

**Determining Cell Markers for the Evaluation of HUVEC and
HUVSMC Phenotypes on Formalin Fixed, Paraffin Embedded
Blood Vessel Mimics**

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By

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Introduction

Minimally invasive procedures have become the treatment of choice for many cardiovascular diseases, leading to a high demand for the development of new intravascular devices such as stents. As a result, a great need exists for the preliminary assessment of tissue responses to these newly emerging stent designs and modifications, but set-up time and a lack of facilities and personnel limit animal studies. A three-dimensional *in-vitro* testing system, or blood vessel mimic (BVM), has been developed, which allows for more efficient initial assessments to be performed on a large number of stent modifications to direct future animal and clinical studies toward only the most promising of these devices [1].

Current research focuses on developing BVMs that are both anatomically and physiologically representative of arterial blood vessels, as they are the focus of many cardiovascular treatments and therapies due to their propensity for disease. Prior to discussing the current research, it is necessary to understand the structure and function of arteries which BVMs aim to mimic.

The typical structure of normal arteries is comprised of three layers; intima, media, and adventitia [2]. The innermost layer, the intima, consists of a monolayer of endothelial cells (EC) known as the endothelium. The endothelium, playing a physiological role, serves as an anti-thrombogenic surface to minimize friction between the arterial wall and blood cells and prevent platelet aggregation [3]. It is also responsible for regulating the permeability of the vessel wall as well as the activity of arterial smooth muscle cells [3].

The middle layer, consisting of elastin sheets and smooth muscle cells (SMC), is referred to as the media. It plays both a structural as well as physiological role as it is responsible for the majority of the strength of the vessel as well as the regulation of circulatory dynamics [4]. As

previously alluded to, smooth muscle cells of the media respond to growth factors that are excreted by the endothelial cells during both normal vessel repair as well as pathological pathways such as atherosclerosis and restenosis [3].

The final, most superficial layer of the arterial vessel, or adventitia, consists mostly of fibroblasts. This layer primarily plays a structural role as these cells produce collagen and elastic fibers, which protect and reinforce the vessel. This layer helps maintain the vessel integrity and ultimately prevents the expansion of the vessel beyond its limit during the systolic, or high pressure period of the cardiovascular cycle [3].

Now that the structure and function of arteries has been reviewed, it is necessary to discuss the initial BVM design and the direction of current and future research. Preliminary BVMs were developed with the use of fat-derived human microvascular endothelial cells (HMVEC) seeded on an expanded polytetrafluoroethylene (ePTFE), tubular scaffold with the use of a perfusion bioreactor [1]. The resulting endothelial cell layer is intended to imitate the endothelium of the tunica intima and is critical in developing an *in-vitro* model of a native artery.

In an effort to create a more physiologically representative model, research has focused on the use of large vessel endothelial cells, specifically human umbilical vein endothelial cells (HUVEC) rather than the previously mentioned HMECs. Similarly, a dual-seeding technique has been developed to introduce a second cell layer onto the construct using human umbilical vein smooth muscle cells (HUVSMC) [5]. This smooth muscle cell layer is intended to mimic the anatomical and physiological function of the tunica media as mentioned earlier.

While research has focused on increasing the physiological accuracy of other components of the BVM system -- including scaffold material selection [6-8], the bioreactor system [9, 10], and more -- the remainder of this paper discusses the selection of cell markers and the initial

development of staining protocols. This is necessary to determine the effectiveness of the dual sodding technique as it pertains to maintaining the desired cellular phenotype of both HUVECs and HUVMSCs sodded onto an ePTFE scaffold to ensure the BVM remains physiologically accurate immediately following the cell-sodding procedure.

Background

In order to discuss the criteria used to select the cell markers to detect both the desired HUVEC and HUVMSC phenotypes on the scaffold following the dual cell-sodding technique, it is necessary to review the antibody targets most commonly used to identify both endothelial and vascular smooth muscle cells.

Vascular Endothelial Cells

While there are dozens of cell markers used to identify vascular endothelial cells, both specific and non-specific to EC, the following discusses four of the more common cell markers used to identify vascular EC in normal and pathophysiologic tissue with an immunohistochemical technique, as found in the peer-reviewed literature. These four cell markers include vonWillebrand Factor (vWF), intercellular adhesion molecule (ICAM-1 or CD54), platelet endothelial cell adhesion molecule (PECAM-1 or CD31), and vascular cell adhesion molecule (VCAM-1 or CD106).

vonWillebrand Factor is a highly multimeric glycoprotein secreted by vascular ECs with two separate biological functions -- the carrier for coagulation Factor VIII, an essential blood clotting factor, and as the cofactor required for platelets to bind to collagen exposed when the vessel wall is damaged [11]. These functions require the constitutive expression of vWF in

endothelium (from Weibel-Palade bodies), megakaryocytes, and connective tissue [12]. The secretion of vWF from Weibel-Palade bodies of ECs is initiated by a very limited range of molecules, mainly thrombin and histamine [11].

Thrombin is generated during the blood clotting process when prothrombin, secreted by the liver into the blood stream, is cleaved [2]. HUVECs, whether in culture or seeded onto a BVM, does not come into contact with blood at any point, and are therefore not expected to contact thrombin anywhere throughout the BVM system. Histamine, a compound involved in local immune response, is mostly generated and secreted in the granules in mast cells or basophils [2], and similar to thrombin, is not expected to be present in the culture of HUVECs or the BVM system since mast cells and basophils are not present. Since the two molecules largely responsible for the secretion of vWF from the Weibel-Palade bodies of ECs do not exist in the BVM system, vWF expression by HUVECs in the BVM system may be minimal, if it occurs at all.

