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RESEARCH ARTICLE

Expansion of retinal stem cells and their progeny using cell microcarriers in a bioreactor

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Abstract

Blindness as a consequence of degenerative eye diseases (e.g., age-related macular degeneration and retinitis pigmentosa) is a major health problem and numbers are expected to increase by up to 50% by 2020. Unfortunately, adult mouse and human retinal stem cells (RSCs), unlike fish and amphibians, are quiescent in vivo and do not regenerate following disease or injury. To replace lost cells, we used microcarriers (MCs) in a suspension stirring bioreactor to help achieve numbers suitable for differentiation and transplantation. We achieved a significant 10-fold enrichment of RSC yield compared to conventional static culture techniques using a combination of FACTIII MCs and relative hypoxia (5%) inside the bioreactor. We found that hypoxia (5% O_2) was associated with better RSC expansion across all platforms; and this can be attributed to hypoxia-induced increases in survival and/or symmetric division of stem cells. In the future, we will target the differentiation of RSCs and their progeny toward rod and cone photoreceptor phenotypes using FACTIII MCs inside bioreactors to expand their populations in order to produce the large numbers of cells needed for transplantation.

KEYWORDS
bioreactor, hypoxia, microcarriers, retina, stem cells

1 | INTRODUCTION

Retinal stem cells (RSCs) are a rare population (1 in 500) of large, multipotent, heavily pigmented cells that reside in the pigmented layer of the ciliary epithelium (CE) of the adult human and mouse eye. Embryonic RSCs can divide symmetrically to expand and maintain the stem cell pool by producing two new stem cells[1–3] or asymmetrically giving rise to a daughter stem cell and a retinal progenitor cell.[1,4–6] Retinal progenitors can differentiate to either retinal pigmented epithelium (RPE) or neural retinal cell types: photoreceptors, horizontal, amacrine, bipolar, retinal ganglion cells and Muller glial cells.[7] There are approximately 10,000 RSCs in a single human eye[3] and 100 per mouse eye.[1,2] It could be said that the stem cell status of the RSC is still contested; in particular, two labs[8,9] have argued against the stemness of RSCs, one of them[8] suggested that RSCs are a product of trans-differentiation from RPE cells to neural cells. This remains, however, a remote possibility given that RSCs can be prospectively isolated and that RSCs can self-renew over multiple passages and produce progenitors that differentiate to all neural retinal cell types.[2,3,7,10–14]

Clinical applications of adult-derived mammalian RSCs are associated with several problems. Unlike fish and amphibians, those mammalian RSC cells are quiescent in vivo in adults and do not regenerate following disease or injury.[4,5] In many fish and amphibians, the ciliary marginal zone is considered a proliferative niche, where RSCs contribute to retinal regeneration throughout life.[15] In mammals, including humans and mice, no such proliferation is observed within the adult CE beyond early postnatal day 10 in mice and after birth in humans.[1,2] Another problem associated with clinical application is that large numbers of cells are needed to overcome cell loss due to degeneration itself, and due to poor cell survival and integration following transplantation.[16] To produce large quantities of retinal precursors and subsequently their progeny of photoreceptors and RPE, some research groups have used pluripotent stem cells, including embryonic

stem cells (ESCs) and induced pluripotent stem cells (iPSCs), which have an infinite ability for self-renewal *as well as* the potential to differentiate into every cell type in the body.[17–21] ESCs are derived from the inner cell mass of blastocyst-stage embryos; hence their ability to differentiate into all adult cell types derived from the three embryonic germ layers. Many labs have been successful in differentiating photoreceptors and RPE from ESCs,[17–20,22] but the ethical issues and immune rejection issues remain problematic, and the clinical application of ESCs carries a risk of teratoma formation as well. Similar to ESCs, iPSCs are pluripotent cells, but they differ from ESCs in being produced in vitro by reprogramming somatic cells through transduction of four transcription factors: Oct3/4, Sox2, Klf4, and c-Myc.[21] The use of defined reprogramming factors for generating specific iPSCs offers: (a) an autologous source, eliminating rejection; (b) an opportunity to repair genetic defects; and (c) absence of ethical problems faced with the use of ESC-derived cells.[21,23] However, issues including the risk of viral integration producing oncogene expression are still concerning.[23] CE-derived human RSCs and RSCs derived from iPCs are potential autologous sources to bypass the ethical and immune rejection concerns associated with the use of ESCs.[24,25]

RSCs traditionally have been grown as clonal spheres in static tissue culture flasks (T-flasks) or in 24 well plates. However, scaling-up cell production by using T-flasks is not effective: it can be very labor intensive and there is no control over many culture parameters. Moreover, there is significant cell loss that results from using the current enzymatic dissociation protocol[1] on clonal spheres. In this report, we studied the effect of systematic variation of factors such as agitation speed and oxygen concentration on RSC behavior inside bioreactors. We hypothesize that using suspension stirring bioreactors (SSBs) that are scalable will enhance RSC expansion through facilitating symmetrical division and allowing greater survival of cells by controlling conditions such as pH, temperature, agitation rate, nutrient level, and O_2 concentration. Another advantage of SSBs over static T-flask cultures is that we can more easily manipulate factors that are known to impact cell behavior (e.g., shear stresses, hydrodynamic pressure, and medium flow patterns).[26] Suspension culture bioreactors have been previously used to expand ESCs,[27–29] neural stem cells,[30] mammary stem cells[31], hematopoietic stem cells[32] and cancer stem cells.[33] To increase viability by reducing the cell death associated with the use of dissociative enzymes, we employed cell microcarriers (MCs).[34] Detaching cells from MCs does not require the use of Trypsin and other aggressive enzymes, from which approximately only 30% of the retinal cells can survive.[35] Instead, 1X TrypLE select solution (Cat. No. 12563-011, Gibco) can be used to detach cells in a safer and faster way because: (a) it is an animal free compound and is less harsh to cells, (b), can be diluted using either buffer or media, (c) is stable at room temperature and can last on the shelf for up to 6 months, and (d) it is free of contaminating viruses and prion proteins.[36] There are several types of MCs that are commercially available, each with different characteristics. For example, several adult cell types have been shown to prefer Cytodex 1 MCs,[37–40] while mouse ESCs have been reported to grow well on Cytodex 3 MCs[41,42] and human ESCs show enhanced attachment and growth on Hillex and CultiSphere MCs.[43] Cytodex 1 and Cytodex 3 MCs have been shown to support human mesenchymal stem cells (MSCs) growth in suspension culture.[39,44] Another method of large-scale expansion using MCs in SSB can be done by using bead-to-bead cell transfer either by adding new bare MCs to already confluent MCs or by transferring old MCs to a new bioreactor containing new MC beads.[45–47] However, this method might not work with all cell types and can lead to MC aggregation, cell multilayering, and heterogeneity within the aggregates.[45]

We have developed a novel RSC enrichment protocol using cell MCs inside suspension bioreactors spinning at lower agitation rates and with lower oxygen concentrations to achieve the best possible cell survival.

