Supplemental Materials

Methods:

1. Identification of primary cultured EC

Prior to use in experimental protocols the EC were identified using multiple approaches, including:

Immunofluorescence Assays for Identification of Cultured EC: Cells were seeded and grown on a Lab-Tek® II Chamber Slide™ (Nunc, Naperville, IL) to confluence. EC were fixed with 4% paraformaldehyde at room temperature for 10 min and then permeabilized with 0.1% Triton-X-100 for 5 min at room temperature. After blocking with 5% normal goat sera (Calbiochem, San Diego, CA) and 5% IgG-free BSA (Jackson Immunoresearch, West Grove, PA) for 1 h at room temperature, EC were incubated overnight with specific mouse monoclonal antibody anti-PECAM (1:50 dilution; Serotec Immunological Excellence, Raleigh, NC) and anti-von Willebrand factor monoclonal antibody (vWF, 1:50 dilution, Novocastra Laboratory Ltd, UK), respectively, at 4ºC, and then washed 3 times with PBS containing 0.05% (v/v) Tween 20 (PBST). EC were incubated with secondary antibody, goat anti-mouse IgG conjugated with Alexa Fluor®-568 (1:300 dilution, Molecular Probes, Eugene, OR), for 1 h at room temperature. EC were washed 3 times with PBST followed by nucleic acid staining with 0.1% DAPI (Pierce, Rockford, IL) incubation for 10 min at room temperature. The slide was mounted with MOWIOL® 4-88 (Calbiochem, San Diego, CA) and then covered with a cover glass. The images were taken by SPOT-2 CCD camera from Zeiss Axioplan microscope. The negative control was performed as described above with the omission of primary antibody reaction (shown in ***Supplemental Fig. 1A***). The images of immunostaining for

vWF and PECAM are shown in ***Supplemental Fig. 1B*** and ***1C***.

Uptake of Dil-Ac-LDL: EC grown on Lab-Tek® II Chamber Slide™ (Nunc, Naperville, IL) were incubated with Dil-Ac-LDL (1:100, Biomedical Technologies, Stoughton, MA) for 4 h in cell culture incubator in a humidified atmosphere of 5% CO_2 and 95% air at 37ºC. Again the images were taken by SPOT-2 CCD camera from Zeiss Axioplan microscope (shown in ***Supplemental Fig. 1D***).

Capillary-like Tube Formation: EC were seeded in 6-well plates coated with Matrigel™ (BD Biosciences, Franklin Lake, NJ) in accordance with manufacturer's instruction for EC functional characterization. The tube formation was observed and images were taken at 4 or 8 h after EC were seeded in a Matrigel™ (BD Biosciences, Franklin Lake, NJ) coated plate by phase contrast microscopy (Olympus, CK40, NA=0.3) (shown in ***Supplemental Fig. 1E*** and ***1F***).

2. Determination of Cultured EC Monolayer Confluence

To study the molecular mechanisms regulating endothelial barrier integrity using *in vitro* model of EC monolayer, it is essential that the cultured EC are confluent and that the monolayer mimics the characteristics of *in vivo* endothelium of intact vessels. Confluence was assessed by lectin labeling of cell surface glycoporteins and by detecting the presence of zona occludens, ZO-1, a selected cell-cell tight junctional protein.

Before EC barrier function was evaluated, EC confluence was examined. Accordingly, EC were seeded at a density of 5×10^3 cells/cm^2 and grown on the 0.2% gelatin (Sigma, St. Louis, MO) coated Transwell® insert with porous polycarbonate filter of 0.4 μm in size (Costar®Transwell®, Corning, Acton, MA) of a 6-well plate to confluence for 5-6

days. EC monolayer confluence was determined by staining lectin with Wheat Germ Agglutinin (WGA)-Alexa 488 (Invitrogen, Carlsbad, CA) at 2µg/ml for 2 min followed by fixation with 4% paraformaldehyde for 5 min at room temperature. The slides were mounted with MOWIOL® 4-88 (Calbiochem, San Diego, CA) and covered with cover glass. Confocal images were then obtained (Olympus IX81, 40X water immersion lens, NA 0.8) as illustrated in ***Supplemental Fig 2A***. Cell-cell junction formation was assessed by immunofluorescence staining with polyclonal antibody against ZO-1 (Invitrogen, Carlsbad, CA), an intercellular junctional molecule, (***Supplemental Fig. 2B***). The concentrations of primary antibody against ZO-1 and secondary antibody of goat against rabbit IgG labeled with Alexa-488 (Invitrogen, Carlsbad, CA) were 1:400 and 1:300 dilution, respectively. Images were taken by confocal (Radiance 2000™ Confocal Microscopy System, Zeiss, Thornwood, NY) laser (Krypton-Argon)-scanning microscopy in the Cytology and Molecular Core of University of Missouri-Columbia.

