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Alternative culture conditions for isolation and expansion of retinal progenitor cells

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Abstract

Purpose. To investigate different *in vitro* model systems for retinal progenitor cell (RPC) isolation and expansion.

Methods. RPCs were isolated from embryonic day (E) 17 Long Evans rat retinas. Three different culture media: (1) modified serum free defined media (2) serum-containing media and (3) embryonic stem cell (ES)-conditioned media were used for RPC isolation and long term expansion Expression of various cellular markers and cell morphologies were compared among the three culture systems at different passages by immunostaining and confocal microscopy.

Results. All three culture systems could maintain RPCs as nestin-positive cells (78–87%) after long-term *in vitro* expansion. However, RPCs appeared to proliferate faster in the serum-free culture system. The ES-conditioned media provided the best RPC survival. Cells appeared smaller at early passages compared with later passages. This morphology change occurred at P9–P10 in the serum-free medium, and at P5–P6 in the other two culture systems.

Conclusions. The serum-free medium may be superior for preventing RPC differentiation during expansion.

Keywords: embryonic stem cell; progenitor, proliferation; retina; serum-free media

Introduction

Recently, there has been an increased interest in the potential application of stem-cell based technologies for the treatment of a variety of diseases, particularly in the central nervous system. As a result, the isolation and *in vitro* expansion of neural stem/progenitor cells, as well as the controlled differentiation and genetic engineering of these cells, have become important focal points of current investigations. In order to maintain stem cell multipotency during *in vitro* expansion and genetic programming, establishment of a stable and reliable *in vitro* culture system becomes of paramount importance. Moreover, having a completely defined media (serum-free) is crucial for understanding the critical elements required for optimal stem cell survival and control of stem cell behavior.

Serum-free culture systems have been successfully developed over the last two decades for neural stem cells originating from the brain and spinal cord.[1,2] Previous studies have shown that neural stem/progenitor cells exist in both embryonic and adult rodent brain and retina.[3–5] These cells can be isolated and extensively expanded in response to EGF and/or bFGF in serum-free defined culture conditions.[6,7] Long-term expansion of retinal progenitor cells in serum free culture systems, however, has not been well-established. Previous investigations have demonstrated poor RPC survival in a serum-free culture system.[8] Recently, Akagi[9] reported the use of a serum-free culture medium for E18 rat retinal progenitor cell (RPC) isolation and expansion. The RPCs, however,

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could not express retinal specific markers following *in vitro* differentiation after long-term expansion.

Previous studies have shown that the early stage embryonic environment presents signals that could delay late stage stem/progenitor cell differentiation.[10] The cell culture supernatant of mouse embryonic stem (ES) cells derived from a very early embryonic stage (3.5 days) is believed to contain certain "early stage development" environmental "signals",[11] which could prevent that stem cells undergo differentiation. The precise nature of these signals is unknown. Although studies have shown that neural stem cells can secrete growth factors that induce ES cell differentiation, it has yet to be shown that ES cell-derived growth factors can inhibit the differentiation of late stage neural stem cell. Therefore, we investigated whether ES conditioned medium could maintain retinal progenitor cell properties.

In this study, we evaluate the effectiveness of a modified serum free defined culture system supplemented with both EGF and bFGF, and an embryonic stem cell-derived growth factor conditioned media system, compared with a traditional serum containing culture system for E17 retinal progenitor cell isolation and expansion. Our purpose is to develop a culture system for RPC isolation and long-term expansion with optimization of cell survival and attachment, and cell multipotency. Such a system will be useful for future application of RPC technology in various therapeutic strategies.

Materials and methods

Retinal progenitor cell culture systems

Three different culture systems were evaluated in these experiments. The composition of each system is described below:

(a) Modified serum-free defined medium (SFM) consisted of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (1 : 1) [Invitrogen, Carlsbad, CA] containing 1% N-2 supplement, 2% B-27 supplement (Invitrogen), 10 ng/ml NT3 (Invitrogen), 100 ng/ml Epidermal Growth Factor (EGF, Invitrogen), 20 ng/ml Fibroblast Growth Factor (bFGF, Invitrogen); 1% L-Glutamine, 100 ug/ml Penicillin and 100 ug/ml Streptomycin (Invitrogen).

(b) Serum-containing culture medium (SCM) consisted of DMEM/F12 containing 1% N-2 supplement, 1% fetal bovine serum (FBS, Invitrogen), 10 ng/ml bFGF, 2 mM L-Glutamine, 100 µg Penicillin and 100 µg Streptomycin.

(c) Embryonic stem cell supernatant conditioned medium (ESM) consisted of 50% serum-containing medium and 50% embryonic stem (ES) cell culture supernatant. ES cell culture supernatant was obtained as follows: Mouse ES cell culture was undertaken as previous described.[12] Briefly, the ES-D3 (Cat: CRL-11632, ATCC, Manassas, VA) cell line was cultured on ESGM medium without a feeder cell layer, but supplemented with mouse leukemia inhibitory factor (LIF) and 2-Mercaptoethanol (2-ME). Cells were passaged every two days. ES supernatant was collected every day. Before use, ES supernatant was filtered using a 0.22 µm pore size filter, and then mixed with 50% traditional serum containing medium.

