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Hydraulic Conductivity of Endothelial Cell-Initiated Arterial Cocultures

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Abstract

This study describes cocultures of arterial smooth muscle cells (SMC) and endothelial cells (EC) and the influences of their heterotypic interactions on hydraulic conductivity (L_p), an important transport property. A unique feature of these cocultures is that ECs were first grown to confluence and then SMCs were inoculated. Bovine aortic smooth muscle cells (BASMCs) and bovine aortic endothelial cells (BAECs) were cocultured on Transwell Permeable Supports, and then exposed to a pressure-driven transmural flow. L_p across each culture was measured using a bubble tracking apparatus that determined water flux (J_v). Our results indicate that arterial L_p is significantly modulated by EC-SMC proximity, and serum content in culture. The L_p of cocultures was also compared to the predictions of a resistances-in-series model to distinguish the contributions of heterotypic interactions between SMCs and ECs. Conditions that lead to significantly reduced coculture L_p, compared to BAEC monoculture controls, have been uncovered and the lowest L_p in the literature for an *in-vitro* system are reported. In addition, VE-cadherin immunostaining of intact BAEC monolayers in each culture configuration reveals that EC-SMC proximity on a porous membrane has a dramatic influence on EC morphology patterns. The cocultures with the lowest L_p have ECs with significantly elongated morphology. Confocal imaging indicates that there are no direct EC-SMC contacts in coculture.

Keywords

Endothelial Cells; Smooth Muscle Cells; Porous Membrane; Coculture; Hydraulic Conductivity

Introduction

Tissues allow for both homotypic and heterotypic cellular interactions and, depending on the proximity of cells, communication may occur through soluble and contact-mediated pathways (Figure 1). Local mechanical and chemical signaling mechanisms are essential for maintaining a tissue's structure and function. Intimal endothelial cells (ECs) and medial smooth muscle cells (SMCs) are neighboring cells in the arterial wall. Their homotypic interactions include contact-inhibited endothelial growth[21], gap junction communication[17,21] and smooth muscle contraction in response to stretch[14,29], while smooth muscle relaxation induced by endothelial nitric oxide is a classic heterotypic interaction[34]. This study examines, for the first time, whether heterotypic interactions of arterial ECs and SMCs influence arterial wall transport properties, specifically hydraulic conductivity (L_p). We focus on co-cultures where the ECs are plated first, followed by the SMCs.

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In-vivo, the order in which ECs and SMCs are established in the vessel wall, and the proximity between ECs and SMCs, varies with the events that are taking place in the localized region of the vasculature. For example, during arteriogenesis, a heterotypic tissue structure develops as SMCs are recruited to form a dense sub-endothelial medial region near a preexisting intact endothelium [4,9,41,47]. In healthy arterial walls, the porous internal elastic lamina lies between the intimal endothelium and a relatively dense medial region of SMCs[20]. Endothelial cells and SMCs can then establish direct heterotypic contacts through the pores of the internal elastic lamina (IEL)[12,22,40]. In the present co-culture study we use a porous membrane separating ECs and SMCs to simulate the IEL.

Previous work with coculture systems[11,18] has clearly demonstrated that ECs can influence SMC function. Coculturing bovine aortic endothelial cells (BAECs) and bovine aortic smooth muscle cells (BASMCs) on opposing sides of a porous membrane (bilayer coculture), as well as through shared media alone (conditioned-media coculture), was shown[18] to cause significant changes in SMC proliferation rates, density, transmembrane projections, and protein synthesis compared to SMC monocultures.

Bilayer coculturing of ECs and SMCs, with a laminar fluid shear stress apparatus, also elicited changes in EC gene expression[8]. Endothelial cell gene expression for adhesion molecules was inhibited when the coculture flow apparatus was used to expose ECs to laminar fluid shear stress. In static (no-flow) conditions, however, SMCs induced EC gene expression for adhesion molecules and lowered EC gene expression for nitric oxide synthase. Incorporating a porous membrane interface between ECs and SMCs in both static and laminar fluid shear stressed conditions was essential to induce those changes in ECs.

Varying both serum content and culture time in bilayer cocultures and monocultures were also shown to cause changes in human aortic SMC cytokine secretions[39]. Cytokine secretions from cocultured SMCs were found to be significantly different from monocultured SMCs when supplied with higher fetal bovine serum concentrations and cultured for longer periods of time. Those findings illustrated that serum supplemented and time-dependent arterial coculturing methods stimulate heterotypic activities. Also, in directly cocultured porcine arterial ECs and SMCs (without an intervening porous membrane), proximity, serum content, and culture time were all demonstrated to be precursors in the chain of events regulating indicators of thrombogenicity[33].

There has been only one previous study of transport in arterial-like coculture models. In porous bilayer cocultures of human umbilical vein ECs and murine smooth muscle-like 10T ½ cells, the diffusive permeability of 70 kDa biotin-dextran was shown to be lower when compared to monoculture controls of ECs and 10T ½ cells[26]. Similar coculture constructs pairing brain ECs and astrocytes have been exploited in blood-brain barrier transport measurements[28]. To date, however, it is unclear how arterial EC-SMC coculture configurations may influence arterial transport.

Hydraulic conductivity of BAECs grown on Transwell Permeable Supports and supplied with 10% fetal bovine serum in culture media has been measured in previous studies[5,7,13,15,24,45] conducted in our laboratory, and there has been a quantitative consistency in these measurements over the last 20 years[44]. The present study determines L_p for various monoculture and coculture configurations of BAECs and BASMCs. Our results identify that the proximity of BASMCs to BAECs on the porous membrane, along with serum content in monocultures and cocultures, modulates arterial L_p. Arterial cocultures that mimic the arrangement of ECs and SMCs in a healthy arterial wall feature an intact monolayer of ECs with an elongated morphology pattern, and lead to the lowest L_p values reported for an *in-vitro* arterial model.

Materials and Methods

Materials

Transwell polyester permeable supports were purchased from Corning, Inc., NY. These membranes are characterized by a pore diameter of 0.4 μm, pore densityof 4.0e6 pores/cm^2, and membrane thickness of 10 μm. T-75 Tissue Culture Flasks were purchased from Becton Dickinson, NJ. A T-25 Flask of primary BAECs was purchased form VEC Technologies, Inc, NY. A cryopreserved ampule of primary BASMCs was purchased from Cell Applications, Inc., CA. Fibronectin (FN) 0.1% from bovine plasma; Triton X-100; Trypsin-EDTA; Penicillin Streptomycin (PS); 200mM L-Glutamine (LG); 30% Albumin solution from Bovine Serum (BSA); and Phenol Red Minimum Essential Medium (MEM) were purchased from Sigma-Aldrich, Inc., MO. Fetal Bovine Serum (FBS) Defined and Paraformaldehyde (PFA) were purchased from Thermo Fischer Scientific, Inc. Phenol Red Free Minimum Essential Medium (PRF-MEM) and Calcium and Magnesium Free Phosphate Buffered Saline (CMF-PBS) were purchased from Mediatech, Inc., VA. VE-Cadherin Primary Antibody (PAb), and Anti-rabbit IgG (H+L), F(ab')$_2$ Fragment (Alexa Fluor 488 Conjugate) Secondary Antibody (SAb) were purchased from Cell Signaling Technology Inc. MA.

Defined Cell Culture Media

Serum-free MEM (SF-MEM) consisted of MEM with 1% LG, and 1% PS. 10% FBS MEM (10FB-MEM) consisted of MEM with 10% FBS, 1% LG, and 1% PS. 2.5% FBS MEM (2.5FB-MEM) was made by combining 1 part 10FB-MEM with 3 parts SF-MEM. 10FB-MEM was used for flask cultures and both 10 and 2.5FB-MEM were used for Transwell cultures. Experimental MEM (E-MEM) consisted of PRF-MEM with 1 % BSA, 1% LG, and 1% PS, and was used when calculating L_p. We used the lower serum concentration of 2.5% to suppress heterotypic interactions between cell types as observed in a previous study [38]. We used 10% serum in most of our previous studies of endothelial L_p [5,7,13].