3. Validation of the Methods for Assessment of in vitro EC Barrier Function

The criterion of optimal concentration of tracer, BSA-Alexa 488, used for assessment of EC permeability was that the fluorescence intensity of BSA-Alexa 488 crossing the EC barrier should be within the linear range of detectable fluorescence intensity of microplate reader (Molecular Devices, SpectraMax M2). Therefore, a range of BSA-Alexa 488 concentrations were tested and the fluorescence intensity of flux of conjugated albumin applied to the transwell insert was measured (***Supplemental Fig. 3A)***. On completion of these controls the amount of BSA-Alexa 488 selected for this permeability study was 2 mg/ml.

In addition, albumin flux was measured using Costar®Transwell® (Corning, Acton, MA) solely coated with 0.2% gelatin in the absence of cells to serve as the control for experiments measuring EC monolayer albumin flux. Albumin flux in the absence of cells was compared to the flux obtained in the presence of the confluent monolayer (***Supplemental Fig. 3B***). On the basis of these experiments we defined functional monolayer confluence on the inserts as having 8-fold or greater resistance to albumin flux than the gelatin coated filter alone (e.g, P_s of the filters was 8-fold or greater than the basal P_s of the monolayer on the gelatin coated filter).

Vehicle control experiments were performed with DMSO (0.002%, v/v) as PDE inhibitor, cilostazol, was dissolved in DMSO. We found that 0.002% DMSO was without influence on basal P_s, therefore all data were compared with their control P_s in starving medium (***Supplemental Fig. 3C***).

