Low-Oxygen Culture Conditions Extend the Multipotent Properties of Human Retinal Progenitor Cells

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Purpose: Development of an effective cell-based therapy is highly dependent upon having a reproducible cell source suitable for transplantation. One potential source, isolated from the developing fetal neural retina, is the human retinal progenitor cell (hRPC). One limiting factor for the use of hRPCs is their *in vitro* expansion limit. As such, the aim of this study was to determine whether culturing hRPCs under 3% O₂ would support their proliferative capacity while maintaining multipotency.

Methods: To determine the effect of low oxygen on the ability of hRPCs to self-renew, rates of proliferation and apoptosis, telomerase activity, and expression of proliferative, stemness, and differentiation markers were assessed for hRPCs cultured in 3% and 20% oxygen conditions.

Results: Culture under 3% oxygen increases the proliferation rate and shifts the proliferation limit of hRPCs to greater than 40 divisions. This increased capacity for proliferation is correlated with an upregulation of Ki67, CyclinD1, and telomerase activity and a decrease in p53 expression and apoptosis. Increased expression of cMyc, Klf4, Oct4, and Sox2 in 3% O₂ is correlated with stabilization of both HIF1 α and HIF2 α . The eye field development markers Pax6, Sox2, and Otx2 are present in hRPCs up to passage 16 in 3% O₂. Following *in vitro* differentiation hRPCs expanded in the 3% O₂ were able to generate specialized retinal cells, including rods and cones.

Conclusions: Low-oxygen culture conditions act to maintain both multipotency and self-renewal properties of hRPCs *in vitro*. The extended expansion limits permit the development of a clinical-grade reagent for transplantation.

Introduction

RETINITIS PIGMENTOSA and age-related macular degeneration, diseases characterized by death of the light-sensing photoreceptor cells of the retina, are leading causes of incurable blindness in the western world. The intrinsic regenerative capacity of the mammalian retina is extremely limited. One potential therapy for these diseases is cellular replacement. A cell type that holds great promise for such purpose is the human retinal progenitor cells (hRPCs). Isolated from fetal neural retina at 16–20 weeks of gestational age, hRPCs possess the capacity to survive, differentiate into retinal neurons, and integrate within the dystrophic host retina following transplantation. However, unlike other less-restricted cell types (such as embryonic stem cells or induced pluripotent stem cells), hRPCs are limited in their proliferative capacity. For instance, we have shown that following isolation these cells can be passaged a maximum of seven times *in vitro*,^{1,2} limiting the number of cells that can be obtained and in turn the number of transplants that can be performed from a single hRPC isolation. As these cells are isolated from developing embryos, increasing the number of usable cells from each donation is

desirable. It is possible to overcome this proliferative limit with immortalization of fetal human retinal cells by SV40 transfection.³ However, these cells have failed to express markers of mature differentiated retinal cells after transplantation. Several other methods like conditional immortalization⁴ or downregulation of pRb⁵ have been described for Muller glial cell lines expressing retinal stem cell genes, but these cells also failed to efficiently differentiate into photoreceptors. Another, more “physiologic,” method that has been shown to increase the proliferative capacity of a variety of cell types, including rat and human neural stem cells,^{6–15} adipose- and bone-marrow-derived mesenchymal stem cells,^{16–18} limbal epithelial stem cells,¹⁹ human embryonic stem cells,^{20–23} and venular endothelial cells,²⁴ is the use of low-oxygen culture conditions.

Oxygen concentration in mammalian tissues varies from 0.5% (retina) to 19% (upper airway epithelia); though, during routine cell culture it is maintained at 20%. While data for developing retina are not available, in adult retina oxygen tension varies from 0.5% (inner nuclear layer) to 7% (outer segments).^{25,26} Previously, it was shown that other factors, such as medium supplements (transferrin and insulin), mitogens (fibroblast growth factor and epidermal

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growth factor), and extracellular matrix, greatly influence survival and proliferation of hRPCs *in vitro*.²⁷ In this work we demonstrate that low oxygen has a similar importance for maintenance of this cell type.

