

The role of endothelial cells in the retinal stem and progenitor cell niche within a 3D engineered hydrogel matrix

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ABSTRACT

Cell–cell interactions are critical to understanding functional tissues. A number of stem cell populations have been shown to receive key regulatory information from endothelial cells (ECs); however, the role of ECs in the retinal stem and progenitor cell (RSPC) niche has been largely unexplored. To gain greater insight into the role of ECs on RSPC fate, a three-dimensional (3D) co-culture model, incorporating cell–cell interactions, was designed by covalently-modifying agarose hydrogels with growth factors and cell-adhesive peptides in defined volumes. Therein ECs adopted tubular-like morphologies similar to those observed *in vivo*, but not observed in two-dimensional (2D) cultures. Unexpectedly, ECs inhibited proliferation and differentiation of RSPCs, revealing, for the first time, the possible role of ECs on RSPC fate. This 3D hydrogel scaffold provides a simple, reproducible and versatile method with which to answer biological questions related to the cellular microenvironment.

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1. Introduction

In recent years, biomaterials have been designed as three-dimensional (3D) scaffolds in order to elucidate stem cell biology [1,2]. Ideally, bioactive materials will mimic the cellular microenvironment or stem cell niche, thereby providing insight into the spatial and temporal cues that govern stem cell fate [2–4]. One of the key considerations in stem cell niche research is interactions among different cell types [5]. Stem cell behavior is influenced by the presence of other cell types, either through direct cellular contact or secreted proteins, and results in differentiation and/or expansion and self-renewal [5,6]. Notwithstanding the importance of culturing multiple cell types in a 3D microenvironment, the relatively few co-culture studies that have been pursued to uncover mechanisms of cellular interactions, have been developed using two-dimensional (2D) cell culture [7–9]. Considering the functional and morphological differences of cells cultured in 2D vs. tissues [10], it is important to develop a 3D co-culture model that allows cells to adopt physiologically relevant phenotypes in order to gain greater insight into cellular interaction and function (Table S1).

Endothelial cells (ECs) have been shown to play fundamental roles in neurogenic niches beyond their traditional vascular roles as suppliers of oxygen and nutrients [11]. For example, neural stem and progenitor cells (NSPCs) in the subventricular zone (SVZ) of the brain are found in close proximity to capillary tips [12] where ECs have been shown to secrete factors, supporting neurogenesis of NSPCs [13,14]. The co-localization of ECs and stem/progenitor cells is not unique to the brain [5]; regulatory signals generated by ECs have also been shown to influence stem cell fate in various tissues, including adipose tissue [9,15] and the hematopoietic system [16,17].

Recent studies have demonstrated that the ciliary epithelium of the eye includes retinal stem and progenitor cells (RSPCs), showing the two cardinal properties of self-renewal and multipotentiality *in vitro* [18,19]. Similar to the NSPC niche, the RSPC niche consists of a highly dense 3D network of capillaries lined by ECs [20,21], suggesting a role for ECs in the RSPC niche. Interestingly, the role of ECs and RSPCs has largely gone unexplored. The RSPCs remain in the quiescent state *in vivo* and show no capacity for endogenous stimulation or regenerative capacity [18]. Elucidating whether ECs play a functional role as inhibitors of RSPC proliferation and differentiation is important to both better understand the cellular microenvironment and provide insight into treatment strategies of retinal degenerative disease. Interestingly, ECs in the microvasculature of the mouse brain [11,12,22] and ciliary body are both

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derived from the central nervous system and similar in phenotype and function [20,21,23], allowing us to use the more accessible brain microvasculature ECs for these studies.

Hydrogels, comprised of agarose [24], hyaluronan [25], alginate [26] or polyethylene glycol [3,27], provide low modulus materials that have been useful to study cellular interactions of the central nervous system. Moreover, these hydrogels allow the diffusion of nutrients and cellular waste through elastic networks [28,29]. Agarose hydrogels can be modified with coumarin-caged thiols to yield un-caged reactive thiol groups upon two-photon irradiation [30]. Agarose provides a blank canvas in which to include specific functionality, thereby allowing precise biological questions to be asked. Agarose is a transparent scaffold, which is critical to multiphoton chemical patterning [24,30] and the 6-bromo-7-hydroxycoumarin thiol-protecting group has been shown to be biocompatible with cells and tissues [31,32]. By taking advantage of two-photon patterning, a linear concentration gradient of photochemically immobilized VEGF165 was created in spatially distinct volumes of agarose hydrogels modified with cell-adhesive peptides, glycine-arginine-glycine-aspartic acid-serine (GRGDS) to guide EC growth [31]. Importantly, ECs were guided by the linear concentration gradient of immobilized VEGF165 and exhibited morphological and functional differences (relative to 2D cultures), forming tubular-like structures with tip-like and stalk-like cells identified [31].

Using this well-defined spatially-controlled EC platform, we describe a method that enables the study of cellular interactions between multiple cell types. We specifically focused on the cellular interactions between RSPCs and ECs within 3D constructs. By taking advantage of the spontaneous aggregation of ECs and RSPCs when co-cultured on the patterned hydrogels, we were able to investigate contact-mediated interactions between ECs and RSPCs on RSPC fate in terms of differentiation and proliferation profiles. The use of optically transparent hydrogels allowed us to visualize cellular behavior of ECs and RSPCs using fluorescent confocal imaging. Thus, this bioengineered scaffold allows cell–cell interactions to be investigated in 3D, thereby providing greater insight into the complex biological mechanisms of the stem cell niche.

2. Materials and methods

Detailed methods are provided in the Supporting Information.

