Stromal cells cultivated from the choroid of human eyes display a mesenchymal
stromal cell (MSC) phenotype and inhibit the proliferation of
choroidal vascular endothelial cells *in vitro*

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Abstract

Mesenchymal stromal cells (MSC), with progenitor cell and immunological properties, have been cultivated from numerous vascularized tissues including bone marrow, adipose tissue and the corneal-limbus of the eye. After observing mesenchymal cells as contaminants in primary cultures of vascular endothelial cells derived from the choroidal tunic of the human eye, we investigated whether the choroid might also provide a source of cultured MSC. Moreover, we examined the effect of the choroidal stromal cells (Ch-SC) on the proliferation of freshly isolated choroidal vascular endothelial cells (ChVEC) *in vitro*. The phenotype of cultures established from five choroidal tissue donors was examined by flow cytometry and immunocytochemistry. The potential for mesenchymal cell differentiation was examined in parallel with MSC established from human bone marrow. Additional cultures were growth-arrested by treatment with mitomycin-C, before being tested as a potential feeder layer for ChVEC. The five unique cultures established from choroidal stroma displayed a phenotype consistent with the accepted definition for MSC (CD34⁻, CD45⁻, HLA-DR⁻, CD73⁺, CD90⁺, and CD105⁺), including the capacity for mesenchymal differentiation when cultivated under osteogenic, adipogenic and chondrogenic conditions. Growth-arrested Ch-SC inhibited the proliferation of ChVEC derived from five separate donors. Cultures of Ch-SC secreted approximately 40-fold higher concentrations of the anti-angiogenic factor pigment epithelium derived factor (PEDF/serpin F1) compared to the pro-angiogenic factor, vascular endothelial growth factor (VEGF), regardless of normal or growth-arrested state. Our results provide first evidence of a resident MSC cell type within the choroid and encourage investigation of new mechanisms for altering the growth of ChVEC.

Keywords: choroid; mesenchymal stromal cell; choroidal stromal cells; vascular endothelial cell; cadaveric eye tissue

1. Introduction

The choroid of the eye is a highly vascularized layer of connective tissue that resides between the retina and sclera (Nickla and Wallman, 2010). In healthy eyes, choroidal blood vessels are essential for the delivery of nutrients and waste products to and from the outer sensory layer of the retina. With increasing age, however, structural changes and dysregulation of the immune response within the retinal-choroidal tissue environment, can facilitate the aberrant growth of choroidal blood vessels into the sub-retinal space (Booij et al., 2010; Chirco et al., 2017; McMenamin et al., 2019). This choroidal neovascularization (CNV) is a major cause of vision loss in the elderly and is a diagnostic feature of the “wet” form of age-related macular degeneration (wet-AMD) (Green, 1999). While many patients with wet-AMD respond well to therapies that block the actions of vascular endothelial growth factor (VEGF) (e.g. ranibizumab and aflibercept) (Solomon et al., 2019), greater than 30% remain refractory to treatment (Tranos et al., 2013; Yang et al., 2016). Further research is therefore required into the mechanisms that control the growth of choroidal blood vessels with the view to identifying alternative treatments for wet-AMD.

While anti-VEGF therapies specifically target choroidal vascular endothelial cells (ChVEC), a number of additional cell types including vascular smooth muscle cells and pericytes contribute to choroidal blood vessel structure and function (Nickla and Wallman, 2010). In addition, a variety of other cell types including melanocytes, fibroblasts and immune cells are known to populate the stromal tissue residing between choroidal blood vessels (Nickla and Wallman, 2010; McMenamin et al., 2019). Not surprisingly, therefore, the use of cloning

rings and other tools are required in order to separate ChVEC from contaminating cell types when attempting to study these cells *in vitro* (Browning et al., 2005; Sakamoto et al., 1995). Nevertheless, relatively little is known regarding the biology and potential clinical significance of the contaminating cells. The goal of the present study, therefore, was to address this gap in the literature by examining the phenotype of contaminating stromal cells present within primary cultures of ChVEC. More specifically, we hypothesize that the majority of stromal cells that emerge in primary cultures derived from choroidal tissue are in fact a form of mesenchymal stromal cell (MSC) derived from either choroidal pericytes and/or fibroblasts.

MSC were originally identified as a population of adherent, non-hematopoietic progenitor cells present in cultures derived from bone marrow (Friedenstein et al., 1970). Cultures with similar properties to bone marrow-derived MSC (BM-MSC) have been subsequently established from a variety of tissues including adipose tissue, lung tissue, and other vascularized tissues (da Silva Meirelles et al., 2006). More recently, several groups have demonstrated that cells with similar properties to MSC can be isolated from the vascularized limbal margin of the corneal stroma (Branch et al., 2012; Bray et al., 2014; Garfias et al., 2012; Polisetty et al., 2008). In each case, the MSC are defined by a similar profile of cell surface antigens (negative expression for CD34 and CD45; positive expression for CD73, CD90 and CD105) as established by the International Society for Cell and Gene Therapy (ISCT) (Dominici et al., 2006; Mushahary et al., 2018). MSC also display capacity for osteogenic, chondrogenic and adipogenic differentiation when cultivated under lineage specific culture conditions. Moreover, MSC have been demonstrated to directly influence the wound healing and immunological properties of a variety of cell types including white blood cells, vascular endothelial cells and epithelial cells (Atkinson, 2017). These properties of

MSC, coupled with their low immunogenicity, have led to MSC and their products, including exosomes, being widely considered as therapeutic agents for tissue repair (Atkinson, 2017). Demonstration of choroidal tissue as a source of MSC might therefore lead to a role for these cells in future treatments for wet-AMD.