In order to determine the extent of RPC cell proliferation *in vitro*, 10 µM bromodeoxyuridine (BrdU, Sigma) was added to each of the three culture media for 48 hours. For the differentiation experiment, based on retinoic acid induction method described by Kelley *et al.*,[13] 5×10^{-7} M *all-trans* retinoid acid (RA, Sigma) and 10% FBS were added. EGF and bFGF were removed from the media. Cells from passages 6 and 9 underwent induction with RA for 8 days to test the multipotent capability of the progenitor cells.

Dissection procedure and retinal progenitor cell isolation

Fetuses derived from E17 timed-pregnant Long-Evans rats (total 16 rats) were used for this study. All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic Research. The eyes were carefully enucleated and kept in 35 mm petri dishes on ice. In order to keep embryonic neural tissue fresh during the dissection procedure, Hibernate TM E medium (Brainbits, US patent No#6180404)[14] plus B-27 supplements (Invitrogen) was used. The retina was dissected free of retinal pigment epithelium (RPE) and mesenchymal tissue. In addition, a 1.5 mm wide zone was removed in the ciliary margin (to remove ciliary epithelium) and around the optic disc (to remove optic nerve glial cells).

After dissection, retinal fragments were incubated with 0.25% trypsin without EDTA-Ca (Invitrogen) for 15–20 minutes at room temperature and neutralized by adding phosphate-buffered saline (PBS). Cells were centrifuged at 5000/rpm for 5 minutes, and the supernatant was aspirated. Cell pellets were re-suspended in the chosen culture medium. Freshly isolated retinal progenitor cells were seeded on Poly-D-Lysine/Laminin (Sigma) coated 6-well culture plates at a density of $4–5 \times 10^4/cm^2$. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Retinal progenitor cell expansion

For each separate experiment with the three different media systems, retinal progenitor cells were seeded at the same cell density ($4–5 \times 10^4/cm^2$). The culture medium was changed every other day and the cells were passaged when they reached 85% confluency. RPC passaging was performed using 0.25% trypsin (Invitrogen, Cat: 15050065; 2.5 g/L trypsin (1 : 250) in Hanks' Balanced Salt Solution without $CaCl_2$, $MgCl_2$, and $MgSO_4$) combined with mechanical trituration. In each experiment, RPC cells were passaged until at least P6. In experiments with the serum-free culture system, cells were passaged up to P15. For immunostaining procedures, passaged cells were seeded/replated on two-chamber slides at a density of 3×10^4 cells/cm^2 for at least 4 hours, fixed with 4% paraformaldehyde in PBS for 15

minutes at room temperature, and then washed three times with PBS. Experiments were repeated 3–5 times for each of the three (serum-free, serum-containing, and ES-conditioned) culture systems.

Immunocytochemistry and confocal microscope evaluation

Immunocytochemistry

Immunostaining procedures were performed as described previously.[8] Briefly, cells were incubated with 20% goat serum in 1% bovine serum albumin (BSA) for 30 minutes at room temperature, then incubated with primary antibodies overnight at 4°C, followed by a PBS wash. Afterwards, cells were incubated with the appropriate secondary antibodies labeled with AlexaFluor 488 and/or AlexaFluor 546 or rhodamine X (Molecular Probes, Eugene, OR) for 1 hour followed by another PBS wash. For double staining, two different antibodies from two different species were mixed at the appropriate concentration and incubated together. The following primary antibodies were used: sheep anti-BrdU (1 : 400, Biodesign, Saco, Maine), mouse anti-nestin antibody, rat104 (1 : 50, DSHB, Iowa), rabbit anti-glial fibrillary acid protein (GFAP, 1 : 1000, Chemicon, Temecula, CA), mouse anti-neurofilament 200 (NF200,1 : 150, Sigma), mouse anti-microtubule associated protein-2 (MAP-2, 1 : 200, Chemicon), rabbit anti-Calbindin (1 : 2000, Calbiochem, San Diego, CA), mouse anti-rhodopsin antibody, Rho-1D4 (1 : 50; generous gift from Robert Molday, Vancouver, CA), Slides were coverslipped with fluorescence mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) to stain the nuclei (Vector labs, Carpinteria, CA).

Confocal microscopic evaluation

Stained slides were viewed under 40X magnification in a Confocal microscope (Carl Zeiss LSM 510, Thornwood, NY). Slides double stained with nestin and GFAP were chosen for the quantitative analysis of nestin-positive cells. For each experiment, nestin-positive cells in each passage were counted by two observers who were masked as to the identity of the culture system (GQ, SA) (see Table 1). In total, about 80–100 cells from 3–5 views were evaluated for each passage, and the observations of the two graders were averaged. The percent of nestin positive cells at P1, P3, and P6 was compared among the three different culture systems. A *t*-test (two tailed, sample unpaired, assuming equal variance) was used for statistical analysis.

