Low-oxygen and knock-out serum maintain stemness in human retinal progenitor cells

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Abstract
Using stem and progenitor cells to treat retinal disorders holds great promise. Using defined culture conditions to maintain the desired phenotype is of utmost clinical importance. We cultured human retinal progenitor cells (hRPCs) in different conditions: such as normoxia (20% oxygen), and hypoxia (5% oxygen) with and without knock-out serum replacement (KOSR) to evaluate its effect on these cells. KOSR is known nutrient supplement often used to replace bovine serum for culturing embryonic or pluripotent stem cells, especially those destined for clinical applications. The purpose of this study was to identify the impact of different environmental and chemical cues to determine if this alters the fate of these cells. Our results indicate that cells cultured with or without KOSR do not show significant differences in viability, but that the oxygen tension can significantly change their viability (higher in hypoxia than normoxia). However, cells with KOSR in hypoxia condition expressed significantly higher stemness markers such as C-myc and Oct4 (31.20% and 13.44% respectively) in comparison to hRPCs cultured in KOSR at normoxia (12.07% and 4.05%). Furthermore, levels of markers for retinal commitment such as rhodopsin were significantly lower in the KOSR supplemented cells in hypoxia culture compared to normoxia. KOSR is known to improve proliferation and maintain stemness of embryonic cells and our experiments suggest that hRPCs maintain their proliferation and stemness characteristics in hypoxia with KOSR supplement. Normoxia, however, results in mature cell marker expression, suggesting a profound effect of oxygen tension on these cells.

Keywords Human retinal progenitor cells · Flow cytometer · Stemness · Hypoxia

Introduction
Eyes are multilayer and multicellular structure organ that can’t be repaired once damaged. Photoreceptor, bipolar, ganglion cells and others neuronal cells along with RPE are few critical retinal cells types that work in sync to perform visual function. In the case of retinal degenerative conditions, such as age-related macular degeneration and retinitis pigmentosa (RP), specific populations of retinal cells are affected [1, 2]. In advanced disease, this can result in irreversible blindness impacting millions of patients worldwide [3]. RP is caused due to many genetic etiologies, all of which always involve death of photoreceptor cells. From treatment prospective, gene therapy is one of the front runners however, prerequisite for its success is living host cells which in the later stages of the disease is almost impossible [4]. Hence, the most promising approach is cell transplantation. Recent advances in stem cell biology indicate that progenitor cells can be used for treating these retinal conditions.

Human retinal progenitor cells (hRPCs) holds great promise as potential candidate that can be isolated from fetal neural retinal tissue and possess the capacity to proliferate, survive, differentiate and ultimately integrate within the host retina following transplantation, as previously described [5-7]. The biggest advantage of using hRPCs, as source
for retinal cells, is that a series of transcriptional factors, needed to act in a multistep cascade to drive cells towards retinal development, are innately present in these cells. They are destined to develop into adult retina, yet they possess multipotent embryonic cells and fetal cells characteristics [8, 9]. This characteristic is critical during transplantation as terminally differentiated cells have not been a successful graft, due to various reasons such as difficulty in migrating and integrating within the host retinal tissue. The balance between stem cells and mature cells could be the impetus factor in a successful retinal regeneration. These cells are presently in clinical trials and have shown groundbreaking results [10]. However, hRPCs have limited proliferation and differentiation index which can restrict its usage in large scale clinical application, hence, different techniques need to be explored to overcome this barrier.

For stem cells to be useful in the clinical medicine the differentiation protocol needs to be devised in a way that allows large scale production of retinal cell types. Various groups have used different nutrients combinations to achieve the desired stem cell differentiation with a high-yield of, for example, photoreceptors, RGCs or RPE cells [11-14]. This indicates that the culture conditions for such cells play a vital role determining the fate and eventual differentiation of these cells. Using a combination of nutrients/growth factors makes it difficult to understand the effect of one condition and to obtain the highest yield of cells that express stage-specific markers. Many types of stem cells depend on different stimuli for in-vitro proliferation and survival. One of the most effective techniques in maintaining stemness of progenitor cells depends upon varying the oxygen tension that directly affect differentiation and functioning of stem cells [15].