We therefore presently examined the phenotype of stromal cell cultures derived from the choroid of cadaveric eye tissue. Cell phenotype was examined by flow cytometry using a standard MSC panel of antibodies. The expression of α -smooth muscle actin (α -sma), a marker for smooth muscle cells, myofibroblasts and pericytes, was examined by immunocytochemistry. The progenitor cell potential of stromal cell cultures was subsequently examined in parallel with that of BM-MSC. Finally, we examine the effect of our putative choroidal MSC on the growth of ChVEC isolated from cadaveric eye tissue. Our results provide evidence for a population of MSC within the choroid, and suggest new strategies for understanding ChVEC growth *in vivo*.

2. Materials and Methods

2.1 Acquisition and dissection of human choroidal eye tissue

Posterior eye segments from cadaveric human donors were obtained from the Queensland Eye Bank (Princess Alexandra Hospital, Brisbane, Queensland) with institutional research ethics approval (PAH Project 07/QPAH/48; QUT Project 0800000807). The age, gender and cause of death for each donor are summarized in Supplementary Table 1. All tissue samples were processed between 50-56 hours post mortem. After removing the lens, vitreous humour and neural sensory retina, the retinal pigment epithelium (RPE) and choroid were gently peeled away from the underlying sclera using forceps and washed in Hanks' balanced salt solution.

The sheets of tissue were subsequently incubated in 1 mg/mL collagenase type 1 (Gibco/Life Technologies, Cat. No.17100-017; diluted in DMEM/F12 medium) for 1 hour at 37 °C to facilitate removal of the RPE. The remaining choroidal tissue was processed further for isolation of either vascular endothelial cells or stromal cells as described below.

2.2 Isolation and cultivation of choroidal vascular endothelial cells (ChVEC)

Human choroidal tissue, as processed above, was minced into approximately 2 mm² pieces using a scalpel, before further treatment for 1 hour with 1 mg/mL collagenase type 1. Larger tissue fragments were removed by passing the digested tissue through a 40 µm filter followed by washing with 7 mL ChVEC isolation buffer consisting of calcium-magnesium free phosphate buffered saline (PBS; Gibco, Thermo Fisher Scientific; Cat. No. 14190-144) supplemented with 2 mM EDTA and 0.5% bovine serum albumin (BSA). Cells within the combined filtrate were subsequently pelleted (300 x g for 10 min) and washed in 2 mL of fresh ChVEC isolation buffer before being processed by magnetic assisted cell sorting (MACS; Miltenyi Biotec). The MACS protocol was conducted according to our previously published method for isolating vascular endothelial cells from the corneal limbus (Gillies et al., 2015). In brief, one round of negative selection for the common leukocyte antigen CD45 was employed, followed by one round of positive selection for the vascular endothelial cell marker CD31. The combined “CD45⁻/CD31⁺” fraction retrieved from each pair of donor eye-cups was subsequently split equally into two wells of a 12-well tissue culture plate that had been pre-coated with Attachment Factor (Gibco, Thermo Fisher Scientific; Cat. No. S-006-100). The cells were seeded and cultivated in MCDB 131 medium (Gibco, Thermo Fisher Scientific; Cat. No. 10372019) supplemented with 10% foetal bovine serum (FBS, HyClone; Cat. No. SH30084.03), 1% glutamine (Gibco, Thermo Fisher Scientific; Cat. No. 25030), 1% penicillin/streptomycin (Invitrogen, Thermo Fisher Scientific; Cat. No. 15070-063), 0.08%

hydrocortisone (Sigma; Cat. No. H0396-100MG), 0.1% EGF (Gibco, Thermo Fisher Scientific; Cat. No. PHG0311), 0.1% recombinant human b-FGF (Gibco, Thermo Fisher Scientific; Cat. No. 13256-029) and 0.1% heparin (Stem Cell Technologies, Thermo Fisher Scientific; Cat. No. 07980).

2.3 Isolation and cultivation of choroidal stromal cells (Ch-SC)

Primary cultures of stromal cells were established from choroidal tissue fragments that were submerged in 3D gels consisting of porcine type I collagen (1 mg/mL; CellMatrix, Nitta Gelatin Inc., Tokyo, Japan). The cultures were maintained for approximately two weeks in collagen gels before digestion in 1 mg/mL collagenase type I and subsequent passaging in 75 cm² culture flasks. The culture medium used for establishment and maintenance of choroidal stromal cell cultures consisted of DMEM (Gibco, Thermo Fisher Scientific; Cat. No. 10313-0211) supplemented with 10% FBS, 1% Glutamine and 1% penicillin/streptomycin (as sourced above for ChVEC cultures). Routine passaging consisted of a brief rinse in PBS (<10 seconds), followed by an equally brief rinse in Versene (Gibco, Thermo Fisher Scientific; Cat. No. 15040-066), followed by incubation in TrypLE (Gibco, Thermo Fisher Scientific; Cat. No. 12563-011) for 5 minutes at 37 °C. Expanded stocks of stromal cells were stored in liquid nitrogen while suspended at 10⁶ cells/mL in 90% FBS/10% DMSO.