Results

RPC proliferation, survival, and neurosphere characteristics in the three experimental culture systems

In response to the mitogens bFGF and EGF, retinal progenitor cells remained undifferentiated and proliferative *in vitro* and formed neurospheres of heterogeneous neural progenitor cells. There was a tendency for RPCs to proliferate faster in the serum-free culture system and ES-conditioned culture system, as it took only 4–5 days to reach 85% confluence after passage. In contrast, in the serum containing culture media, 5–7 days were required to reach 85% confluence following seeding at an identical cell density ($4–5 \times 10^4/cm^2$) (Fig. 1). Regardless of the culture condition, there appeared to be no significant change in cell number increase and periods in later passages as compared to earlier passages. In the ES-conditioned system, however, RPCs were observed to attach more easily to the Poly-D-Lysine/laminin coated substrate after replating, compared with the serum-containing and serum-free culture systems.

Retinal progenitor neurosphere characteristics were also found to be different between the three culture systems. In the serum-free culture system, RPCs formed neurospheres with a very uniform size (see Fig. 2A). In contrast, in the ES-conditioned culture system, RPC neurospheres appeared irregular and flat in shape, and cells at the edge of a neurosphere tended to spread outward (Fig. 2B), particularly in the late passages. In the serum-containing culture system, RPCs did form typical three-dimensional neurospheres (Fig. 2C), but their size varied considerably. Some were only clusters of a few cells, while others had already developed into large three-dimensional neurospheres 5 days after replating. In this study, for the serum-free system, cells were expanded *in vitro* for more than 2 months, and passaged up to P15. At P10, retinal progenitor cells still retained their proliferative capacity and the ability to form neurospheres. However, cell spheres began to show differences in size and shape (Fig. 2D). At P15, retinal progenitor cells appeared to lose the capacity to form 3-D neurospheres, although they still maintained their capacity to proliferate and continued to express the neuroectodermal marker, nestin (data not shown). For ES-conditioned and serum-containing culture systems, retinal progenitor cells were passaged up to P10. Cells maintained their proliferative capacity and nestin expression (data not shown), but lost the ability to form 3-D neurospheres at P10.

Change in RPC morphology over time

Regardless of the culture system used, retinal progenitor cell morphology at passage 1 (P1) was very similar, with cells appearing smaller in size and more uniform in shape (Fig. 3A) compared to late passage cells. About 60% of retinal progenitor cells incorporated BrdU over a 48 hour period indicating cell division, and 80% expressed nestin (Fig. 4) at passage 2. With subsequent passages, retinal progenitor cells tended to become progressively larger and more pleiomorphic (with variations in both size and shape). In serum-containing and ES-conditioned culture systems, this morphology change (i.e., to larger, pleiomorphic cells) occurred around P5–P6 (Fig. 3B, 3C), whereas, in the serum free culture system, it occurred at later passages, around P9 to P10 (Fig. 3D).

Table 1. Percentage of nestin positive cells in different culture systems at different passages (P).

Groups: Field No.	SFM-P1 Ratio (%)	SFM-P3 Ratio (%)	SFM-P6 Ratio (%)	ESM-P1 Ratio (%)	ESM-P3 Ratio (%)	ESM-P6 Ratio (%)	SCM-P1 Ratio (%)	SCM-P3 Ratio (%)	SCM-P6 Ratio (%)
1	84.6	88.2	77.4	75.0	73.7	100	93.8	100	100
2	75	100	87.5	76.9	78.9	83.3	100	88.7	100
3	92.9	80	81.3	70.6	81.8	100	80.0	77.3	57.1
4	85.1	84.6	79.1	92.9	81.8	75.0	88.9	76.3	100
5	100	90	86.2	71.4	90.5	75.0	72.7	75.0	83.3
6	90	92.9	77.8	76.5	87.0	83.3	88.9	62.2	60.0
7	78.2	92.9	80.6	98.2	85.7	70.0	65.2	46.7	100
8	73.9	80.8	78.9	73.3	79.2	67.7	88.9	72.7	100
9	87.9	80	82.8	95.0	88.1	75.8	91.7	74.0	100
10	89.1	86.4	84.1	80.0	77.8	82.4	80.0	70.0	100
11	80.6	94.4	85.7	74.7	65.0	91.7	100	74.0	80.0
12	91	94.1			80.8	100	81.5	77.0	100
13		82.4			81.0	100	87.5	100	62.5
14		81.5				81.8	84.6	93.7	100
15		84.2				23.1	65.2	100	100
16		84.2				88.2		100	61.5
17						75.0		80.0	87.5
18								33.0	100
19									63.6
20									60.0
21									100
Total fields (n)	12	16	11	11	13	17	15	18	19
mean ± SD	85.6 ± 0.60	87.3 ± 0.38	82.0 ± 0.13	80.4 ± 1.0	80.9 ± 0.44	80.7 ± 3.38	84.5 ± 1.16	77.8 ± 3.35	86.5 ± 3.12