Cell Culture in Flasks

A primary BAEC culture was expanded up to passage 2 subcultures in T-75 tissue culture flasks and then cryopreserved in 1 mL cryovials at a concentration of 1.0e6 cells/mL. A primary BASMC culture was expanded to passage 3 subcultures in T-75 tissue culture flasks and then cryopreserved in 1 mL cryovials at a concentration of 7.5e5 cells/mL.

Cryopreserved vials of either passage 2 primary BAEC cultures or passage 3 primary BASMC cultures were thawed for 2 minutes in a 37°C waterbath and the cell suspensions were transferred to separate sterile T-75 tissue culture flasks with 14 mL of 10FB-MEM and incubated for 30 minutes. That media was then aspirated and replaced with 15 mL of 10FB-MEM and the cultures were incubated until grown to be 80% confluent. Each culture was then passed with cell suspensions being split equally into three new sterile tissue culture flasks and grown in 10FB-MEM until 80% confluent. BAEC and BASMC cultures were each passed three times and finally passage 5 BAECs and passage 6 BASMCs were inoculated on Transwell inserts or companion wells in specific monoculture and coculture arrangements and supplied with 10FB-MEM or 2.5FB-MEM.

Cell Culture with Transwell Permeable Supports

Transwell insert membranes—Transwell inserts containing a 10 μm thick polyester (PET) membrane with a total growth area of 1.12 cm^2 were used. While the PET membrane is almost 10X thicker than a normal internal elastic lamina[1], the 0.4 μm diameter membrane pores and membrane pore density of 4.0e6 pores/cm^2 fall within the normal ranges found in

internal elastic lamina[41]. Prior to seeding cells on either the apical or basal side of the membrane, 224 μL of FN diluted to 30 μg/mL with MEM was pipetted on the appropriate region. FN-coated Transwell inserts in companion plates were incubated for 2 hours and then excess FN was removed.

Cell culture media for Transwell Permeable Supports—To culture cells with 10FB-MEM, 1.5 mL and 0.5 mL of that media were placed in the companion well and Transwell insert, respectively. To culture cells with 2.5FB-MEM, 1.5 mL of SF-MEM was placed in the companion well and 0.5 mL of 10FB-MEM was placed in the Transwell insert.

BAEC and BASMC plating densities, locations, and culture times—BAEC and BASMC cultures were inoculated with a 1:1 plating density ratio of 1.25e5 cells/cm^2. BAECs in monoculture and coculture were always the most apical culture on the Transwell membrane. BASMCs in monoculture or coculture were either inoculated on the basal side of the Transwell membrane or on the bottom surface of the companion well. The total BAEC culture time in all monoculture and coculture arrangements was 5 days, and the total BASMC culture time in all culture arrangements was 2 days.

Monoculture and coculture notations—The apical and basal locations of the Transwell membrane will be denoted as (a) and (b), respectively, and the bottom surface of the companion well is denoted as (c). BAEC and BASMC culture inoculums are abbreviated as (EC) and (SMC), respectively. Monoculture formats are described by [Location (Inoculum)]. Coculture formats are described by [Location (First Inoculum); Location (Second Inoculum)]. These monoculture and coculture notations are presented in Figure 2.

EC and SMC monocultures and cocultures

[a(EC)] monocultures—An EC culture was inoculated on the apical side of a Transwell membrane that was pre-coated with FN. The culture was supplied with either 10FB-MEM or 2.5FB-MEM and incubated for 5 days.

[b(SMC)] monocultures—An SMC culture was inoculated on the basal side of an FN-coated membrane while the insert was inverted. The inverted insert was incubated for 30 minutes while SMCs settled and attached to the membrane. The insert with basal SMC culture was then placed upright, supplied with either 10FB-MEM or 2.5FB-MEM, and incubated for 2 days.

EC-SMC cocultures

i) [a(EC);b(SMC)] membrane coculture: An EC culture was inoculated on the apical side of an FN-coated membrane, supplied with either 10FB-MEM or 2.5FB-MEM, and incubated for 3 days. The insert was then inverted and an SMC culture was inoculated on the basal side of the membrane, which was pre-coated with FN. The inverted insert was incubated for 30 minutes to allow for SMC settling and attachment to the membrane. The membrane coculture was then placed upright, supplied with either 10FB-MEM or 2.5FB-MEM, respectively, and incubated for 2 days.

ii) [a(EC);c(SMC)] shared media coculture: An EC culture was inoculated on the apical side of an FN-coated membrane, supplied with either 10FB-MEM or 2.5FB-MEM, and incubated for 3 days. An SMC culture was then inoculated in a separate companion well and allowed to settle and attach to this surface during a 30 minute incubation period. The insert with the EC culture was then paired with the companion well containing the SMC culture. The coculture was supplied with either 10FB-MEM or 2.5FB-MEM, respectively, and incubated for 2 days. These cocultures were dissociated on day 5 to measure endothelial L_p.

Calculating L_p of Transwell cultures: Fluid flux (J_v) across Transwell insert cultures was measured in pairs with a bubble tracking apparatus and those measurements were then used to calculate L_p. Both the bubble tracking apparatus and the equations for calculating L_p have been previously described[10] and are briefly explained here. Following the prescribed monoculture and coculture times, the Transwell insert was transferred to the bubble tracking apparatus. E-MEM was added above and below the Transwell insert to eliminate the development of an osmotic pressure gradient. The L_p was calculated by Equation 1 with $\Delta P = 10$ cmH_2O. Each measurement was repeated 6 times.

$$L_p = J_v / \Delta P \quad \text{(Eqn. 1)}$$

Monoculture resistances-in-series models of coculture L_p: Hydraulic conductivity measurements for EC and SMC monocultures were combined to provide a resistances-in-series model of the coculture systems that was compared with coculture L_p measurements. Deviations between model and experimental values of L_p could be attributed to heterotypic interactions. Monoculture L_p values were first inverted to represent hydraulic resistances (Equations 2 and 3),which were then summed up in series (Equation 4). Note that since the PET membrane was included as a substrate in each monoculture, its hydraulic resistance ($R_{[PET]}$) was duplicated during series summation. Therefore, a value for the hydraulic resistance of one porous membrane, $R_{[PET]} = 1.196e4$ $cmH_2O/cm/s$ ($L_{p[PET]} = 8.361e\text{-}5$ $cm/s/cmH_2O$), was subtracted from the combination of monoculture resistances in order to account for the single PET membrane present in the actual coculture.

$$R_{[a(EC)]} = 1 / L_{p[a(EC)]} \quad \text{(Eqn. 2)}$$

$$R_{[b(SMC)]} = 1 / L_{p[b(SMC)]} \quad \text{(Eqn. 3)}$$

$$L_{p[a(EC)]+[b(SMC)]-[PET]} = 1 / \left(R_{[a(EC)]} + R_{[b(SMC)]} - 1R_{[PET]} \right) \quad \text{(Eqn. 4)}$$

Immunofluorescence

Immunofluorescence solutions—Paraformaldehyde fixative was diluted with CMF-PBS to 1% PFA and filtered through a 0.45 μm syringe filter on the day of use. Triton X-100 was diluted with CMF-PBS to a 0.2% Triton X-100 permeabilizing solution. Blocking buffer consisted of BSA and Triton X-100 diluted in CMF-PBS to 10% BSA and 0.1% Triton X-100. Primary antibody (PAb) was diluted 15:1000 in blocking buffer. Secondary antibody (SAb) was diluted 2:1000 in blocking buffer.

Immunofluorescence of VE-cadherin in Transwell cultures—CMF-PBS was immediately removed after being added for each rinse. All solutions were added to the insert first and then, when required, to the companion well. Vacuum aspirations were performed without touching the membrane. The volume of CMF-PBS for each rinse was 0.5 mL/insert and 1 mL/companion well. All steps were carried out at room temperature (RT) and in a laminar flow hood.

First, remaining cell culture media was aspirated. The culture was quickly rinsed once and, immediately, 0.5 mL of fixative was added to the apical side of the insert. Fixative was removed after 10 minutes; the culture was rinsed once and 0.5 mL of permeabilizing solution was added to the apical side of the insert and left for 10 minutes. The culture was

rinsed once and 0.5 mL of blocking buffer was added to the apical side of the insert and left for 60 minutes. After one rinse, 200 μL of diluted PAb was added the apical side of the insert and incubated at RT for 3 hours. The PAb was removed and the culture was rinsed five times.