2.4 Immunocytochemistry

Cultures of either ChVEC and/or Ch-SC were routinely fixed in neutral buffered formalin (20-30 minutes), permeabilized with 0.01% (v/v) Triton X-100 diluted in HEPES buffered saline (0.85% w/v NaCl with 20 mM HEPES buffer at pH 7.2) for 10 minutes and blocked using 2% normal goat serum diluted with PBS for 30 minutes. Mouse monoclonal antibodies to either human von Willebrand Factor (vWF; 1:50 dilution of clone F8/86, DakoCytomation;

Cat. No. M0616) or α -SMA (1:1000 dilution of DakoCytomation; Cat. No. M0857) were applied for 1 hour at room temperature in PBS. Following at least three rinses in PBS, an Alexa 488-conjugated goat anti-mouse IgG (1:1000 dilution of Life Technologies, Thermo Fisher Scientific; Cat. No. A11001) was applied for 1 hour. Cell nuclei were stained by applying 2 μ g/mL Hoechst 33342 nuclear dye (Life Technologies, Thermo Fisher Scientific; Cat. No. H1399) for approximately 30 minutes. Controls were performed by omission of the primary antibody. The percentage of cells with stress fibers staining positive for α -SMA was determined by manual examination of at least 100 cells per culture for each donor. Fluorescence micrographs were captured using a Nikon TE2000-U microscope equipped with an X-Cite fluorescence power source and CoolSNAP Cooled CCD camera (Photometrics). Coloured overlays of staining for vWF (green), α -SMA (pink) or cell nuclei (blue) were created by conversion of grayscale images to indexed colour LUTs using Adobe Photoshop CC (Version 19.0).

2.5 Flow Cytometry

Vials of cryopreserved Ch-SC from 5 unique tissue donors were rapidly thawed and washed in fresh culture medium before being grown to 80-90% confluency in 75 cm² culture flasks. The cells from each donor were subsequently harvested and resuspended in fresh medium at a concentration of 10⁶ cells/mL. The harvested samples were immediately transported on ice for analysis by the Immunology Department at Mater Pathology (Mater Hospital, South Brisbane). Upon arrival, samples were washed and re-suspended at a concentration of 10⁷/mL in BD FACSFlow buffer (BD Biosciences, Cat. No. 324003) supplemented with 1% fetal bovine serum. Labelling of cells with antibodies (100 μ L of cell suspension plus 5-20 μ L of antibody) was performed for 15 min in the dark before washing and resuspension in fresh buffer at approximately 0.2-0.4 x 10⁶ cells/mL. Donor samples were analyzed individually on

a BD FACSCanto II (BD Biosciences) using a validated panel of 8 antibodies summarized in Table 1. Voltage adjustments and compensation were performed on samples labelled with individual antibodies, before subsequently analyzing samples labelled with all eight antibodies.

2.6 Mesenchymal cell differentiation assays

The osteogenic, adipogenic and chondrogenic potential of Ch-SC derived from 3 unique donors (Donors 3, 4 and 4) were examined in parallel with a BM-MSC culture established previously from a living male donor (24-years of age, Mater Hospital Project Clearance: 1541A-023) (Futrega et al., 2015). For osteogenic, adipogenic and control cultures, 1.2×10^5 cells were seeded in 1 mL of induction medium into each well of a 24-well culture plate. For chondrogenic induction, 2×10^5 cells were resuspended in 1 mL of chondrogenic medium and centrifuged at $500 \times g$ for 3 minutes into v-bottom well plates (Sigma; Cat. No. CLS3960). The supplementation details for each of the induction mediums are summarized in Supplementary Table 2. Adipogenic, osteogenic and control cultures were incubated at 20% O₂, 5% CO₂ at 37°C, and chondrogenic induction cultures were incubated at 2% O₂, 5% CO₂ at 37°C. Induction cultures were incubated for 21 days, with medium exchanged every 3–4 days. Outcomes of osteogenic and adipogenic induction were assessed by fixing the cells with 4% paraformaldehyde (PFA; Sigma; Cat. No. 158127) for 30 min, and then staining with Alizarin Red S (Sigma; Cat. No. A5533) for mineralization and Oil Red O (Sigma; Cat. No. O0625) for fat vacuoles. To assess chondrogenic induction, spheroids were frozen in OCT (Tissue-Tek), cryo-sectioned at 7 µm onto poly-lysine coated slides (Thermo Fisher Scientific; Cat. No. MENSF41296PL). Sections were fixed to slides with 4% PFA and stained with Alcian Blue (Sigma; Cat. No. B8348) for the presence of glycosaminoglycans and Nuclear Fast Red (Sigma; Cat. No. N3020) was used to stain cell nuclei.

2.7 Preparation of growth-arrested stromal cells

A single vial of frozen choroidal Ch-SC (p2; Donor 3) was rapidly thawed, seeded into 5 x 75 cm² flasks, and grown to approximately 90% confluency in regular stromal cell culture medium. On day of treatment, the culture medium was replaced with fresh medium supplemented with 20 µg/mL of mitomycin C. The mitomycin C stock solution (1 mg/mL in sterile MilliQ water) was freshly prepared from 2 mg dry powder (Sigma; Cat. No. M4287). Treatment was conducted for 3 hours under regular cell culture conditions (5% CO₂ at 37 °C). Following removal of medium containing mitomycin C, each culture was rinsed twice in PBS and once in Versene, before being harvested using TrypLE (5 min at 37 °C). The harvested cells were washed further *via* centrifugation (300 x g for 5 min) in culture medium, then stored frozen in 90% FBS/10% DMSO.

2.8 Co-culture experiments

For each experiment, one frozen vial of growth-arrested Ch-SC (Donor 3) was rapidly thawed, washed once in culture medium *via* centrifugation (300 x g for 5 min), and seeded into two wells of a 12-well culture plate at a density of 100,000 viable cells/3.5 cm² well. The first well was used as a control to monitor the behavior of the growth-arrested stromal cells. The second well was subsequently seeded with half the CD45⁻/CD31⁺ fraction of choroidal cells that had been freshly isolated from a pair of left and right donor eye cups. The remaining donor cells were added alone to a third well within the plate. All wells were pre-coated with Attachment Factor. Each culture was maintained in full endothelial cell medium with feeding every second day. After two weeks, the cultures were fixed in neutral buffered formalin and examined for potential differences in relative ChVEC growth *via* immunostaining for vWF as

described above. This experiment was performed 5 times using ChVEC isolated from a unique donor on each occasion (Donors 8 to 12).