When oxygen level offered to stem cells is not at the same level as its niche, it induces cellular alterations such as metabolism turnover, increased oxidative stress and reduced cellular proliferation and self-renewal properties [16]. Furthermore, it effects its motility and changes differentiation potentials along with loss of its potential stemness. Changes in oxidative stress results in altered reactive oxygen species (ROS) level also known as intracellular messenger. ROS plays a key role as it determines the fate of normal stem cells. Normally, low level of ROS is needed for maintaining quiescence and self-renewal in stem cells and any increase in ROS level can change proliferation/differentiation potential along with inducing senescence and apoptosis leading to cell exhaustion [17]. Hence, ROS production in stem cell is a tightly regulated process to ensure the stem cells can maintain homeostasis and repair tissue throughout the life span of organism [18]. Similarly, when cells are cultured in hypoxia condition, any available oxygen diffuses through the cytoplasm to the mitochondria creating a lack of oxygen condition in its cytosol which in turn inhibits prolyl hydroxylases, a component that controls the activation of hypoxia-inducible factor (HIFs). It is a heterodimer that has oxygen regulated α subunit (1 and 2) and importantly β express subunit. HIF is known to play a critical role in angiogenesis but have recently been found to contribute towards the stemness maintenance, homeostasis and differentiation ability of stem cells. In hypoxia culture, HIF-1α is not hydroxylated and is stabilized to start HIF- transcriptional activity [19]. Furthermore, hypoxia is a critical stimuli for many specific cell lines by acting as a cytokine enhancer [20].

The most commonly used small molecule component in stem cell culture is knock-out serum (KOSR) which has been shown to facilitate the generation of embryonic stem cells. It serves as a replacement for fetal bovine serum that can greatly enhance alkaline-phosphate positive colonies [21]. Furthermore, using KOSR decreases the time needed for Oct4 expression in the case of reprogramming induced pluripotent stem cells (iPSCs) [22]. However, the effectiveness of KOSR and hypoxia for retinal progenitor cells has not yet been clearly defined.

In our study, we compared the effect of hypoxia and KOSR on human retinal progenitor cells (hRPCs). Cells were cultured in defined media with and without KOSR in normoxia and hypoxia conditions and cellular expression and viability, using live dead assay, immunostaining and, flow cytometry, were assessed to evaluate the effect of environmental and chemical conditions on these cells.

**Materials and methods**

**Human retinal progenitor cell culture**

All the human material work was performed in accordance to institutional guidelines. hRPCs were gifted by ReNeu-group and were cultured in fibronectin coated T-75 in Ultraculture medium containing 0.4 mM Primocin (Invitrogen) 2 mM l-glutamine (Invitrogen), 20 ng/mL recombinant human basic EGF (Peprotech), 10 ng/mL recombinant human basic fibroblast factor (Peprotech) in hypoxia condition (37 °C, 5% O2, 5% CO2, 100% humidity) until confluency, as previously described [6]. Cells are dissociated using TrypZean (Sigma-Aldrich) diluted in Hank’s Balanced Salt Solution (HBSS, Thermo-Fisher) and viability was assayed by Trypan blue (Sigma-Aldrich).

**Knock-out serum replacement and normoxia**

To test the effect of different conditions, hRPCs were cultured in Ultraculture medium in hypoxia condition (37 °C, 5% O2, 5% CO2, 100% humidity), Ultraculture supplemented with KOSR in hypoxia, hRPC in ultra-culture medium in normoxia condition (37 °C, 20% O2, 5% CO2, 100%
humidity), and Ultraculture supplemented with KOSR in normoxia: Normoxia, KOSR-Normoxia, Hypoxia and, KOSR-Hypoxia. Cells were seeded with cell surface density of 20,000 cells/cm².

**Viability assay**

Live-dead assay was performed on the hRPC by using 2.5 μM calcein-AM and 10 μM ethidium bromide (Life technologies) staining solution diluted in HBSS without calcium and magnesium. Cells were washed with HBSS to remove any trace of medium and incubated with staining solution for 10 min at 37 °C. Post-incubation cells were washed thoroughly three times using HBSS and imaged with epi-fluorescence microscope. Random sections were selected from field of view (N = 15) and counted using 20 × objective lens.

**Cell marker analysis**

For cell marker analysis immunofluorescence staining for performed. Cells were washed and fixed using 4% paraformaldehyde for 10 min at room temperature. Fixed cells were permeabilized using Phosphate Buffer Saline (PBS) containing 0.1% triton-x and tween 20 following this was blocking with goat serum containing PBS (ThermoFisher Scientific, cat 50197Z). Samples were incubated with primary antibodies Oct4, C-myc, rhodopsin and Ki67 overnight. Following day slides were washed three times using PBS and incubated with secondary antibody (FITC and TRITC) along with counterstaining of cell nuclei was done using DAPI. Slides were incubated for 1 h in dark room. As final step, slides were washed with PBS, mount media was used to cover the slides before visualizing using fluorescent microscope.