2.1. Scaffold Fabrication and immobilization of cell-adhesive peptides and VEGF165

Agarose was modified with a photolabile thiol protected-6-bromo-7-hydroxycoumarin as described previously [30]. Modification and immobilization of VEGF165 was performed as previously described [31]. A solution of photolabile agarose (0.3 wt%) containing maleimide- and Alexa Fluor-modified VEGF165 (MI-VEGF165-f) solution (8 µg/mL) was pipetted into a 1 mm glass cuvette (Starna Cells, Inc, Atascadero, CA). After cooling at 4 °C for 40 min to ensure complete gelation, the cuvette was mounted on the stage of a Leica TPS SP2 confocal microscope equipped with a Spectra-Physics Mai Tai broadband Ti-Sapphire laser, tuned to 740 nm [30]. After focusing the laser to a plane in the interior of a gel at low power, the Leica software was used to define a region of interest (300 µm × 300 µm) and the laser power was increased through software controls to the maximum available. A macro program was previously written using Leica software to create concentration gradient patterns of the desired shape and dimension [30]. After patterning, gel samples were immersed in PBS buffer to remove unreacted MI-VEGF165-f for 2 days. Maleimide-GRGDS (MI-GRGDS), synthesized as previously described [31], was then added to each cuvette with tris(2-carboxyethyl) phosphine hydrochloride (Sigma–Aldrich) in PBS (100 µL, 0.5 mg/mL) and immobilized, as described above, using confocal laser patterning. Unreacted mi-GRGDS was rinsed by successive washing in PBS for 12 h.

2.2. Co-culture of ECs and RSPCs and characterization of the effects of ECs on RSPC proliferation and differentiation

Descriptions of the isolation and culture scheme of primary endothelial cells (ECs) from the brain of adult mice and primary retinal stem and progenitor cells

(RSPCs) from the ciliary epithelium of adult mice are available in the Supporting Information. 3D co-culture was performed in the co-culture medium comprised of retinal serum-free media (SFM) [18,19] containing 2% fetal bovine serum (FBS), 2% horse serum (Gibco-Invitrogen), 20 µg/mL heparin (Sigma–Aldrich), 20 µg/mL endothelial cell growth supplement (ECGS) (Sigma–Aldrich) and 100 µg/mL penicillin/streptomycin (P/S). Dissociated primary ECs (10,000 cells per cuvette) and RSPCs (10,000 cells per cuvette) were seeded on top of the cell-adhesive agarose hydrogels including VEGF165 gradient or no VEGF165 gradient. In order to distinguish RSPCs from ECs, RSPCs were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) using CellTrace™ CFSE Cell Proliferation Kit (Invitrogen), and ECs were labeled using the CellTrace™ Far Red DDAO-SE Kit (Invitrogen). We maintained the hydrogels for 14 d in 0.5 mL medium per cuvette, changed every 4 d, in a humidified 5% CO₂ incubator at 37 °C. As a control, RSPCs were cultured alone separately for the same period in the identical cell culture medium. We investigated RSPC proliferation and differentiation with immunofluorescence staining, described in detail in the Supporting Information. Next, we investigated the effects of ECs on RSPC fate in 2D culture by co-culturing ECs and RSPCs either directly or indirectly. Descriptions of direct and indirect RSPC and EC co-culture systems are available in the Supporting Information. We investigated RSPC proliferation as well as differentiation with immunofluorescence staining and reverse transcription polymerase chain reaction (RT-PCR), described in detail in the Supporting Information.

2.3. Characterization of cellular interactions between ECs and RSPCs

Based on the observation that laminin expression was detected where RSPCs and ECs were in contact, we examined whether adult RSPCs express the laminin receptor α6β1 integrin by immunofluorescence staining, described in the Supporting Information. In addition, in order to test whether α6β1 integrin was important for RSPCs to bind to ECs, ECs (Passage 2–4) were grown in 48-well plates in DMEM/F12 plus 10% FBS, 10% horse serum, 100 µg/mL heparin, 100 µg/mL ECGS to 80% confluence. Medium was changed to serum-free medium 2 d before co-culture. RSPCs labeled with CFSE were pre-incubated with GoH3-blocking antibody 1:50 (Beckman Coulter), β1 integrin-blocking antibody (BD), or IgG control antibody (BD). To assess adhesion, the medium was changed 1 h after plating, thereby removing non-adherent cells. The remaining adherent CFSE positive RSPCs were counted.

2.4. Statistical analysis

Differences among groups were assessed by one-way ANOVA with the Tukey's *post hoc* analysis to identify statistical differences among three or more treatments at $p < 0.05$. A student's *t*-test was used to statistically compare between two treatment groups. All data are presented as mean ± standard deviation (SD).

3. Results

3.1. Development of a linear VEGF165 gradient in GRGDS-modified agarose hydrogels

In order to create an endothelial cell niche, a linear VEGF165 concentration gradient in GRGDS-modified agarose hydrogels was created (Fig. 1). VEGF165 was modified with 4-(4-N-maleimidophenyl) butyric acid hydrazide (MPBH) and Alexa Fluor 488 hydrazide sodium salt [31,33]. A solution of photolabile agarose (0.3 wt%) containing maleimide-modified VEGF165 solution was pipetted into a 1 mm glass cuvette. After cooling at 4 °C to ensure complete gelation, the cuvette was mounted on the stage of a Leica TPS SP2 confocal microscope equipped with a Spectra-Physics Mai Tai broadband Ti-Sapphire laser, tuned to 740 nm. By taking advantage of photolabile coumarin-protected agarose-sulphide groups and maleimide-modified VEGF165, a gradient of VEGF165 was photochemically immobilized within a defined volume in the agarose hydrogel, using a multiphoton Ti/sapphire confocal laser [30]. The gradients were created by controlling the number and volume of two photon-scanned regions and quantified based on the fluorescence intensity (Supporting Information Fig. S1). After VEGF165 patterning, maleimide-modified GRGDS peptides were immobilized at a uniform concentration of 2.08 µM in the same volume using the confocal multiphoton technique, as determined by constant fluorescence intensity with depth (Supporting Information Fig. S1). Vertical, linear concentration