2.9 Growth factor secretion by Ch-SC The relative levels of vascular endothelial growth factor (VEGF) and pigment epithelium derived growth factor (PEDF/Serpin F1) secreted by Ch-SC were determined using commercial enzyme-linked immunosorbent assays (ELISAs; Cat. Nos. DY293B and DY1177 respectively; R&D Systems) according to methods reported previously (Shadforth et al., 2017). Secretion was examined from both growth-arrested (mitomycin-C treated) and normal (non-treated) cells under both standard and serum-free growth conditions. Untreated cells were seeded at a density of 7,500/cm² and growth-arrested cells were seeded at 75,000/cm². Culture medium was sampled for testing on culture day 8 and day 15, allowing for 48 hours of secretion prior to collection. This experiment was conducted three times using Ch-SC cultures derived from a unique donor on each occasion (Donors 13 to 15). The data was analyzed for statistical significance by using an analysis of variance (ANOVA).

3. Results

3.1 Characterization of stromal cell cultures derived from human choroidal tissue.

Choroidal vascular endothelial cells (ChVEC) isolated from cadaveric tissue using magnetic assisted cell sorting (MACS; CD45⁻/CD31⁺) generated small clusters of presumptive endothelial cells within the first week in culture (Figure 1A). These clusters subsequently expanded to produce larger islands of cells within a few weeks (Figure 1B). Immunostaining for von Willebrand Factor (vWF) confirmed the presence of presumptive ChVEC in primary cultures (Figure 1C). Nevertheless, large numbers of non-stained cells (i.e. vWF-negative

cells) were also observed adjacent to each cluster of vWF-positive stained cells, suggesting contamination with non-vascular endothelial cell types. The close association between expanding islands of ChVEC and surrounding contaminating cells suggested to us the potential for a feeder-cell effect similar to that displayed by stromal cells for skin keratinocytes and corneal epithelial cells. We therefore progressed to establishing larger cultures of non-ChVEC cells for analysis of their cell phenotype and for use in co-culture experiments with ChVEC.

Choroidal stromal cell (Ch-SC) cultures were established from tissue fragments suspended in collagen gels. Significant cellular outgrowth was observed from tissue explants within 7 days (Figure 2A) with the majority of cells displaying negative immunostaining for vWF (Figure 2B). The cells emerged orthogonally to the tissue surface, giving rise to a series of radiating trails of migrating cells. This pattern was subsequently lost, however, as the cells proliferated within the adjacent collagen gel. Following collagenase digestion and seeding into culture flasks, the majority of cells displayed a mesenchymal morphology and retained this phenotype after reaching confluency (Figure 2C and D). Analysis of second passage cultures derived from 5 unique donors (Donors 3 to 7) by flow cytometry consistently demonstrated an MSC profile of cell surface antigens as defined by the ISCT (Dominici et al., 2006; Mushahary et al., 2018). More specifically, approximately 98% of cells in all 5 donor cultures were negative for CD14, CD34, CD45, CD79a and HLA-DR, while being positive for CD73, CD90 and CD105 (Table 2). In addition, fewer than 10% of cells in any culture displayed α -smooth muscle actin-positive stress fibers when analyzed by immunocytochemistry (Figure 3; Donors 3 to 6).

Given the MSC-like phenotype for Ch-SC revealed by flow cytometry, we proceeded to examine whether these cells may also display a similar pattern of cellular differentiation to that displayed by MSC derived from bone marrow (BM-MSC). In order to perform this analysis, 3 of the previously tested unique donor cultures of Ch-SC (Donors 3 to 5) were cultured in parallel with BM-MSC under either osteogenic, adipogenic or chondrogenic growth conditions for 21 days. Subsequent analysis by histochemical methods confirmed evidence of osteogenic and adipogenic activity for Ch-SC when compared to control cultures grown under non-inductive growth conditions (Figure 4 Parts C-J). In addition, cellular spheroids derived from Ch-SC displayed histochemical evidence of chondrogenic differentiation after being maintained for 21 days in the appropriate inductive medium in parallel with BM-MSC (Figure 4. Parts K and L).

3.2 Influence of Ch-SC on ChVEC growth in vitro

In order to study the influence of Ch-SC on ChVEC growth *in vitro*, we first created a stock of growth-arrested Ch-SC for Donor 3 by treatment with mitomycin-C. The growth-arrested Ch-SC displayed a similar morphology to that of non-treated Ch-SC and maintained a sub-confluent density when seeded into culture (Figure 5). We subsequently examined the effects of these growth-arrested Ch-SC on the establishment of primary ChVEC cultures derived from the next 5 pairs of donor eye cups (Donors 8 to 12). Our hypothesis, was that the Ch-SC would encourage more rapid expansion of ChVEC islands in culture by providing a support layer of feeder-cells, akin to fibroblast feeder cells traditionally used for cultivation of epithelial cells (Rheinwald and Green, 1975) and embryonic stem cells (Schwartz et al., 2011). The resulting effects of growth-arrested Ch-SC on primary ChVEC cultures established from the 5 unique tissue donors after 14 days in culture are displayed in Figure 6. A substantial decrease in ChVEC growth (as monitored by staining for vWF) was observed

across all 5 donors, when the freshly isolated CD45⁻/CD31⁺ fraction of donor cells were seeded in the presence of growth-arrested Ch-SC. Residual mitomycin-C was ruled out as a contributing factor to these results since no inhibition of ChVEC growth was observed when using growth-arrested MSC cultivated from the corneal-limbal stroma (L-MSC; Supplementary Figure 1).