**Flow cytometer analysis**

Flow cytometer analysis was performed by trypsinizing cells into single cells and filtering using 30 um filter. Cells collected were fixed at 4 °C for 15 min with Perm/Fix buffer (BD Biosciences). Furthermore, cells were then washed and blocked at room temperature using block buffer containing 2% goat serum (ThermoFisher Scientific) for 30 min. Cells were seeded onto a flat bottom 96-well plate and stained with conjugated primary antibodies in 1: 500 dilution of antibody buffer containing TBS, 0.3% Triton X-100 and 1% goat serum. Primary antibody used were DAPI-VioBlue, Oct4-APC, C-myc-APC, Ki67-APC, Rhodopsin-FITC, Recoverin-FITC and incubated overnight at 4 °C. The following day, cells were washed three times for 15 min using wash buffer and stained with secondary antibodies in 1:200 dilution in antibody buffer at room temperature for 3 h. MACSQuant flow cytometer was used to measure light scatter and fluorescence signals from each sample (2 × 10⁵ events were recorded). Generated results were further analyzed using the MACSQuantify software (https://www.miltenyibiotec.com). For each primary antibody, DAPI-positive single cell population was gated. Population 1 was used as identifying positive cells in the gated population which was estimated in comparison with blank and species-specific isotype control.

**Statistical analysis**

The power calculation was based on detecting a significant difference in the means between groups. Values were expressed as mean ± standard error mean (SEM) using Graphpad software (https://www.graphpad.com/). Analysis of variance (two-way ANOVA) followed by Tukey’s multiple comparison test were performed for statistical analysis. Statistical significance was set at p < 0.01.

**Results**

**Cell viability and proliferation assay**

Cells cultured in different condition were assessed for viability using calcein-AM and ethidium bromide for live/dead staining (N = 4 replicates). Four different hRPCs groups are analyzed throughout all the experiments: Normoxia, KOSR-Normoxia, Hypoxia, KOSR-Hypoxia. Live & dead results (Fig. 1) show that hRPC cultured in hypoxic conditions have a significantly higher number of viable cells compared to normoxia condition, both with or without KOSR. However, no difference was found between cells cultured with or without KOSR supplement. Statistical analysis performed using two-way ANOVA, followed by Tukey’s test, indicates a significantly higher viability (5–7%, p = 0.006) in KOSR-hypoxia and hypoxia when compared, respectively, to the KOSR-normoxia and normoxia groups (Fig. 1b). At all-time points the viability of cells was above 80%. The cells were more elongated and fibroblastic, morphologically, in both Normoxia and KOSR-Normoxia groups when compared to the hypoxic conditions.

Proliferation of hRPCs in all groups was monitored using Ki67 (N = 4 replicates). By day 3, immunostaining suggest that the percentage of proliferating cells decreased in normoxia condition (with or without KOSR) when compared to cells in hypoxia (Fig. 2). A quantitative measurement of the percentage of Ki67 expression is performed using flow cytometry analysis.

**Stemness and maturation markers assay**

Markers for stemness, like Oct4 and C-myc, are key genes that maintain and regulate cells in the progenitor state [23-25]. A qualitative difference in expression of these markers
was found between groups of different oxygen tension. hRPCs cultured in hypoxic conditions, with and without KOSR, showed the highest Oct4 expression and cells in normoxia showed the least expression (Fig. 3). The opposite trend can be seen for Recoverin expression, where cells in normoxia shows a higher expression than cells in hypoxic conditions, suggesting that lower oxygen tension tends to decrease the maturation of hRPCs (Fig. 4).

**Cell profile analysis with flow cytometer**

Flow cytometry analysis showed that less than 5% of the cells cultured in normoxia (with or without KOSR) were expressing C-myc and even lesser percentage of cells expressed Oct 4 which was significantly different from hRPCs cultured in hypoxic condition (from 4 to 7%, p = 0.0001). No significant difference was found between hRPCs with KOSR (in both oxygen tension conditions) and without KOSR (Figs. 5, 6). These results, in addition to the immunostaining, suggest that stemness is driven by the oxygen tension (hypoxic conditions increasing the stemness in hRPCs cultures) while KOSR has little effect on the differentiation.