2 | MATERIALS AND METHODS

2.1 | Mouse strain

RSCs were derived from the CE of adult C57BL/6 mice (7–8 week old). All animal procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals developed by the Canadian Council on Animal Care and approved by the Animal Care Committee at the University of Toronto.

2.2 | Primary cell isolation and culture

Cells were plated in serum-free media (SFM) on nonadherent tissue culture plates (Nunc; Thermo Fisher Scientific, Rochester, NY) at a density of 20 cells/μL with FGF2 (10 ng/mL, human recombinant; Sigma-Aldrich) and heparin (2 ng/mL; Sigma-Aldrich) in humidified 5% CO_2 incubator with temperature of 37°C.

For low-density experiments, we dissected 7–10 mice (14–20 eyes) per individual bioreactor experiment. For high-density bioreactor and MCs experiments, we dissected 15–17 mice (30–34 eyes) per individual experiment.

2.3 | Bioreactor preparation

Following 7 days of primary culture in SFM, clonal spheres were dissociated into a single cell suspension using an enzymatic solution (trypsin 1.33 mg/mL, hyaluronidase 0.67 mg/mL, kynurenic acid 0.2 mg/mL, 0.5 mg/mL collagenase I, 0.5 mg/mL collagenase II, 0.1 mg/mL elastase, Sigma-Aldrich, Oakville, ON). Cells were then transferred to (125 mL/paddle) stirring bioreactor vessels (DASGIP, SR0250ODLS, Eppendorf) with a 65 mL working volume of serum-free medium with FGF2/heparin (FH). Prior to use, all inner surfaces of the glass suspension bioreactor vessels and outer surfaces of the impellers were siliconized using a 1:9 ratio of Sigmacote (Cat. No. SL-2, Sigma-Aldrich, Toronto, Canada) to hexane (Cat. No. B90210, Omnisolv from VWR International), sterilized, and calibrated (oxygen and pH calibration). Bioreactor parameters were set as following for all experiments: pH: 7.3, temperature: 37°C, and O_2 concentration: 21% and 5%. Semi-fed batch culture (30%) was used by adding 15 mL every 2 days. Cells were cultured at a density of 7 cells/μL. Static

control culture was kept in a humidified 5% CO_2 incubator at 7 cells/μL density for 7 days.

2.4 | Tertiary RSC clonal sphere assays

Samples (15 mL) were taken every 2 days over 7 days duration. A 37 μm filter (Cat. No. 27215, Stem cell technology) was used to separate single cells from aggregates. Dissociative enzymes similar to those used above were added to aggregates only. Then, cells (single cells and aggregates) were cultured separately at 10,000 cells/well density in a 5% CO_2 humidified incubator to determine the tertiary clonal spheres percentage.

2.5 | Cells counts and viability

After dissociating bioreactor and static samples, 10 μL samples were prepared for cell counting performed by hemocytometer (Cat. No. B3175, VWR) using Trypan blue dye (Cat. No. T8154, Sigma-Aldrich). Viability was analyzed using Ethidium homodiamer-2 (Cat. No. E3599, Invitrogen) and Hoechst staining. Samples were diluted, if necessary, using 1X Dulbecco's phosphate-buffered saline (DPBS) (Gibco).

2.6 | Flow cytometry

Secondary spheres from 5% O_2 bioreactors were dissociated. EdU (Click-iT EdU kit, Cat. No. C10083, Invitrogen) was diluted in dimethylsulfoxide (DMSO) giving 2 ml stock concentration of 10 mM. Working dilutions were made in 1X phosphate buffered Saline (PBS) at 10 μM. Cells were treated with 10 μM EdU for 3 hr duration, after 24 hr of initial culture. Cells then were washed using DBPS 1X and dissociated using the previously described enzymes, after which they were fixed using 4% paraformaldehyde (PFA) for 15 min and permeabilized using 0.5 Triton X-100. Reaction cocktails were prepared according to protocol instructions and cells incubated for 30 min. Cells were then analyzed using flow cytometry (BD LSR Fortessa, BD Biosciences).

2.7 | MC surface screening

We conducted the screening experiment using three different coatings: FACTIII (cationic charged collagen), ProNectin F (recombinant protein coated), and HillexII (modified polystyrene) purchased as a starter kit from SoloHill Engineering, Inc. These MCs do not require the overnight soaking step and can be sterilized along with the bioreactor during the autoclaving step. For preliminary testing of the MCs, primary RSCs were dissociated and seeded on different types of MCs (FACTIII, ProNectin F, HillexII, SoloHill Engineering, Inc., Ann Arbor, MI) in six well plates seeded with 20,000 cells/mL (4–5 cells/bead) in a 21% static humidified incubator with the shaker adjusted to 50 rpm, in addition to static control culture without MCs. Two milliliter samples were taken every 2 days to monitor cell growth, attachment, viability, and do RSC sphere clonal assay (Table 1). MCs were allowed to settle down in the falcon tube then washed two times using 1X DPBS. Cells then were detached from the MCs using 1X TrypLE select solution (Cat. No. 12563-011, Gibco) for 15–20 min in 37°C, and then they were filtered and washed with SFM. Static control samples were dissociated using the trypsin/collagenase enzyme mix. Cell counts were done using Trypan blue stain and viability was assessed using Ethidium homodiamer-2 and Hoechst staining. Cells then were cultured in SFM + FH at a density of 10,000 cells/well at 21% oxygen concentration in incubators for 7 days for tertiary clonal spheres assay.

2.8 | Continuous versus intermittent agitation

To test the effect of agitation mode (continuous vs. intermittent) on RSCs attachment and expansion, we dissociated primary RSC spheres and seeded them on FACTIII MCs (10,000 cells/mL) in either continuous agitation (50 rpm) or intermittent agitation (50 rpm, 3 min agitation followed by 30 min no agitation for 6 hr followed by continuous agitation thereafter) under conditions of 5% oxygen, pH 7.34, and temperature 37°C. Samples were taken every 2 days for growth analyses and tertiary clonal spheres assay. MCs were allowed to settle down in the falcon tube, and then washed two times using 1X DPBS. Cells were detached from the MCs using 1X TrypLE enzyme, then

TABLE 1 Retinal stem cell growth compared using three different microcarrier types

	FACTIII	ProNectin F	HillexII	Control
Maximum cell density (cells/mL)	495,000 (Day 2)	275,000 (Day 2)	267,500 (Day 6)	207,500 (Day 6)
Viability percentage (%)	81.47	81.03	74.76	83.66
Attachment percentage (%) (Day 4)	75.13	41.88	51.75	
Average specific growth rate	0.0174	0.015	0.0179	0.0162
Doubling time (hours)	39.82	46.2	38.7	42.77
Expansion fold	12.37	8.8	13.37	10.37
Number of tertiary RSCs/10,000 cells	0.442	0.246	0.193	0.193

Note. MCs = microcarriers; RSCs = retinal stem cells.