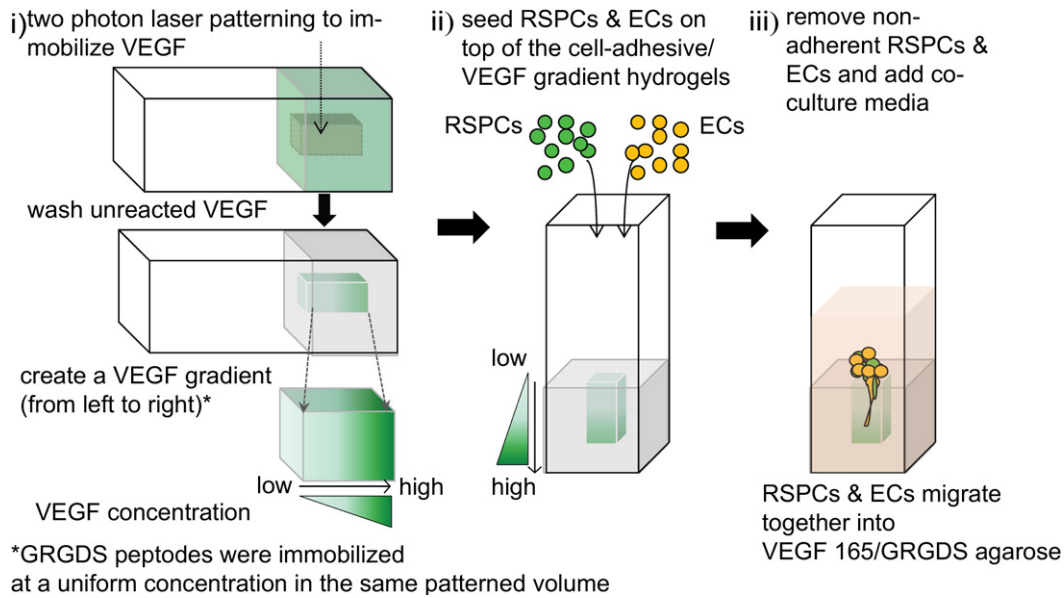


Fig. 1. Schematic of the 3D patterning and co-culture of RSPCs and ECs. i) Using a multiphoton laser patterning technique, maleimide-modified VEGF165 gradient and maleimide-modified GRGDS were immobilized within agarose-sulphide hydrogels. The VEGF165 gradient increases from white to green. Green represents fluorescently-modified VEGF165. ii) RSPCs and ECs were plated on the cell-adhesive, VEGF165 gradient agarose in the co-culture medium. iii) both RSPCs and ECs formed aggregates on top of the patterned volumes and then penetrated the agarose hydrogels, following the VEGF165 concentration gradient. The green gradient represents the VEGF165 gradient which increases with depth from the top surface towards the bottom. The pale pink box represents the co-culture media. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

gradient volumes of fluorescently-tagged VEGF165, having dimensions of $300 \mu\text{m} \times 300 \mu\text{m} \times 600 \mu\text{m}$ ($L \times W \times D$), were synthesized in GRGDS-functionalized agarose [31]. A schematic of the experimental setup is also shown in Fig. 1. An optimized VEGF165 concentration gradient to guide ECs into the agarose hydrogels was used [31], having a surface VEGF165 concentration of 250–300 ng/mL and a VEGF165 concentration gradient of 1.5–1.8 ng/mL/ μm for this co-culture experiment (Supporting Information Fig. S1).

3.2. Growth of EC tubular-like structures from EC and RSPC aggregates

In order to examine the cellular behavior of RSPCs and ECs, dissociated adult mouse primary brain microvascular ECs (10,000 cells per cuvette) and adult mouse RSPCs, dissected from the ciliary epithelium (10,000 cells per cuvette), were seeded on top of the GRGDS cell-adhesive, VEGF165 gradient agarose hydrogels using the co-culture medium of: 2% fetal bovine serum (FBS), 2% horse serum, 20 $\mu\text{g}/\text{mL}$ heparin, 20 $\mu\text{g}/\text{mL}$ endothelial cell growth supplement (ECGS) and 100 $\mu\text{g}/\text{mL}$ penicillin/streptomycin (P/S). In order to distinguish RSPCs from ECs, RSPCs were labeled with CFSE using the CellTrace™ CFSE Cell Proliferation Kit. By taking advantage of the low cell-adhesive properties of agarose hydrogels, ECs and RSPCs seeded on top of the agarose hydrogels formed small aggregates on top of the cell-adhesive patterned volumes only within the first 24 h of culture. This aggregation process promotes cell–cell contact, allowing the interactions between ECs and RSPCs to be investigated.

When ECs and RSPCs were cultured on patterned GRGDS peptide hydrogels in the absence of VEGF165, ECs and RSPCs migrated only a short distance ($32.4 \pm 14.5 \mu\text{m}$, mean \pm standard deviation, $n = 12$) into the hydrogels, just below the surface after 3 d of culture (Supporting Information Fig. S2). Moreover, in the absence of the VEGF165 gradient, both ECs and RSPCs retracted

their processes toward the aggregates on top of the hydrogels after 5 d of culture (data not shown).

