Rho GTPases Control Ciliary Epithelium Cells Proliferation and Progenitor Profile Induction In Vivo

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PURPOSE. Rho GTPases play a central role in actin-based cytoskeleton reorganization and regulate multiple signaling pathways that control gene transcription, cell survival, and proliferation. We investigated the effect of Rho GTPases on cell cycle regulation and progenitor genes expression on mouse ciliary epithelium (CE), a potential source of progenitor/stem cells in the adult retina.

METHODS. Rho GTPases were activated by intraocular injection of lysophosphatidic acid and inactivated by Clostridium difficile Toxin A (general Rho GTPase inhibitor), NSC23766 (Rac1 activation inhibitor), or Y27632 (Rho-associated protein kinase [ROCK] inhibitor). Thereafter, we assayed for RhoA, Rac1, and ROCK protein localization in CE cells. Proliferation was examined by the expression levels of cell cycle regulators p27Kip1, p16INK4a, and Ki67 and the effects on progenitors by determining the changes in Pax6 and Chx10 progenitor markers expression.

RESULTS. All GTPases investigated were expressed in mouse CE cells. Activation increased the coexpression of Pax6 and Chx10, but had no significant effect on the proliferation of CE cells. In contrast, Rho GTPases inactivation increased cell proliferation and potentiated the proliferative effect of growth factors. Specific inactivation of Rac1 or ROCK increased the levels of Ki67 and decreased the expression of the cell cycle inhibitors p27Kip1 and p16INK4a.

CONCLUSIONS. This study reports that Rho GTPase modulation (activation and inactivation) controls the expression of retinal progenitor genes and proliferation, respectively, in the adult ciliary epithelial progenitor/stem cells of rodent eyes. The modulation of these two different mechanisms (proliferation and reprogramming) may provide a potential new approach in retinal repair.

Keywords: ciliary epithelium, retinal progenitor cells, proliferation, Rho GTPases

The developing optic cup is subdivided in central retina and a peripheral region called “ciliary margin” that gives rise to two nonneural structures: the folded proximal ciliary epithelium (CE) and the distal iris. The CE can be further subdivided in two zones: a flat region called “pars plana” and a folded one called “pars plicata,” consisting of two cell layers, an inner nonpigmented epithelium and an outer pigmented epithelium1–3 (Fig. 1A).

The CE is a specialized secretory tissue that produces the aqueous fluid, responsible for nourishment of the lens and cornea and maintenance of eye pressure.1,4 The regulatory peptides secreted by CE are responsible for multiple functions, such as the immune status of the anterior segment, the diurnal circadian rhythms of aqueous humor secretion, and intraocular pressure. Because of these specialized functions, the ciliary body is considered as a neuroendocrine system by some authors.4 Furthermore, the ciliary muscle fibers are responsible for vision accommodation through lens deformation.

In the past, the mammalian CE was not known for its ability to proliferate. However, two independent groups5,6 have presented compelling evidence of the proliferative potential in mammalian CE. Single cells from pigmented CE of rodent eyes are able to form neurospheres in vitro in the presence of mitogens and to give rise to new retinal-like cells after differentiation stimulus. These data have set an important milestone in CE research and include CE cells as a source of optic stem cells.5,6

Since then, many researchers have investigated the quiescent potential of these cells and their capacity to differentiate into various types of retinal neurons. Their accumulated results indicate that the avian and mammalian CE cells, including those of humans, also harbor a pool of neural progenitor/stem cells with properties homologous to those found in the ciliary marginal zone of nonmammalian vertebrates.7–14

Although the proliferative and reprogramming capacity of these cells are well established, the efficiency of their differentiation toward retinal fates is considered low for therapeutic purposes. This might be due to low rates of proliferation and/or enrichment of retinal progenitors, which might be poorly stimulated by the current culture condition protocols. Furthermore, CE cells do not lose completely their pigmentation and epithelial features,15,16 suggesting that they do not achieve complete reprogramming. Several factors are known to activate/improve cell reprogramming and proliferation of CE cells, including Sonic Hedgehog,17 fibroblast and epidermal growth factors (FGFs, EGFs),5,6,13,18,19 Wnt3a,20