Then, in a dark environment, 200 μL of diluted SAb was added to the apical side of the insert and incubated at RT for 60 minutes, followed by removal of SAb and four rinses. 0.5 mL of rinse solution was added to the apical side of the insert that was transferred to a clean glass slide set on a Nikon TE 2000 microscope stage equipped with epi-fluorescence and MetaVue Imaging Software (Universal Imaging Corp. PA). The culture was imaged in the center field and then in four peripheral fields with a 10x objective. Five more similar fields were imaged with a 20x objective.

Cell Tracker Green Staining and Confocal Imaging—Co-cultured BAECs and SMCs were briefly washed with PRF serum-free medium. The Transwell inserts were then inverted and the SMCs were incubated in PRF serum free media containing 5μM of Celltracker green for 15 min. The excess media was first aspirated , before incubating the BAECs for 15 minutes with cell tracker green. The co-cultured cells were quickly washed with PBS before incubation in growth media containing 10 % FBS, 1% penicillin and streptomycin for 30 min at 37°C. After incubation the inserts were flushed for 15 minutes (3x, 5 min each) by adding 1ml and .5ml of PBS to the apical and basal side of the inserts respectively, to remove any free dye from the filter pores. They were then fixed in 3.7% fixative for 10 minutes. The cultures were washed twice, and mounted on glass coverslips for imaging. Positive controls were prepared using blank filters incubated with Celltracker green and fixed as indicated above. Confocal z-stacks of the cell tracker green stained co-cultures were obtained on a LSM 510 or LSM 710 confocal laser scanning system, using the Plan-Neofluar 40×/1.3 Oil DIC objective and analyzed using the Zeiss LSM software.

Morphometric Analysis

EC shape factors—The shape factors of ECs in VE-cadherin immunostained monocultures and cocultures were calculated using the MetaVue software. Briefly, a trace region tool was used to outline individual ECs. MetaVue's Region Statistics of the outline generated calibrated pixel areas and perimeters for each cell. The shape factors for ECs were then calculated with the formula $(4\pi * area)/(perimeter)^2$, where values ranged between zero and one; a value of one is a perfect circle and a value near zero is a flattened or elongated object.

Statistical analysis—Hydraulic conductivity measurements and shape factors are presented as the mean +/− the standard error of the mean (SEM). Each coculture experiment was normalized against its paired EC monoculture experiment to account for the variations in Lp from plate to plate of the cultures. When comaparing normalized coculture experiments to controls (EC monocultures) a one sample t-test on the null hypothesis was performed , i.e. we tested whether the average normalized values were different from 1. This approach has been used in other studies [37,42]. A two sample t-test was performed when comparisons were made between different coculture formats . Comparisons were considered statistically significant if $p < 0.05$. Where multiple comparisons were made the bonferroni correction was used.

Results

EC and SMC monoculture L_p

Note that all of the Lp values reported for monocultures and cocultures include the PET membrane. The L_p measurements of [a(EC)] monocultures grown in 10FB-MEM were paired with L_p measurements of [a(EC)] monocultures grown in 2.5FB-MEM and are presented in Figure 3A. [a(EC)] monocultures grown in 2.5FB-MEM had a significantly lower L_p than monocultures grown in 10FB-MEM. The L_p measurements of [b(SMC)] monocultures grown in10FB-MEM were paired with the L_p measurements of [b(SMC)] monocultures grown in 2.5FB-MEM and are presented in (Figure 3B), respectively. These measurements show that [b(SMC)] monoculture L_p was significantly lower when grown in 2.5FB-MEM. Note that [b(SMC)] L_p was more than 10 fold higher than [a(EC)] L_p.

EC-SMC coculture L_p

The L_p measurements of each coculture grown in either 10FB-MEM or 2.5FB-MEM were paired with the corresponding L_p measurements of [a(EC)] monocultures. The L_p of each coculture was normalized to the paired L_p of the monoculture. The normalized mean L_p values of cocultures grown in 10FB-MEM or 2.5FB-MEM are presented in (Figure 4). For cocultures grown in 10FB-MEM, L_p of [a(EC);b(SMC)] was significantly lower than either [a(EC)] L_p and [a(EC);c(SMC)] L_p. In addition, L_p of [a(EC);b(SMC)] grown in 10FB-MEM was significantly lower than the same coculture format grown in 2.5FB-MEM. Both coculture formats grown in 2.5FB-MEM had L_p values that were significantly lower than [a(EC)] L_p, but not significantly different from each other.

Monoculture resistances-in-series models of coculture L_p

Resistances-in-series models of coculture L_p (Equation 4) representative of each membrane coculture configuration were normalized to paired endothelial monoculture L_p. The resistances-in-series L_p values were plotted next to the L_p measurements of the cocultures for both10FB-MEM and 2.5 FB-MEM (Figure 5) in order to distinguish heterotypic interactions in the cocultures. Resistances-in-series L_p was significantly higher than the [a(EC);b(SMC)] coculture L_p for 10FB-MEM, but resistances-in-series L_p was not significantly different from coculture L_p for 2.5FB-MEM.

Immunostaining of VE-cadherin

Endothelial cell VE-cadherin was immunostained for each coculture and EC monoculture. Center field 10x objective images for each coculture and EC monoculture grown in 10FB-MEM or 2.5FB-MEM are presented in Figure 6. VE-cadherin was highly expressed in each culture format and was localized at the cell border as expected. Endothelial cells imaged in coculture [a(EC);b(SMC)] appeared more elongated when compared to any of the other cultures.

Analysis of EC and SMC processes across PET membrane

ECs and SMCs were stained with cell tracker green for monocultures and EC/SMC cocultures grown in 10FB-MEM. Cell tracker green staining of 10FB-MEM BAEC and BASMC monocultures (Figure 7) and cocultures (Figure 8), showed no evidence of processes extending across the membrane from ECs or SMCs in any of the formats. See Figure 9 for the positive control.

EC morphometry

The sample mean, standard deviation (SD), and SEM for random samples of 10 EC shape factors from each culture format are presented in Table 1. The mean shape factor for ECs in

the [a(EC);b(SMC)] coculture was much closer to zero, compared to any other culture, indicating that these ECs were the most elongated. In addition, the EC shape factors in that coculture were significantly lower in comparison to shape factors for ECs in the 10FB-MEM [a(EC)] monoculture.

Discussion

Serum concentration modulates EC L_p

The influence of serum concentration on EC Lp response was determined by first growing EC monocultures in either 2.5FB-MEM or 10FB-MEM for 5 days and then measuring Lp. Hydraulic conductivity of [a(EC)] in 2.5FB-MEM was significantly lower than the Lp of [a(EC)] in 10FB-MEM (Figure 3A) by a factor of 4. It is known that serum (protein) – free media increases permeability/hydraulic conductivity [36], and that some protein/serum is needed to maintain barrier function [2,27,30]. However, above a certain minimum level of serum, low serum levels result in decreased EC motility and turnover that are associated with lower permeability/hydraulic conductivity, [6,16]. It has also been shown in other studies that factors in serum weaken the intercellular junctions of endothelial and epithelial cells [10,32] and these factors may also play a part in the increased Lp at higher serum concentration.

Hydraulic conductivity of [b(SMC)] grown in 2.5FB-MEM was found to be significantly lower than [b(SMC)] grown in 10FB-MEM (Figure 3B), but still 1-2 orders of magnitude higher than L_p of [a(EC)]. To our knowledge, this is the first study to describe the serum effect on L_p.