In the co-culture of ECs and RSPCs in VEGF165 gradient/GRGDS-agarose hydrogels, ECs migrated into the hydrogels, up the VEGF165 concentration gradient after 3 d of culture and, RSPCs migrated with the ECs as shown in Fig. 2A. ECs retained this tubular-like structure deep within the hydrogel for 14 d (Fig. 2B). In contrast, RSPCs cultured alone formed aggregates on top of the patterned volumes after 1 d, and did not migrate into the hydrogels, even after 14 d of culture (Supporting Information Fig. S3). Interestingly, ECs cultured alone penetrated the VEGF165 gradient/GRGDS-agarose hydrogels and formed tubule-like structures therein after 3 d of culture; however, even the ECs retracted their leading process and reversed direction toward the EC aggregates localized on the surface after 5 d of culture (in 11 out of 12 samples) (Supporting Information Fig. S4). Thus only when ECs and RSPCs were co-cultured together, did the RSPCs grow into the hydrogel scaffold and were the EC tubule-like structures stable for the 14 d culture period.

To gain greater insight into the 3D spatial interaction between ECs and RSPCs co-cultured in the VEGF165 gradient/GRGDS-agarose hydrogels, EC specific junctional proteins were immunostained with VE cadherin and the EC extracellular matrix was stained for laminin. With CFSE-labeled RSPCs, we clearly observed the co-localization of ECs and RSPCs in fluorescent confocal image stacks taken along the z-axis of the hydrogels (Fig. 2B). VE cadherin has been shown to be crucial for establishing and maintaining the cell–cell contacts necessary for the formation of new capillary tubes [34,35]. Furthermore, it has been shown that EC tubules are quiescent once VE cadherin is expressed and, under these circumstances, ECs neither migrate nor sprout filopodia in response to VEGF stimulation [35,36]. This pattern of EC tubular-like formation and VE cadherin expression is mirrored in our EC-RSPC co-culture system where we observe that stable cell–cell junctions increased VE cadherin expression of ECs forming tubular-like structures after 14 d of culture (Fig. 2B) vs. after 3 d of culture

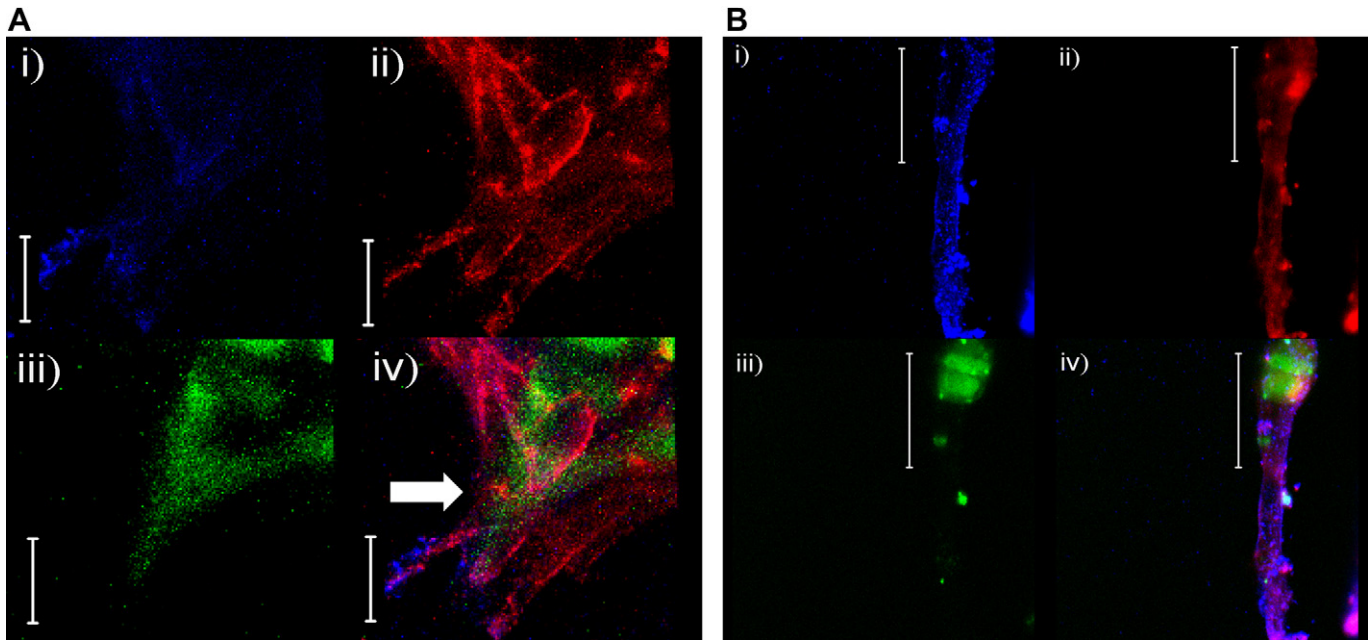


Fig. 2. Representative images of RSPCs migrating along ECs in 3D agarose hydrogels. (A) Confocal 3D images show both ECs migrating into the VEGF165 gradient/GRGDS-agarose hydrogels and RSPCs closely associated with the ECs after 3 days of co-culture. Cells were immunostained with primary antibodies: i) VE cadherin (blue) for junctional proteins expressed by ECs; ii) laminin (red) for the extracellular matrix of ECs; iii) CFSE (green) labeled RSPCs; and iv) a merged image of the combined staining for ECs with VE cadherin and laminin with CFSE-labeled RSPCs shows the close association of ECs and RSPCs (scale bar = 20 μm). The white arrow indicates the top surface of the hydrogel. Cells are aggregated together on the surface (above the arrow) and are beginning to migrate together into the gel (below the arrow). (B) Confocal 3D images of ECs that had migrated and formed tubular-like structures in the VEGF165 gradient/GRGDS-agarose hydrogels after 14 days of culture. RSPCs were detected where laminin expression was highly abundant. Cells were immunostained with primary antibodies as in (A): i) VE cadherin (blue) for EC junctional proteins; ii) laminin (red) for EC ECM; iii) CFSE (green) for RSPCs; and iv) merged staining of EC VE cadherin and laminin with CFSE-labeled RSPCs (scale bar = 50 μm). The top of the hydrogel is not shown as it is located above the image. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 2A). The mean \pm standard deviation of EC tubule extension into VEGF165 gradient/GRGDS-agarose gels at 14 d was $205 \pm 11 \mu\text{m}$ (mean \pm standard deviation, $n = 12$) for the EC-RSPC co-culture system vs. $45.6 \pm 19.8 \mu\text{m}$ (mean \pm standard deviation, $n = 6$) for the EC monoculture system, demonstrating significantly greater EC migration in the presence of RSPCs. The difference at 14 d may reflect a stabilizing effect that the RSPCs have on the ECs in the gels as we observed an average of 2.4 ± 0.6 RSPCs on each EC tubular structure (mean \pm standard deviation, $n = 12$).