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Figure 1. Rho GTPases expression in adult mice CE cells. (A) Schematic diagram of mice CE. Briefly, the ciliary body can be subdivided in two zones: a flat region called "pars plana" (1), and a folded one called "pars plicata" (2), consisting of two epithelial cell layers, an inner nonpigmented epithelium (yellow circles) and an outer pigmented epithelium (red circles). The delineation between pars plana and plicata is not clear in all the histologic sections. (B) Confocal micrographs of transverse sections of mouse retinas processed for immunofluorescence for RhoA, RhoB, and Rac1, and higher magnification images from the same sections (right column). RhoA and RhoB proteins distribution were homogeneously diffused in outer (pigmented) and inner (nonpigmented) epithelium cell layers, whereas Rac1 was only observed in outer epithelium. Staining was similar in both pars plana and plicata in all cases. Arrowheads indicate the limits between the peripheral edge of the retina–pars plana (white), pars plana–pars plicata (red), and pars plicata–iris (yellow). PE, pigmented epithelium (outer); NPE, nonpigmented epithelium (inner).
stem cell factor (SCF), and pigment epithelium-derived factor. However, it is still unclear the role that some of these factors play during late retinogenesis, and the proteins possibly involved in cell cycle regulation. Therefore, the search for factors capable of efficiently removing CE cells from their quiescence state and of stimulating cell differentiation becomes crucial, and the Rho GTPase family of proteins has shown potential in this field.

Rho family small GTPases are signaling proteins implicated in the regulation of cell migration, actin organization, focal adhesion formation, adherent and gap junction assembly, and cell cycle regulation. They cycle between an inactive GDP-bound state and an active GTP-bound form. Only in the GTP-bound state are these proteins able to bind effector proteins in response to signals from a large variety of membrane receptors, including cytokine and growth factor receptors, adhesion receptors (integrins), and G-protein–coupled receptor.

Some of the best known proteins in the family are the GTPases RhoA, RhoB, and Rac1. In the retina, the distribution of Rho proteins suggests an important role in the differentiation of specific cell types during synaptogenesis and Müller cell morphology maintenance.

The effect of Rho GTPases in cell cycle progression is variable and depends on cell type and age (adult melanoma, prostate cancer, NIH3T3 cells, adult tumor cell lines, among others). They are important in the regulation of G1 progression through cyclin D1, as well as p21cip and p27kip, two cyclin-dependent kinase inhibitors. Inactivation of RhoA results in increased expression of p21cip and p27kip, two cyclin-dependent kinase inhibitors. Activation of Rac1 GTPase stimulates cyclin D1 expression, and activation of RhoA results in decreased transcription from the p21cip promoter.

Inhibition of the Rho effector ROCK (Rho-associated protein kinase) improves cloning efficiency, increases the number of cells in S phase, and the uniformity of human embryonic stem cells in vitro. In fact, the ROCK inhibitor Y27632 has been used in mouse and human embryonic stem cells and induces pluripotent stem cell cultures to prevent cell differentiation and improve clone formation.

Activation of Rac1 GTPase stimulates cyclin D1 expression, Rb phosphorylation, and E2F promoter activity.

Together, the evidence points toward an active role for Rho GTPases in the modulation of stem cell proliferation and fate determination; the mechanisms underlying these processes, however, are still unclear. Therefore, we decided to investigate the role of RhoA, RhoB, and Rac1 on proliferation and retinal progenitor profile expression of adult quiescent progenitor CE cells in vivo. We report here that adult CE cell proliferation increased after inactivation of Rho GTPases by intracocular injection of Toxin A from Clostridium difficile, and after specific inactivation of ROCK and Rac1. Conversely, activation of GTPases by lysophosphatidic acid (LPA) positively regulated the progenitor profile. In addition, the combination of Toxin A and growth factors had a synergistic effect on the proliferation rate.

METHODS

Animals and Tissue Preparation

All experiments were conducted in accordance to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Biomedical Sciences Institute/University of São Paulo Ethical Committee for Animal Research (No. 052-16-2).