3.3 Growth factor secretion by Ch-SC

In view of the above findings, we decided to investigate the relative quantity of vascular endothelial growth factor (VEGF; a pro-angiogenic growth factor) and pigment epithelium derived growth factor (PEDF/Serpin F1; an anti-angiogenic growth factor) secreted by Ch-SC *in vitro*. A further three unique cultures of Ch-SC were established from separate donors (Donors 13 to 15) for these experiments, owing to stocks having been depleted during earlier studies. Conditioned medium derived from each unique donor culture of growth-arrested Ch-SC as well as untreated Ch-SC, were analyzed in the presence and absence of serum (Figure 7). While less than 30 pg/mL of VEGF were detected across all conditions (Supplementary Figure 2), 10-40 times greater levels of PEDF were detected. The quantity of PEDF secreted over a 48 hour period was directly proportional to Ch-SC density, with confluent cultures of normal Ch-SC (Figure 7 Part A at 15 days) secreting similar amounts of PEDF (>1000 pg/mL over 48 hours) to cultures of growth-arrested Ch-SC that had been seeded at a confluent density (Figure 7 Part B at 8 days).

4. Discussion

While anti-VEGF therapies have proven to be valuable for the management of wet-AMD (Solomon et al., 2019), the limited effectiveness of these drugs (Tranos et al., 2013; Yang et al., 2016), combined with the multi-cellular composition of CNV membranes removed from patients (Grossniklaus and Green, 1998; Schnurrbusch et al., 2001), encourages research into alternative and/or complementary, molecular and cellular targets. To this end, we have presently investigated the phenotype of cells present in cultures established from the human choroidal stroma and have examined the effects of these cells on the growth of choroidal vascular endothelial cells (ChVEC) *in vitro*. Our data indicates that cells with a similar immunophenotype to MSC can be cultivated from human choroidal tissue (based upon the current definition of MSC established by the International Society for Cell and Gene Therapy). Moreover, these putative “Ch-MS” secrete significant quantities of the anti-angiogenic growth factor PEDF/Serpin F1 and inhibit the proliferation of ChVEC in culture. These findings provide fresh insights into the biology of choroidal stromal cells.

Our reporting of an MSC-like phenotype for cultures established from the choroidal stroma (as defined by immunophenotyping and differentiation assays) is consistent with the findings from a broad body of literature concerning the discovery of MSC in cultures derived from other organs and tissues including bone marrow, adipose tissue, lungs, kidneys and the cardiovascular system (Atkinson, 2017; da Silva Meirelles et al., 2006). In each case, it is generally accepted that MSC cultures arise from some form of stromal progenitor cell in the form of either pericytes or fibroblasts. To this end, the morphology of Ch-SC presented in our study is consistent with that described previously for human choroidal fibroblasts (Klyve et al., 1996). The level of immunotyping in this earlier study was based upon positive staining for vimentin and a lack of staining for cytokeratin (a marker for epithelial cells), S100 (a

marker for melanocytes), desmin (a marker for smooth muscle cells), α -sma (pericytes and myofibroblasts) and vWF (endothelial cells).

More recently, Djigo et al. (2019) have described creation of a tissue engineered choroidal tissue construct containing cultured choroidal stromal fibroblasts (CSF). The CSF were determined by immunostaining to express vimentin and cytokeratin 8/18. While it is possible that the later marker was detected owing to contaminating retinal pigment epithelial (RPE) cells, the study identified a 20-fold difference in the expression for over 200 genes by microarray when comparing 3 unique choroidal fibroblast cultures to a reference culture of RPE cells. The supplementary data provided by Djigo et al. (Gene Expression Omnibus accession number GSE122885), however, demonstrates a number of interesting observations. To begin, the RPE cells used by Djigo et al. were evidently poorly differentiated, as demonstrated by high levels of gene expression for the mesenchymal cell marker vimentin (VIM) and low to negligible levels of expression for genes expressed by mature RPE cells (e.g. RPE65, CRALBP, BEST1, TJP1/ZO-1 and CDH1/E-cadherin). More significantly, however, analysis of Djigo's supplementary data for MSC panel markers reveals a gene expression pattern for CSF cultures that is consistent with our presently reported flow cytometry data (i.e. negative for HLA-DR and CD34, with expression for CD73, CD90, CD105 and α -sma). Not surprisingly, the mesenchymal-like RPE cultures analyzed by Djigo et al. tended to share this profile, with the exception of significantly lower expression for α -sma and essentially negligible expression for CD105. Of course, the potential for cross-contamination of either RPE cultures or choroidal fibroblasts/Ch-SC in either the study by Djigo et al. or the present study must be considered a possibility. Further analyses of gene expression at the level of individual cells will therefore be required to not only distinguish Ch-SC cultures from RPE, but to also distinguish Ch-SC from the growing list of MSC types.