ICAM-1 is a transmembrane, cell-surface glycoprotein typically expressed in ECs as well as the cells of the immune system such as macrophages and lymphocytes [13]. ICAM-1 is present in very low concentrations in the membranes of EC, and its expression is only upregulated on cytokine activated endothelium [14]. ICAM-1 is most often involved in the promotion and binding of leukocytes to the endothelium during a local immune response [15].

While staining ECs for ICAM-1 is common in the literature, it is often done in conjunction with an array of other adhesion molecules and performed when immune-mediated cell-cell adhesion reactions are of interest [16-19]. Since ICAM-1 is only continuously expressed at very low concentrations, rarely utilized on its own in peer-reviewed literature, not

entirely specific to vascular EC, and typically involved in immune-mediated reactions, it may not be the optimal cell marker for identifying HUVECs in the BVM system.

VCAM-1 is another cellular adhesion molecule that is only expressed on the surface of cytokine-activated endothelium in both large and small blood vessels [20] and, unlike ICAM-1, is restricted to the vascular tissue [21]. It promotes the adhesion of lymphocytes, monocytes, eosinophils, and basophils to the vascular endothelium. It is also believed to play a role in the development of atherosclerosis and rheumatoid arthritis [20].

While the natural baseline concentrations of VCAM-1 in normal, non-pathological ECs of the endothelium may be undetermined, it is believed that the upregulation of VCAM is highly dependent on the presence of Tumor necrosis factor- α (TNF- α) and Interleukin-1 (IL-1) [21, 22]. Since VCAM may only be expressed on cytokine-activated endothelium and its regulation may require the presence of specific factors and cytokines, its use for identifying HUVECs in the BVM system may not be ideal. This is because TNF- α and IL-1 are not present in the BVM cell culture or BVM media and the degree of endothelium activation in the presence of the cell culture media used in the BVM system would need to be characterized prior to using antibodies for VCAM. VCAM-1 expression has also been observed in other cell types, including vascular SMCs [23]. Since the antibody chosen to identify HUVECs in the BVM system will ultimately be used in the presence of HUVSMCs as well, VCAM-1 is not an ideal choice.

PECAM-1 is a single-membrane-spanning glycoprotein found on platelets, leukocytes, and endothelial cells [24]. With regards to EC, it is concentrated on cell-cell borders [11], has been shown to mediate cell-cell adhesion [25], and makes up a very large portion of the endothelial cell intercellular junction [26]. The minimal concentration of PECAM-1 at the EC junctions of the endothelium has been shown to be in the range of 10^6 molecules [24]. *In-situ*,

PECAM-1 is constitutively expressed on continuous endothelium of all blood vessel types [24] and is not expressed on fibroblasts or epithelial cells [27]. PECAM-1 has been used extensively as an immunostaining marker for identifying the ECs of blood vessels from a variety of species [24-28]. Similarly, PECAM-1 has been utilized as an EC cell marker for a variety of tissue engineering applications [29, 30], such as vascular grafts [31, 32], in which ECs are used, including the HUVEC cell line [29, 33, 34].

Due to the high concentration of PECAM-1 at EC junctions and its high specificity to vascular endothelial cells making up the endothelium of blood vessels, it may be an ideal cell marker for its use in identifying HUVECs in the BVM system, and its expression could be indicative of a physiologically accurate mimic of an endothelium on the scaffold. Its extensive use in the literature for both natural tissue and engineered constructs has provided a large amount and variety of staining protocols that could serve as a starting point for developing a protocol suitable for the BVM system. Lastly, its use with the specific HUVEC lineage that is used in the BVM lab ensures that successful results obtained in the past by other researchers could be duplicated. Antibodies to PECAM-1 will be used in the remainder of this paper.

Vascular Smooth Muscle Cells

While the literature identified many cell markers used to stain for the vascular endothelial cells of the endothelium in both pathological and non-pathological states, and though SMCs share many lineage-specific markers, only a small amount of markers were found to be commonly used for distinguishing *vascular* smooth muscle cells. The most consistently used markers found were calponin, smooth muscle myosin heavy chain (SM-MHC), SM22- α , and α -smooth muscle actin (α -SMA). The following discusses a brief background of each cell marker.

Calponin is a filament-associated protein that is involved in the regulation and modulation of smooth muscle contraction and is specific to smooth muscle tissue [35]. While calponin has been used extensively in the laboratory for identifying SMCs, a review of the literature will find that it is rarely, if at all, used on its own for staining SMCs, but rather with a cocktail of other SMC-specific markers such as SM22 α , and α -SMA [36-38]. Also, the literature demonstrating the use of calponin has been associated with staining malignant tissue such as malignant myoepithelioma, a variety of carcinomas, and lesions. Since calponin does not stand on its own as a SMC marker of *normal* smooth muscle tissue in the literature, it may not be ideal for identifying HUVSMCs of the BVM system.

Smooth muscle myosin heavy chain is a major contractile protein whose expression is believed to be exclusive to the SMC lineage [39] and highly prevalent toward the end of vascular smooth muscle cell differentiation [40]. It's been used, most often in conjunction with α -SMA, to identify the smooth muscle tissue of vasculature, respiratory and gastrointestinal tracts, uterus, bladder, and eye [41]. Antibodies to SM-MHC have not been shown to cross-react with the myosin of skeletal muscle tissue [42]. Due to its extensive use in the literature, specificity to SMCs, and high prevalence in *vascular* SMCs, SM-MHC may be suitable for identifying HUVSMCs in the BVM system and should be considered for its use in the laboratory.