Adult Balb/c mice (8–10 weeks) were kept in a 12-hour light/12-hour dark cycle (light intensity, 80–100 lux), with food and water ad libitum. Before intracocular injections, mice were anesthetized with ketamine (12 mg/100 g body weight, intramuscularly; Parke-Davis, Ann Arbor, MI, USA) and xylazine (0.8 mg/100 g intramuscularly; Miles, Inc., West Haven, CT, USA). At the end of the treatments, mice underwent transcardial perfusion with saline, followed by 2% paraformaldehyde in phosphate buffer (PB) 0.1 M (pH 7.4). Their eyes were dissected and cryoprotected in 30% sucrose in PB for at least 24 hours at 4°C, and sectioned perpendicular to the vitreal surface on a cryostat (12-μm sections).

Intravitreal Injections and Factors

Balb/c mice were anesthetized as described, and the vitreous of the right eye (treated) was injected with Rho GTPases activator or inactivators and/or growth factors (GFs), concentrated in 1-μL volume, using a 10-μL Hamilton syringe with a 32-gauge needle (Hamilton Company, Reno, NV, USA). The vitreous of the left eye (control), received a 1-μL injection of sterile vehicle (PBS). Growth factors used were purified human insulin (2 μg/eye; Gibco, Gaithersburg, MD, USA), and human recombinant basic fibroblast growth factor (bFGF, 100 ng/eye; Invitrogen, Carlsbad, CA, USA). Other factors injected included the general inhibitor for Rho GTPase, Toxin A, from C. difficile (10 ng/eye, No. 152C; List Biological Laboratories, Inc., Campbell, CA, USA), Rac1 inhibitor (400 ng/eye, NSC23766; Calbiochem, San Diego, CA, USA), ROCK inhibitor (100 ng/eye, Y27632, (1(–)–(R)–trans–4 – 1-aminoethyl) – N – (4–pyridyl) cyclohexanecarboxamide dihydrochloride); Calbiochem), and Rho GTPase activator LPA (400 μg/eye, Sigma-Aldrich, St. Louis, MO, USA). From injection paradigms used in other studies, animals received four consecutive injections of the factors (once a day) and were killed on the fifth day.

Immunohistochemistry

Ciliary epithelium sections were incubated overnight at room temperature with different primary antibodies: rabbit anti-RhoA, anti-RhoB, or anti-Rac1 (1:200, 1:100, and 1:20, respectively; Santa Cruz, Dallas, TX, USA), sheep anti-Chx10 (1:100; Chemicon, Temecula, CA, USA), rabbit anti-Ki67 (1:100; Vector, Burlingame, CA, USA), rabbit anti-Pax6 (1:100; Babco/Covance, Princeton, NJ, USA), rat anti-pro-inflammatory markers CCL22 and CXCL11 (1:200; R&D Systems, Minneapolis, MN, USA), and rat antimicroglia marker OX42 (1:300; Abcam, Cambridge, MA, USA), diluted in PB 0.1 M containing 3% normal donkey or goat serum, and 0.3% Triton X-100. After several washes in PB, sections were incubated for 2 hours with antisera against rabbit, sheep, or rat IgG (1:50), tagged to fluorescein isothiocyanate or rhodamine isothiocyanate (Jackson Laboratories, West Grove, PA, USA). Slides were then coveredlipped with VectaShield (Vector Laboratories, Burlingame, CA, USA), analyzed with a Nikon PCM2000 (New York, NY, USA) or Zeiss LSM780 (Jena, Germany) confocal microscope. Figures were mounted with Adobe Photoshop (San Jose, CA, USA). Manipulation of the images was restricted to threshold and brightness adjustments to the whole image. Controls for the experiments consisted of the omission of primary antibodies; no staining was observed in these cases.

Western Blot

After intracocular injection of specific factors, the total retina was dissected and immersed in ice-cold 20 mM Tris/HCl (pH 8.0), in the presence of protease inhibitors (0.4 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 0.005 trypsin inhibiting U/mL aprotinin, 2 μg/mL soybean trypsin inhibitor), and homogenized. Cell debris was discarded by centrifugation...
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**Table.** List of Specific Primers

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(500g for 10 minutes at 4°C), and the supernatants were centrifuged at 25,000g for 30 minutes to separate the cytosolic from the crude membrane fraction. Protein was determined by using Bradford assay (Bio-Rad Laboratories, Cambridge, MA, USA). Samples were subjected to SDS-PAGE (15%) gel, and the proteins transferred to nitrocellulose membranes. Membranes were blocked with Superblock blocking solution (Pierce, Rockford, IL, USA) containing 3% BSA and 5% nonfat dried milk, incubated overnight at 4°C with antibodies against RhoA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and then with goat anti-rabbit IgG-peroxidase. Detection of labeled proteins was achieved with the enhanced chemiluminescent system (Amersham, Piscataway, NJ, USA).