Low oxygen alters cell metabolism by several pathways (Smad, MASH, Ca⁺⁺, JNK, AP, Src, Ras/Raf, PKC α , PI3K, p42/44, EGR-1, PKC- β , and reactive oxygen species), although the main mediators are hypoxia inducible factors (1 α and 2 α), which are constitutively expressed but hydroxylated and ubiquitinated in oxygen tensions higher than 6%. If the oxygen level is decreased, then HIF α subunits dimerize with HIF β subunit (ARNT). This complex is transported to the nucleus, where it functions as a basic helix-loop-helix transcription factor.⁵⁹ While HIF1 α expression is found in most cell types, HIF2 α expression is limited²⁸ to the brain, heart, lung, kidney, liver, pancreas, intestine, and retina. The targets of these complexes are also different²⁹; HIF1 α regulates metabolic pathways (shift to glycolysis),^{29–31} tissue remodeling (increase in metalloproteases and decrease in ECM production), migration (chemokine expression), prosurvival pathways, and genome methylation (3h3mCoenzA and JMJD1A). Growth factor signaling is affected both via HIF1 α and HIF2 α , and HIF2 α activates multipotency transcription factors Oct4, c-Myc, and Sox2 and Cyclin (D1, D2, and E2F) expression.²⁰ Most of these “positive” effects are observed in oxygen tensions between 2% and 3%, while 1% and lower is considered “anoxic.” We have chosen 3% concentration for our experiments and the aim of this study was to determine whether culturing hRPCs under low-oxygen conditions (3% O₂) would extend the proliferative limits placed on these cells and allow one to obtain a population of potent cells in numbers suitable for clinical application.

Materials and Methods

Retinal morphology

To investigate the morphology of the neural retina at 18 and 20 weeks of gestation, one eye cup was fixed in Karnovsky fixative, embedded in plastic, cut (2 μ m), and stained for H&E. One eye cup was fixed in 4% PFA, embedded in OCT, cryosectioned (6 μ m), and immunostained with antibodies directed against Ki67, Sox2, Pax6, Recoverin, β 3-tubulin, and laminin.

Cell isolation

hRPCs were isolated as previously described^{32–34} with small modifications. Briefly, whole neuroretinas from human fetal eyes (16–20 weeks gestational age) were dissected, dissociated in 0.1% collagenase I (Sigma) during four cycles (1.5 h of dissociation in total), and plated in modified Ultraculture media (10 ng/mL rhEGF, 20 ng/mL rhbFGF, Pen/strep, Nystatin, and L-glutamine) or frozen. The amount and viability of single cells and clumps were estimated using Trypan blue and a hemocytometer.

Cell culture

Cells were plated at a density of $\sim 10,000$ cells/cm² and cultured under either physiologic oxygen (3%) or normoxic (20%) condition at 37°C, 100% humidity, and 5% CO₂ in

modified Ultraculture media (10 ng/mL rhEGF, 20 ng/mL rhbFGF, Pen/strep, Nystatin, and 2 mM L-glutamine) on flasks coated with bovine serum fibronectin (Akron). Cells were passaged at 75–85% confluence (usually each 2–5 days) using a 0.05% Trypsin-EDTA solution. At each passage cells were counted and plated at the density mentioned just now. Low-oxygen conditions were created using a Thermo 150i incubator.

Cell proliferation and growth curve

Cell proliferation in both conditions was assessed during routine passaging by cell count via a hemacytometer (three times for each of two separate flasks for each passage/source). For all further studies we have used only two cell lines, expanded up to passage 16.

Apoptosis

hRPCs (p1–p9) for TUNEL assay (Roche) were plated in 16-well chamber slides coated with fibronectin as per the maintenance conditions (4000 of alive cells in each well, hRPC media with supplements); 48 h after plating, cells were fixed, permeabilized (0.01% Triton-X and 0.01% sodium citrate), and stained for double-strained DNA breaks. Slides were mounted, and cell counts were performed in nine fields of view for each condition. The number of cells varied from 20 to 150 per field of view.

Immunocytochemistry

Cells were assessed via immunocytochemical analysis at passages 1, 3, 5, 7, and 10 in both 3% and 20% oxygen conditions and at passage 16 in the 3% oxygen condition for stemness and proliferation marker expression; Otx2, Sox2, and Pax6—eye field development transcription factors; CyclinD1, Ki67, and hTERT—proliferative markers; cMyc, Klf4, and Oct4—“stemness” transcriptional factors; and SSEA4—surface antigen, characteristic for undifferentiated cells. For this purpose, 4000 cells were plated in each well of 16-well fibronectin-coated chamber glass slides (Nunc); after 24 h of incubation in appropriate conditions, cells were washed in PBS, fixed (cold, freshly prepared 4% PFA), permeabilized (0.02% Triton X-100 in 5% BSA), blocked, and stained with primary antibodies overnight (Supplementary Table S1, Supplementary Data are available online at www.liebertpub.com/tea) at 4°C and secondary antibodies (1:50, Goat Cy3-conjugated anti-rabbit or anti-mouse; Jackson ImmunoResearch) for 1 h at room temperature.

