is the first study to investigate its relationship to Müller cells. While the ability to generate Müller glia in vitro from passaged late RPCs may have potential clinical implications, a better understanding of the changes underlying Müller cell activation and gliosis is required. We also suggest that the accumulating evidence for an important role of Müller cells in retinal regeneration demand that we further probe the developmental potential of this cell type.

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