**Polymerase Chain Reaction**

After treatment, animals were euthanized, the eyes enucleated, and the CE cells (both inner and outer layers) were manually dissected from the other ocular structures with an Olympus SZ40 (Center Valley, PA, USA) stereo zoom microscope. Total RNA from CE-dissected cells was isolated from the cells by using the MininRnasy Kit (Qiagen, Hilden, Germany), and cDNA (3 μg) was synthesized as previously described. Briefly, specific transcripts were amplified with gene-specific forward and reverse primers, by using a step cycle program on a RoboCycler (Stratagene, La Jolla, CA, USA) or Quantifast SYBR Green PCR kit (Qiagen), or a RotorGene 6,000 (Corbett Robotics, San Francisco, CA, USA). The gene-specific primers used for RT-PCR and quantitative-PCR are described in the Table. Semi-quantitative PCR products were resolved on 2% agarose gels. Quantitative PCR measurements were performed in triplicate; a reverse transcription–negative blank of each sample and a no-template blank served as negative controls. Amplification curves and gene expression were normalized to the housekeeping gene Gapdh, used as an internal standard.

**Cell Counting and Statistical Analysis**

Cell counting was performed in a Leitz Aristoplan microscope (Wetzlar, Germany) equipped with a Nikon DS-R1 digital camera. At least 15 sections/eye of the entire CE from 3 to 5 different animals (N = 3–5) were analyzed for each experimental group with a ×40 objective. Results were presented as percentage of immunoreactive cells in relation to the number of 4′,6-diamidino-2-phenylindole (DAPI)-labeled nuclei. Polymerase chain reaction samples consisted of three different CE pools (N = 3), and each pool was obtained from 7 to 10 different animals. Statistical differences are given between treatments and respective controls. Statistical analysis was performed by using GraphPad Prism Software (GraphPad, Inc., San Diego, CA, USA), calculated by unpaired Student’s t-test (two-tailed). Results are expressed as mean ± SEM (standard error of the mean), and a threshold of P < 0.05 was used for each test.

**RESULTS**

**RhoA, RhoB, and Rac1 Are Differently Expressed in CE Cells**

We showed here, for the first time, the presence of Rho GTPases RhoA, RhoB, and Rac1 in adult CE cells. The common nomenclature “pigmented” and “nonpigmented” epithelial cells will be replaced with “outer” and “inner” layers of the CE, respectively, because the experimental subjects were albinos (see schematic representation of mice CE in Fig. 1A). Our results indicated that all GTPases studied were expressed in CE cells of adult mice, with distinct distribution patterns (Fig. 1B). RhoA immunoreactivity was mainly observed in the cytoplasm of CE cells, similarly expressed in all cells from both *pars plana* and *plicata*, whereas RhoB was diffusely expressed in the cytoplasm and nucleus of the cells from outer and inner layers throughout the CE, also in both *pars plana* and *plicata*. Rac1 was strongly observed in the cytoplasm of the outer layer cells (pigmented) of the CE, similarly to the expression found in retinal pigmented epithelium cells from the central retina.

**Activation of Rho GTPases Induced Progenitor Genes Expression in CE Cells, and Rho GTPase Inhibition Increased Cell Proliferation**

To determine the role of Rho GTPases in adult CE cells, we interfered pharmacologically with these proteins with intraocular injection of the inhibitor Toxin A (10 ng/eye), or the activator LPA (400 μg/eye; Fig. 2A). We confirmed the effect of these drugs on GTPase activation/inactivation through Western blot analysis of fractionated membrane and cytoplasm (Fig. 2B). Activated GTPases migrate to the membrane to interact with the respective effectors, whereas inactive GTPases remain in the cytoplasm. Our results confirm that Rho proteins were in the membrane fraction after LPA injection, and in the cytoplasmic fraction after inactivation by Toxin A, suggesting that our intraocular injection protocols were effective in interfering with Rho GTPase activity. For this analysis, retina