Arterial cocultures exhibit a range of L_p

In healthy arteries, the intimal and medial regions, which consist of an endothelial monolayer and dense multilayered SMCs, respectively, share the same porous internal elastic lamina[20]. Our coculture [a(EC);b(SMC)] best mimics this organization. The total culture time of ECs in monocultures and cocultures was 5 days, and the total culture time of SMCs in monocultures and cocultures was 2 days. Using the 10 μm thick porous membrane to separate ECs and SMCs in the [a(EC);b(SMC)] coculture (Figure 2) brings both cell types into close proximity, compared to the [a(EC);c(SMC)] coculture, which distances ECs from SMCs by a 1 mm layer of media (Figure 2) ensuring only soluble component interaction. Pairwise comparisons of coculture L_p normalized to paired [a(EC)] L_p (Figure 4, *left and right*) reveal that the [a(EC);b(SMC)] L_p was significantly lower than [a(EC)] L_p for both the 10FB-MEM and 2.5FB-MEM cases. The L_p value of 5.507e-8 +/− 4.080e-10 cm/s/ cmH_2O for the 2.5FB-MEM [a(EC);b(SMC)] coculture (Figure 4, *right*), which included the porous membrane, is the lowest value reported in the literature for an *in-vitro* model. This value is also the same order of magnitude as L_p in intact arteries[19,38,46].

A lower [a(EC);b(SMC)] L_p, compared to [a(EC)] L_p, suggests that directing vascular SMCs to form a medial region in the presence of an existing endothelial monolayer, as in arteriogenesis, is one of the localized approaches to improving arterial wall barrier function. The significant differences in arterial coculture L_p, compared to EC monocultures, also suggests their potential application in generating unique tissue-engineered blood vessels that have physiological transport barrier properties.

Coculturing in shared media influences EC L_p

Coculture configuration [a(EC);c(SMC)] differs from *in-vivo* vascular wall anatomy and restricts heterotypic interactions to those mediated by soluble components. In 2.5FB-MEM, ECs that were cultured in shared media had a significantly reduced L_p compared to EC

monocultures (Figure 4, *right*). That trend was preserved in 10FB-MEM shared media cultures although statistical significance was not achieved (Figure 4, *left*). Also, the L_p of ECs derived from shared media cocultures was significantly higher in comparison to the porous membrane interface coculture, [a(EC);b(SMC)], in 10FB-MEM conditions (Figure 4, *left*). However, this trend was not preserved in low serum concentrations (Figure 4, *right*) where there was no significant difference. The significant changes in endothelial L_p resulting from shared media coculturing methods indicate that heterotypic soluble interactions and serum concentration regulate L_p.

Comparison of coculture to monoculture resistances-in-series modeled L_p

Resistances-in-series modeled L_p values were derived from series combinations of monoculture hydraulic resistances, and were nearly identical to the values of [a(EC)] L_p for ECs alone for both serum levels (Figure 5). These models emphasize that in the absence of heterotypic interactions, the EC component provides the dominant hydraulic resistance to water flux, compared to the membrane and SMC components. In 10FB-MEM conditions, the significant reduction in L_p for coculture [a(EC);b(SMC)], compared to resistances-in-series model prediction, reveals the presence of heterotypic interactions (Figure 5, *left*). In 2.5FB-MEM, the coculture L_p was also lower than the resistances-in-series model prediction, but not quite significant ($p = 0.0553$) (Figure 5, *right*). Comparisons presented in Figure 5 clearly demonstrate that the degree of heterotypic regulation of L_p is dependent on the serum concentration in the growth media. This is consistent with earlier studies that showed enhanced heterotypic interactions in higher serum[25]. Reductions in Lp in co-culture, as we have observed, would be expected when a growth factor or cytokine is reduced in concentration since growth factors are generally associated with increases in permeability. Our observations are consistent with a previous study in which ECs were directly cultured on top of preexisting SMCs[23]and it was shown that the protein levels of the growth factor PDGF-BB were significantly lower when compared to control groups (EC and SMC monocultures). PDGF-BB is known to degrade tight junctions and impair the EC transport barrier[48].

Since the [a(EC);b(SMC)] coculture configuration most closely represents the arrangement between ECs and SMCs in an intact arterial wall, it is tempting to conclude that this heterotypic configuration enhances the transport barrier for normal blood vessels. The specific mechanism of the heterotypic interactions that lead to a reduction in L_p for the [a(EC);b(SMC)] coculture remains to be investigated. We have considered whether direct cellular contact between EC and SMC plays a role.

Processes involved in EC and SMC interactions

Cell tracker green staining of 10FB-MEM BAEC and BASMC monocultures (Figure 7) and cocultures (Figure 8), showed no evidence of processes within the pore structure from either ECs or SMCs. On the other hand, electron micrographs of small arteries have identified EC and SMC process invasions in pores of the intervening internal elastic lamina, which also extended to form intact endo-myothelial and myo-endothelial junctions[40]. Differences between our observations in culture and those in small arteries may be related to the much thinner internal elastic lamina (order 1 μm) compared to our PET membrane thickness (10 μm), and to differences in materials. The pore density and diameter of the PET membranes, however, are similar to those of arterial IEL [25,43]. However, in a bilayer coculture study[18], electron micrographs also revealed intact myo-endothelial junctions, formed by SMCs extending their cell processes through 0.4 μm diameter pores and across a 13 μm thick membrane to make intimate contact with ECs. That coculture closely resembled the heterotypic cell arrangement in our [a(EC);b(SMC)] coculture, the main difference being the time of co-culture – ours being 5 days compared to 7 or 14 days in that study.

Coculture arrangements regulate patterns in EC morphology—Contact-inhibited formation of EC monolayers is a characteristic homotypic function. A 10x microscope objective was used to image wide fields of view and we observed uniformly intact EC monolayers in every culture configuration (Figure 6). Figure 6, and Table 1 show that the shape of ECs in the shared media coculture, [a(EC);c(SMC)], is similar to that in the [a(EC)] monoculture. However, the ECs in the porous membrane interface coculture, [a(EC);b(SMC)], in 10FB-MEM and 2.5FB-MEM conditions, were unique because they displayed a more elongated morphology in comparison to all other cell culture arrangements (Figure 6). Endothelial cell shape factor comparisons shown in Table 1, confirmed that the ECs in [a(EC);b(SMC)] conditions were in fact significantly elongated. The differences in shape factor between [a(EC)] and [a(EC);b(SMC)] may be due to differences in some growth factor that is altered in co-culture such as the increased VEGF observed in a previous study[23] .

An increase in the intercellular junction perimeter per unit of surface area that is apparent in Figure 6 for the elongated pattern of ECs in [a(EC);b(SMC)] arterial cocultures suggests a greater junctional area for water transport that would lead to increased J_v if junction integrity or other factors were not altered. Yet the [a(EC);b(SMC)] coculture displayed a significantly lower L_p than the [a(EC)] monoculture (Figure 4). Therefore, within the [a(EC);b(SMC)] coculture, heterotypic interactions induce enhanced intercellular junction integrity.

Various coculturing methods have been developed to capture more *in-vivo*-like tissue features, in comparison to monoculture cases. For example, elongated EC shapes were noticed when they were cultured directly above a matrix of collagen type I gel that was embedded with SMCs[49]. Previous studies[23] have also shown that coculturing techniques modulate a variety of protein secretions from both ECs and SMCs. It was demonstrated that directly coculturing ECs on top of preexisting SMCs led to different VEGF, PDGF-BB, TGF-β and bFGF secretions from both ECs and SMCs[23]. The proximity and inoculation order of SMCs and ECs in that study[23] differed from our [a(EC);b(SMC)] coculture (Figure 2) and did not include basal culture media and a porous membrane allowing for water flux.

Coculturing methods in another study[39], which incorporated an intervening porous membrane between ECs and SMCs, induced changes in endothelial secretions of IL-1 and MCP-1. Smooth muscle cells were also characterized as being either ‘more’ or ‘less secretory’ as a result of changes in serum content in culture media[39]. The distance between ECs and SMCs in that coculture was most similar to what is found in coculture format [a(EC);b(SMC)] (Figure 2), however, both the coculture inoculation order and the culture time varied from our present study, and a transmural water flux was not present.