Upon first review, SM22- α , similar to SM-MHC, appeared to be a potential cell marker for the HUVSMCs in the BVM system, as it is commonly used to identify the SMCs in smooth muscle tissue. However, upon further investigation, it was discovered that SM22- α is a calponin-related protein and it is expressed in conjunction with calponin [43]. It was also learned that SM22- α expression, though present during embryological development, is not upregulated in vascular smooth muscle tissue until adulthood [44]. Since the SMCs of the BVM

system are isolated from *umbilical* vein blood, its concentration in HUVSMCs could be questionable. SM22- α expression has also been discovered in cardiac and skeletal muscle cells [43]. Since SM22- α expression is associated with calponin, a cell marker that has already been determined to be non-ideal for its use in the BVM system, is not specific to only vascular smooth muscle cells, and concentrations are not increased until adulthood, it too should not be pursued for the BVM laboratory to identify HUVSMCs.

Alpha smooth muscle actin is a smooth-muscle specific intermediate-sized filament prevalent in vascular smooth muscle tissue [45]. While different smooth muscle cell types contain distinct and unique species of both contractile and cytoskeletal proteins, the greatest variation between cell types exists in these intermediate-sized filaments [46]. By taking advantage of this variation and the specificity of the intermediate-sized filaments to a given cell type, an intermediate filament, or a component of the filament, specific to vascular smooth muscle cells could be used as a cell marker to stain for the HUVSMCs in the BVM lab.

It has been shown that the smooth muscle cells of the digestive, respiratory, and urogenital tract contain desmin and γ -type smooth muscle actin as the predominant and exclusive filaments; α -type smooth muscle actin is not expressed in these tissue types. The intermediate filaments of vascular smooth cells differ in that they contain abundant amounts of vimentin and no desmin. A component believed to be exclusive to vimentin filaments, and therefore specific to vascular smooth muscle cells, is α -smooth muscle actin [46]. Smooth muscle tissue of different blood vessels types such as aorta, small arteries, arterioles, venules, and the vena cava from a variety of species including human, bovine, porcine, and murine have all shown a high expression of α -SMA [46]. Its high specificity to only vascular SMCs of all types of blood

vessels in a large variety of species suggests that α -SMA may serve as an ideal candidate for a cell marker toward HUVMSCs in the BVM lab and should be further investigated.

Upon additional review, the reliability of α -SMA as a marker for staining vascular SMCs has been demonstrated and is extensively used in research. Similar to the use of PECAM-1 in identifying EC in tissue-engineering applications, α -SMA is commonly used to stain for SMCs, including the HUVMSC lineage, in a variety of engineered vascular constructs as well [36, 47]. Due to its specificity to vascular smooth muscle cells, reliability in previous research, and demonstrated use in staining for HUVMSCs in vascular tissue-engineering applications, antibodies toward α -SMA will be pursued for its use in the BVM lab.

Procedure

Prior to developing an immunohistochemical staining protocol for detecting the expression of PECAM-1 by HUVECs and α -SMA by HUVMSCs on the ePTFE scaffold following the dual sodding method, it is necessary to determine if HUVECs and HUVMSCs express their respective cell markers when in culture in the first place. After staining ECs and SMCs in culture for PECAM-1 and α -SMA, respectively, both positive and negative controls need to be established prior to using the antibodies for the IHC analysis of BVM samples.

Cell Culture of HUVECs and HUVMSCs

Both cell types were thawed and incubated separately in their respective growth media in a 6-well plate overnight at 37°C and 5% CO₂. Prior to cell thawing and incubation, coverslips were placed on the bottom of each well so cells would grow on the coverslips rather than the surface of the wells. This will allow for the easy transfer of coverslips to glass slides for the

necessary microscopy after immunohistochemical staining. HUVSMCs were thawed and incubated after their seventeenth passage while HUVECs were taken after having undergone nine passages. Cell confluence was allowed to reach approximately 70-80% for both cell cultures prior to immunofluorescent staining.

Immunofluorescent Staining of HUVECs and HUVSMCs in Culture

Following cell culture, both 6-well plates with each cell type was removed from the incubator and placed on the laboratory bench top for staining procedure. Media was aspirated and each well was lightly washed with PBS twice. Samples were then fixed with 5mL of Histochoice, per well, for 30 minutes. Samples were again washed twice with PBS then incubated for 20 minutes in 10% normal goat (Santa Cruz Biotechnology, sc-2043) and donkey (Santa Cruz Biotechnology, sc-2044) blocking serum, for SMCs and ECs, respectively. All dilutions were made with PBS. Wells were washed twice with PBS, then samples were incubated for 60 minutes with a mouse monoclonal primary α -SMA antibody (Santa Cruz Biotechnology, sc-56499) for SMCs or a goat polyclonal primary PECAM antibody (Santa Cruz Biotechnology, sc-1506) for ECs, at a concentration of 1 ug/mL in 1% of their respective blocking serums. To serve as negative controls, two wells per 6-well plate were incubated with PBS rather than the primary antibody solution.

After the primary antibody incubations, samples were washed with three changes of PBS for five minutes each. All wells were then aspirated and incubated for 45 minutes with goat anti-mouse (Santa Cruz Biotechnology, sc-2010) or donkey anti-goat (Santa Cruz Biotechnology, sc-2024) fluorescein-conjugated secondary antibodies for the SMCs and ECs, respectively. Secondary antibody concentrations were 1 ug/mL with 1.5% normal blocking serum and

incubation occurred with minimal light exposure. Cells were then washed three times in PBS for five minutes. A 2 ug/mL concentration solution of Hoechst dye was prepared and 200 uL transferred to each well to stain nuclei. Cells were incubated for 15 minutes with the Hoechst dye and were then washed for five minutes, three times with PBS. PBS was aspirated and all coverslips with the stained cells were removed from the wells with a razor blade. Coverslips were transferred to glass slides, glued, and labeled accordingly.