Growth-arrested stromal cells are a well-established tool for encouraging the growth of epithelial cells and embryonic stem cells in culture (Rheinwald and Green, 1975; Schwartz et al., 2011). A common and effective method for arresting growth includes pre-treatment with mitomycin-C (Llames et al., 2015). It was therefore hypothesized that our growth-arrested cultures of Ch-SC would provide a useful tool for encouraging the establishment and serial expansion of ChVEC from human cadaveric tissue. Nevertheless, the consistent reduction in ChVEC proliferation observed across 5 unique donors, led us to consider the alternative hypothesis that Ch-SC *inhibit* rather than *stimulate* ChVEC proliferation *in vitro*. This hypothesis is supported by a number of additional factors. To begin, we initially considered that residual free mitomycin-C could have directly influenced the growth of ChVEC. Nevertheless, mitomycin-C is known to degrade by a factor of 50% per hour in serum-supplemented culture medium (Proctor and Gaulden, 1986). In addition, from the time of removing the culture medium supplemented with mitomycin-C, to the time of seeding into co-cultures with ChVEC, the Ch-SC had been extensively washed (including the additional washing steps performed during retrieval from storage in liquid nitrogen). Moreover, we were unable to replicate the inhibitory effects of the growth-arrested Ch-SC when using MSC-derived from corneal limbal stroma (Supplementary Figure 1).

Based upon the above, we concluded that the inhibitory effects of growth-arrested Ch-SC on ChVEC proliferation have a biological origin and one that is most likely related to the balance of pro-angiogenic and anti-angiogenic factors secreted by the stromal cells. It should be noted that other growth factors could be involved in this inhibitory effect. However in this study we proceeded to investigate the relative secretion of VEGF and PEDF/Serpin F1 since their relative balance of secretion has been shown previously to dictate the angiogenic properties of

other stromal cell types including dermal fibroblasts (Pollina et al., 2008). Prior studies have established that cultured choroidal fibroblasts express two isoforms of VEGF (Kvanta, 1995; Nagineni et al., 2012) and that the levels of expression are up-regulated in response to treatment with inflammatory cytokines (ranging from 70-100 pg/mL to approximately 700 pg/mL in stimulated cultures). Allowing for minor differences in experimental design (e.g. culture volume and duration), the observation of relatively low levels of VEGF detected in our Ch-SC cultures (up to 30 pg/mL) is close to that reported previously for un-stimulated cultures of choroidal fibroblasts. Likewise, our observation of relatively higher levels for PEDF (>1000 pg/mL) is consistent with that made previously for control cultures of choroidal fibroblasts (>6000 pg/mL) (Nagineni et al., 2012). We had, however, expected to see a significant difference in the ratio of growth factor secretion according to the proliferative status of Ch-SC, based upon prior studies of dermal fibroblasts (Pollina et al., 2008). Nevertheless, as our study design relied on permanent growth-arrest via Mitomycin-C, and not reversible cellular quiescence (induced by removal of serum or other sources of growth factors in Pollina et al. 2008) there could be further biological considerations that are having an influence on growth factor ratio, beyond the scope of the present study. In any case, the consistent observations of relatively higher PEDF expression for Ch-SC compared with VEGF may explain the suppression of ChVEC proliferation observed in the present study.

A further important issue for consideration when viewing the results of our “feeder cell” experiments, is that the serially-expanded, mitomycin C-treated cultures of Ch-SC may very well display different properties to the non-vWF-expressing cells originally observed in our test cultures of ChVEC. Indeed, having only examined the p0 ChVEC cultures for the presence of vWF, it remains possible that a number of additional non-vascular choroidal cell types may have been present in these test cultures; each with the potential to affect ChVEC

growth. It is therefore possible, that the inhibitory effects we have attributed to Ch-SC in our study, may be due in part to an effect on additional non-vascular cell types that may be present alongside the ChVEC. In short, we cannot fully exclude the possibility of cells other than ChVEC or Ch-SC (as characterized in our passaged cultures) contributing to this data.

In conclusion, our study demonstrates for the first time that MSC-like cultures can be derived from human choroidal tissue. While the capacity of Ch-SC for mesenchymal differentiation was markedly less than for bone marrow derived MSC (based upon histochemical stains) it seems likely that this difference can be related to a combination of donor age (62-80 years of age compared to 24 years of age), generally poor health status of cadaveric donors immediately prior to death (refer to Supplementary Table 1), and the specific microenvironment differences of these cell populations *in situ*. The inhibitory effects of Ch-SC on proliferation of ChVEC are consistent with the ratio of VEGF and PEDF secreted by these cells, but further studies are required to confirm this link. In any case, the ability of Ch-SC to dramatically alter the growth of ChVEC opens a fresh research pathway. As a starting point, we propose that a more detailed analysis is warranted of the genes expressed by choroidal derived MSC, compared to bone marrow derived MSC, using single-cell RNA sequencing and other emerging techniques.

Author Contributions

Nadine Alexander helped design the experiments and performed majority of the study. She contributed to the data analysis and helped write the manuscript.

Jennifer Walshe contributed to data analysis and edited the manuscript.

Neil A. Richardson contributed to data analysis and edited the manuscript.

Kathryn Futrega performed the study for Fig. 4 and edited the manuscript.

Michael R. Doran performed the study for Fig. 4 and edited the manuscript.

Damien G. Harkin helped design the experiments, contributed to data analysis and helped write the manuscript.

Audra M.A. Shadforth helped design and perform the experiments, managed the project, contributed to data analysis, and helped write the manuscript.

Declaration of competing interest

The Authors report no conflicts of interest.

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Tables

Table 1. Antibodies used for phenotyping of choroidal stromal cells by flow cytometry.

Antigen	Clone	Isotype	Supplier	Catalogue No.
CD14	MφP9	IgG _{2b} , κ	BD Biosciences	641394
CD34	8G12	IgG ₁ , κ	BD Biosciences	348057
CD45	HI30	IgG ₁ , κ	BD Biosciences	560777
HLA-DR	L243	IgG _{2a} , κ	BD Biosciences	347363
CD73	AD2	IgG ₁ , κ	BD Biosciences	562431
CD79a	HM47	IgG ₁ , κ	BD Biosciences	341643
CD90	5E10	IgG ₁ , κ	BD Biosciences	561558
CD105	266	IgG ₁ , κ	BD Biosciences	562408

Table 2. Immunophenotyping of choroidal stromal cell cultures after second passage.