While the co-culture models of this study simulate several aspects of a an artery or an artery construct, there are several physiological features that were not investigated. We used a static model without flow over the EC surface as used in most culture studies of the EC transport barrier. There have been in vitro transport studies using ECs exposed to arterial levels of fluid shear stress that show a permeability or L_p response to shear stress[44]. Additional studies will be required to determine shear effects on co-culture transport properties. As described above, the porous polymer membrane, while capturing the pore size and the pore area fraction of the IEL, does not match the thickness or compliance or chemical composition of the IEL. We did, however, simulate arterial transvascular flow as required for measurements of L_p, and the J_v values utilized (order 10^{-6} cm/s) are in the range of physiological transvascular [43] flows.

Concluding remarks—Serum level in cultures had a significant influence on L_p. Lowering the serum concentration by four fold resulted in a proportionate reduction in

[a(EC)] L_p, which was the largest serum-dependent shift in L_p that we observed. Also, [a(EC);b(SMC)] coculture, which mimics the EC-SMC arrangement in a healthy arterial wall, led to a significantly lower L_p than ECs alone. Since soluble factors produced in [a(EC);c(SMC)] did not reduce L_p to the same level as [a(EC);b(SMC)], and we did not see any evidence of extended processes in the pores that might have increased the resistance to J_v or provided direct contact between ECs and SMCs, it seems likely that increased transport rates and concentrations of soluble mediators induced by reduced transport distances (10 μm for [a(EC);b(SMC)] compared to 1 cm for [a(EC);c(SMC)]) are key factors distinguishing the two cases.

Overall, we have demonstrated that L_p magnitude is a direct result of EC and SMC coculture arrangements. In particular, we have produced an arterial coculture system, [a(EC);b(SMC)], that includes an intervening porous membrane and mimics the arrangement of ECs and SMCs in a healthy arterial wall. This coculture produced a pattern of significantly elongated ECs, and the lowest *in-vitro* L_p values yet reported in the literature.

Acknowledgments

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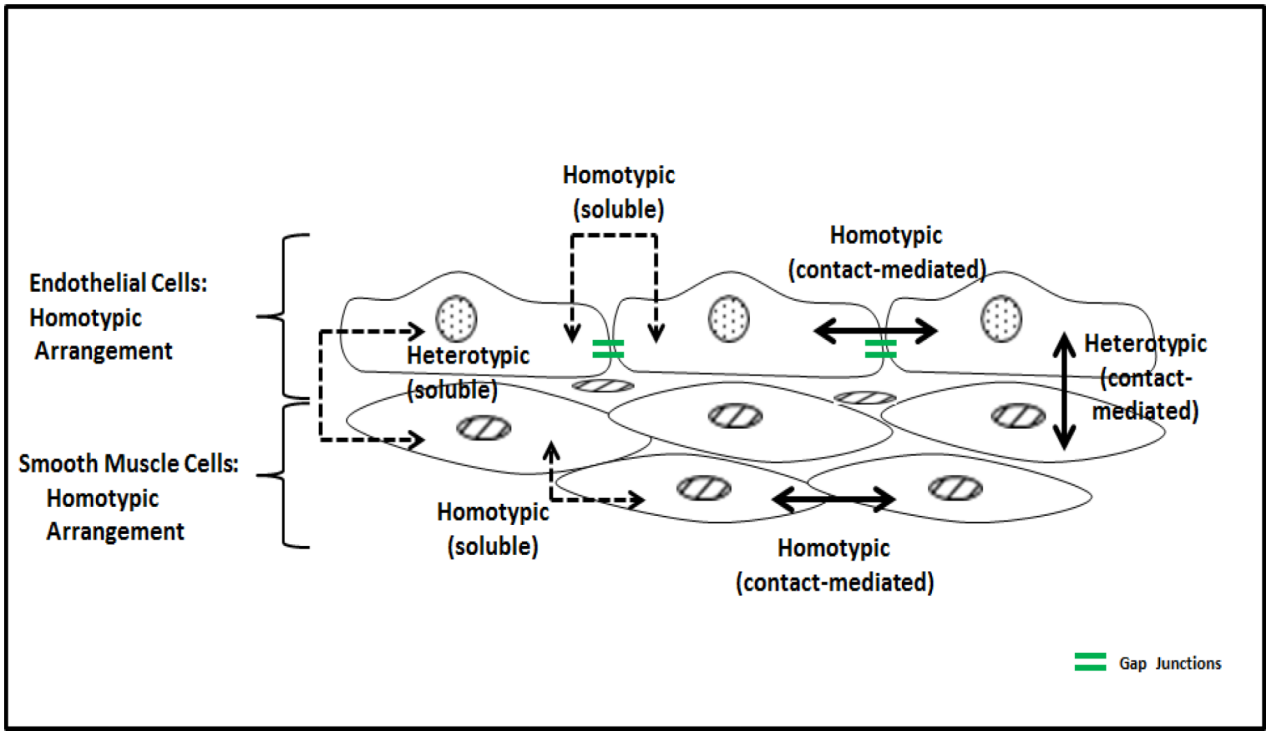

Figure 1.
Homotypic and heterotypic arrangements of BAECs and BASMCs showing combinations of soluble (dashed arrows) and contact-mediated (continuous arrows) signal pathways for cell interactions. Double-sided arrows indicate that cell signaling is also bidirectional.

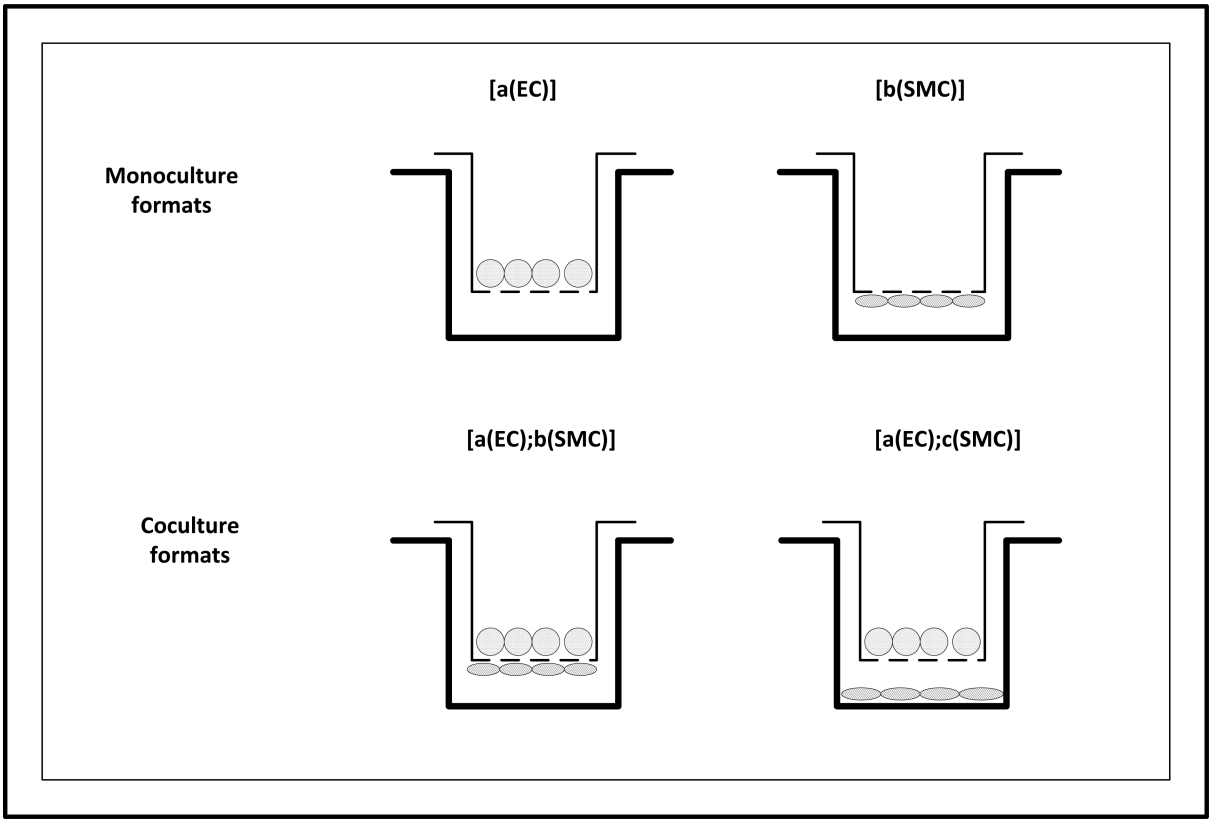

Figure 2.
Schematic of BAEC and BASMC monoculture and coculture formats on Transwell membranes and companion wells. The horizontal dashed line indicates the location of the porous Transwell membrane. The black band indicates the location of BAEC cultures. The grey band indicates the location of BASMC cultures. Monoculture formats include [a(EC)], and [b(SMC)]. Coculture formats include [a(EC);b(SMC)], and [a(EC);c(SMC)].