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Figure 2. Effect of LPA and Toxin A in proliferation and progenitor genes expression. (A) Schematic representation of intraocular injection of factors. (B) After the injection of LPA (activation), Toxin A (inactivation), or PBS (control), protein extracted from the total retina was fractionated into cytoplasm (C), and membrane (M) portions, and analyzed by Western blot for RhoA GTPase antibody. The activated GTPase (LPA) migrates to the membrane to interact with the respective effectors, and proteins located in the cytoplasm are indicative of nonactivated proteins (Toxin A). PBS injections generated bands in both cytoplasm and membrane fractions. Immunofluorescence analysis and cell counting of retinal sections demonstrated that, after injection of 400 μg/eye LPA, the number of cells expressing the cell cycle protein Ki67 (green) was similar to the control (C, E, I). However, the number of Pax6 (red) and Chx10 (green) doubled-stained cells was significantly increased (D, F, J). Injection of 10 ng/eye Toxin A significantly increased the numbers of Ki67+ cells (G, I), but not the Pax6+ Chx10+ cells (H, J). Quantitative PCR analysis revealed that the transcriptional level of ki67 decreased after LPA injection (K), and the levels of chx10 and pax6 significantly increased (L, M), in comparison to control. On the other hand, ki67 transcripts increased (K), whereas chx10 and pax6 decreased or showed no difference compared to the controls (L, M) after Toxin A injection. Arrows indicate the end of the retina on the left side, and the beginning of the CE on the right side. Arrowheads indicate some Ki67+ cells (C, E, G) or Pax6+ Chx10+ cells (D, F, H). Values are given in fold change after equating PBS to 1. P value was assigned after statistical analysis (t-test) between treatment and respective controls. *P < 0.05; **P < 0.005; ***P < 0.0001. N = 5. GAPDH, housekeeping gene.
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was used instead of CE, owing to the amount of tissue necessary to perform the fractioning. The PBS injections generated bands in both cytoplasm and membrane fractions.

To investigate the proliferative response of the CE cells after modulation of Rho GTPase activity, we used Ki67 immunostaining and PCR in the absence of stimulus, normal adult CE cells do not express Ki67. Immunofluorescence analysis and cell counting of eye sections revealed that PBS injections induced a minimal increase in proliferation (3.07% ± 1.12%), probably related to the injection procedure (Figs. 2C, 2J). Lyposphatidic acid did not alter in a statistically significant manner the number of Ki67+ cells compared to control (5.10% ± 0.87%; Figs. 2E, 2I). Interestingly, quantitative PCR indicated that LPA decreased the expression of ki67 (Fig. 2K).

In contrast, Toxin A induced a significant increase in both the percentage of Ki67+ CE cells (32.8% ± 0.20%; Figs. 2G, 2I), and transcript levels (Fig. 2K). To rule out the possibility that proliferating cells were infiltrating inflammatory cells, we immunostained the CE tissue after Toxin A injections with the pro-inflammatory markers CXCL11 and CCL22. CXCL11 is a cytokine involved in immune response and activation of pro-inflammatory pathways, including in retinal pigmented epithelium inflammatory diseases.48–50 CCL22 is involved in the migration of various types of leukocytes to inflammation sites51 and has also a direct pro-inflammatory role in certain pathologic conditions such as postoperative proliferative vitreoretinopathy.52 Here, we show that no cells were CXCL11 positive. In contrast, few CCL22+ cells were detected outside the CE structure, but none of them coexpressed Ki67 (Supplementary Fig. S1).

Double-labeling experiments with antibodies against Pax6 and Chx10, transcription factors known to be coexpressed by embryonic retinal progenitors cells,53 indicated that a few inner CE cells coexpressed these two factors (9.8% ± 1.5%; Figs. 2D, 2J), suggesting that some rare proliferative and progenitor cells could be observed in control conditions. Lyposphatidic acid injection increased significantly the number of Pax6+Chx10+ cells in inner CE of pars plana and plicata (Figs. 2F, 2J). Quantitative PCR showed that the expression of both markers was increased in general after LPA injection (Figs. 2L, 2M). Toxin A did not alter significantly the number of Pax6+Chx10+ cells (Figs. 2H, 2J), and the chx10 transcript levels (Fig. 2L), but decreased pax6 levels (Fig. 2M).