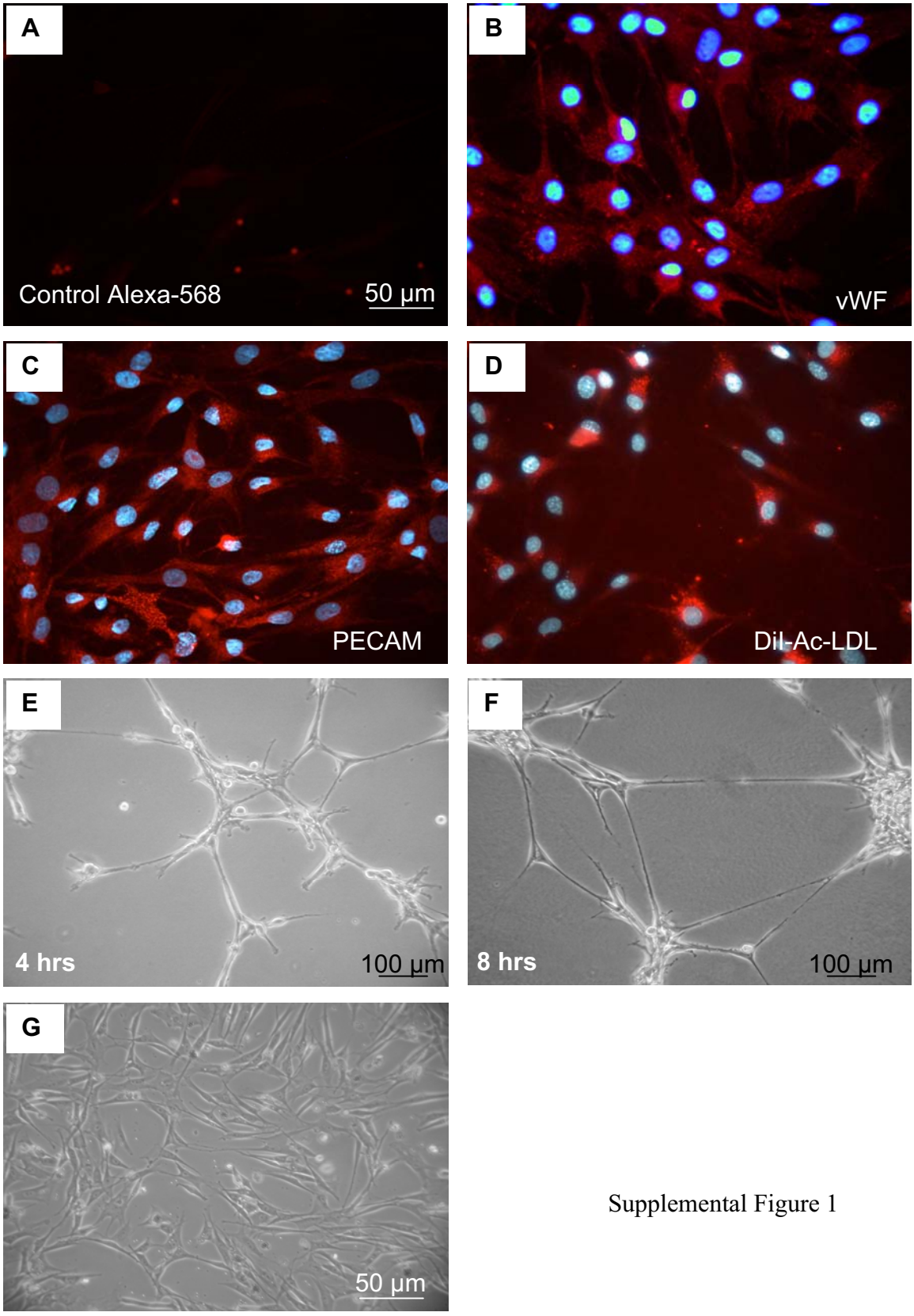
Supplemental Figure Legends

Supplemental Figure 1. Characterization of primary EC derived from abdominal skeletal muscle microvessels in rats. The negative control for immunofluoresence staining with omission of primary antibody reaction is given in panel 1A and immunofluorescence staining (red) with selected EC markers, Von-Willebrand Factor (vWF, panel 1B), PECAM (panel 1C), and acetylated LDL (panels 1D) are shown. The blue dots denote the nuclei of cells detected by DAPI nucleic acid stain. The exclusive function of EC, capillary-like tube formation, after seeding on Matrigel™ for 4 and 8 hours is displayed in panels 1E and 1F, respectively. Panel 1G is the representative image taken by phase contrast microscopy to demonstrate EC growing in the culture plate coated with 0.2% gelatin.

Supplemental Figure 2. EC monolayer confluence was demonstrated by binding EC with a lectin (Wheat Germ Agglutinin conjugated with Alexa Fluor 488) (A), and immunofluoresence staining with antibody against ZO-1, a protein located in the intercellular junction (B). The scale is 40 μm shown in the Fig. 2A and 2B.

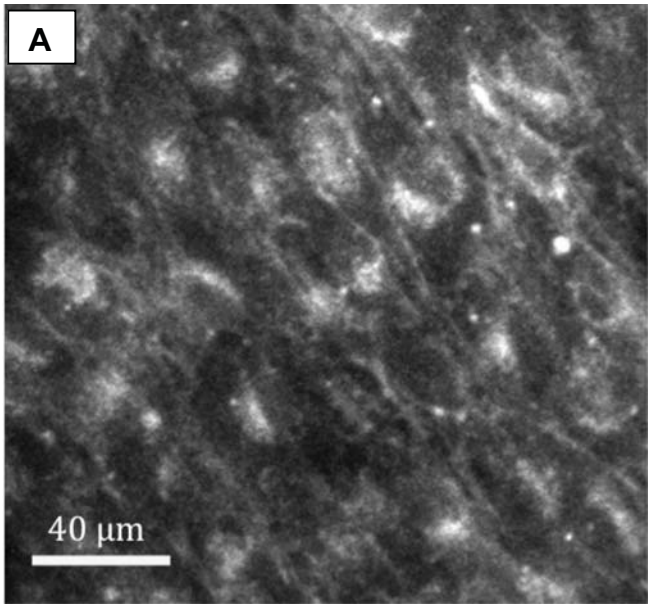
Supplemental Figure 3. Validation of the method for measuring EC monolayer P_s. To choose the optimal concentration of BSA-Alexa 488 for assessment of albumin flux through an EC monolayer, fluorescent intensity of BSA-Alexa 488 crossed the EC monolayer and the filter was measured as EC were exposed to different concentrations of BSA-Alexa 488 (A). Absolute P_s ($x10^{-6}$ cm/sec) measured in the absence of EC monolayer (filter plus gelatin) compared with that in the presence of EC monolayer seeded on the top of the gelatin-coated porous filter (B) resulting in a >8 fold change in

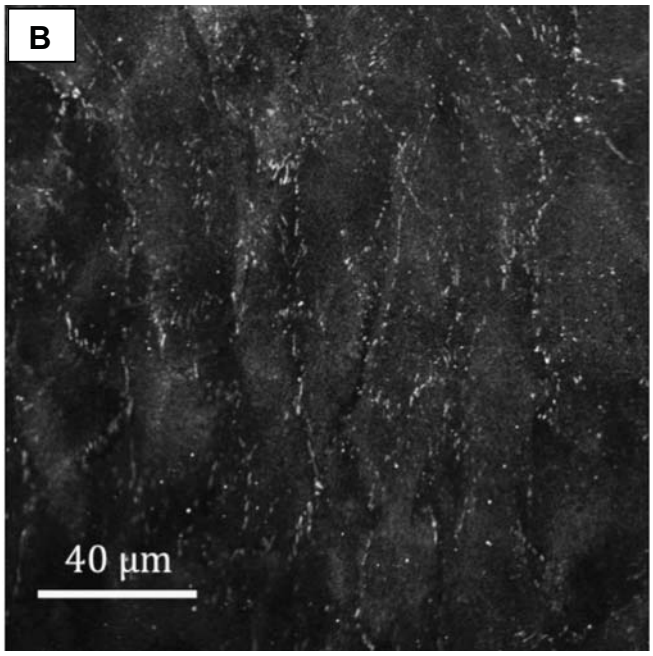
P_s. Vehicle solution, DMSO (0.002%), used for this study did not change EC P_s (C) relative to the baseline in the culture medium alone.



Supplemental Figure 1

Supplemental Figure. 2





Supplemental Figure 3.