Western blot

hRPCs cultured in 3% and 20% oxygen for 4 days were harvested for protein analysis on passages 1, 3, 5, 7, 10, and 16; lysed in RIPA buffer; and analyzed for protein expression by western blot. Proteins were separated on an 8% SDS-PAGE gel, transferred to a PVDF membrane (Bio-Rad), blocked with 5% nonfat milk (Bio-Rad) in TBS-T, and stained with antibodies diluted in 5% BSA in TBS-T, targeted against HIF1 α , HIF2 α , hTERT, Nestin, Sox2, Oct4, Klf4, and cMyc (Supplementary Tables 1). Resulting bands were imaged with ECL Plus (Perkin Elmer) and CL-Xposure film (Thermo scientific). An anti- β Actin HRP-linked

antibody (Abcam) was used as a loading control. Band density was measured using ImageJ and normalized by internal loading control for all markers.

Telomerase activity assay

Telomerase activity was assessed in both experimental conditions on passages 1, 3, 5, 7, 10, and 16 by the TRAPeze method according to the manufacturer's (Millipore) instructions. Briefly, cells were harvested and lysed in CHAPS buffer for 30 min on ice and telomeres were amplified for 30 min at 30°C. The products were amplified using Platinum Taq (Invitrogen), separated by PAGE gel electrophoresis (nonreducing conditions), and stained with SYBR Gold (1:10,000; Invitrogen) for 20 min at room temperature.

Differentiation abilities in vitro

To assess the ability of hRPCs to differentiate *in vitro* hRPCs expanded in 3% oxygen condition from passages 1, 5, 10, and 16 were plated on fibronectin- and laminin-coated 16-well chamber slides. Cells were cultured in differentiating media (DMEM/F12, 1XNEAA, L-glu, 5% HI FBS, Pen/Strep, and Nystatin) in 3% oxygen. On days 2, 5, and 9, cells were fixed and stained for blue opsin (short-wave cones), red/green opsin (long-wave cones), rhodopsin (rods), recoverin (photoreceptors), and CRX (pan-photoreceptor transcription factor). Control staining was performed for hRPCs of the same passages but after 24 h in maintenance conditions. Cells were counted in three fields of view (number of cells varied from 20 to 150 per field of view) for

FIG. 1. Morphology of human fetal retina. H&E and immunohistochemistry of human fetal retina. All scale bars are 100 μ m. **(a)** Twenty-week gestational age: outer and inner nuclear layers are not completely separated and outer segments are not present, while the inner limiting membrane (black arrows) and ganglion cell layer have been formed. Total retina thickness is consistent at about 250 μ m. **(b–e)** Eighteen-week gestational age. **(b)** Proliferative marker Ki67 is present in the middle of the inner and outer nuclear layers, but not in the layers expressing recoverin. **(c)** Neural progenitor marker Sox2 is expressed in all cells within the neural retina, with slightly decreased levels in the outer nuclear layer. **(d)** Recoverin (photoreceptor precursor marker) expression was limited to the outer nuclear layer and single cells in the ganglion cell layer (white arrows). **(e)** Pax6 is present in both the outer and inner nuclear layers. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Color images available online at www.liebertpub.com/tea

three independent experiments, with the mean and standard deviation of expressing cells calculated. The ability to differentiate was estimated by comparison of the number of cells expressing mature photoreceptor markers in differentiating versus maintenance conditions (paired *t*-test, $p < 0.05$).

For RT-PCR and WB analysis, we differentiated cells in the same medium for 4 and 7 days and control samples were collected 24 h after placing cells in the differentiation medium.

Total RNA was collected using QIAGEN RNEasy kit (Qiagen) and reverse transcriptase reaction was performed using SuperScript III First Strand kit (Life Technologies). The amplification was performed for 30 cycles using Platinum Taq Polymerase (Life Technologies) according to manufacturer's suggestions with annealing at 56°C. Primer sequences were obtained through PrimerBank database (Supplementary Table S2).