From the CE, LPA or Toxin A did not induce significant modifications in the retina, except for rare rod-like Ki67+ cells observed in plexiform and ganglion cell layers. They coexpressed Otx2, a reactive microglia marker, indicating a minor response to the injection procedure (data not shown).

To verify if the LPA effects observed here were due to activation of other pathways besides GTPases, we coinjected LPA together with the GTPases inhibitor Toxin A. Our results indicated that, although LPA can induce some changes in GTPases expression pattern in central retina, these changes disappeared when the proteins were inactivated by Toxin A (Supplementary Fig. S2A). Likewise, in the presence of Toxin A, the effect of LPA on Pax6 and Chx10 coexpression was attenuated (Supplementary Fig. S2B).

Taken together, these results indicated that in vivo inactivation of Rho GTPases induced cell cycle re-entry of a subset of CE cells in adult mice. In addition, Rho GTPase activation by LPA increased progenitor gene profile, as seen by Pax6 and Chx10 coexpression.

Rho GTPase Inhibition by Toxin A Potentiates Cell Proliferation Induced by GFs

To verify if Rho GTPases are also involved in proliferation induced by other stimuli, we investigated Ki67 expression pattern and levels after four intracocular injections of GFs (insulin and bFGF) combined or not with Toxin A. Consistent with previous findings,5–8 our results indicated an increase in CE cell proliferation (9.02% ± 0.15%, Figs. 3B, 3E) after GF injections compared to the control (2.91% ± 0.61%, Figs. 3A, 3E). In contrast, Toxin A inhibited a Rho activator protein (guanine nucleotide exchange factors [GEFs]), with intraocular injections of Y2763254 or NSC23766,55 respectively (Fig. 4A).

Four-day consecutive injections of the specific ROCK inhibitor (Y27632) stimulated a significant increase in Ki67+ cells (9.94% ± 0.79%) in comparison to the control (3.05 ± 1.10%, Fig. 4B). An increase of the ki67 transcripts, and a decrease in the cyclin-dependent kinase inhibitor p16INK4a and p27kip transcript levels (Fig. 4C), were also detected. We observed a significant decrease in the expression of the progenitor markers pax6 and chx10 (Fig. 4C). No significant difference was observed in the transcriptional expression levels of progenitor genes nestin and mash1 (Fig. 4C).

Rac1 GEF inhibitor (NSC23766) was twice as effective in the induction of cell proliferation (19.32% ± 0.30% Ki67+ cells) as Y27632 (Fig. 4B). The extent of NSC23766-mediated inhibition was statistically similar to Toxin A treatment (18.24% ± 0.20%). In support of this, NSC23766 increased ki67 and decreased p16INK4a and p27kip transcript levels (Fig. 4D). Again, quantitative PCR indicated significant decrease in pax6 transcripts (Fig. 4D), but no significant difference was observed in transcriptional expression of progenitor genes nestin, mash1, and chx10 (Fig. 4D).

DISCUSSION

It is known that CE cells can proliferate as neurospheres in serum and growth factor–free conditions,5,6 and the supplementation with growth factors significantly increases this capacity. However, although significant, the proliferative potential of CE cells is limited under these conditions. The same limited proliferative property and retinal cell differentiation were observed in vivo, and it has been proposed that this limited in vivo activity of CE stem/progenitor cells is hampered by intrinsic limitations, lack of permissive factors in their microenvironment, and/or the presence of local inhibitory cues, which remain to be determined.12 Thus, the search for experimental approaches capable of increasing the stem cell properties of these cells is indispensable for therapeutic purposes.

Several studies have shown the crucial influence of Rho GTPases on progenitor/stem cells regulation in different tissues. For example, Rho GTPases are involved in the regulation of interkinetic nuclear migration in neocortical progenitor cells of developing mouse brain.56 and self-renewal and proliferation in neural crest stem cells.57 Recent reports
have demonstrated that ROCK inhibitor promotes the survival of dissociated human embryonic stem cells and induces pluripotent stem cells, improving the efficiency and reproducibility of the clonal growth without affecting the cell capacity to differentiate into the three embryonic germ layers. Although this compound is widely used in stem cell research, little is known about the mechanism of action and involvement of Rho GTPases in embryonic and pluripotent stem cell maintenance. Here, we provided evidences that the inactivation of Rho GTPases significantly stimulated/increased adult CE cell proliferation in situ. We demonstrated an important increase in the number of Ki67+ cells after general Rho GTPases inhibition by Toxin A, as well as with the specific inhibitors Y27632 and NSC23766.