Marker	Method	Expression (% +/- SD)
CD14	Flow cytometry	<1
CD34	Flow cytometry	<1
CD45	Flow cytometry	<1
HLA-DR	Flow cytometry	<1
CD73	Flow cytometry	98.5 +/- 0.5
CD79a	Flow cytometry	<1
CD90	Flow cytometry	98.0 +/- 1.0
CD105	Flow cytometry	97.5 +/- 1.5
α-sma*	Immunocytochemistry*	<10

Results are expressed as either the upper percentage value or mean +/- SD of percentage values obtained for individual cultures generated from 4 unique donors for immunocytochemistry (Donors 3 to 6) and 5 unique donors for flow cytometry (Donors 3 to 7; refer to Supplementary data for raw data).

*Positive cells defined by demonstration of positive staining within stress fibers. Results are based upon manual examination of at least 100 cells for each unique donor culture.

Figure legends

Figure 1. Typical outcome observed when attempting to isolate vascular endothelial cells from cadaveric choroidal tissue with the aid of magnetic assisted cell sorting (MACS). (A) Small clusters of cells are evident 7 days after seeding the CD45⁻/CD31⁺ eluted fraction into culture dishes. (B) Larger islands of smaller and more densely packed cells are evident by 14 days. (C) Immunostaining for von Willebrand factor (vWF) at 14 days reveals large clusters of presumptive vascular endothelial cells (vWF⁺ cells) surrounded by contaminating, non-stained cells (vWF⁻ cells). All images are of a culture derived from Donor 1 (Refer to Supplementary Table 1 for full donor details).

Figure 2. Technique used to establish cultures of choroidal stromal cells (Ch-SC) from cadaveric tissue. (A) Typical example of cellular outgrowth observed from a choroidal tissue fragment seeded into collagen gel after 14 days. (B) The majority of cellular outgrowth was devoid of staining for von Willebrand factor (vWF). (C) Example of culture 24-hours after passaging into culture flasks. (D) Appearance of first passage culture at 7 days. (Images A and B were acquired using tissue from Donor 2; Images C and D were acquired using tissue acquired from Donor 3).

Figure 3. Demonstration of α -smooth muscle actin (α -sma) expression in choroidal stromal cell (Ch-SC) cultures. Parts A to D display examples of positively stained cells observed in cultures derived from four unique cadaveric tissue donors. Actual level of expression across

each donor is <10% (based upon assessment of at least 100 cells per culture). (Images were acquired using p3 cultures derived from Donors 3, 4, 5 and 6 respectively in order).

Figure 4. Evaluation of tri-lineage differentiation potential of choroidal stromal cells was performed using 3 unique tissue donors (Donors 3 to 5). Data displayed for Donor 3 (Ch-SC; 62-year old cadaveric female donor) compared to MSC derived from bone marrow (BM-MSC; 24-year old living male donor). (A & B) Phase contrast images of cultures prior to testing. (C to L) Bright field microscopy images of induced (D, F, H, J, K and L) and control cultures (C, E, G and I) after day 21. Panels D and F indicate evidence of osteogenic activity by staining with Alizarin Red for calcified extracellular deposits. Panels H and J indicate evidence of adipogenic activity by staining for lipid inclusions with Oil Red-O. Panels K and L provide evidence of chondrogenic activity by virtue of positive staining of sectioned cell spheres with Alcian blue (with nuclear fast red as the counterstain).

Figure 5. Visual confirmation of choroidal stromal cell growth-arrest following treatment with mitomycin C. Phase contrast images display the same field of cells at: (A) 24-hours, (B) 4 days, and (C) 7 days after seeding into culture. The culture was derived from Donor 3.

Figure 6. Effect of growth-arrested choroidal stromal cells (Ch-SC) on the establishment of ChVEC cultures over 14 days. The freshly isolated CD45⁻/CD31⁺ MACS fractions from 5 unique tissue donors (Donors 8 to 12) were individually seeded into culture in the absence (A,

C, E, G and I) and presence of growth-arrested Ch-SC derived from Donor 3 (B, D, F, H and J). While large clusters of vWF+ cells developed over 14 days in control wells, only small sporadic clusters of stained cells were observed in the presence of the growth-arrested Ch-SC.

Figure 7. Assessment of PEDF expression by Ch-SC. Normal Ch-SC (seeded at 7,500/cm²) and growth-arrested Ch-SC (seeded at 75,000/cm²) were cultivated for up to 15 days in the presence and absence of serum. Conditioned medium (collected 48-hours after refreshing the growth medium) was removed and tested by ELISA for PEDF and VEGF on culture days 8 and 15. Less than 30 pg/mL of VEGF were secreted (Supplementary Figure 2). Bars represent mean $\pm$ SEM (n = 3 experimental replicates using unique Ch-SC cultures derived from donors 13 to 15). (A) Normal Ch-SC, when cultured in the presence of serum, expressed increasing amounts of PEDF as the culture achieved confluency (as confirmed by phase contrast images at right). Asterisk indicates significantly higher levels of PEDF secretion for serum-treated cultures at 15 days compared to all other conditions (p <0.001; by one-way ANOVA). (B) Growth-arrested Ch-SC expressed similar levels of PEDF in either the presence or absence of serum.

Supplementary Table 1. Summary of donor information for cadaveric eye tissue used.