Note. Three different MCs with different coatings were used for the screening experiment: FACTIII (cationic charged collagen), ProNectin F (recombinant protein coated) and HillexII (modified polystyrene). Cells were seeded on MCs in six well plates in 21% static humidified incubator with shaker adjusted to 50 rpm in addition to static control culture without microcarriers. FACTIII reached its maximum cell density on day 2 and had a higher attachment compared to other types of microcarriers and produced 2.4 times RSCs compared to static culture.

filtered and washed with SFM and seeded at 10,000 cells/well clonal density in a 5% oxygen incubator in SFM + FH.

2.9 | Low versus high seeding density

Primary spheres were dissociated using the Trypsin/Collagenase enzymes mix and then transferred to a bioreactor vessel filled SFM + FH and FACTIII MCs (sterilized and autoclaved along with the bioreactor). Cells were cultured either at 10,000 cells/mL (2 cells/bead) or 50,000 cells/mL (9–10 cells/bead). The bioreactor stirring speed was adjusted to 50 rpm continues agitation in both experimental assays. Samples were taken every 2 days for growth analyses and tertiary clonal spheres assay. MCs were allowed to settle down in the falcon tube, and then washed two times using 1X DPBS. Cells were detached from the MCs using 1X TrypLE enzyme, then they were filtered and washed with SFM and seeded at 10,000 cells/well clonal density in 5% oxygen incubator in SFM + FH.

2.10 | 1X TrypLE versus 10X TrypLE

Primary spheres were dissociated using Trypsin/Collagenase enzymes mix and then transferred to a bioreactor vessel filled with SFM + FH and FACTIII MCs (sterilized and autoclaved along with the bioreactor) at 50,000 cells/mL (9–10 cells/bead) seeding density. Samples were taken every 2 days and cells on the MCs were incubated in 1X TrypLE or 10X TrypLE Select (Cat. No. A1217701, Gibco) for 10–15 min at 37°C. After counting, cells were seeded at a density of 10,000 cells/well (SFM + FH) for 7 days in a 5% oxygen incubator for tertiary clonal spheres assay.

2.11 | RSC differentiation and immunostaining

RSC colonies cultured in 5% SSB were plated on laminin-coated (50 ng/mL, Sigma-Aldrich) 24-well plates (Nunc) in a humidified 5% CO_2 incubator, and differentiated using a protocol for rod photoreceptor differentiation involving taurine (100 μm; Sigma-Aldrich) and retinoic acid (RA) (500 nM; Sigma-Aldrich), plus FH for 50 days.[7] Pan-retinal differentiation media supplemented with 1% fetal bovine serum (FBS) (Invitrogen, Burlington, ON) and FH was used as a control. Media and growth factors were replaced every 4 days. The cells were then rinsed with PBS and fixed using 4% PFA for 10 min at room temperature. Cells were permeabilized with 0.3% Triton X-100 for 10 min and preblocked with 2% normal goat serum and bovine serum albumin (BSA) for 1 hr at room temperature. Cells were then incubated overnight at 4°C in the following primary antibody: anti-Rhodopsin (MAB5316, RetP1, 1:250; Millipore) and next day cells were washed three times with PBS and incubated in the following secondary anti-body: Alexa fluor 568 (1:400; Invitrogen) for 1 hr at room temperature. Nuclei were stained with Hoechst dye (1:1,000; Sigma-Aldrich). Stained cells were then examined under a fluorescence microscope (Axio Observer D1; Carl Zeiss) using AxioVision 4.8 software (Carl Zeiss).

2.12 | Statistics

Statistical analysis was performed using Student's *t* test through the program GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA) and Microsoft Excel. Data are presented as mean ± *SEM*. The significance value was set at $p < 0.05$.

3 | RESULTS AND DISCUSSION

3.1 | A lower agitation rate increased the yield of RSCs by eight times over faster agitation

To engineer the RSC microenvironment in stirring bioreactors, several factors should be taken in consideration: physical forces (mechanical stress and hydrodynamic forces), oxygen tension, extra cellular matrix, and the regulation of autocrine and paracrine signaling. The hydrodynamic forces generated in a bioreactor depend on agitation rate, seeding density, impeller and vessel geometry, as well as the presence of oxygen and pH probes that can disturb the radial flow.[48] We dissociated RSC colonies from primary clonal spheres and then cultured the cells at 7,000 cells/mL density under high and low speeds (50 and 80 rpm) in 21% oxygen, 5% CO_2, pH 7.3, and at a temperature of 37°C in a DASGIP stirring bioreactor vessels (Cat. No. SR0250ODLS, Eppendorf). Thirty percentage of fresh media and growth factors were added every 2 days (30% semi-fed batch). Other studies previously have illustrated the advantages of using semi-fed batch (i.e., adding fresh media and growth factors regularly) over batch culture (maintaining the initial starting media inside the bioreactor for the entire experiment length without adding fresh media) on cell growth and proliferation.[49] Semi-fed replacement of culture media will ensure an adequate supply of nutrients as well as removal of any byproducts or cytokines that can negatively affect cell growth and proliferation.[49] In our study, samples were taken every 2 days over the 6-day culture period. The average percentage of viable cells for each sample from the three experiments is shown in Figure 1a. We observed less viability in cells grown under high agitation compared to those cultured under low agitation or in a static culture, which can be attributed to the effects of shear stress on the cells in the high agitation culture in addition to the negative effects of the dissociating enzymes. We dissociated the cells from each bioreactor sample and cultured them at clonal density in a humidified static incubator for 7 days (tertiary clonal assay)[1,50] in order to calculate the numbers and percentages of RSCs. Although single RSCs start clonal sphere growth, the percentage of RSCs in a sphere is very low; the vast majority of sphere cells are retinal progenitor cells with less proliferative and differentiation abilities. The average tertiary sphere percentages from all samples (days 2, 4, and 6) were significantly higher at 50 rpm 0.01 ± 0.00182 compared to 80 rpm (0.00133 ± 0.00023), but the numbers of RSC spheres produced under 50 rpm conditions were not statistically greater than those seen in the static control cultures (0.00653 ± 0.00323) (Figure 1b). The percentages of clonal RSC spheres of total cells cultured per day are shown in Supporting Information Figure S1.We conclude that culturing RSCs under the higher agitation rate of 80 rpm negatively affected cell

viability and the number of tertiary RSCs. Thus, 50 rpm was chosen as a preferred stirring speed for all of the following experiments.