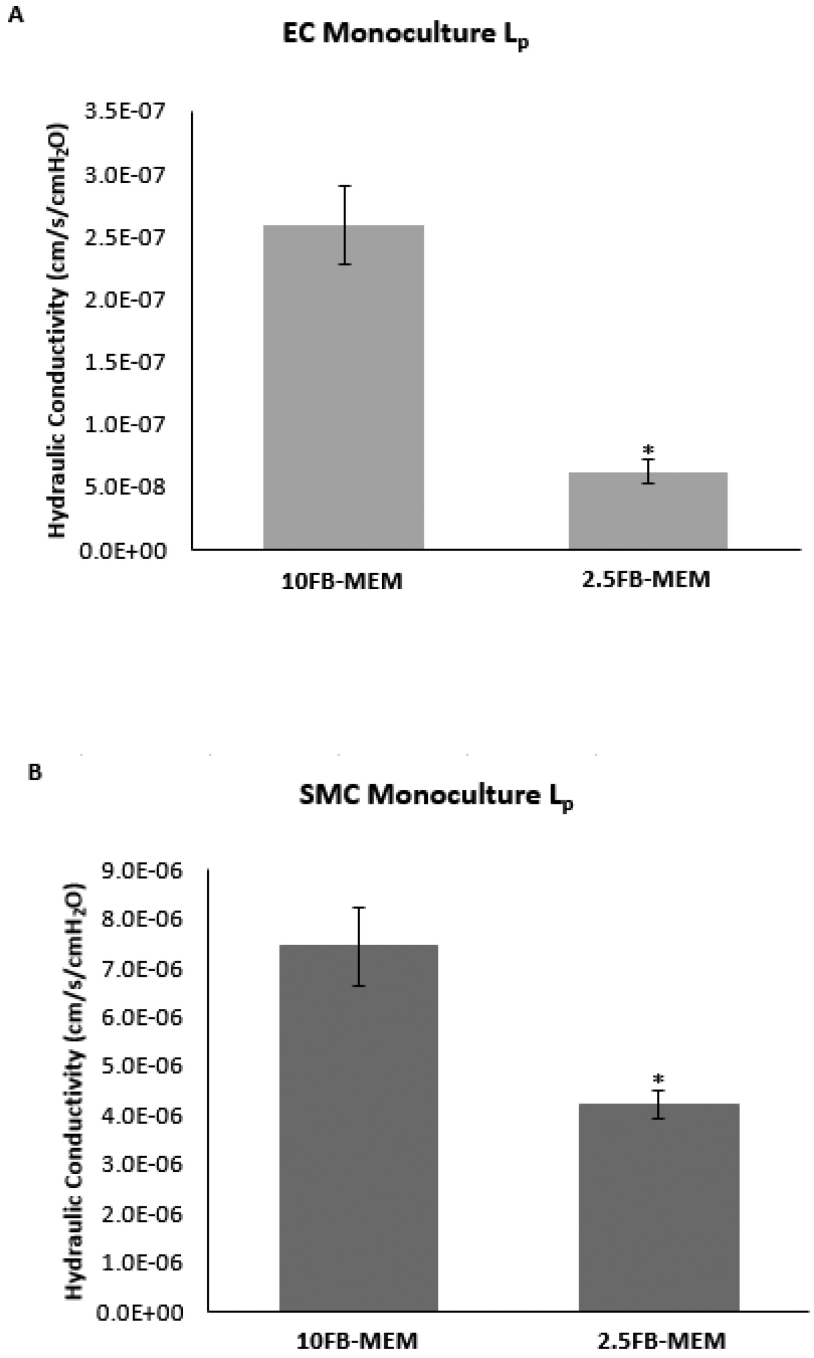

Figure 3.

Hydraulic conductivity of 5 day apical EC ([a(EC)]) (A) and 2 day basal SMC ([b(SMC)]) (B). Monocultures were supplied with 10FB-MEM or 2.5FB-MEM. Data are presented as mean +/− SEM. * - $p < 0.05$ compared to 10FB-MEM; n = 6. (B): Data are presented as mean +/− SEM. * - $p < 0.05$ compared to 10FB-MEM; n = 6.

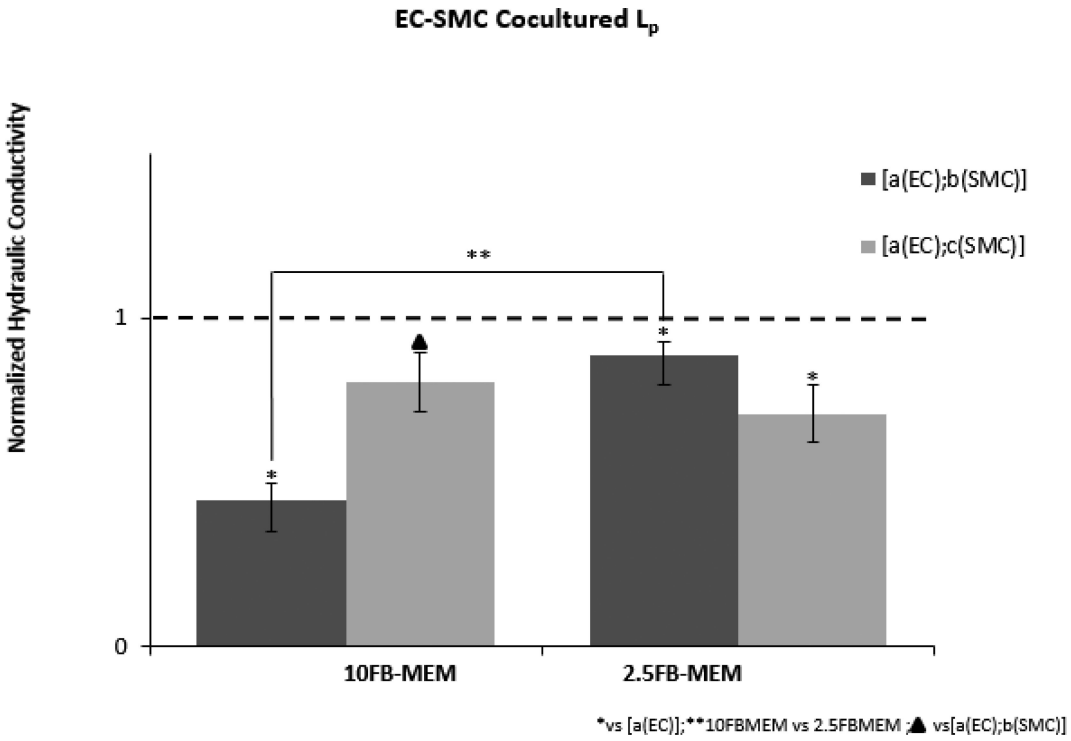

Figure 4.

Hydraulic conductivity of each coculture [a(EC);b(SMC)] and [a(EC);c(SMC)] normalized to the mean L_p of paired endothelial monocultures [a(EC)]. Each culture format was supplied with 2.5FB-MEM and 10FM-MEM . Statistical significance ($p < 0.05$) of coculture L_p normalized to paired endothelial L_p was denoted (* *vs* [a(EC)]). (**10FB-MEM vs 2.5FB-MEM[a(EC);b(SMC)]). (▲ *vs* [a(EC);b(SMC)]). All experiments were n = 6.