Previous reports have indicated a correlation of Rho GTPase activity in p27kip1 transcription and protein regulation. Rac1 inhibitor NSC23766 induces transcriptional down-regulation of p27kip1 in vascular endothelial cells, by truncating p27kip1 promoter region. Activated RhoA alone promotes p27kip1 degradation by increasing cyclin E-CDK2 activity. Besson and collaborators have found that p27kip1 forms a complex with both activated and dominant-negative RhoA forms, and suggested that p27kip1 could coordinate a diverse array of cellular responses through its effect on Rho-dependent cytoskeletal structure. However, Rho activation does not influence significantly p27kip1 levels in Ras-Rho–transformed Swiss 3T3 cells or in Ras mutation–positive human colon carcinoma cell lines, suggesting that GTPase influence on p27kip1 may be different depending on cell type or stage.

Similarly, Rho GTPase influence on cell proliferation is also quite ambiguous in the literature, and may induce both pro- and anti-proliferative responses, depending on the cell type and stage of cell differentiation. For example, while GTPase activation is required for cell cycle progression in fibroblasts, carcinoma, and endothelial cells, it reduces proliferation of Swiss 3T3 and S26 (NIH 3T3 derivatives) cells. On the other hand, GTPase inactivation can induce cell proliferation in h5-3T3 cells and hematopoietic progenitor cells.
Figure 5 shows a summary of the results in our experimental paradigm. Rho GTPase inactivation induced adult CE cell proliferation by decreasing the expression of p27<sup>Kip1</sup> and p16<sup>INK4a</sup>. Moreover, we also demonstrated that inhibition of Rho GTPases potentiated GF-induced proliferation. Traditionally, activation of GTPases is required for GF-induced proliferation.

In corneal endothelial cells Rac1 is downstream to the PI3-kinase/Akt pathways triggered by FGF<sub>68</sub>. After treatment with FGF Rac1 is involved in p27<sup>Kip1</sup> phosphorylation at the Thr187 residue. Phosphorylation of p27<sup>Kip1</sup> at Thr187 is known to be a prerequisite for binding to Skp2, the F-box component of an SCF (SCFSkp2) ubiquitin ligase complex, resulting in degradation of p27<sup>Kip1</sup> through the ubiquitin-proteasome machinery.<sup>69,70</sup> However, to elucidate the relationship between GFS, Rho, and cell cycle proteins in adult CE cells, further studies are needed.

Finally, we also provided here evidence that activation of Rho GTPases by LPA stimulated/increased coexpression of progenitor genes <i>pax6</i> and <i>chx10</i>. <i>Pax6</i> and <i>Chx10</i> homeodomain transcription factors are known to be coexpressed in embryonic retinal progenitors,<sup>35</sup> as well as in progenitors from the retinal margin,<sup>71</sup> FGF-treated retinas,<sup>7</sup> and Müller glia-derived of acutely damaged chicken retina. Here, the increased expression was observed as an augmentation of the number of double-positive

![Figure 4](image_url)