3.2 | Hypoxia (5% O_2) increased the survival and the symmetric division of RSCs with no effect on proliferation rate

Next, we examined the effect of different oxygen concentration on RSC growth and proliferation in stirring suspension bioreactor cultures. RSCs within their niche reside in relatively hypoxic conditions (1–5% O_2).[51] Low oxygen tension is critical for multiple stem cell types to maintain their self-renewal and proliferation.[52] Hypoxia also is known to stabilize hypoxia-induced factor alpha (HIF-1), which in turn activates genes that are implicated in many different cellular functions such as cell survival, cell proliferation, self-renewal, apoptosis, glucose metabolism, and angiogenesis.[53] To investigate the effect of relative hypoxia on RSCs, we dissociated primary clonal retinal spheres and transferred them to a DASGIP bioreactor system. The average tertiary sphere percentages (days 2, 4, and 6) from aggregate samples were 0.01% ± 0.00311 in 5% SSB, 0.0018% ± 0.0009 in 21% SSB, and 0.0014% ± 0.00088 in the static cultures (Figure 2a). The tertiary clonal sphere percentages from aggregate samples only per day are shown in Supporting Information Figure S3a, and data from single cell samples only are shown in Supporting Information Figure S3b. The average tertiary sphere percentages (days 2, 4, and 6) from aggregate and single cell samples were (0.0068% ± 0.00197) in 5% SSB, (0.0013% ± 0.00055) in 21% SSB, and (0.002% ± 0.00099) in the static cultures (Figure 2b). By culturing in SSB and hypoxia, we recovered five times more clonal spheres compared to 21% SSB and seven times more than that obtained from 21% static cultures. No significant difference was observed between the numbers of tertiary clonal spheres derived from 21% SSB and 21% static cultures. We conclude that culturing in relative hypoxia was associated with a significant increase in clonal stem cell yields in both static and bioreactor cultures. We suggest that relative hypoxia could have exerted this effect through one or more of the following mechanisms: improving cell survival, enhancing proliferation, or increasing the symmetric division of RSCs.

To further analyze the effect of relative hypoxia on the survival of RSCs, we tested the viability of single cell and aggregate samples every 2 days using Ethidium homodiamer-2 and Hoechst staining. The average 5% SSB viability (single cells + aggregates) over 6 days was similar to 21% SSB culture and 21% static culture (77.54% ± 15.15, 77.11% ± 9.53, and 62.29% ± 12.31, respectively) (Supporting Information Figure S2a). On the other hand, there was 1.5-fold increase in the viability of cells in the aggregate samples in hypoxia (Supporting Information Figure S2b) compared to normoxia in SSB cultures. To investigate the effect of 5% oxygen on the proliferation of RSCs, cell

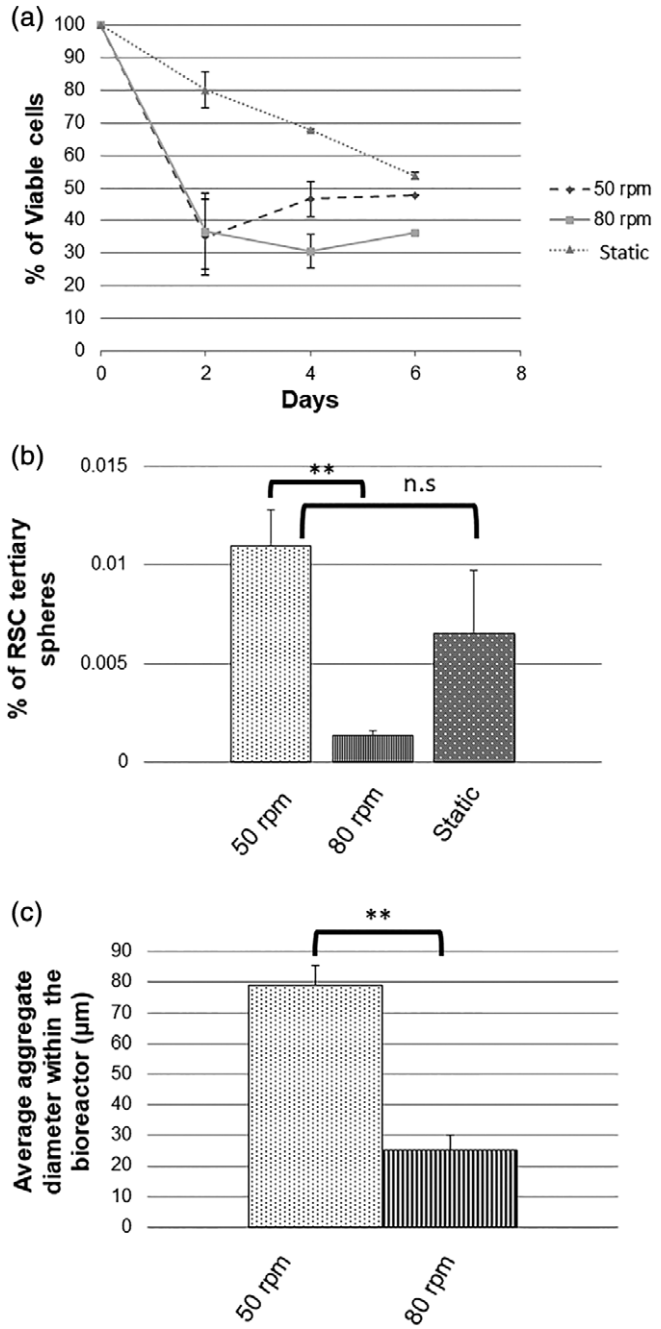

FIGURE 1 Viability of retinal stem cells (RSCs) and their progeny in spinning suspension (50 and 80 rpm) versus stationary cultures. (a) Average percentage of viable cells cultured in a stirring suspension bioreactor for 6 days under two different agitation rates compared to static control culture. (b) Average percentage of tertiary sphere forming cells in 50 and 80 rpm bioreactors versus static culture. Average tertiary sphere percentages from all samples (day 2, 4, and 6) were significantly higher at 50 rpm compared to 80 rpm ($p = 0.02$, $t = 3.731$, $df = 4$, $N = 2$) but not to the static control culture ($p = 0.4407$, $t = 0.855$, $df = 4$). Each bar represents the average percentage of RSCs retrieved from day 2, 4, and 6 samples in each experiment from $N = 2$ individual bioreactor experiments and $n = 8–24$ technical replicates per sample in each experiment. Fourteen eyes from 7 mice were dissected for primary spheres growth to seed each bioreactor. (c) Average diameter of aggregates formed inside the bioreactor at both speeds on day 6. Average aggregate diameter was significantly smaller in 80 rpm cultures compared to 50 rpm cultures ($p < 0.004$, $t = 5.76$, $df = 3$). Results represent the means ($N = 2$ individual bioreactor experiments per condition and $n = 2–9$ technical replicates in each experiment) ± *SEMs* (**$p < 0.01$)