Maturation markers of retinal lineage like rhodopsin and NRL, which are characteristic of rod photoreceptor cells, recoverin (rod and cone photoreceptor and some bipolar neurons) and, PAX6 (retinal marker) was used to identify and quantify effects of culture condition on maturation and differentiation of hRPCs. Expression of these key differentiation markers were significantly higher in normoxia conditions (rhodopsin, recoverin and, PAX6 positive 15%, 10% and, 14% respectively, p = 0.0003) compare to hypoxia groups (respectively 7%, 4% and, 9%). A significant difference was seen by the addition of KOSR for the rhodopsin expression. hRPCs cultured in Normoxia-KOSR shows a significantly lower expression of rhodopsin markers compare to Normoxia (respectively 15% and 18%, p = 0.003). However, no other difference in differentiation markers was found by the addition of KOSR supplement.

Proliferation measurement (Ki67) shows higher value for cells cultured in hypoxic conditions with KOSR supplement than without KOSR (respectively 18% and 15%, p = 0.01).

hRPCs in normoxia with and without KOSR shows a significantly lower proliferation (only 7%, p = 0.0001).

**Discussion**

The principal objective of this study was to investigate the effects of oxygen tension (especially hypoxia) and knock-out serum replacement (KOSR) on human retinal progenitor cells (hRPCs). The role of these two factors on viability, proliferation, maintenance of stemness and, driven differentiation was evaluated in vitro in four different conditions: Normoxia, KOSR-Normoxia, Hypoxia and, KOSR-Hypoxia.

Hypoxic conditions (usually 5–10% O₂) are known to be beneficial for stem and progenitor cell culture [26]. They are, in fact, physiologically normal for many stem cells niches [27]. A number of studies have shown significant benefits in terms of cellular expansion and viability using lower than 20% oxygen tension. In the case of embryonic stem cells specifically, low oxygen tension appears to favor cell growth more efficiently than standard oxygen concentration. For example, in bovine blastocysts significantly higher inner cell mass formation was found when cultured in hypoxia [28]. Further benefits of hypoxic conditions have been seen in the growth and expansion of adult stem cells especially in bone marrow derived mesenchymal stem cells. Grayson et al. showed a 30-fold increase in cell expansion in hypoxia compared to normoxia conditions [20]. Our current work confirmed this effect with a significantly higher viability seen in hRPCs cultured in both Hypoxia conditions (with or without KOSR). However, the addition of KOSR seems to have a low effect on hRPCs viability in both oxygen tension conditions.

It is well established that mammalian cells have limited proliferative potential when in culture condition. There is progressive loss of mitotic activity with increased cellular division that is referred to as senescence [29]. Due to this, many researchers have developed protocols to maintain and increase the proliferative capacity of cells in culture conditions [30, 31]. However, both genetic and environmental factors play vital roles in cellular behavior in in vitro conditions. Rubin et al., postulated that the limited proliferative capacity of cells in vitro could be attributed to inability of cells to survive dissociation and culture conditions [32]. These and other such studies suggest that improving the methods used to culture progenitor cells would have a major influence on their proliferative and differentiation efficiency [20]. In this study, we have examined chemical (KOSR) and environmental (Hypoxia vs Normoxia) effects on human retinal progenitor cells. Flow cytometry and immunostaining, in our study, suggest that hypoxia can significantly improve the proliferation
(measured with Ki67 expression) of hRPCs: expression doubles by culturing cells in hypoxic conditions compared to both normoxia conditions (with and without KOSR). Furthermore, KOSR supplement enables a higher proliferation by combining it with hypoxic conditions (KOSR-Hypoxia) compared to all other conditions.

In addition, hypoxia can improve differentiation of stem and progenitor cells into specific lineages, and has been shown to increase differentiation of adipose stem cells into functioning smooth muscle cells [33], implying that the impact of hypoxia is complex, and a more complete understanding its influence of cultured cells would benefit the field of developmental biology, and improve our ability to translate stem cells into treatment for disease. While improving the viability and proliferation of cell cultures, it has been shown that hypoxia can also preserve the stemness expression of embryonic stem cells [23, 27]. Here, we have evaluated the effect of hypoxia and KOSR on specific stemness markers (Oct 4 and C-myc) for hRPCs. Stemness markers were significantly decreased in both normoxia conditions which agrees with other studies that suggested hypoxia is critical for maintaining the undifferentiated state of progenitor cells [15, 34, 35]. We confirm that hRPCs specifically require hypoxic conditions to maintain proliferation and may be providing a biomimetic environmental niche for these cells by mimicking

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**Fig. 2** Ki67 staining for proliferation assay using immunohistochemistry for different culture conditions. Hypoxia shows more proliferative cells than normoxia. Cells cultured in KOSR shows higher proliferative cells indicating its effect on cells. Images taken on 3rd day of culture at 20× magnification. Scale bar 75 um
in vivo conditions [27]. There was no significant effect seen using KOSR on the preservation of hRPCs stemness.