These results suggest that the association of RSPCs with ECs stabilizes EC growth and this is further supported by the longer stability observed of the ECs deep within the hydrogel when co-cultured with RSPCs (14 d) vs. alone (3 d). To gain greater insight into the mechanism of this association, the extracellular matrix proteins secreted by the ECs were investigated.

3.3. Characterization of cellular interactions between ECs and RSPCs

It is well known that the ECM plays a critical role in the stem cell niche [4,37]. The ECM interacts with cells via cell-surface receptors such as integrins and provides a site for growth factor binding. The ECM provides a substrate for cell attachment and spreading, contact guidance for cell migration, and a scaffold that serves as a building block of tissues [4,38].

We found that laminin was expressed by ECs and also evident at the EC-RSPC interface after both 3 and 14 days of culture (Fig. 2A and B, respectively). Importantly, the $\alpha 6 \beta 1$ -integrin, one of the laminin cell-surface receptors, was widely expressed by RSPC spheres (Supporting Information Fig. S5 for $\beta 1$ expression and Fig. S6 for $\alpha 6$ expression) and has also been reported for ECs [39]. The GoH3 antibody, which recognizes the $\alpha 6$ integrin, was found in

close association with the most laminin-positive elements and labeled RSPCs associated with ECs (Fig. 3A). Similarly, the $\beta 1$ -integrin was observed at the laminin-positive elements where RSPCs interacted with ECs (Fig. 3B). To determine the importance of the RSPC $\alpha 6 \beta 1$ -integrin expression for RSPC binding to ECs, CFSE-labeled RSPCs were pre-incubated with either anti- $\alpha 6$ or anti- $\beta 1$ prior to plating on 80% confluent EC monolayers. The number of CFSE-labeled RSPCs that bound to ECs was significantly reduced when pre-incubated in either GoH3 (anti- $\alpha 6$) or anti- $\beta 1$ antibodies prior to plating relative to controls (which were pre-incubated in an IgG isotype control) (Fig. 3C, $p < 0.001$). Thus, RSPCs express a key integrin receptor that enables them to bind to the laminin-rich endothelial cell ECM. The close association observed between ECs and RSPCs suggests that they interact via laminin which is expressed by the ECs in their ECM and for which RSPCs express receptors. These results provide new insights into the mechanisms by which ECs may regulate RSPC behavior through contact-mediated cues.

3.4. Contact-mediated effects of ECs on RSPC proliferation and differentiation in 3D co-cultures

In order to investigate whether ECs guide RSPC proliferation and differentiation through contact-mediated signaling, the autocrine and/or paracrine factors secreted by ECs were examined in terms of RSPC proliferation and differentiation. To distinguish RSPCs from ECs in our co-culture experiments, RSPCs were labeled with CFSE, and ECs were labeled using the CellTrace™ Far Red DDAO-SE Kit. After 14 days of co-culture in VEGF165 gradient/GRGDS-agarose hydrogels, ECs formed tubular-like structures that were apparently stabilized by the close association with RSPCs, as described above. The co-cultured cells were fixed and immunostained with Ki67 for