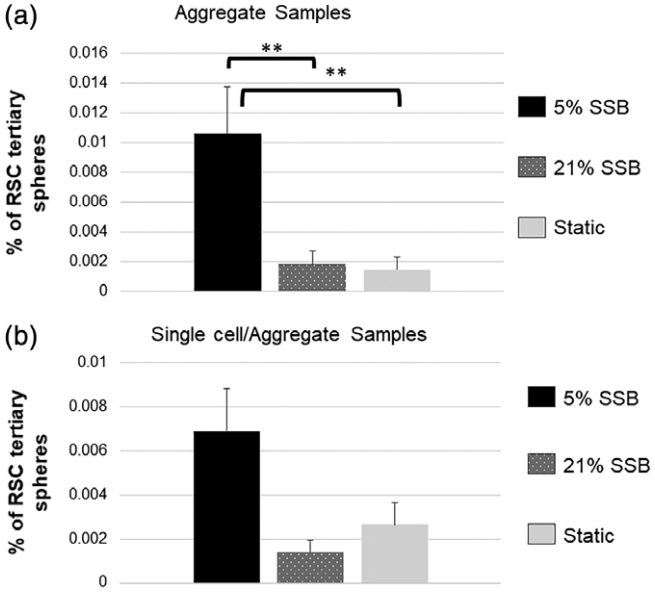

FIGURE 2 Tertiary clonal retinal stem cell (RSC) spheres assay. Cells from bioreactor and static culture samples were dissociated and plated at 10,000 cells/500 μL/well in SFM + FGF2 and heparin (FH) for 7 days in 21% oxygen static humidified incubator. (a) Average percentage of clonal sphere forming cells in 5% suspension stirring bioreactor (SSB), 21% SSB, and 21% static culture. Culturing in SSB and hypoxia resulted in a fivefold increase in spheres compared to 21% SSB (p = 0.0265, t = 2.719, df = 4, N = 3 individual bioreactor experiments) and sevenfold increase when compared to 21% static culture (p = 0.0235, t = 2.837, df = 4, N = 2–3 individual bioreactor experiments). Results represent the means ± *SEMs*. Each bar represents the average percentage of RSCs retrieved from day 2, 4, and 6 samples in each experiment from N = 2 individual bioreactor experiments and n = 8–24 technical replicates per sample in each experiment. Fourteen eyes from 7 mice were dissected for primary spheres growth to seed each bioreactor). (b) Average percentage of RSC clonal spheres from aggregate and single cell samples. Average tertiary spheres percentage (days 2, 4, and 6) from aggregate and single cell samples in 5% SSB, 21% SSB, and static culture

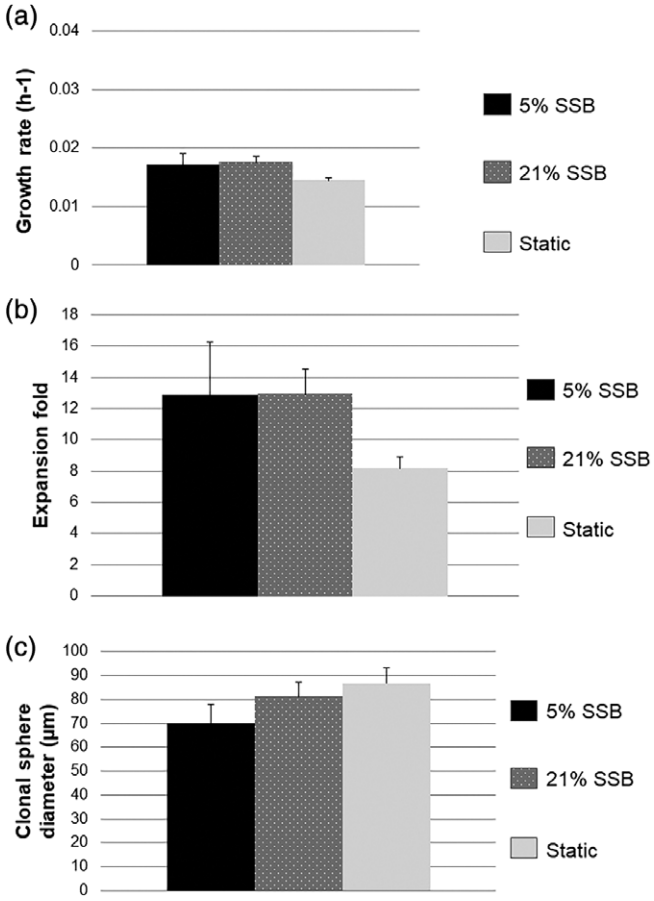

FIGURE 3 Growth kinetics of retinal stem cell (RSCs) grown in hypoxic and normoxic suspension stirring bioreactor (SSB), and static conditions. (a) Growth rate. (b) Expansion fold. (c) Average clonal sphere diameter for tertiary spheres derived from 5% SSB, 21% SSB, and 21% control static culture. Results represent the means of N = 2–3 of individual bioreactor experiments ± *SEMs*

density was measured every 2 days using trypan blue and a hemocytometer for both single cell and aggregate samples. The growth rate chart showed nonsignificant differences between 5% and 21% SSB with expansion of 12.9 ± 3.31-fold and 12.92 ± 1.59-fold, respectively (Figure 3a,b). The average sphere diameters also were similar at 70 μm ± 8.08 in 5% SSB, 81.33 μm ± 5.81 in 21% SSB, and 86.66 μm ± 6.65 in static culture (Figure 3c). To test the proliferative capacity of RSCs in hypoxic and normoxic cultures, the population doubling rate for each condition was calculated using this equation: N = [log (NH) – log (N1)]/log (2), where N = population doublings, N(H) = cell harvest number, and N(I) = plating cell number. Population doubling time (PDT) was calculated at each passage, PDT = time of culture (hours)/N, where N = population doubling level. Minimal differences were observed between normoxic and hypoxic cultures suggesting that relative hypoxia did not affect the proliferation of RSCs and their progeny. To further document this observation and test the alternative explanation of increased survival in hypoxia, hypoxic and normoxic RSC spheres were inoculated with Edu 24 hr postculture initiation. The percentages of cells labeled with Edu were determined using a click-it kit (Cat. No. C-10420, Invitrogen) 3 hr postinoculation. Edu incorporates into DNA during S-phase and labels all dividing cells (Figure 4a). No significant difference was seen in Edu labeling. Cells (5.55%) grown in hypoxia were Edu positive compared to 3.95% in normoxia.

To investigate if hypoxia affects the mode of RSC division, we added the Notch receptor ligand Delta3 (Dll3) to tertiary RSCs grown from both hypoxia and normoxia in static cultures (Figure 4b). Delta 3 previously was shown to increase RSC symmetrical division in adult and PND2 RSCs, and a number of previous studies has also indicated its critical role in maintaining the precursor cell pool in the developing eye.[4,54,55] Dll3 increased the number of clonal RSCs by 2.4 times when added to cells from normoxic cultures (0.875 ± 0.144 in normoxia alone and 2.14 ± 0.340 in normoxia + Dll3). When added to cells from hypoxia, Dll3 caused a nonsignificant increase in the number of RSCs compared to hypoxic culture alone (hypoxia