The western blot was performed as described previously and membranes were stained for Rhodopsin, Recoverin, and GAPDH.

Results

Eye morphology

At 20 weeks gestational age, the human neural retina consists of three nuclear layers and is ~250- μ m thick (compared with 500 μ m for adult retina) (Fig. 1a). While the inner and outer nuclear layers are beginning to diverge (i.e., the plexiform layer is not present at this time in development), the ganglion cell layer is distinctly separated by the inner plexiform layer (Fig. 1a). Due to the absence of rod and cone outer segments, the outer limiting membrane is not present at this time, while the space between the ganglion cell layer and the inner limiting membrane (black arrows,

FIG. 2. Primary culture of human retinal progenitor cells (hRPCs). Immunocytochemical expression of progenitor and eye field developmental markers in primary culture 3 days after isolation. While Otx2 (c) and Pax6 (b) were expressed only in some of the cells, Sox2 (f) was expressed in almost all of the cultured cells. Ki67 (a) expression in primary culture was low compared with higher passages, while some recoverin- (d) positive cells were observed. Tight junctions were present after isolation (e) After passage 1, cell clumps (g) were dissociated and cultured cells became more homogenous, obtaining higher levels of Ki67 expression, losing recoverin and increasing in size (h) Scale bars are 200 μ m. Color images available online at www.liebertpub.com/tea

Fig. 1a) is wider than the inner plexiform layer. At 18 weeks gestational age, the innermost portion of the ganglion cell layer is represented by single recoverin-positive cells (white arrows, Fig. 1d). Recoverin expression was also identified in within the presumptive outer nuclear layer (Fig. 1d). The proliferative/progenitor cell marker Ki67 is present in the middle of the combined inner and outer nuclear layers, but not in the region expressing recoverin (Fig. 1b). This correlates with previous studies that show Ki67 immunoreactive cells in developing human retina up to 13 weeks of gestational age.^{35–37} The retinal progenitor cell marker Sox2 is expressed in all cells within the neural retina at this time, however, at lower levels in cells close to the outer retina (Fig. 1c).

The expression of progenitor cell markers and absence of outer segments makes the neural retina at this age a suitable candidate for retinal progenitor cell isolation.

Cell isolation and cell culture

We found that hRPC isolation can be performed up to 24 h after enucleation. From each pair of eyes, ~17.8 million (± 1.5 million) single cells (viability $52\% \pm 16\%$) and $1.5E6$ ($\pm 0.5E6$) cell aggregates, consisting of 10–100 cells, were obtained. The viability of the aggregates, indirectly assessed by adhesion to fibronectin during the first hour after plating, was about 70% and does not vary between low- and

FIG. 3. Growth kinetics, proliferation, and apoptosis of hRPCs. (a) Estimated number of hRPCs, expanded in 3% (red) and 20% (green) oxygen conditions, established for each of six different donations (A–F). Each point on the graph represents cell counts at the time of passaging. Expression of proliferative marker Ki67 (c) and cell cycling marker CyclinD1 (d) in 3% (red) and 20% (green) oxygen conditions for passages 1, 3, 5, 7, 10, and 16 (for the 3% oxygen condition only). Cycle checkpoint protein expression p53 (e) and phosphorylated Rb were higher in 20% oxygen conditions on all passages investigated (H1, H5, and H10—3% oxygen passages 1, 5, and 10; N1, N5, and N10—20% oxygen passages 1, 5, and 10). Percentage of apoptotic cell death (as determined by TUNEL labeling) in 3% (red) and 20% (green) oxygen conditions on passages 1–9 (b). There was no difference in the percentage of apoptotic cells in low-oxygen conditions on passages 1–7, and a significant increase on passages 8 and 9 (paired *t*-test with Bonferroni correction). For 20% oxygen conditions there was no difference between passages 1–4. However, apoptosis significantly increases at passage 5 in 20% oxygen, which correlates with pRb upregulation. Starting from passage 3 the ratio of TUNEL-positive cells was significantly higher in 20% oxygen compared with 3% oxygen. Color images available online at www.liebertpub.com/tea

normal oxygen conditions. Most of the small single cells die on the second to third day after plating, with the majority of the proliferative cells obtained from outgrowing cells from the aggregates (Fig. 2g). This suggests that the starting material consists of $\sim 45,000,000$ viable cells (assuming an average number of cells in each aggregate of 30). After the first passage, cells increased in size (Fig. 2h) but maintained a high nucleus/cytoplasm ratio, obtained a triangular or spindle morphology, and in general became more homogeneous in morphology. Despite some reports that cell adhesion decreases in low-oxygen conditions due to integrin down-regulation,^{38,39} we did not observe any difference in hRPC adhesion to fibronectin-coated (1 h coating with 75 $\mu\text{g}/\text{mL}$) culture surfaces. Proper adhesion is critical for survival and expansion of these cells in a monolayer culture.