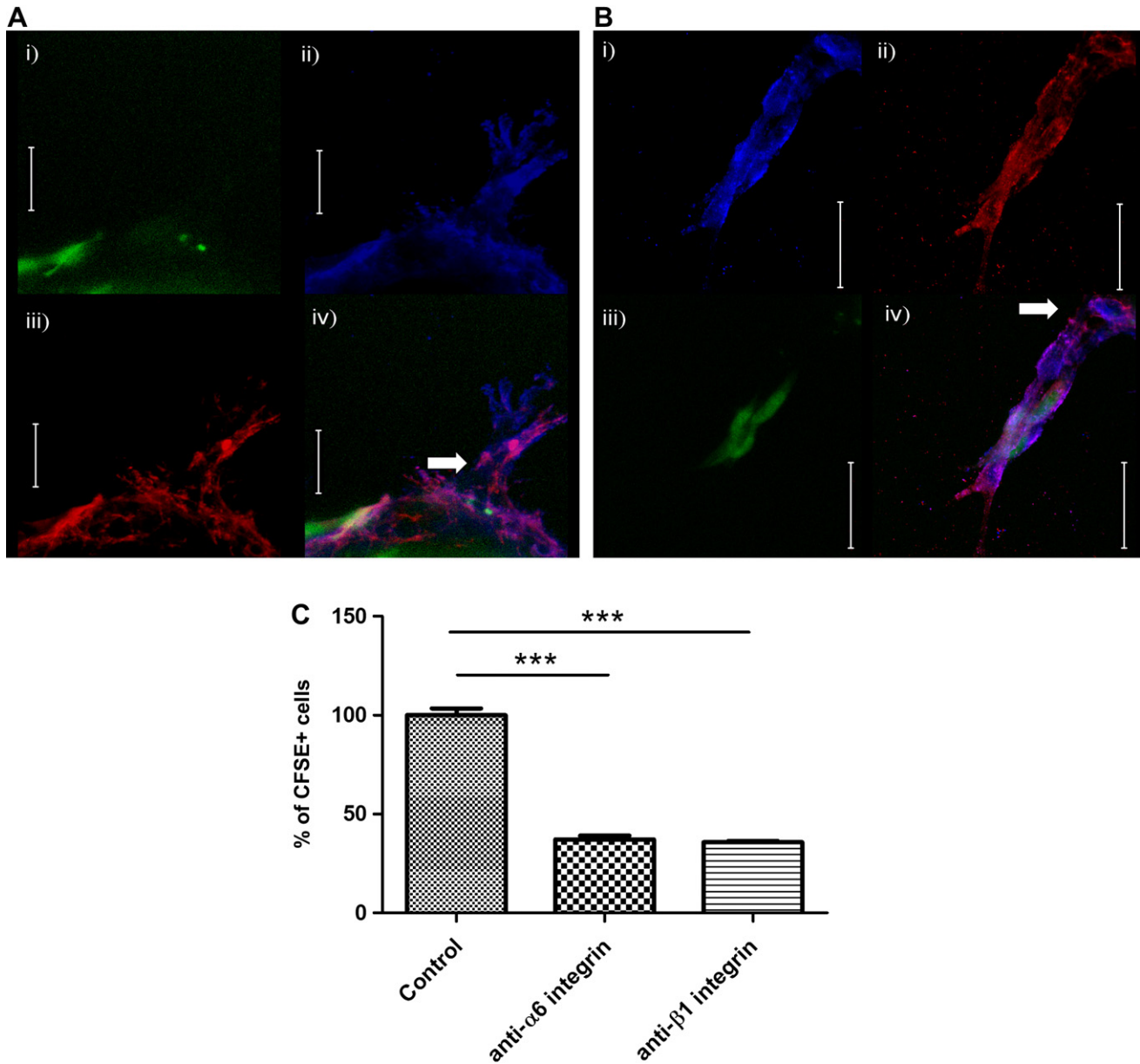


Fig. 3. $\alpha 6\beta 1$ integrin is expressed in RSPCs in close association with ECs which express laminin. (A) Representative 3D confocal images of $\alpha 6$ integrin immunostaining of RSPCs co-localized with tubular-like ECs in VEGF165 gradient/GRGDS-agarose hydrogels. The $\alpha 6$ integrin labeled the most laminin-positive elements and also labeled RSPCs closely associated with ECs: i) RSPCs labeled with CFSE (green); ii) $\alpha 6$ integrin (blue); iii) laminin for extracellular matrix (red); and iv) merged staining of laminin, $\alpha 6$ integrin and CFSE-labeled RSPCs (scale bar = 20 μm). Arrow indicates top surface of the gel. (B) Representative 3D confocal images of $\beta 1$ integrin immunostained RSPCs co-localized with tubular-forming ECs in the VEGF165 gradient/GRGDS-agarose hydrogels. $\beta 1$ integrin labeled the most laminin-positive elements and also labeled RSPCs in close association with the ECs: i) $\beta 1$ integrin (blue) expressed by RSPC; ii) laminin (red) for EC extracellular matrix; iii) CFSE (green) labeled RSPCs; and iv) merged staining of laminin, $\beta 1$ integrin and CFSE-labeled RSPCs (scale bar = 20 μm). White arrow depicts the top surface of the hydrogels. (C) Blocking with anti- $\alpha 6$ integrin (GoH3) or anti- $\beta 1$ integrin inhibited the adhesion of RSPCs (CFSE + cells) to EC monolayers demonstrating the importance of EC-laminin and RSPC-laminin receptors, $\alpha 6\beta 1$, for EC-RSPC interactions. In the control, the identical blocking study was done with an IgG isotype control antibody instead of anti- $\alpha 6$ or anti- $\beta 1$. Data are mean \pm SD (***) $p < 0.001$, $n = 3$ separate experiments, with each experiment done in triplicate and a minimum of 200 cells counted). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proliferation and with a series of markers to determine the differentiated retinal progeny (as described in the Supporting Information). Interestingly, $84.5 \pm 6.7\%$ of RSPCs were Pax6+ progenitor cells (Fig. 4) with only a very low percentage of Otx2+ mature bipolar cells or immature rod/bipolar progenitors ($1.2 \pm 0.5\%$). To obtain a quantitative differentiation profile, we counted CFSE + cells, which lie on EC tubular structures, 10 μm below the surface of the hydrogels as well as cells doubly labeled with a specific retinal marker and CFSE ($n = 12$ separate samples, with a total of 148 cells counted). We observed neither proliferative Ki67 + RSPCs nor mature differentiated retinal cell phenotypes,

suggesting that ECs may regulate the differentiation and proliferation of RSPCs.

Importantly, RSPCs have been shown to differentiate into rod photoreceptors, bipolar neurons, retinal ganglion cells and Müller glia on laminin-coated tissue culture plates [18]. In addition, RSPCs cultured on GRGDS-agarose hydrogels exhibited mRNA expression specific to both undifferentiated nestin + retinal progenitor cells after 14 days of culture and differentiated retinal cells including: Brn3b + ganglion cells, rhodopsin + photoreceptors, GFAP+ Müller glia cells and PKC α + bipolar cells [40]. The apparent regulation of proliferation and differentiation of RSPCs observed by their 3D co-