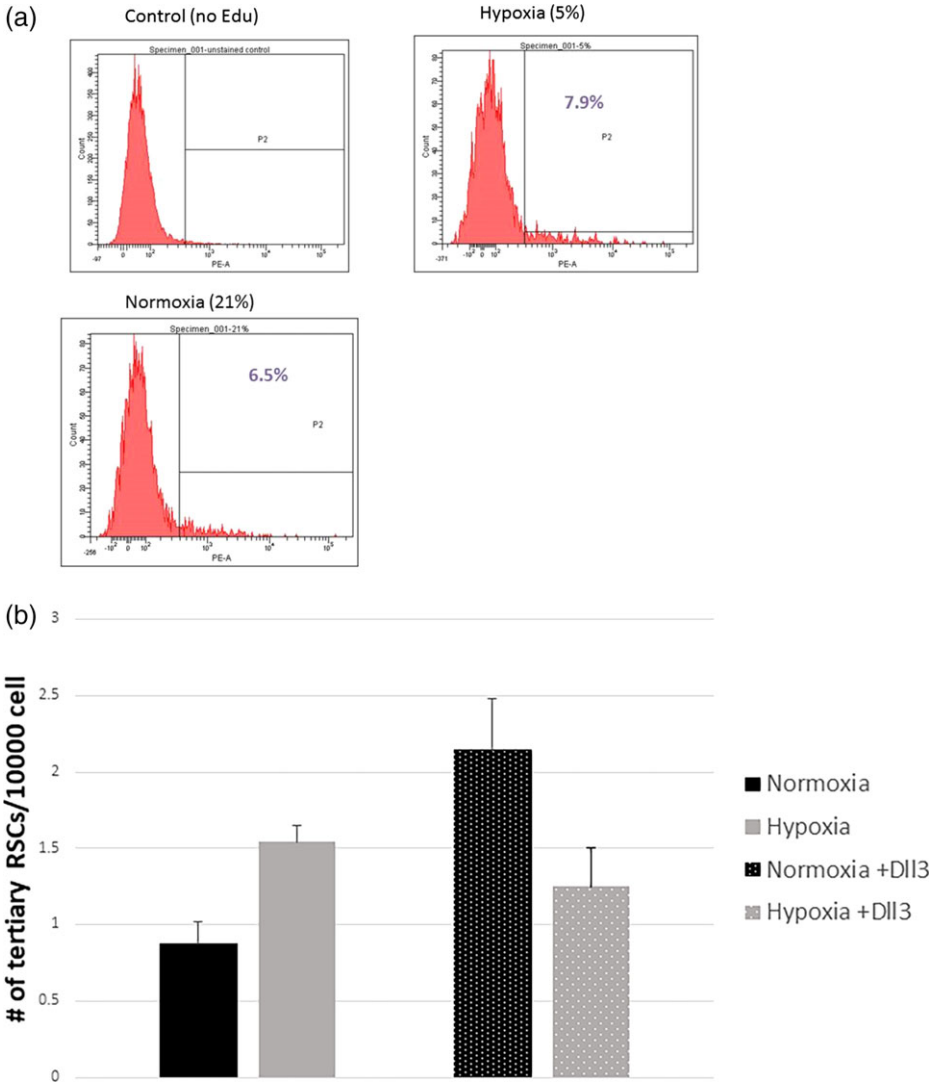

FIGURE 4 Effects of hypoxia on retinal stem cells (RSCs) (symmetrical division rather than proliferation). (a) Flow cytometric analysis of adult RSC cultured in hypoxic (5%) and normoxic (21%) oxygen. No significant difference in Edu expression (red) was seen between 5 and 21% oxygen cultures. Data shown were gathered on a BD LSR Fortessa flow cytometer. (b) Effect of adding Dll3 to tertiary RSCs cultured in normoxia and hypoxia. We found 2.4 times increase in the percentage of clonal RSCs when Dll3 was added to normoxic culture ($p = 0.002$, $t = 4.719$, $df = 7$, $N = 3$, $n = 8$) and 1.7 times when we cultured in hypoxia. Adding Dll3 to hypoxic cultures produced no differences in RSC percentages, $p = 0.402$, $t = 0.891$, $df = 7$, $N = 3$, $n = 8$. Results represent the means ± *SEMs*. N = number of biological replicates and n = technical replicates

1.54 ± 0.110, hypoxia + Dll3 1.25 ± 0.25). We suggest that hypoxia already had increased symmetric divisions of RSCs and that adding Dll3 had no further effect on enhancing symmetric divisions.

From the above observations, we conclude: (a) relative hypoxia did not increase RSC and progenitor proliferation compared to normoxia and (b) relative hypoxia improved cell survival and facilitated RSC symmetrical division. However, RSCs are quite rare cells in our cultures and thus differentiating selective survival versus symmetric proliferation effects on RSCs remains difficult. In light of the above results, relative hypoxia (5% O_2) will be used for all of the following experiments.

3.3 | A specific cell MC resulted in a fivefold increase in clonal RSC spheres compared to SSB alone and a 10-fold compared to static culture

To expand the RSCs using MCs, we conducted a screening experiment using three different coatings: FACTIII (cationic charged collagen), ProNectin F (recombinant protein coated), and HillexII (modified polystyrene) purchased as a starter kit from SoloHill Engineering, Inc. We observed that the viability in all MCs stayed above 80% throughout the culture period. FACTIII and HillexII had similar growth rates and expansion (Table 1). These values were higher than those observed in ProNectin F and control culture. FACTIII reached its maximum cell density on day 2 (495,000 cells/mL) and had a higher attachment with an average of 75.13% of cells attached compared to other types of MCs. The average clonal RSC percentages from cells taken at day 2, 4, and 6 were 0.0022% ± 0.00046 in FACTIII cultures, 0.001 ± 0.00042 in ProNectin F, 0.0009% ± 0.00028 in HillexII, and 0.0009% ± 0.00028 in static control (Figure 5a). Using FACTIII in shaking static culture produced 2.4 times the RSCs compared to static culture. Thus, the following bioreactor experiments used the FACTIII MCs, as they were shown to affect positively RSC attachment and expansion. We then tested the effects of agitation mode, seeding density, and different dissociation protocols on RSCs seeded on the FACTIII MCs and compare them to the Stirring SSB alone and static control cultures. We found that the average percentage of RSCs (days 2, 4, and 6) was higher in continuous agitation culture compared to intermittent agitation with 0.01 ± 0.00225 and 0.00638 ± 0.00144, respectively (Figure 5b). More clonal RSC spheres were observed in the continuous agitation culture