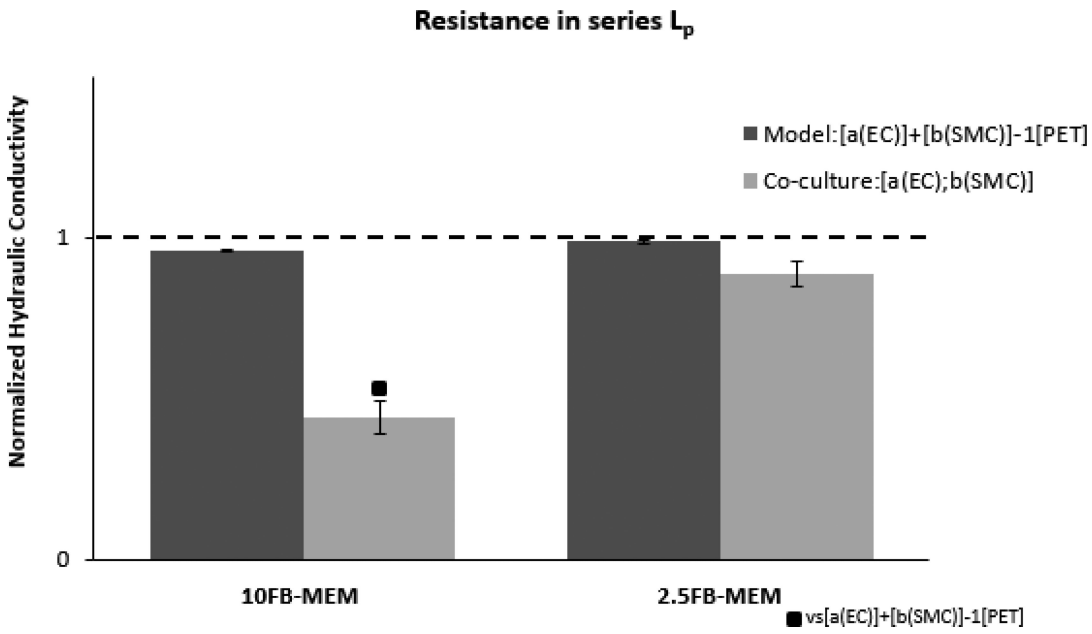

Figure 5.

Hydraulic conductivity of membrane coculture [a(EC);b(SMC)], and resistances-in-series model predictions [a(EC)] + [b(SMC)] normalized to the mean L_p of paired endothelial monocultures [a(EC)]. Each culture was supplied with 10FB-MEM or 2.5FB-MEM. Statistical significance ($p < 0.05$) of membrane coculture L_p compared to resistances-in-series L_p was denoted by (■ *vs* [a(EC)] + [b(SMC)]). All experiments were n = 6.

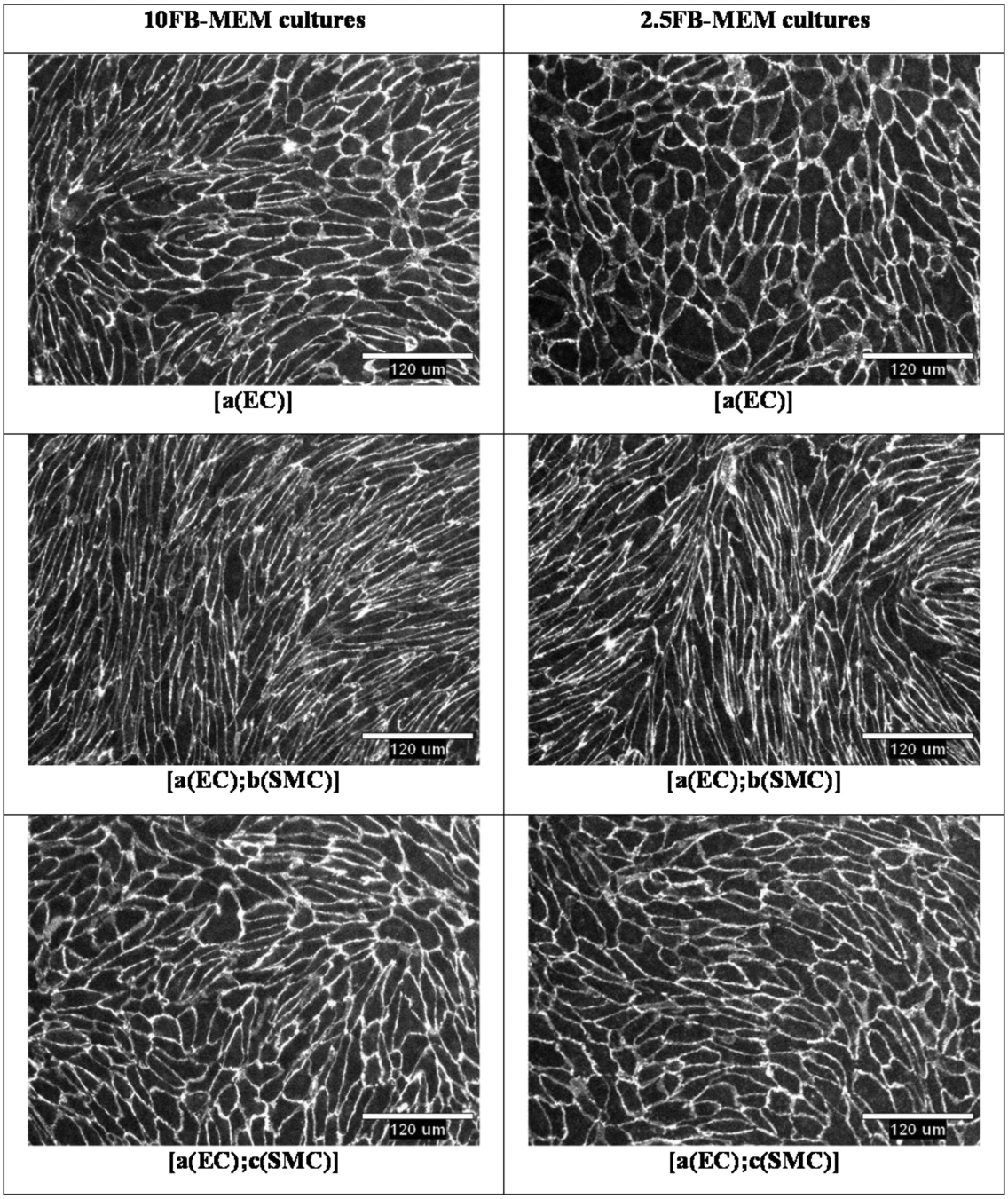

Figure 6.
Representative images of VE-cadherin immunostaining in EC monocultures and cocultures supplied with either 10FB-MEM or 2.5FB-MEM. 10x objective. Scale bar 120 um.