Cell proliferation and growth curve

The rate of hRPC proliferation in the 20% oxygen condition was constant during approximately the first 20 days of culture, after which expansion plateaued, and the cells senesced (Fig. 3a). In contrast, in the 3% oxygen condition, the proliferation rate maintained a higher level for up to 60 days in culture, with no evidence of slowed growth or senescence up to 40 population doublings, indicating that the potential number of cells expanded from two donor eyes is 4.9×10^{19} .

Proliferative markers

The observed increase in hRPC proliferation in the 3% oxygen condition correlates with an increase in expression of the cell cycle markers Ki67 and CyclinD1 (Fig. 3c, d). An increase in p53 expression at all passages in the 20% oxygen condition as compared with the 3% oxygen condition was also observed.

Apoptosis

The increase in p53 expression together with growth kinetics at passages 8 and 9 was linked with a higher level of apoptotic cell death in the 20% oxygen condition as determined by TUNEL assay (Fig. 3). It did not exceed 2% in the low-oxygen group at passages 1–7, and increased to 5% at passage 8. In the 20% oxygen condition, the level of apo-

ptotic cell death increased from 2% at passage 1, to 6% at passages 5, 6, and 7, and 20% at passage 8 (Fig. 3e).

Telomerase expression and activity

hRPCs express hTERT in both the 3% and 20% oxygen conditions on all passages. Telomerase activity was decreased with passage in both conditions but was higher in the 3% versus the 20% oxygen condition (Fig. 4). In the 3% oxygen condition, telomerase activity was preserved until passage 16.

Stemness markers

By immunocytochemical and western blot analyses (Fig. 5), we found that culture in 3% oxygen tension stabilizes HIF1 α and HIF2 α , and upregulates cMyc, KLF4, Oct4, and Sox2. Expression of specific eye field development transcription factors (Pax6, Sox2, and Otx2) as well as nestin is maintained up to passage 16 in the 3% oxygen condition and up to passage 10 in the 20% oxygen condition. Sox2 and Oct4 were upregulated in 3% oxygen conditions (Supplementary Figure S1), in part due to HIF2 alpha activity.^{40,41} Sox2 expression decreased during differentiation. SSEA4 upregulation in low-oxygen conditions, previously shown for MIAMI¹⁶ and hESCs,²⁰ was seen in hRPCs after passage 3.

Differentiation

As was shown previously, hRPCs isolated from 10- to 20-week neural retina are able to differentiate into specialized retinal cells.^{2,27,35} In previous work we found that cells expanded in 20% oxygen tension were able to generate photoreceptors only at low passages (<4).² Here we examined hRPCs expanded in 3% oxygen, and found that this ability was extended to the highest passage examined (passage 16). Cell expansion in 3% oxygen did not induce spontaneous differentiation of hRPCs into rods, cones, retinal ganglion, bipolar, or glial cells (<0.5% in maintenance conditions according to ICC staining; Fig. 6, data not shown).

As shown in Figure 6, hRPCs expanded in 3% oxygen are able to generate specialized retinal cells, including rods ($\sim 6\%$) and cones ($\sim 4\%$). Further, this ability is not lost through expansion of over 40 population doublings, but

FIG. 4. Telomerase activity (TRAPeze method). Telomerase activity (**b**) in hRPCs, obtained for passages 1, 3, 5, 7, 10, and 16 in 3% and 20% oxygen conditions. Fifty-base-pair telomerase products are marked with white arrows (**a**) and are not present in negative controls (right of each sample). Telomerase activity in hRPCs at passage 1 in 20% oxygen is set as 100%, and other bars (normalized by internal loading control) show the relative telomerase activity.