**Figure 4.** Specific inhibition of Rho (Y27632) and Rac1 (NSC23766) affect the cell cycle and progenitor genes expression. (A) Schematic diagram of Rho GTPase activation/inactivation cycle, and the specific inhibitor drug's effect. Briefly, Rho GTPases act as molecular switches, cycling between an inactive, GDP-bound state and an active, GTP-bound state. The activated conformation interacts with specific effectors to propagate downstream signaling events that influence many aspects of cell biology. GEFs activate the switch by catalyzing the exchange of GDP for GTP whereas GTPase-activating proteins (GAPs) increase the intrinsic GTPase activity and inactivate the switch. NSC23766 interacts with Rac1-binding sites for GEFs, and Y27632 selectively targets p160ROCK from the family of Rho-associated protein kinases. (B) Ki67<sup>+</sup> cell counting demonstrated increase in proliferation after intraocular injection of Y27632 (100 ng/eye), NSC23766 (400 ng/eye), and Toxin A (10 ng/eye), in comparison to the control. Error bars indicate standard error of the mean. *<i>P</i> value was assigned after statistical analysis (t-test) between treatment and respective control. (C) After Y27632 injection, semiquantitative PCR indicated increased ki67 transcripts, and decreased p16<sup>INK4a</sup> and p27<sup>Kip1</sup> (a); no significant changes were observed on nestin and mash1 (b). Quantitative PCR indicated increased expression of ki67 (c), and decreased expression of pax6 and chx10 (d, e). (D) After NSC23766 injection, semiquantitative PCR indicated increased ki67 transcripts and decreased p16<sup>INK4a</sup> and p27<sup>Kip1</sup> (a); no significant changes were observed for nestin and mash1 (b). Quantitative PCR indicated increased expression of ki67 (c), decreased expression of pax6 (d), and no difference in chx10 (e). A.U., arbitrary unit. *<i>P</i> < 0.05; **<i>P</i> < 0.005; ***<i>P</i> < 0.0001.

Figure 5. Schematic representation of the results. Rho GTPase inactivation increased CE cell proliferation, indicated by the increase in Ki67 and decrease in p27<sup>Kip1</sup> and p16<sup>INK4a</sup> levels. On the other hand, activation of Rho GTPases increased progenitor profile in CE cells, observed by coexpression of Pax6 and Chx10.
cells in the CE cells as well as in their transcript levels in the total population. Lysophosphatidic acid has been shown to have a range of effects on neural stem/progenitor cells, through mechanisms mediated by LPA receptors encoded by distinct genes. The named LPAR1–LPAR5 in humans and Lpar1–Lpar5 in mice.73–75 In 2008, Dotti and collaborators76 indicated that LPA sustains the undifferentiated state of human embryonic stem cells without interfering with the cell cycle, by activating the PI3K/Akt and the Rho/ROCK pathways.

Many of the signaling effects of LPA are also mediated through the activation of phospholipase C, Ca2+ mobilization, extracellular signal-regulated kinases 1/2, and adenylate cyclase activation or inhibition.77 For that reason, we cannot completely exclude the possibility that the effect of LPA observed here might be mediated through other signaling pathways besides Rho GTPases. However, our results from the use of LPA together with Rho inhibitor Toxin A suggests that LPA is acting through GTPases to induce progenitor profile.

Although there is still no information on the influence of Rho GTPases in CE cell differentiation or migration, their role is well established in corneal epithelium cell migration and wound repair.78–80 RhoA is known to regulate corneal epithelial cell migration and focal adhesion formation through ROCK, and its inhibition with Y27632 promotes basal and heparin-binding EGF-like growth factor–enhanced human CE cell migration and adhesion to matrices.80,81 Y27632 disrupts E-cadherin– and β-catenin–mediated cell–cell junctions,82 improves corneal healing, and has been used for the treatment of corneal endothelium disorders.82,83

In summary, our results demonstrated that Rac1 and Rho kinase inhibition increased CE cell proliferation, and Rho-specific inhibition might control the cell cycle entry by the down-regulation of cyclin-dependent kinase inhibitors. In contrast, activation of Rho increased progenitor profile in CE cells, with no influence on cell cycle. These data suggest that the proliferation and cell progenitor profile induction may be processes regulated by different and/or complementary mechanisms that can occur in the same cell at different time points. We have recently demonstrated that CE in culture responds rapidly to GF treatment by expressing proliferation markers (ki67 and cyclin D1), whereas the progenitor genes expression (otx2, lhx10, and pax6) is detectable with longer periods of exposure.84 Together, these data suggest that the state of Rho GTPase activation or inactivation varies with time. Future experiments are necessary to confirm this hypothesis.

While definitive treatments for retinal degenerative diseases are still needed, and transplant therapies are actually limited by stem cell supply, tissue rejection, teratoma formation, and ethical issues, we suggest here a new viewpoint for CE cell cycle entry and retinal progenitor gene manipulation. It is likely that Rho GTPase inactivation is necessary to induce CE cell cycle entry in adult stages, whereas their activation is necessary for the next step, for example, the regulation of the expression of progenitor genes, coordinating and orchestrating the possible tissue repair in a controlled way.

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