* In each column, the percentage of nestin-positive cells was counted from 3–5 repeat experiments. SFM = serum-free medium; ESM = ES-conditioned medium; SCM = serum-containing medium. For each separate experiment, a total of 80–100 cells were counted in confocal images (×40 magnification) of 3–5 non-overlapping fields. If the seeded cell density was low, additional fields were counted, such as for SCM-P3 and SCM-P6. The fields from all experiments were summed and averaged.

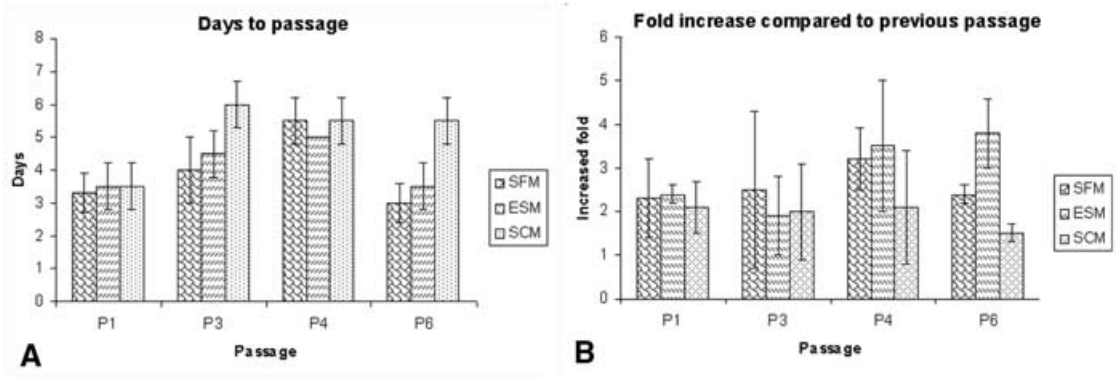

Figure 1. Retinal progenitor cell proliferation properties in three different culture systems. (A): Time until passage when confluency was reached in serum-free (SFM), ES-conditioned (ESM) and serum-containing (SCM) culture at passages (P) 1, 3, 4, and 6. (B): Retinal progenitor cell proliferation rate at different passages in ESM, SCM and SFM culture systems.

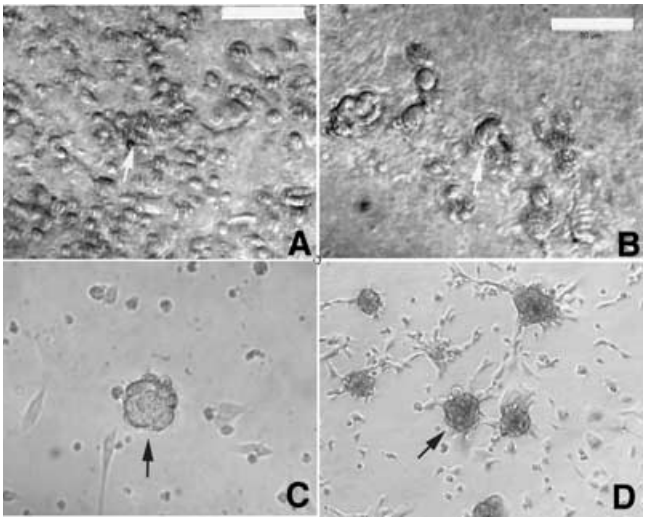

Figure 2. Phase-contrast photographs of characteristic E17 rat retinal progenitor cell neurospheres in different culture systems. Passage 3 RPC neurospheres (indicated by arrows) on the 4th day in the: serum-free culture (A), ES-conditioned culture (B), and serum-containing culture (C). (D): Passage 10 RPC neurospheres on the 4th day in the serum-free culture. Magnification bar: 50 µm.