FIG. 5. Expression of stemness markers. Relative expression of immature markers in 3% (red) and 20% (green) oxygen conditions on passages 1, 5, and 10. Beta-actin was used as loading control. H1, H5, and H10—passages 1, 5, and 10 in 3% oxygen. N1, N5, and N10—passages 1, 5, and 10 in 20% oxygen, respectively. Oct4, cMyc, and Sox2 were upregulated at all passages in low-oxygen conditions and HIF transcription factors were stabilized in 3% oxygen.

rather increases with successive passaging. On passage 1 compared with other passages (5, 10, and 16), we observed higher levels of specialized cells after differentiation, suggesting that the culture at early passage is enriched with somewhat differentiated precursors that are lost during expansion. The possibility of these primary differentiated cells surviving through extended passaging was ruled out by

staining the cultures for mature photoreceptor markers prior to differentiation. During differentiation we observed downregulation of Pax6 and Sox2, and the staining pattern changed from nuclear to perinuclear and cytoplasmic.

Discussion

One of the limiting factors in the translation of cell therapy from *in vitro* research to clinical application is the inability to significantly expand the donor cells in order to obtain sufficient cells for characterization and evaluation of safety and efficiency profiles, as well as use in transplantation. The pioneering work in this field by Kelley *et al.* demonstrated the feasibility of isolating and maintaining human retinal progenitors *in vitro*.²⁷ We improve proliferation rate by modifying the original protocols,^{2,33} which allowed us to obtain several billion (10^9 – 10^{10}) cells during *in vitro* expansion following a single isolate. Using low-oxygen culture conditions, we have now overcome this limit: with each cell source providing up to 10^{18} – 10^{19} hRPCs *in vitro*. This proliferation limit withdrawal correlates with the doubling of the proliferation rate, which should also decrease the accumulation of genome changes and promote positive selection for rapidly dividing progenitors. This positive selection is confirmed by high levels of Ki67 and Cyclin D1 expression compared with passage 0 (isolation time point). We suggest that Ki67 expression may be used as a “quality marker” for hRPCs, as it is used for corneal limbal stem cells.^{42–44} The number of Ki67-expressing cells decreased greatly with passage in 20% oxygen condition, but this did not occur in 3% oxygen condition. A modest increase in expression of these markers (Ki67 and CyclinD1) at passage 7 in 20% oxygen culture conditions can be explained by positive “selection” at passages 5 and 6, when actively proliferating progenitors replace “mature” cells. However, this increase is lost after two additional passages in 20% oxygen. Also, this pause in hRPC division on passage 5 is characterized by an increase in the expression of cell cycle control protein p53 that is downregulated in the 3% oxygen condition on all passages. The fact that p53 is upregulated in severe hypoxic conditions in most observed cell types and stabilized via a p300-dependent pathway by HIF α ^{45–47} highlights the importance of this mechanism in the observed results. We detected both HIF α isoforms in hRPCs, with each of these expected to play different roles in cell fate. While HIF1 α mainly mediates the “hypoxic” effect, stabilizing p53,⁴⁶ blocking cell cycle progression and proliferation⁴⁸ (HIF1 α independent⁴⁹), and activating apoptosis,⁵⁰ HIF2 α mediates the “physiologic” effects of low-oxygen concentrations: increased proliferation, multipotency gene expression, TERT upregulation, and cell cycle progression. The level of this effect of low-oxygen culture conditions on different cell types depends upon their ability to express HIF2 α in response to O₂ levels lower than 5%. We have shown that HIF2 α is stabilized in hRPCs at 3% oxygen and that this stabilization is maintained during passaging. In the case of hRPCs, low-oxygen culture conditions drive the expression of Sox2, Klf4, cMyc, and Oct4 (possibly in an HIF2 α -dependent way), which maintains the “multipotent” state and shifts the proliferative limit. One of the most important findings is the maintenance of functional properties of

FIG. 6. Differentiation pattern of hRPCs. Percentage of cells expanded in 3% oxygen expressing specialized photoreceptor cell markers in maintenance conditions (red bars) and 2 days (orange bars), 5 days (green bars), and 9 days (blue bars) postdifferentiation on passages 1, 5, 10, and 16. Representative pictures of cells that were expanded up to passage 16 and differentiated for 9 days are shown in (a). All scale bars are 200 μ m. The RT-PCR (b) and WB (c) analysis confirm the expression of photoreceptor markers after differentiation. Color images available online at www.liebertpub.com/tea

hRPCs—the ability to form mature retinal cell types after prolonged expansion in 3% oxygen conditions. We have recently developed more efficient differentiation protocols, using specific molecular substrates, and have obtained much higher yields of photoreceptors *in vitro*.⁵¹