Figure	Donor	Age	Gender	Cause of death	Notes
1.	D1	71	F	Cholangiocarcinoma	Typical growth of Ch-VEC isolated via MACS.
2. A and B C and D	D2	68	F	Peritonitis/colon cancer	Typical explant outgrowth.
	D3	62	F	Bowel obstruction	Typical appearance of passaged cultures.
3.	D3	62	F	Bowel obstruction	Examples of positive staining for alpha-smooth muscle actin observed in passaged cultures.
	D4	63	F	Small cell lung cancer	
	D5	80	F	Pneumonia	
	D6	72	F	Stroke/Glioblastoma	
Table 2.	D3	62	F	Bowel obstruction	All cultures analyzed for evidence of MSC phenotype via flow cytometry. D3 to D6 stained for alpha-sma,
	D4	63	F	Small cell lung cancer	
	D5	80	F	Pneumonia	
	D6	72	F	Stroke/Glioblastoma	
	D7	64	M	Tongue cancer	
4.	D3	62	F	Bowel obstruction	Cultures used for differentiation assays. (Data displayed for D3).
	D4	63	F	Small cell lung cancer	
	D5	80	F	Pneumonia	
5.	D3	62	F	Bowel obstruction	Growth-arrested Ch-SC.
6.	D3	62	F	Bowel obstruction	Growth-arrested Ch-SC.
	D8	38	M	Colorectal cancer	Utilized as a source of freshly isolated Ch-VEC for cultivation in the presence and absence of growth-arrested Ch-SC.
	D9	79	F	Small cell lung cancer	
	D10	81	M	Stroke	
	D11	69	M	Hypoxic brain injury	
	D12	44	F	Carcinoma of breast	
7. and Supp. Fig. 2	D13	36	M	Asphyxia	Used as a source of Ch-SC for measurements of growth factor secretion.
	D14	72	M	End stage COPD*	
	D15	67	M	Oesophageal cancer	
Supp. Fig 1.	D16	80	M	Brain cancer	Growth-arrested L-MSC.
	D17	54	M	Glioblastoma	Utilized as a source of freshly isolated Ch-VEC for cultivation in the presence and absence of growth-arrested L-MSC.

*COPD = chronic obstructive pulmonary disorder.

Supplementary Table 2. Summary of medium supplementation for induction cultures.

Induction method	Culture medium supplementation
Base medium (also used for control cultures)	High glucose DMEM (Life Technologies, Thermo Fisher Scientific; Cat. No. 10569010) supplemented with GlutaMax (Life Technologies, Thermo Fisher Scientific; Cat. No. 10569010), 10% FBS (Life Technologies, Thermo Fisher Scientific; Cat. No. 10099141), and penicillin/streptomycin (Life Technologies, Thermo Fisher Scientific; Cat. No. 15140122).
Osteogenic medium	Base medium supplemented with 50 mM ascorbic acid 2-phosphate (Sigma; Cat. No. A8960), 10 mM β -glycerol phosphate (Sigma; Cat. No. G9422), and 10^{-7} M dexamethasone (Sigma; Cat. No. D4902).
Adipogenic medium	Base medium supplemented with 200 mM indomethacin (Life Technologies, Thermo Fisher Scientific; Cat No. 18280), 10 mg/mL insulin (Sigma; Cat. No. 13536), 500 mM 3-isobutyl-1-methyl xanthine (Sigma; Cat. No. 17018) and 10^{-7} M dexamethasone.
Chondrogenic medium	High-glucose DMEM supplemented with GlutaMax, penicillin/streptomycin, insulin-transferrin-selenium-ethanolamine (ITS-X), 110 mg/mL sodium pyruvate, 200 mM ascorbic acid 2-phosphate, 40 mg/mL L-proline, 10^{-7} M dexamethasone, and 10 ng/mL recombinant human transforming growth factor- β 1 (TGF- β 1) (PeproTech; Cat. No. 100-21).

Supplementary Figure 1. Effect of growth-arrested limbal MSC on the establishment of ChVEC cultures. Cultures of growth-arrested corneal-limbal MSC (L-MSC; derived from Donor 16) were grown in parallel with cultures of freshly isolated ChVEC (CD45⁻/CD31⁺ fraction derived from one pair of donor posterior eye cups (derived from donor 17) grown in the presence and absence of L-MSC. The marked proliferation of ChVEC in the presence of growth-arrested L-MSC suggests that residual mitomycin C is an unlikely contributor to the inhibition observed when using growth-arrested Ch-SC (Figure 6).

Supplementary Figure 2. Assessment of VEGF secretion by Ch-SC. Normal Ch-SC (seeded at 7,500/cm²) and Ch-SC that had been growth-arrested by treatment with mitomycin C (seeded at 75,000/cm²) were cultivated for 15 days in the presence of serum. Conditioned medium (collected from over last 48-hours of culture) was removed and tested by ELISA for VEGF. Bars represent mean +/- SEM (n = 3 experimental replicates using Ch-SC unique cultures derived from donors 13 to 15). No difference in secretion was observed between cultures.

BM-MSC

Ch-SC

Monitoring of growth-arrested culture of Ch-SC

Donor ChVEC ChVEC+Ch-SC (D3)

7 July 2020

“Stromal cells cultivated from the choroid of human eyes display a mesenchymal stromal cell (MSC) phenotype and inhibit the proliferation of choroidal vascular endothelial cells *in vitro*”

Highlights

- There is a resident population of mesenchymal stromal cells in the human choroid
- Cultures established from the human choroidal stroma displayed a phenotype consistent with the accepted definition for MSC including the capacity for mesenchymal differentiation.
- Growth-arrested choroidal MSC inhibit the proliferation of choroidal-derived vascular endothelial cells in primary culture.