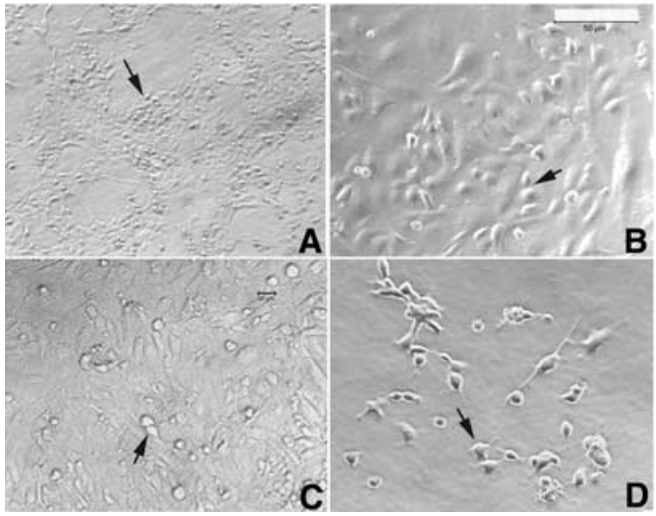

Figure 3. Phase-contrast photographs depicting change in E17 rat retinal progenitor cell morphology over time. RPC neurospheres showed different morphologies in the different culture systems on the 4th day after replating. (A): Passage 1 RPCs (arrow) in serum-free medium; (B): Passage 5 RPCs(arrow) in serum-containing medium; (C): Passage 5 RPCs (arrow) in ES-conditioned medium; (D): Passage 10 RPCs (arrow) in serum-free medium. Magnification bar: 50 µm.

Immunohistochemical studies further demonstrated that retinal progenitor cells of varying size and shape were nestin-positive cells in different passages (Fig. 5A, 5B, 5C, 5D). At P6 in ES-conditioned and serum-containing culture systems, and P10 in the serum-free culture system, nestin-positive cells had similar morphological characteristics, with abundant cytoplasm and long cellular process. Although retinal progenitor cell morphology did change with long-term *in vitro* expansion, these cells continued to express nestin and exhibit mitotic capacity (data not shown). Differentiation experiments demonstrated that retinal progenitor cells could maintain their multipotent capacity in the three different culture systems. Cells at Passage 6 and Passage 9 underwent *in vitro* induction with all-trans retinoic acid. Immunocytochemistry showed that the retinal progenitor cells exhibited retina neuronal cell characteristics, expressing neuronal cell markers including microtubule-associated protein (Map)-2 and neurofilament (NF) 200, as well as retinal specific neuronal markers including rhodopsin (label for rods) and Calbindin (label for horizontal and amacrine cells) and protein kinase C (PKC, label for bipolar cells). However, very few cells (less than 1%) were positive for the glial marker glial fibrillary acidic protein (GFAP). Figure 6 demonstrates NF200, calbindin, rhodopsin, and GFAP expression following *in vitro* induction of passage 6 retinal progenitor cells that were expanded in the SFM system. Similar results were also

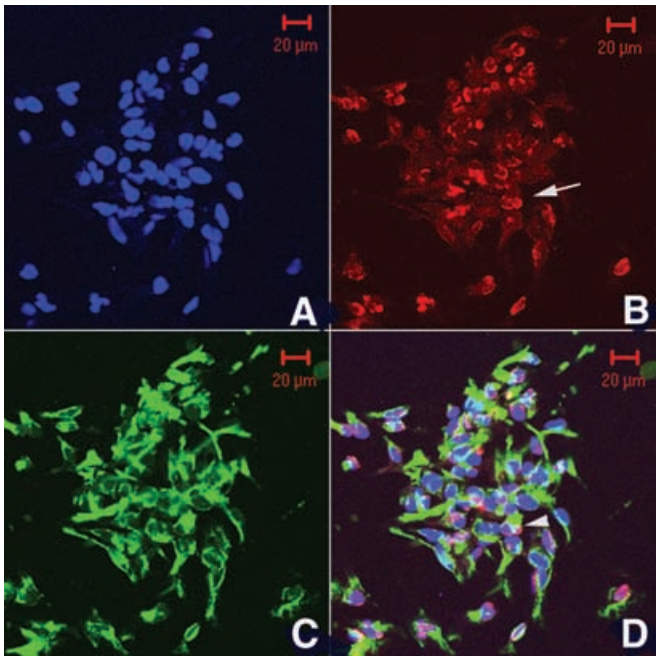

Figure 4. Confocal microscopic images of Passage 2 retinal progenitor cells maintained in the ES-conditioned culture system, and incubated in 10 µM BrdU for 48 hours. Cells were stained with BrdU and nestin antibodies. (A): DAPI (nuclear staining, blue staining); (B): BrdU (arrow, red staining); (C): Nestin (green staining); (D): Merged image (arrow). Magnification bar: 20 µm.