Fluorescent Microscopy

Slides were imaged using a wide-field fluorescent microscope. Slides were viewed first under bright light to find an area of high cell density. This area was then exposed to light using a filter of excitation wavelength 365 nm and emission 480 nm for the Hoechst dye (nuclei) immediately followed by exposure using a filter of excitation wavelength 494 nm and emission 518 nm for FITC, which shows the secondary antibody bound to α -SMA or PECAM, depending on the cell type. Using ImageCapturePro, the slides' exposure to light was recorded for one second for Hoechst stain and ten seconds for FITC, to enable a more accurate representation of the total fluorescence.

Image J Analysis

Images were processed using ImageJ by subtracting background and enhancing brightness and contrast for each image. Corresponding images of Hoechst and FITC were then merged and a compiled image was generated showing both stained nuclei and α -SMA or PECAM-1 expression for HUVSMCs and HUVECs, respectively.

IHC Analysis of Formalin Fixed, Paraffin Embedded Murine Tissue using PECAM-1

To generate both positive and negative controls for staining with the PECAM-1 primary antibody, an IHC protocol was performed on murine vascular tissue with and without the presence of the antibody, respectively. An avidin-biotin complex (ABC) staining technique was used (Santa Cruz Biotechnology, sc- 2017). Samples previously fixed in formalin and embedded in paraffin were prepared by sectioning a thickness of six microns, incubated in xylene to remove paraffin, and rehydrated with decreasing concentrations of ethanol. All samples were incubated with 1.5% blocking serum for one hour. Positive controls were incubated with the same primary antibody used during the fluorescent technique with concentrations ranging between 0.5 – 5.0 ug/mL for 30 minutes, while negative controls were incubated in only PBS. Then all samples were incubated with a 1 ug/mL concentration of biotinylated secondary antibody for 30 minutes and incubated with an avidin-biotin (AB) enzyme for an additional 30 minutes. Finally, samples were incubated with a peroxidase substrate for one – ten minutes, dehydrated with increasing concentrations of ethanol, and washed in xylene prior to mounting with a coverslip. All samples were washed with PBS three times for five minutes each between incubating in each reagent and an aspirating pump was used to remove PBS and reagents during the entire process. After a 24-hour drying period, samples were viewed and imaged using brightfield microscopy. Comparisons were made between positive and negative controls, and optimal concentrations of the primary antibody and peroxidase incubation times were determined for future use.

IHC Analysis of Formalin Fixed, Paraffin Embedded Murine Tissue using α -SMA

The IHC procedure of murine vascular tissue using α -SMA to generate both negative and positive controls for the primary antibody were identical to those used with PECAM-1. A mouse

ABC staining kit (Santa Cruz Biotechnology, sc-2023) was used rather than the goat ABC kit used for PECAM, since the α -SMA primary antibody was a mouse monoclonal antibody. Again, samples were incubated with and without the primary antibody. Samples receiving the primary antibody (the same α -SMA primary antibody used during the immunofluorescent staining) used concentrations varying between 0.5 – 5.0 μ g/mL and the peroxidase incubation period varied between one - ten minutes to determine the optimal parameters for future use. Both controls were viewed and imaged using brightfield microscopy.

Results

Immunofluorescent staining of HUVECs and HUVSMCs in culture

Figure 1 corresponds to the immunofluorescent staining of HUVSMCs. Figure 1A, taken at 100X magnification, is representative of the smooth muscle cells that were incubated without the presence of the α -SMA primary antibody to serve as negative controls. The blue staining corresponds to the Hoechst dye used to identify nuclei and confirms the presence of cells. However, there is no green staining apparent throughout any of the entire sample.

Figures 1B and 1C correspond to the positive controls in which HUVSMCs were stained with the α -SMA primary antibody with images taken at 100X and 400X magnification, respectively. Again, the blue staining corresponds to cell nuclei and, unlike the negative controls, there is obvious green staining corresponding to the presence of α -SMA. The merged image displays the smooth muscle actin in the cytoplasm surrounding the nuclei of each cell.

Figure 2 is representative of the immunofluorescent staining of HUVECs. Results found with the EC staining were similar to the results obtained during the SMC staining. Figure 2A corresponds to the negative controls in which EC were incubated without the PECAM-1 primary