The decrease in apoptosis observed in physiologic oxygen culture conditions can be explained by the decrease in the events leading to programmed death (loss of genome integrity, membrane damage, and cytoskeleton malfunction), or alternatively by downregulation of apoptotic signaling cascades. While both are important for stem cell expansion, a decrease in apoptosis via antiapoptotic protein overexpression may in fact be detrimental to the overall population. Low-oxygen culture conditions (but not anoxic¹¹) are known to decrease apoptosis in neural precursors,^{9,14,15,22,52–54} endo-

thelial progenitors,⁵⁵ an SVEC 4–10 cell line,⁵⁶ and bone marrow cells.²² While downstream antiapoptotic signaling cascades (PI3K-Akt, p38, ERK 1/2, and Bcl-2) are upregulated, proapoptotic signaling cascades (Fas, Bax, and p21) are downregulated via HIF-dependent cMyc activation and p53 suppression.^{57–59} Genomic integrity is also known to be improved in low-oxygen culture conditions, mainly due to lower levels of ROS.²⁰ The prosurvival effect of low oxygen is further supported by an HIF-dependent upregulation of breast cancer resistance protein gene expression (Abcg2),^{60–62} erythropoietin and the erythropoietin receptor.^{22,63} HIF1 α is also known to protect retinal neurons from light-induced degeneration.^{64–66}

One of the main limiting factors in extending cell proliferation in culture is the telomere length. One possible

mechanism for the observed hTERT upregulation is HIF1 α stabilization⁶⁷ that has been shown to mediate TERT upregulation in human placenta⁶⁷ and human MIAMI cells¹⁶ in 3% oxygen culture conditions.

Most of the low-oxygen effects on cell function *in vivo* and *in vitro* are mediated by hypoxia-inducible factors, which are stabilized at oxygen levels lower than 5%. This allows them to dimerize with the ARNT subunit, translocate to the nucleus, and function as transcription factors to bind specific promoters (hypoxia-responsive elements) of their gene targets.⁵⁹ Since the discovery of HIF1 α as a specific transcription factor for erythropoietin⁶⁸ and HIF2 α ,²⁸ the amount of HIF1 α and HIF2 α targets greatly increased.²⁹ Despite some controversy in this area,^{69,70} most studies have shown that physiologic oxygen tension (3–4%) upregulates Oct4, Sox2,^{16,40,41,71,72} Nanog,^{71,72} SSEA4,¹⁶ and cMyc.^{73,74} Upregulation of “pluripotency” factors (cMyc, Klf4, and Sox2) in low-oxygen conditions affects not only the multipotency properties of these cells, but also the ability to proliferate *in vitro*. The expression of the eye field development markers (Pax6 and Otx2) in low-oxygen conditions was extended until passage 16. Our previous work found that expansion of hRPCs leads to a loss in their ability to differentiate into mature retinal cell types, such as photoreceptors.² Low oxygen has been shown to have a dramatic impact on differentiation for several types of stem and progenitor cells, with huge variations in its effect. The 5% oxygen is required for complete differentiation of hESCs to endoderm,⁷⁰ dopaminergic differentiation in NSCs,^{13,14} and carcinoma cells.⁷⁵ But hypoxia (3%) inhibits differentiation of ESCs⁷⁶; endothelial,⁷⁷ myogenic, and neural precursor cells^{78,79}; and MIAMI cells.¹⁶ This difference may be explained by the primary signaling cascades involved (mainly HIF1 α or HIF2 α).⁸⁰

Conclusions

We found that hRPCs show an increase in proliferation, multipotency marker expression, and maintenance of the ability to form specialized retinal cells (including photoreceptors) as well as a decrease in apoptosis in 3% oxygen tension, suggesting that this microenvironment is physiologically appropriate for expansion of this cell type *in vitro*.

Author Contributions

Petr Y. Baranov: conception and design, collection and/or assembly of data, data analysis and interpretation, article writing, and final approval of article. Budd A. Tucker: conception and design, collection and/or assembly of data, data analysis and interpretation, article writing, and final approval of article. Michael J. Young: conception and design, financial support, data analysis and interpretation, article writing, and final approval of article.

Disclosure Statement

No competing financial interests exist.

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