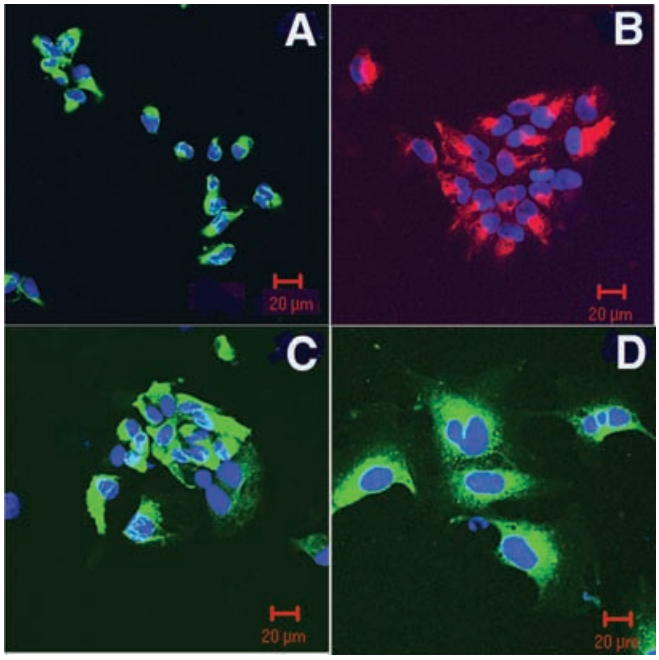

Figure 5. Representative confocal microscopic images of nestin-positive cells from different culture systems at different passages (P). Nestin-expressing retinal progenitor cells change their morphology at different passages. Cells from (A): serum-containing medium at P 1; (B): serum-free medium at P3; (C): serum-free medium at P6; (D): ES-conditioned at P6. Note that cells in serum-free culture maintain their morphology, whereas cells in ES-conditioned medium change their morphology at P6. Nestin stained with red or green; nuclei stained with DAPI (blue). Magnification bar: 20 µm.

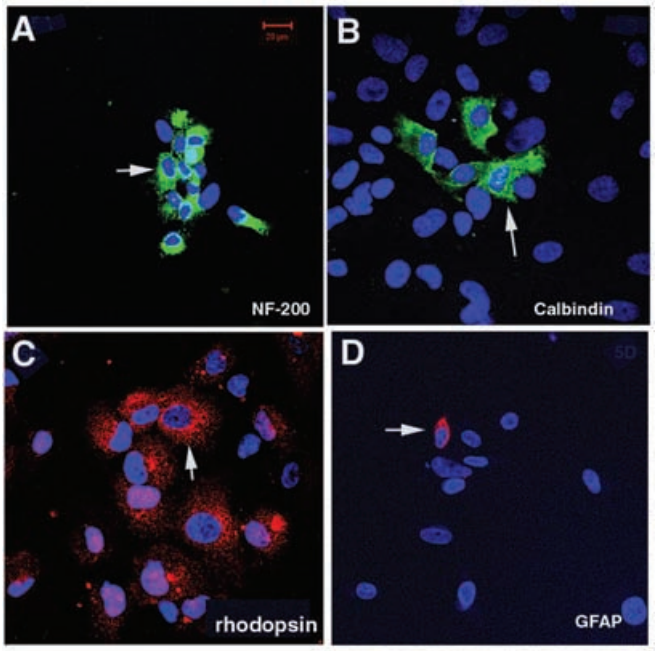

Figure 6. Representative confocal images showing the differentiation capacity of Passage 6 RPC maintained in serum-free medium. After 8 d *in vitro* induction with retinoic acid and 1% FBS, the cells were able to express multiple neuronal cell markers and a glia cell marker. (A): NF-200 (green, arrow); (B): Calbindin (green, arrow); (C): rhodopsin (red, arrow); (D): GFAP(red, arrow). Blue: DAPI staining.

found in ES-conditioned and serum-containing culture systems (data not shown), except that cells maintained in serum containing culture media showed a greater tendency for glial cell differentiation following induction (20% of cells expressing GFAP at P9).

Nestin positive cell percentages at different culture systems and different passages

In these studies, all three culture systems were able to maintain retinal progenitor cells as nestin-positive cells after long-term *in vitro* expansion. The percentage of nestin-positive cells ranged from 77%–87% depending on the culture system and passage, as shown in Table 1.

In order to investigate the difference in nestin expression among cells in the different culture systems, the nestin positive cell percentage in serum-free, ES-conditioned, and serum-containing culture systems at passages P1, P3, and P6 were compared using an unpaired t-test (two samples assuming equal variance). Statistically significant differences were found in comparisons between: serum-free-P3 (87.3%) vs serum-free-P6 (81.9%) ($P = 0.0077$), serum-free-P3 vs ES-conditioned-P3 (80.9%) ($P = 0.0058$), and serum-free-P3 vs serum-containing-P3 (77.8%) ($P = 0.029$), as shown in Figures 7 and 8. There were no statistically significant

differences in comparisons between other combinations of groups.

Discussion

In this study, we were able to use a serum free defined media for E17 rat retinal progenitor cell isolation and long-term expansion. We also compared the performance of embryonic stem cell-derived growth factor conditioned and serum-free media with standard serum containing culture media for use in maintaining a stable population of retinal progenitor cells. We observed that both ES-conditioned and serum-free media could sustain excellent survival and *in vitro* expansion of RPC cells. With long-term *in vitro* expansion, a high percentage of retinal progenitor cells maintained expression of nestin, an intermediate filament protein typical for neural precursor/stem cells[15,16] that is down-regulated in the postnatal rodent brain. Although nestin re-expression has also been observed in reactive astrocytes after injury in adult brain,[17,18] our study observed that fewer than 1% of nestin-positive cells were positive for GFAP, a useful marker for identifying reactive Muller glial cells.[19] Moreover, it is generally accepted that nestin-expression is a useful marker for identification of retinal progenitor cells.[5,8,15] Changes in retinal progenitor cell morphology over time, however, was observed during *in vitro* expansion in all three culture systems.