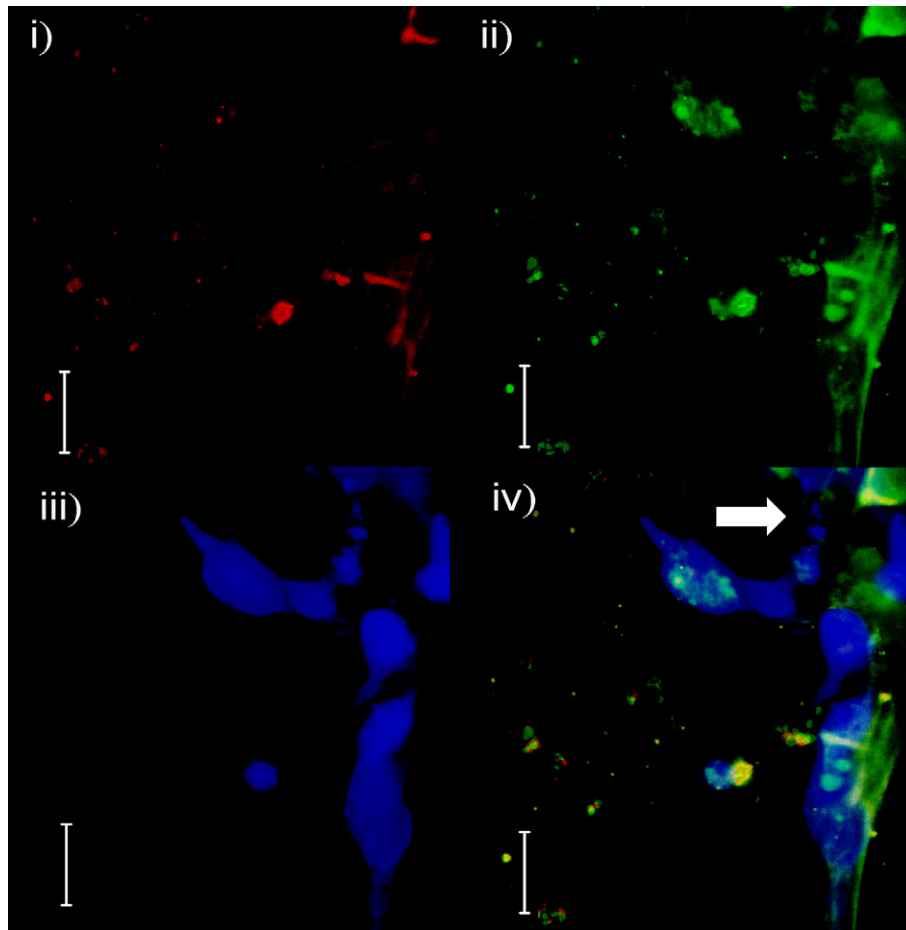


Fig. 4. RSPCs remain undifferentiated when they are co-cultured with ECs. Representative 3D confocal images of Pax6 immunostaining of RSPCs migrating along tubular-forming ECs in VEGF165 gradient/GRGDS-agarose hydrogels at day 14: i) RSPCs express the retinal progenitor marker Pax6 (red); ii) RSPCs labeled with CFSE (green); iii) ECs labeled with far Red DDAO-SE (blue); and iv) merged staining of Pax6 and CFSE-labeled RSPCs with DDAO-SE labeled ECs (scale bar = 20 μ m). (White arrow points to the top surface of the hydrogel). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

culture with ECs has not been previously reported and suggests an important role for ECs in the niche.

3.5. Effects of ECs on RSPC proliferation and differentiation in 2D co-culture studies

To gain a greater understanding of whether ECs and RSPCs interact through contact-mediated interactions or secreted signals, they were co-cultured together in either direct contact or indirect contact (i.e. in the same medium, but without cell–cell contact). Since RSPCs would not migrate into the modified agarose gels in the absence of ECs (Supporting Information Fig. S3), we reverted to traditional 2D co-culture systems to provide some insight into how ECs and RSPCs were interacting with each other. As controls, RSPCs were either cultured alone but still in the same co-culture medium. After 5 weeks of culture, the RSPCs were analyzed using immunofluorescence staining and RT-PCR. In the direct co-culture study, RSPCs were plated directly on EC cultures whereas in the indirect co-culture study, RSPCs were plated on laminin-coated 24-well plates and ECs were plated on collagen-coated transwell inserts placed in the same well. RSPCs in control monocultures differentiated to Otx2+Rho1D4-mature bipolar cells or immature rod/bipolar progenitors, Calbindin + horizontal cells, and Rho1D4+ photoreceptors at 5 weeks; the expression of Pax6 was significantly reduced to $33.6 \pm 3.5\%$ in control cultures at 5 weeks compared to that of $78.6 \pm 6.3\%$ at day 0 (Supporting Information Fig. S7 and

Table S1). In contrast to the differentiation profile observed for RSPC monocultures, most of the RSPCs growing in both the direct EC contact and indirect EC co-cultures remained undifferentiated, exhibiting $78.4 \pm 4.1\%$ of Pax6+ cells in direct and $76.9 \pm 1.0\%$ in indirect co-culture systems (Supporting Information Table S1). In these co-culture systems, there was no significant difference in the Pax6+ progenitor cell population compared to day 0 ($n = 3$, $p < 0.001$). Low levels of Otx2 expressing cells were observed after 5 weeks of co-culture; however, $10.8 \pm 3.1\%$ of Otx2+ cells were observed prior to co-culture studies. There was some evidence of differentiation of RSPCs in the indirect cultures that was absent in the direct contact RSPC-EC co-culture system. The immunofluorescence data (presented in Table S1) were consistent with mRNA expression for all markers (Supporting Information Fig. S8) except cells showed very low levels of Rho1D4+ immunostaining yet no rhodopsin mRNA expression in the indirect co-culture system. This may be due to differences in sensitivity of these analyses or due to post translational modification or degradation of the mRNA.