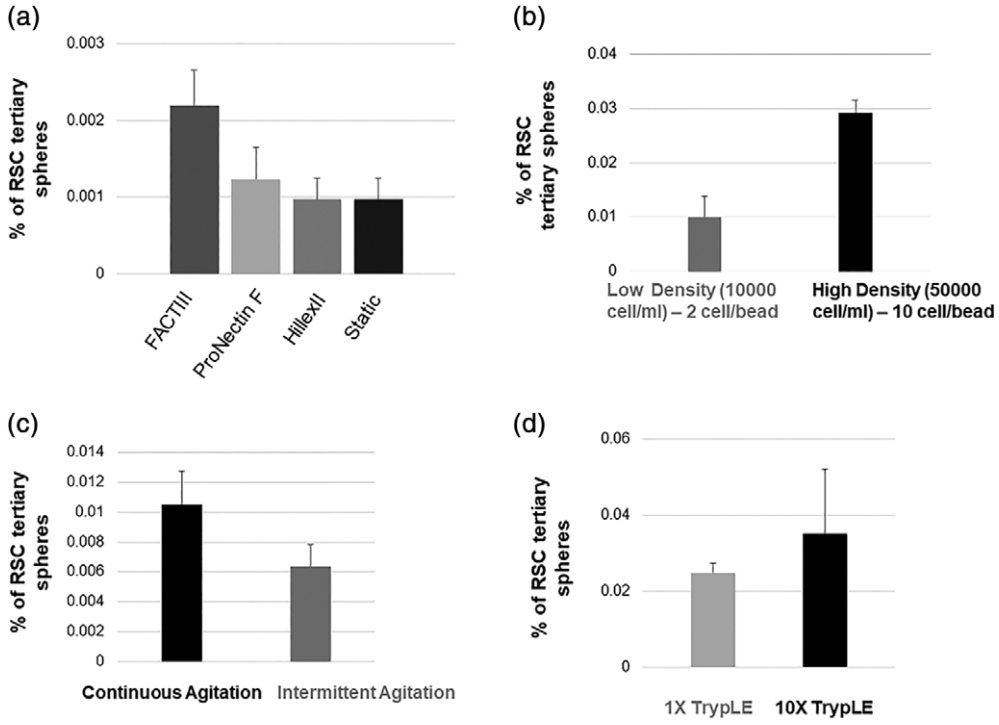

FIGURE 5 Microcarrier (MC) screening and testing under different agitation and oxygen tension. (a) Average tertiary sphere forming cell percentage. Primary retinal stem cells (RSCs) were dissociated and seeded on different types of MCs (FACTIII, ProNectin F and HillexII, SoloHill engineering, Inc., MI) in six well plates placed on a shaker adjusted to a speed of 50 rpm in 21% oxygen humidified incubator. A static culture without MCs was used as a control. (b) Percentage of clonal tertiary sphere forming cells: Continuous agitation versus intermittent agitation. Average percentages of RSCs were higher in continuous agitation culture compared to intermittent agitation. (c) Percentage of clonal tertiary sphere forming cells: Effect of seeding density (high vs. low) on growth and behavior of RSCs. We observed 2.5 times more RSC spheres arising from high-density culture compared to low-density culture ($p = 0.0262$, $t = 2.731$, $df = 4$, $N = 2–4$). (d) Effects of cell detaching enzymes (1X TrypLE vs. 10X TrypLE) on growth and behavior of RSCs. A nonsignificant RSC percentage increase was observed in the 10X TrypLE experiment compared to 1X TrypLE ($p = 0.365$, $t = 0.394$, $df = 2$). Results represent the means ± *SEMs*. Each bar represents the average percentage of RSCs retrieved from days 2, 4, and 6 samples in each experiment from 2–4 individual bioreactor experiments ($N = 2–4$ individual bioreactor experiments, $n = 8–24$ technical replicates per sample in each experiment). For high-density bioreactor and MCs experiments, we dissect 15–17 mice (30–34 eyes) for primary spheres growth to seed each individual bioreactor experiment

on day 4, but there were no significant differences on days 2 or 6 (Supporting Information Figure S4b). We also observed three times more clonal RSC spheres (as a percentage per total number of cells) arising from high-density culture (0.02911 ± 0.0009) compared to low-density culture (0.01 ± 0.00225) (Figure 5c). In all experiments, culturing in higher density (50,000 cells/mL) gave better RSC yields. Also, a higher but not significantly different frequency of RSCs for the same numbers of cell plated was retrieved from 10X TrypLE experiments (0.0351 ± 0.0169) compared to 1X TrypLE (0.0291 ± 0.00371) (Figure 5d). Given that no significant difference was observed in the total cell expansion between the two conditions (Supporting Information Figure S5a), we used 10X TrypLE for the following experiments. We then studied the effects of using FACTIII MCs in hypoxia, high density, continuous agitation and 10X TrypLE on RSCs expansion, static and SSB preparations without MCs were used as controls. Samples were taken every 2 days to monitor cell viability (Figure 6a); SSB and static samples were dissociated using a Trypsin/Collagenase enzyme mix, while MCs were treated with 10X TrypLE. 1X and 10X TrypLE failed to dissociate spheres from static and bioreactor samples, but did detach cells from MCs. Using collagenase enzyme to detach cells from MCs was associated with much lower stem cell yield than 1X and 10X TrypLE (data not shown). Cells from each sample were then seeded at 10,000 cells/500 μL/well in a 5% oxygen and 5% CO_2 humidified incubator for 7 days for clonal sphere assay. The overall viability of the cells on the MCs was higher compared to SSB and static cultures (Figure 6a). The use of MCs resulted in five times more tertiary RSCs compared to hypoxic SSB and 10 times more tertiary RSCs in comparison to normoxic static culture (Figure 6b–d), with the most RSC spheres observed from day 4 samples (Supporting Information Figure S6). We noticed that in MC experiments, the numbers of stem cell spheres decreased between days 4 and 6 (Supporting Information Figure S6a,b). Potential explanations for this observation are: (a) an increase in toxic metabolites or a decrease in nutrients in the media between day 4 and 6, (b) more cell expansion was seen on day 4 which could have negatively affected the stem cells through contact inhibition (Supporting Information Figure S7), or (c) RSCs could have increasingly divided asymmetrically between day 4 and 6. We conclude from the above results that continuous agitation, higher density culture,