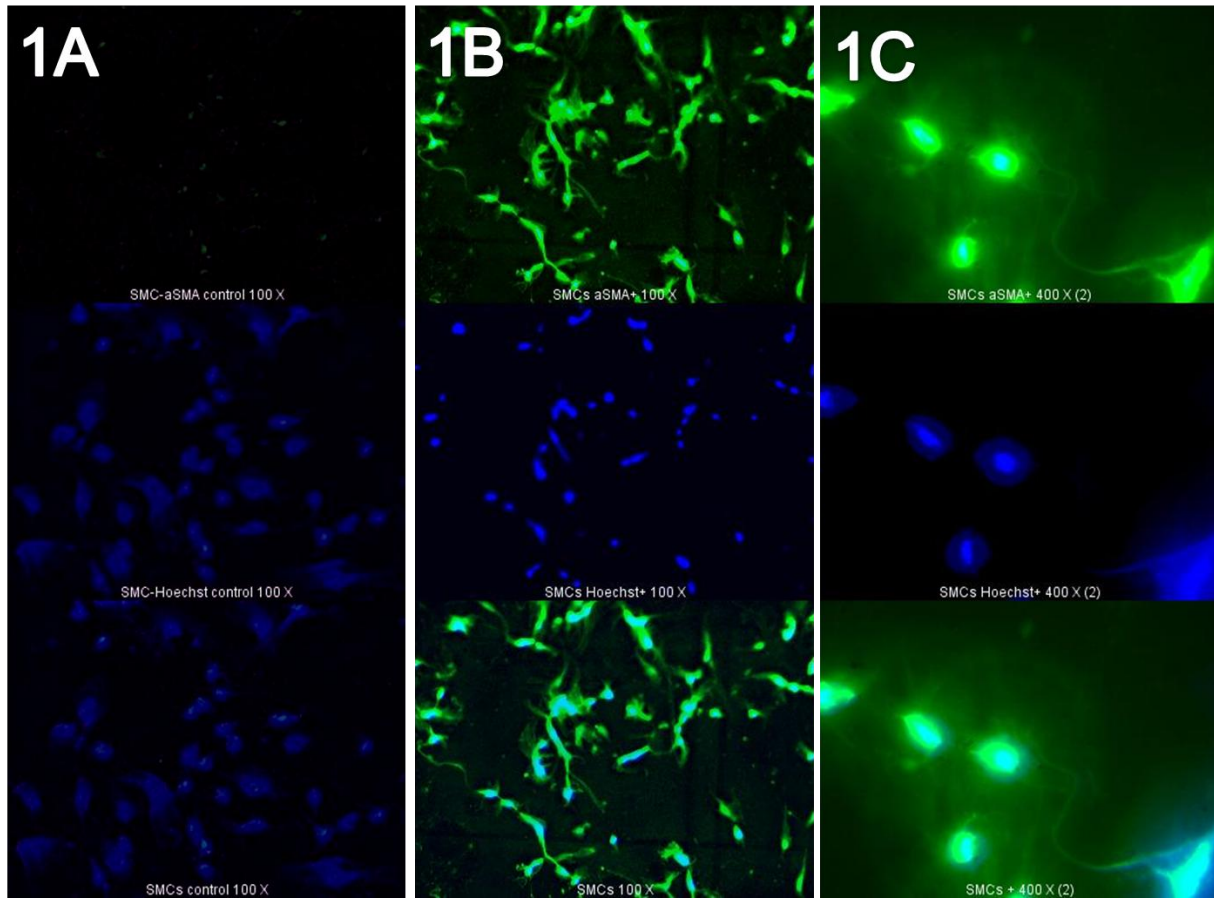


Figure 1: Immunofluorescent staining of HUVMSC in culture with α -SMA. A) Negative control, HUVMSCs incubated without a primary antibody, demonstrates no false positives. Images of HUVMSCs stained using the primary antibody to α -SMA at B) 100X and C) 400X magnification show positive identification of HUVMSCs in culture expressing α -SMA

antibody. The blue stain confirms the presence of cells. There appears to be a small amount of light green staining as well, which is not expected when incubated without a primary antibody. There is a minimal presence and lack of intensity of this green staining. Also, the merged image in the bottom of Figure 2A shows the presence of this green fluorescent where there are no cell nuclei (no blue staining). Images shown for the negative controls were taken at 40X magnification. However, the images obtained at 100X magnification show identical results as those represented in the figure.

Figure 2B and 2C correspond to HUVECs stained with the PECAM-1 primary antibody serving as positive controls with images taken at 100X and 400X magnification, respectively.