Oxygenation of the inner retina is usually achieved through the intraretinal microvasculature and RPE and photoreceptor oxygenation is by diffusion from the choriocapillaris [36]. Due to these different vascular sources there is an uneven oxygen tension across the retina with significantly less oxygenation at the level of the outer nuclear layer (ONL). This fluctuation is also due to divergent oxygen utilization by retinal neurons and glial cells. Since photoreceptors are highly metabolically active, they utilized high amounts of retinal oxygen [37, 38]. As hRPCs are primary rod progenitor cells, there is likely to be a significant effect of oxygen tension on these cells. hRPCs and their rhodopsin expressing progeny are cells that are most active during dark conditions with high cytoplasmic calcium levels maintained through cGMP-gated calcium channels. This process is extremely ATP-dependent and it has been suggested these retinal neurons can consume about 4 times more oxygen under scotopic condition [39]. Expression of mature retinal lineage markers such as rhodopsin and recoverin was evaluated to examine the cell differentiation profile under different physiological conditions. Immunostaining and flow cytometric analysis show there is a significant increase in rhodopsin (15%) positive cells in both normoxia conditions compared to hypoxia, with less than 8% positive cells found. Similarly,

**Fig. 3** Stemness marker C-myc was used to identify the effect of culture condition of hRPCs. Oxygen gradient affects the stemness of hRPCs as significantly fewer positive cells were seen in hRPCs in normoxia in comparison to hypoxia and KOSR-hypoxia conditions. Images taken on 3rd day of culture, at 20× magnification. Scale bar 75 um
recoverin expression was significantly higher in the normoxia conditions than hypoxia. In retinitis pigmentosa, loss of rod photoreceptors leads to a reduced metabolic demand that in turn causes reduction of retinal oxygen consumption [40]. Higher expression of both rhodopsin and recoverin in normoxia conditions indicates the tendency of hRPC to leave the cell cycle and differentiate into mature cells when exposed to higher oxygen tension. There was no significant difference with the addition of KOSR, which may be due to the fact these are normally cultured in serum-free condition hence, KOSR shows no differentiation effect on hRPCs.

This study demonstrates the effect oxygen tension has on hRPCs. Hypoxia may provide environmental cues that helps hRPCs maintain their progenitor cell state, while the addition of KOSR can improve their proliferation. Normoxia might be used as one mechanism to differentiate these cells into mature phenotypes without their need for exogenous chemical stimulus.
Fig. 5 Statistical analysis for flow cytometry assay for hRPCs expression in different culture conditions. Data shown as mean ± SEM of N = 3 replicates. C-myc expression was found highest in both Normoxia conditions compared to both Hypoxia conditions. The stemness marker Oct4 shows a similar trend. The mature retinal markers Rodopsin and Recoverin was significantly increased in Normoxia, compared to KOSR-Normoxia, and even more compared to both Hypoxia conditions. This suggests that differentiation of hRPCs from progenitor to mature state is driven by normoxia conditions and that KOSR helps in keeping cell in an undifferentiated state. The opposite trend is seen for proliferation (Ki67), where both Hypoxia conditions have an increased expression of Ki67 compared to Normoxia. Two-way ANOVA was performed followed by Tukey’s multiple comparison test. (*p = 0.01, **p = 0.003, ***p = 0.0003, ****p = 0.0001)

Fig. 6 Flow cytometry assay was performed to quantify the hRPCs expression under different culture conditions. Flow cytometry analysis of hRPCs suspension. Isotype control antibodies were used as control. Gating strategy consisted in gating cells from the forward scatter (FSC) vs side scatter (SSC). This cell population was then gated to find single cells (FSC-A vs FSC-H) and with DAPI is used to identify single cell without debris. Single staining of C-myc, Ki67, PAX6 and, recoverin are shown for each group. Double staining of Oct4 with Rodopsin is shown for all groups. The experiment was performed in triplicate N = 3.
Conclusion

Culturing cells in vitro is critical for many tissue engineering and regenerative medicine strategies and it is therefore important to understand the effect of different conditions on cells in culture. KOSR is the most commonly used serum replacement especially for the stem cell culture, and varying oxygen concentrations can aid in maintaining stemness of these cells. Our experiments confirm that hypoxic condition along with KOSR aids in the maintenance of cells in progenitor state. Furthermore, increasing the oxygen concentration alters the cellular phenotype and push them towards differentiation. hRPCs shows increase in the expression of mature markers indicating cells differentiate when subjected to normoxia conditions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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