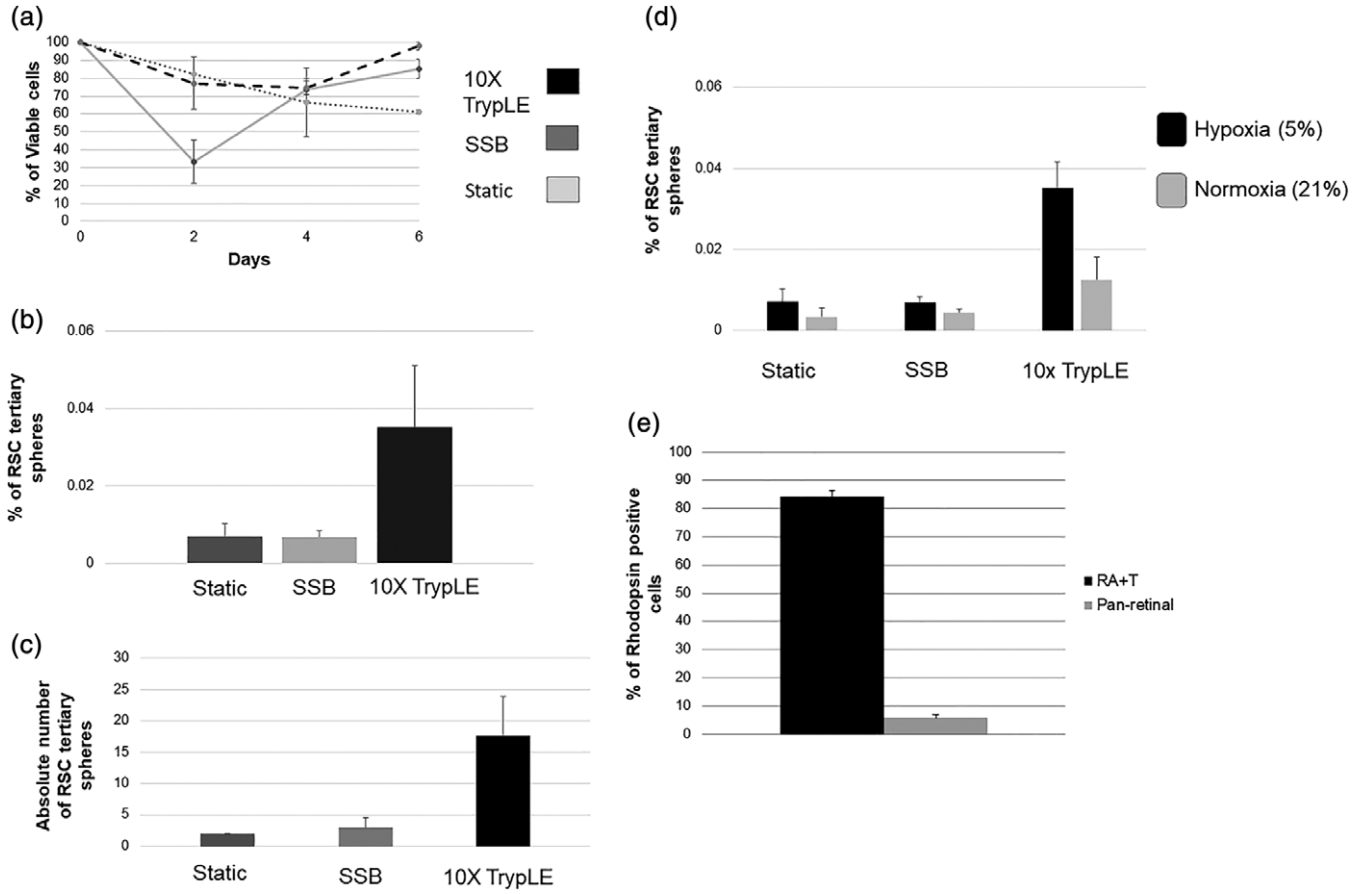

FIGURE 6 (a) Viability of cells inside microcarrier (MC), suspension stirring bioreactor (SSB), and static culture. (b) The average numbers of clonal tertiary retinal stem cells (RSCs) as percentages of total viable cells in different platform (days 2, 4, and 6). MC experiment produced five times more tertiary RSCs compared to hypoxic SSB and 10 times more when compared to normoxic static culture, p values 0.0327 (t = 3.715, df = 2) and 0.02 (t = 3.92, df = 4), respectively. Each bar represents the average percentage of RSCs retrieved from day 2, 4, and 6 samples in each experiment from two individual bioreactor experiments (N = 2 individual bioreactor experiments, n = 8–24 technical replicates per sample in each experiment). 15–17 mice (30–34 eyes) were dissected for primary spheres growth to seed each individual experiment. Results represent the means ± *SEMs*. (c) Average total number of RSCs in each condition. (d) Average numbers of clonal tertiary RSCs as percentages of total viable cells observed in MC, bioreactor, and static culture in hypoxia versus normoxia. (e) Differentiation of RSC derived from hypoxic bioreactor culture in taurine and retinoic acid (RA) for 50 days compared to pan-retinal control. RSCs differentiated into rods using a protocol developed in the lab.[6] The percentage of rod photoreceptors (stained for anti-Rhodopsin antibody) was 84.31 ± 2.08 in taurine and RA, significantly higher (p = 0.00052, t = 31, df = 2) compared to 5.82% ± 1.23% rhodopsin positive cells in pan-retinal differentiation control cultures. Results represent the means of N = 3–5 biological replicates ± *SEMs*

10X TrypLE (or 1X TrypLE), and 5% O_2 increased the expansion of RSCs over static culture.

3.4 | Bioreactor-derived RSCs differentiate effectively to rod photoreceptors using RA and taurine

We then cultured bioreactor-derived spheres on laminin in RA and taurine (RA + T) to test their differentiation potential. The percentage of rod photoreceptors (stained for anti-Rhodopsin Immunocytochemistry) was 84.31 ± 2.08 (Figure 6e) in taurine and RA, and significantly different (p = 0.00052, N = 3–5) compared to 5.82% ± 1.23% rhodopsin positive cells in pan-retinal differentiation control cultures. These results were similar to previous published data from our lab growing rod photoreceptors from adult-derived mouse RSCs using RA and taurine.[7]

4 | CONCLUSION

The present results provide an RSC expansion protocol using MCs in SSB to overcome problems associated with growing stem cells in static culture. We found that our bioreactor expansion protocol enhanced RSCs survival through optimizing culture conditions and using less aggressive MC detaching enzymes. Using FACTIII MCs in hypoxia increased the yield of RSCs 10 times compared to static normoxic culture conditions. We found that hypoxia (5% O_2) was associated with better RSC expansion across all platforms; and this can be attributed to hypoxia-induced increase in survival and/or symmetric division of stem cells. RSCs within their niche in vivo reside in relatively hypoxic conditions (1–5% O_2).[51] A number of previous studies has shown that low oxygen tension is important for multiple stem cell types to maintain

self-renewal and proliferation of the resident precursor cells,[52] including the retina.[56–58] For example, earlier formation of neural rosettes was observed under hypoxia in addition to higher number of Pax6/Chx10 positive retinal precursor cells in cultures derived from human ESCs and iPSCs embryoid bodies in a static culture.[56] Other studies also observed better growth of retinal organoids in hypoxia, both in stirring bioreactor[57] and static cultures.[59] Several studies showed that hypoxia exerts this effect by stabilizing hypoxia-induced factor 1 alpha (HIF-1), which in turn activates genes that are implicated in many different cellular functions such as cell survival, proliferation, and self-renewal.[53] HIF upregulates the Notch signaling pathway and its downstream cascade including Hes genes.[60,61] Our previous demonstration that the Notch ligand Delta 3 increased symmetric divisions by both adult and PND2 RSCs also has highlighted the critical role of Notch signaling in maintaining the precursor cell pool in the developing eye.[4,54,55] We suggest that hypoxia activated Notch signaling and its downstream Hes pathway through stabilizing HIF1 alpha, and this may explain why adding Dll3 to our hypoxic culture conditions did not produce any further expansion of the RSCs through symmetric divisions. In the future, it will be important to target the differentiation of RSCs and their progeny toward rod and cone photoreceptor phenotypes using FACTIII MCs inside bioreactors to expand their populations in order to produce large numbers of cells needed for transplantation. A similar expansion of human RSCs may allow us to differentiate large numbers of postmitotic cells (rods, cones, and RPE) that can be transplanted to patients with degenerative eye diseases.

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AUTHOR CONTRIBUTIONS

D. v. d. K. and T. B. designed the experiments. T. B. preformed the experiments and analyzed the data. T. B. wrote the manuscript and D. v. d. K. revised the manuscript.

CONFLICT OF INTEREST

The authors declare no potential conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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