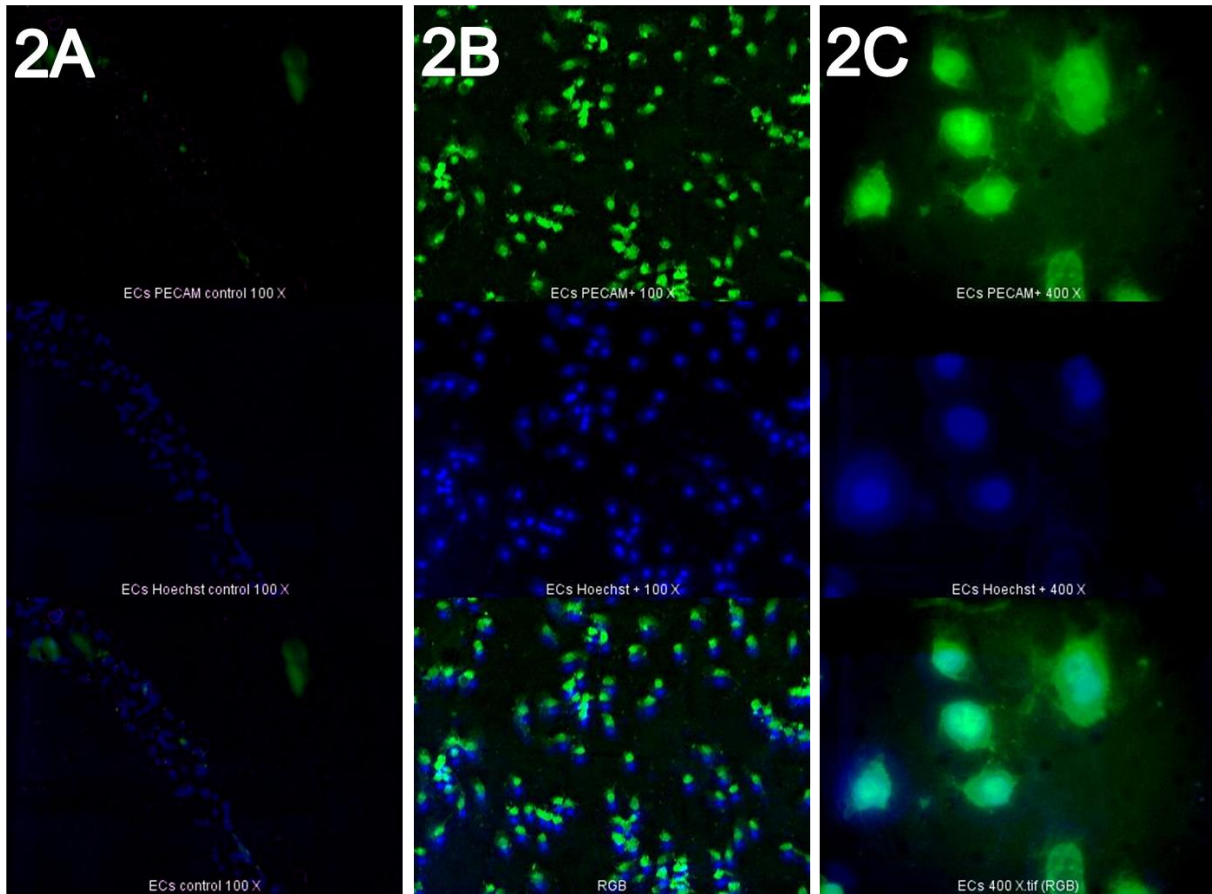


Figure 2: Immunofluorescent staining of HUVECs with PECAM-1 antibody. A) Negative control, HUVECs incubated without a primary antibody, demonstrates no false positives. Images of HUVECs stained using the primary antibody to PECAM-1 at B) 100X and C) 400X magnification show positive identification of HUVECs in culture expressing PECAM-1.

Again, the blue staining of the cell nuclei confirm the presence of cells. Unlike the negative controls, the green staining, representative of the presence of PECAM-1, is much more intense. When merged with the Hoechst stain, the green fluorescent surrounds the cell nuclei rather than randomly throughout the image, as was the case with the negative controls.

IHC Analysis of Murine Vascular Tissue with PECAM-1

Figure 3 corresponds to the immunohistochemical staining of murine tissue fixed with formalin and embedded in paraffin. The top and bottom images in Figure 3B, taken at 100X and 400X magnitudes, respectively, correspond to the negative controls in which samples were

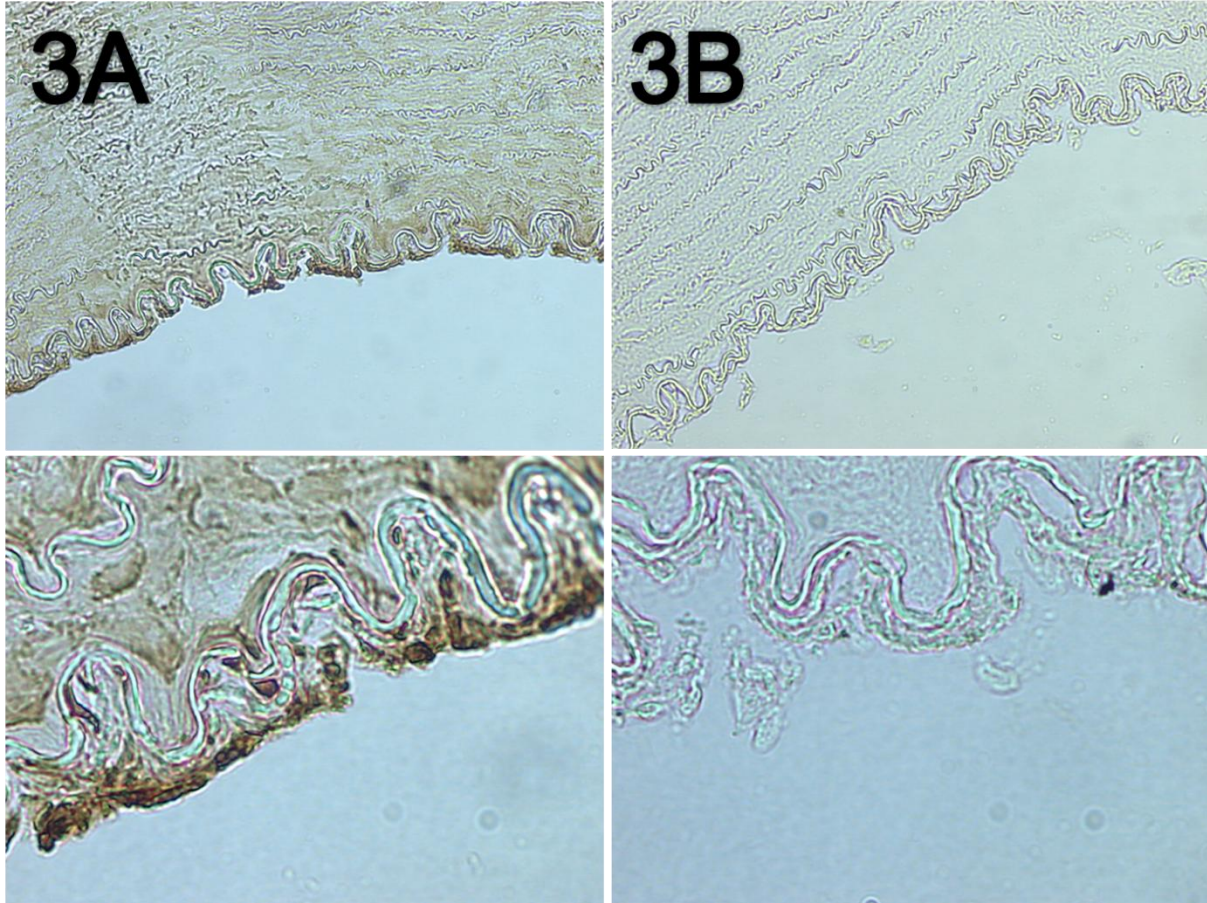


Figure 3: Immunohistochemical staining of formalin-fixed, paraffin-embedded murine tissue using PECAM-1. A) Murine vascular tissue incubated in PECAM-1 antibody shows successful staining of the endothelial monolayer (images at 100X and 400X mag.). B) Negative control; identical murine tissue stained in the absence of the PECAM-1 antibody demonstrates no false positive staining.