Maintenance of a stem cell in an undifferentiated, multipotent state (i.e., as a stem cell) during *in vitro* cell expansion is a challenging, yet critical task. Similar to the maintenance of a differentiated state, maintenance of the stem cell state is dependent in large part on the critical balance among the signals present in the culture media ("environmental influences"). Indeed, several studies have shown that the fate of the retinal stem cell is controlled by both extrinsic (environment) and intrinsic cues both *in vivo* and *in vitro*.[20–22] Previous studies have also shown that co-culture of E16 retinal progenitor cells with postnatal retinal cells inhibits production of amacrine cells,[10] which differentiate early in retina development, suggesting that the postnatal environment presents certain negative signals that prohibit the production of earlier born cell types.[10] Zhao and coworkers also reported that retinal progenitor cells could induce ES cell differentiation into retinal neurons when ES cells were co-cultured with retinal progenitor cells.[23] This observation suggests that the mature extracellular environment presents signals that induce early stage stem cell differentiation.

In this study, we used ES cell-derived growth factor conditioned media to culture E17 rat retinal progenitor cells and observed that this culture system could maintain retinal progenitor cell in an undifferentiated state, with continued expression of nestin after long-term expansion. Following *in vitro* induction, retinal progenitor cells obtained multiple retinal specific neuronal makers. Our studies also indicated that the ES-conditioned system appeared to increase cell attachment. As we know, retinal progenitors are unlike CNS-derived neural progenitors that can easily proliferate and expand in suspension culture.[24,25] Instead, RPCs rely on an adherent substrate to survive and grow. Indeed, our previous study indicated that RPCs survived poorly in suspension culture.[8] Therefore, better attachment is generally associated with improved RPC survival.

Traditional serum – containing culture media are not only incompletely defined with respect to their constituents, but are also known to stimulate Müller/glial cell growth.[14] The development of a serum free culture system requires the inclusion of factors that not only increase the cell viability and plating efficiency, but also promote cell proliferation. It is well-known that there are both EGF and bFGF responsive stem cells in the central nervous system, and that neural progenitor cells can proliferate and expand in EGF and/or bFGF supplemented culture media.[6] During retinal development, there is a wave-like pattern of expression of EGF and bFGF

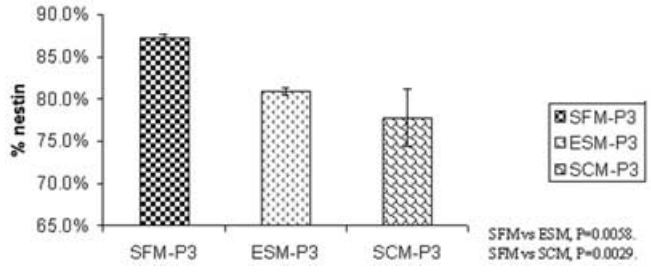

Figure 7. Percentage of nestin positive cells in different culture systems at passage (P) 3. A *t*-test (one tailed sample, unpaired, assuming equal variance) was used to compare between SFM and ESM, SFM and SCM, ESM and SCM at P3. A statistically significant difference was found between the serum-free and the other media at P3 [SFM-P3 vs ESM-P3 ($p = 0.0058$), SFM-P3 vs SCM-P3 ($p = 0.029$)]. There was no statistically significant difference between the other groups.

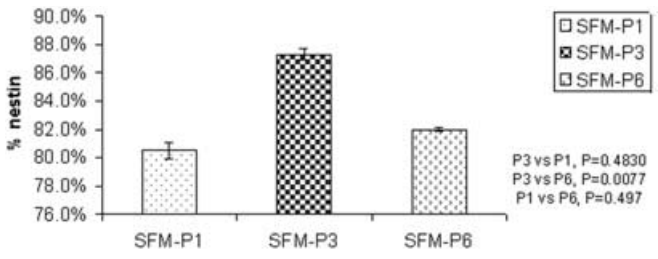

Figure 8. Percentage of nestin positive cells in serum-free culture at different passages. A *t*-test (one tailed sample, unpaired, assuming equal variance) was used to compare between P1 and P3, P1 and P6, P3 and P6. A statistically significant difference was found between P3 vs P6 ($p = 0.0077$). There was no statistically significant difference between the other groups. (P3 vs P1, $p = 0.4830$, P1 vs P6, $p = 0.497$).

receptors.[26,27] In the early embryonic stage (E12-E15) of rat retinal development, RPCs mainly express bFGF receptors.[27,28] Subsequently, the level of EGF receptor expression in RPCs increases steadily from E15 to postnatal day 14, reflecting a shift in responsiveness to EGF.[27] Therefore, at stage E17, rat RPCs express both EGF and bFGF receptors. Thus, we chose to supplement the serum free defined media for RPC expansion with both EGF and bFGF, in addition to N-2 and B-27 supplements, which are also known to promote neural stem cell growth.[29–31] We found retinal progenitor cells could proliferate and expand *in vitro* in response to both EGF and bFGF.