In terms of the proliferation profile, Ki67 + proliferative RSPCs were not detected in direct contact co-culture systems after 1, 2 and 5 weeks of culture. In contrast, a low percentage of proliferating cells was observed in both indirect co-culture and control RSPC monoculture systems after 5 weeks of culture, although the percentage of proliferative RSPC populations decreased from the initial time of plating: $34.1 \pm 4.0\%$ at day 0 decreased to $5.6 \pm 1.7\%$ in indirect co-culture and to $2.5 \pm 0.3\%$ in RSPC monocultures at 5

weeks (mean \pm standard deviation, $n = 3$ samples) (Supporting Information Table S1). Taken together, these data indicate that ECs influence the RSPC differentiation prolife: ECs maintain RSPCs in an undifferentiated state and direct physical contact between ECs and RSPCs inhibits RSPC proliferation. Both the 3D agarose and 2D co-culture systems support this statement.

4. Discussion

Adult stem cells are regulated by signals from both adjacent differentiated cell types and extracellular matrix molecules, which collectively define the stem cell niche [6,41,42]. In order to elucidate the complex biological mechanisms of the stem cell niche, biomimetic strategies must evolve to include some of this complexity including 3D presentation of the extracellular matrix (ECM) and culture of multiple cell types. Although commercially available Matrigel[®] matrix has been used in 3D studies [3], Matrigel[®] is ill-defined and inconsistent in composition, making results difficult to interpret and reproduce.

Recent studies show promise for reconstituting the damaged cellular populations of the retina with cellular transplantation strategies [43,44]. One of the key advantages of using retinal stem cells is that they have the capacity to differentiate *in situ* and integrate (at least in part) into the retinal pigmented epithelium [45]. With clinical studies planned for RSPC delivery, understanding the cellular microenvironment is critical to their successful survival and integration with the host tissue. By better understanding how RSPCs and ECs interact, we may gain greater insight into the niche. The well-defined VEGF-gradient/GRGDS-immobilized agarose hydrogel allows us to investigate the influence of ECs on RSPC fate and the mechanism of their interactions in 3D.

Our understanding of the importance of endothelial cells and the vascular system within the stem cell niche is still emerging [11,12]. For example, cell–cell interactions through ECM and integrins are critical in the neural stem cell niche of the adult forebrain SVZ [11,12]. Despite similarities in the structures of capillary vessels within both the retinal stem cell niche and the neural stem cell niche, studies of functional interactions between ECs and RSPCs have been investigated neither *in vitro* nor *in vivo*. Furthermore, the lack of RSPC-specific markers *in vivo* hampers functional studies on the retinal vascular niche - that is, it is difficult to identify RSPCs from other cell types in the ciliary margin by immunostaining.

Primary brain-derived ECs were utilized in this study because both the endothelial blood–retina and the blood–brain barriers share common features such as low fluid-phase endocytosis and tight junctions with high electrical resistance, limiting transcellular and paracellular flux [46]. Notwithstanding similarities in function and structure, we acknowledge that there may be differences between the endothelial cells in the brain and retina. While technical limitations prohibited the use of retina-derived ECs in these studies, we are confident that the similarities in these two cell types have allowed us to probe the interactions between ECs and RSPCs and that these interactions are meaningful.

Importantly, most of the RSPCs were associated with strong laminin-containing ECM derived from ECs. RSPCs express laminin receptor, $\alpha 6\beta 1$ integrin, and this receptor plays a critical role in RSPC adhesion to ECs. RSPCs cultured alone did not migrate into the hydrogels, suggesting that the ECM derived from ECs plays a pivotal role in regulating RSPC behavior, which in turn impacts EC behavior. Thus it is highly plausible that the functional reciprocal signaling between RSPCs and ECs, governed by common growth factors, stabilizes EC tubular-like formations. While not pursued herein, we acknowledge that other cell types may also be able to stabilize EC tubular-like structures.

Surprisingly, our 3D co-culture results demonstrate that direct cellular contact with ECs inhibit RSPC differentiation. Correspondingly, our 2D co-culture studies showed that RSPCs remain undifferentiated in both direct and indirect co-culture systems. These results suggest that paracrine interactions regulate RSPC differentiation. Moreover, our results demonstrate that direct cellular contact with ECs inhibits proliferation of RSPCs when co-cultured in 3D as well as 2D. Interestingly, we did not observe this same result with indirect co-cultures or of RSPCs in EC-conditioned media (see Supporting Information), indicating that EC-derived soluble factors did not inhibit proliferation. Overall, our results suggest that ECs regulate RSPC differentiation through soluble factors; however, it is possible that cell contact may also play a role yet is masked by the absence of proliferation in the EC-RSPC direct co-cultures.

To the best of our knowledge, this is the first demonstration to show that ECs contribute to maintaining RSPCs in a quiescent state, inhibiting differentiation and proliferation of RSPCs using a 3D co-culture model. This suggests that ECs localized in the ciliary margin may have a functional role as inhibitors of RSPC proliferation and differentiation, the biological mechanisms of which can be further investigated in future *in vivo* studies.

5. Conclusions

Engineered polymers can be used as platforms to better mimic the stem cell niche, allowing for multiple stimuli and many cell types to be explored individually or in combination. We demonstrate here that our 3D *in vitro* scaffold provides important insights into the effects of ECs on RSPCs to be probed in terms of proliferation and differentiation. Considering the importance of ECs in other stem cell niches, these results create a foundation for future studies on stem cell therapies. Moreover, this 3D co-culture model can be further extended to examine other cell–cell interactions with or without endothelial cells to provide insight into the strategies required for successful cell delivery leading to regeneration.

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Appendix A. Supporting information

Supplementary data related to this article can be found online at [doi:10.1016/j.biomaterials.2012.03.062](https://doi.org/10.1016/j.biomaterials.2012.03.062).

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