incubated without the presence of PECAM-1 antibody. There is no staining throughout any portion of the tissue, including the inner wall of the lumen where ECs should be present. The images in Figure 3A correspond to the positive controls -- samples that were incubated with the PECAM-1 primary antibody. The bottom image of Figure 3A, taken at 400X magnification, shows a very distinct staining of the endothelium of the tunica intima. This correlates to a successful binding of PECAM-1 to the ECs of murine tissue and will serve as quality positive controls.

IHC Analysis of Murine Vascular Tissue with α -SMA

Figure 4 corresponds to the immunohistochemical staining of formalin fixed murine tissue embedded in paraffin using the α -SMA antibody to detect the vascular SMC phenotype. Images in Figure 4B correspond to the negative controls in which the samples were not incubated with the primary antibody. The top and bottom images of Figure 4B, taken at 100X and 400X magnification, respectively, show no staining throughout any of the tissue. The images in Figure 4A are representative of the samples serving as positive controls in which the tissues were incubated in the presence of the α -SMA primary antibody. Unlike the negative controls, there is a very distinct staining throughout the majority of the tissue, indicative of the

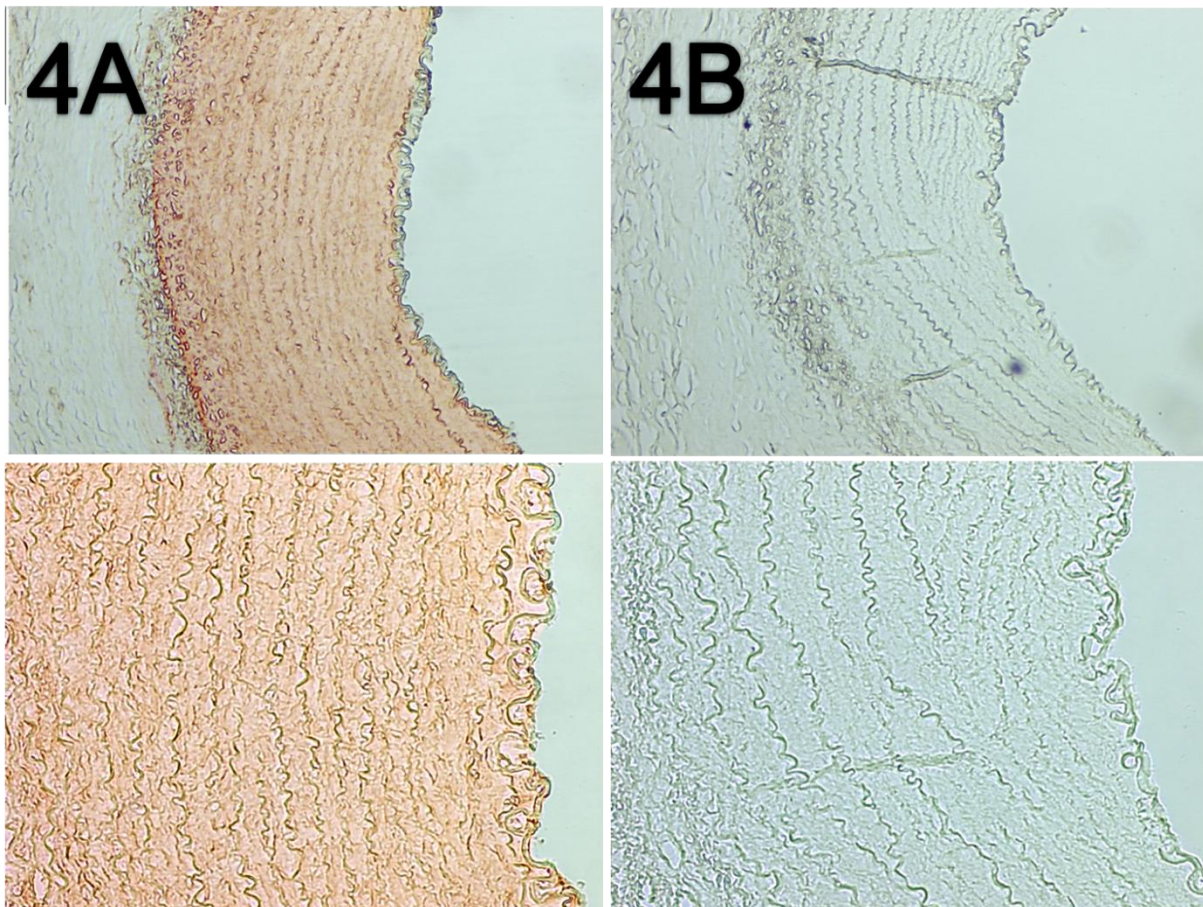


Figure 4: Immunohistochemical staining of formalin-fixed, paraffin-embedded murine tissue using α -SMA antibody. A) Murine vascular tissue incubated in α -SMA antibody shows successful staining of the smooth muscle cells of the tunica media (images at 100X and 400X mag.). B) Negative control; identical murine tissue stained in the absence of the α -SMA antibody demonstrates no false positive staining.

presence of SMCs in the tunica media. However, the staining that occurred is not nearly as dark as was found when staining the endothelium with PECAM-1. This could be a concern and requires further investigation.