Yang *et al.* reported that retinal progenitor cell survived poorly in serum-free culture media. In the current study, we modified the isolation media and culture media as follows. Firstly, we used hibernation medium plus B27 for maintaining the fetal retina during the dissection procedure. Hibernation medium plus B27 has been used previously to maintain fetal retina tissue for transplantation,[32] and has been observed to prolong embryo tissue survival and improve neuronal cell viability. Secondly, in the current study, the modified serum-free medium was supplemented with EGF, B-27 supplement and L-Glutamine based on our previous formula (DMEM/F12 with bFGF).[8] B27 supplement is a serum-free supplement for growth and long-term viability of hippocampal neurons[14,31,33] which has also been used in hippocampal derived neural progenitor cell culture and expansion.[29]

Our study also demonstrated that RPCs at P3 developed uniformly high-density neurospheres. Statistical analysis of the data also revealed that the nestin-positive cell percentage at P3 in serum-free culture system was significantly higher than at P1 or P6. Moreover, the nestin-positive cell percentage at P3 in the serum-free system was also higher than that observed in serum-containing and ES-conditioned culture systems at the same passage stage (P3). It is possible that E17-derived RPCs at passage 3 up-regulated both EGF and bFGF receptors that allowed most retinal progenitor cells to proliferate in a similar pattern and rate.

Maintaining the multipotency of stem cells in long-term culture is particularly challenging with E17 neural progenitor cells. E17 represents the beginning of late retinal neurogenesis in rodents. Retinal progenitors from this stage have "stem cell properties" (passagability, capability for self-renewal, and capacity to form neurosphere), but these cells are heterogeneous in their mitosis rate and extent of proliferation.[10,34,35] RPCs could divide symmetrically and produce two progenitor cells capable of cell division; or they could divide asymmetrically and produce one progenitor cell and one cell committed to differentiation (neurons or glial cells). Some progenitor cells maintain a high ratio of symmetric/asymmetric cell division and yield a higher percentage of neurospheres. Increasingly asymmetric cell division eventually results in differentiation of the population into mature cells. In clonal density culture systems, Jensen[36] observed that the cell cycle time of retinal progenitor cells varies considerably from one to five days. In our study, we found that only 60% of cells incorporated BrdU. This suggested that some retinal progenitor cells probably had not divided within this time frame, and therefore could not incorporate BrdU within the 48 hours of application However, we cannot exclude the possibility that some cells had begun to differentiate and had lost their mitotic property.

In this study, we also observed that retinal progenitor cell neurospheres are different in size and shape among the different culture systems. In the serum-free culture system, neurosphere size was uniform, compared to the serum-containing culture system, which showed extensive variation in size. In the ES-conditioned culture system, the neurospheres appeared flat and irregular, compared to the round shape in the serum-free culture system. In addition, in ES-conditioned and serum-containing systems, progenitor cells lost their capability to form 3-D neurospheres at P10, whereas in the serum-free culture system, this property was maintained until P15. This observation suggests that the pattern (symmetric/asymmetric ratio) and rate of cell division as well as the proliferation properties of the cells were affected by some unique attribute(s) of the various cell culture systems. Although the precise mechanism is unknown, it is possible that the higher concentrations of EGF and bFGF in the serum-free medium were sufficient to reduce the rate of differentiation and increase proliferation. Alternatively, some component of the serum may have slightly hastened differentiation. One caveat, in making conclusions based on the size and shape of the neurospheres in this experimental design is the uncertainty of the origin of the cells composing the cell clusters. It is possible that some neurospheres were not a clonal group of cells, but rather were aggregates of single cells which had migrated together or simply clusters of post-mitotic cells that had lost the capacity to divide. Clonal cultures were outside the scope of this study. It should be noted, however, that during cell passage, most of the retinal progenitor cells were dissociated into either single or two-to-three cell clusters (confirmed by microscopic inspection after passage).

In summary, the modified serum free defined culture medium was found to support retinal progenitor cell proliferation and expansion, as well as better maintenance of desired RPC morphology compared to the embryonic stem-cell conditioned and serum-containing culture media. The ES cell-conditioned medium, however, was associated with the best overall RPC attachment (and presumably survival). These observations may be useful in choosing the optimal culture system for future studies requiring *in vitro* expansion of retinal progenitor cells as part of a broader therapeutic strategy.

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