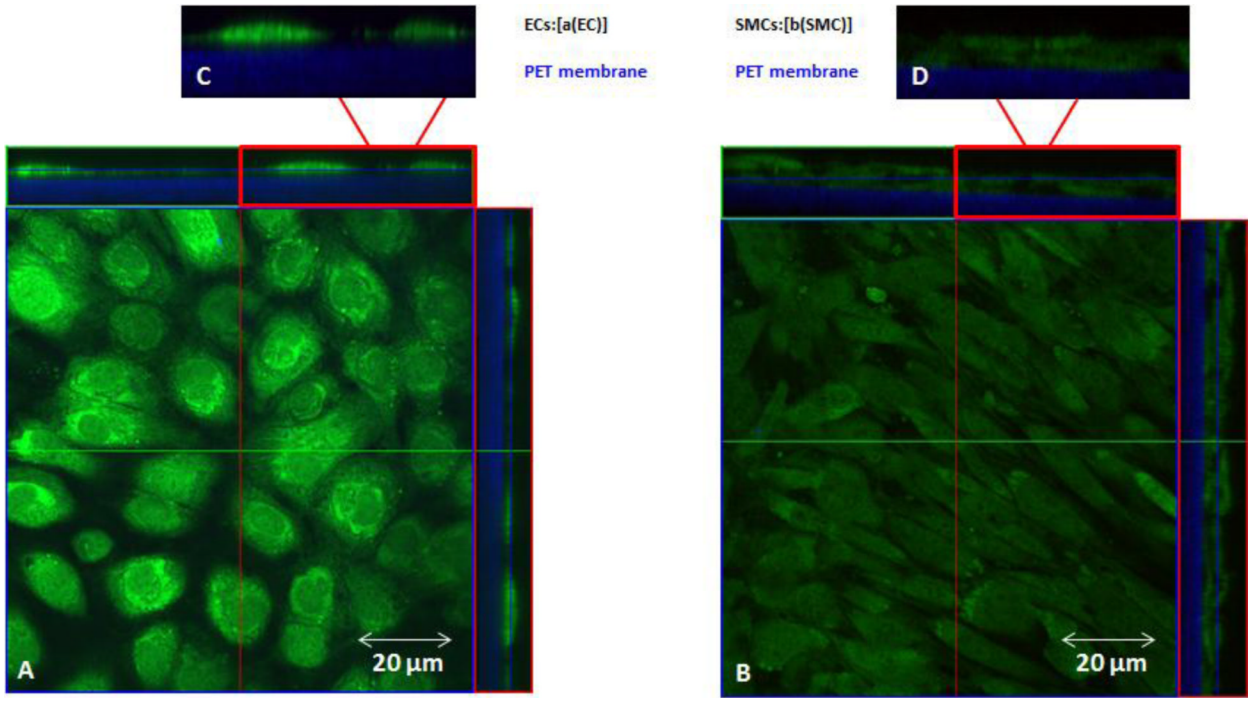

Figure 7.

Confocal images of cell tracker green localization in BAEC and BASMC monocultures grown in 10FB-MEM on PET membrane. The central portion of each panel is an en face view of the BAEC A, (left panel) and BASMCs ,B (right panel), shown from the Z-axis ,C-D (*top*,*left* and *right*) are cross sectional views in the Z-plane of each panel, and the PET membrane (blue). Note that there is no Celltracker green in the membrane region.

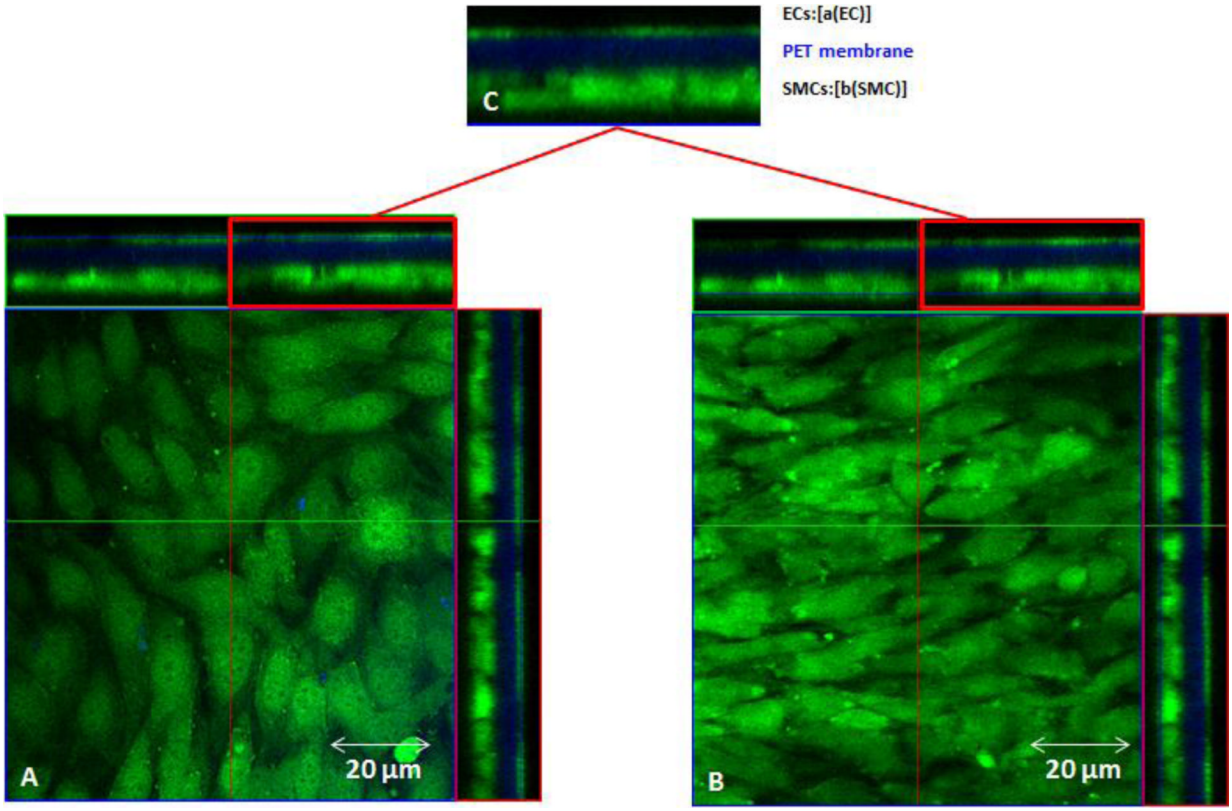

Figure 8.

Confocal images of cell tracker green localization in BAEC and BASMC cocultures (green) grown in 10FB-MEM on PET membrane (blue). The central portion of each panel is an en face view of the BAEC s , A (left panel) and BASMCs ,B (right panel), shown from the Z-axis, C(*top*)is a cross sectional view in the Z-plane of each panel; BAECs on the apical side and BASMCs on the basal side (green) of the PET membrane (blue). Note that there is no Celltracker green in the membrane region.

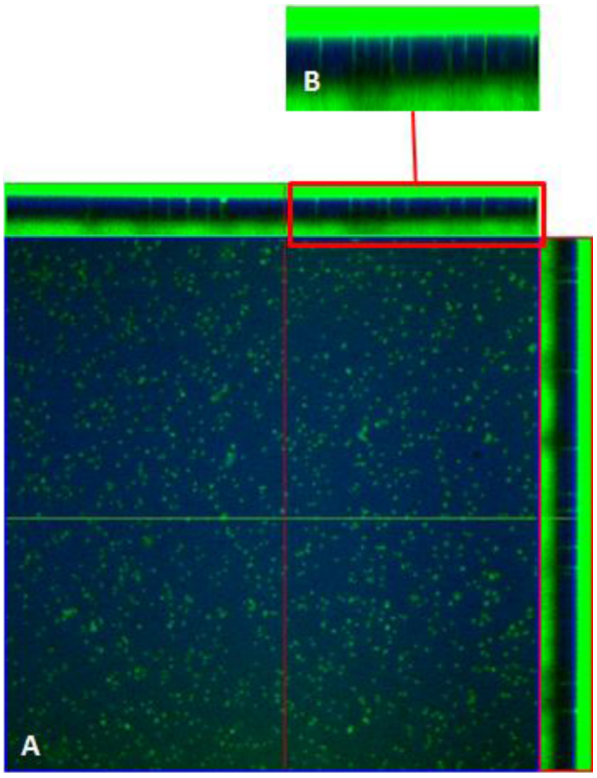

Figure 9.
Celltracker green was incubated on blank PET membranes as a positive control. Confocal images of cell tracker green localization on blank PET membrane (blue). The central portion of the panel is an en face view of the PET filter ,A, shown from the Z-axis, B(*top*)is a cross sectional view in the Z-plane of each panel; Celltracker green on the apical and basal side (green) of the PET membrane (blue). Note that pores are filled with Celltracker green.

Table 1

Sample means, SDs, and SEMs for EC shape factors (n = 10) for each culture format.

	Culture Format					
	[a(EC)]		[a(EC);b(SMC)]		[a(EC);c(SMC)]	
Media(MEM)	10FB	2.5FB	10FB	2.5FB	10FB	2.5FB
Sample mean (n=10)	0.5291	0.5865	0.2378*	0.2497*	0.5574	0.5450
Sample SD (+/−)	0.0847	0.1179	0.0547	0.0472	0.1333	0.1399
Sample SEM (+/−)	0.0268	0.0373	0.0173	0.0149	0.0422	0.0442

* $p < 0.05$ compared to [a(EC)] 10FB-MEM; n = 10.