Conclusion

The immunofluorescent staining of HUVMSCs in culture when incubated without the presence of the α -SMA primary antibody showed no staining throughout any of the samples. This is to be expected and confirms that the FITC-conjugated secondary antibody did not bind to the SMCs and that no false positives will be created when using the primary antibody. When samples were incubated with the primary antibody, there was a distinct and intense presence of the green fluorescent surrounding the cell nuclei of each cell. This confirms that the HUVMSCs express α -SMA when in culture prior to any cell-sodding process. The α -SMA primary antibody can potentially be used for the IHC analysis in the BVM lab to determine whether or not HUVMSCs remain in the desired phenotype after the pressure-sodding technique used in the BVM lab.

While the results for the immunofluorescent staining of HUVECs in culture were similar to those of the HUVMSCs, the presence of green fluorescent in the negative controls could be a concern and require further discussion. When comparing the green fluorescents between the negative and positive controls, there are several distinct differences which will be addressed.

First, the green fluorescent of the negative controls is very dim and lacks the intensity of the positive controls. Second, the minimal green fluorescent of the negative controls do not align with the cell nuclei and can be found throughout the glass slides where there are no cells.

These two observations suggest that the green fluorescent of the negative controls are not indicative of the FITC-conjugated secondary antibody binding to the ECs without the primary antibody and that these are not false positives. If the secondary antibody was binding to the ECs without the primary antibody, then the green staining would align at the same locations as the blue staining of the cell nuclei, which was not the case. Rather, the very slight presence of dim green fluorescent is likely due to poor washing after the secondary antibody incubation period and that all of the non-bound secondary antibody was successfully removed in the washes prior to imaging. This procedure should be attempted again, taking extra care to ensure that the PBS washes after the secondary antibody incubation period are performed diligently.

The very high intensity of the green fluorescent in the positive controls, as well as its alignment with all EC nuclei suggests that HUVECs express PECAM-1 when in culture prior to any cell-seeding process and that the antibody can potentially be used for IHC analysis of HUVECs on BVMs.

The IHC analysis of murine vascular tissue with PECAM-1 successfully generated both positive and negative controls for the primary antibody to be used when analyzing HUVECs on the BVM. The negative controls showed absolutely no staining throughout any of the tissue. This also confirms that, when using the proper protocol, no background or non-specific staining occurs. The positive controls shows what a successful staining of EC on an inner lumen will look like and all that is required next is to optimize the protocol for staining in the presence of an ePTFE scaffold rather than the native tissue. Complications include ensuring the scaffold remains adhered to glass slides during incubation periods and determining optimal primary antibody concentrations and peroxidase incubation periods as they will more than likely differ from results obtained with the murine tissue.

The IHC analysis of the murine vascular tissue with the α -SMA primary antibody used to identify the vascular SMCs successfully generated negative controls. When incubated without the primary antibody, there is no staining throughout any of the tissue. This confirms that the secondary antibody did not bind anywhere in the tissue and that no background or non-specific staining occurred. While the positive controls differed significantly from the negative controls, the nature of the staining could be a concern.

Rather than the darker brown staining common with a peroxidase technique, and shown in the PECAM-1 positive controls, the staining that occurred with α -SMA is a lighter red tint, even at the highest antibody concentrations and longest peroxidase incubation periods. These results could not be a consequence of secondary antibody cross-reactivity, otherwise the negative and positive controls would be identical and the staining, if anything, would be expected to be even darker than those obtained when staining the endothelium with PECAM-1. The same is true of primary antibody cross-reactivity, plus the staining occurs only in the location where SMCs are expected to reside. If primary antibody cross-reactivity was occurring, we would expect to see staining throughout the entire tissue, not just concentrated in the tunica media. Endogenous biotin activity could not be the factor either because, again, the negative and positive controls would look identical.

The lighter staining that occurs with the positive controls using α -SMA is likely a result of poor enzyme activity or primary antibody potency. It should be noted that one vial of the primary antibody was handled poorly at some point during the shipping process from the vendor and that a fresh vial of α -SMA antibody could result in better staining. If the problem persists, a higher concentration of the primary antibody exceeding 5.0 ug/mL and/or a peroxidase incubation period greater than ten minutes could be attempted. Another suggestion would be to

use an α -SMA primary antibody that was raised in a different species and would also require a different ABC staining kit, utilizing a different secondary antibody as well. This is preferred over using higher concentrations of the current antibody, as the reagents are expensive and need to be used sparingly. Another suggestion would be to use antibodies toward smooth muscle myosin heavy chain rather than the α -SMA primary antibody.

While these suggestions may generate better positive controls, they may not be necessary when staining HUVSMCs on an ePTFE scaffold for several reasons. First, the SMCs in the BVM system are human rather than murine. This could provide different results than those of the murine tissue that was used for the controls, so the current mouse antibody may still work well with BVMs. Second, there would not be a presence of a large variety of tissue types found in the murine samples (such as different types of connective tissue) as the BVM would consist of only the HUVSMCs, HUVECs, and the polymer scaffold. So, if the large presence of other tissue types in the sample was a source of the problem, this would not occur with the BVM. Lastly, the seeding density of HUVSMCs onto the BVM, and the sum of any cell division that may occur immediately afterward is likely to be less than the total density of SMCs in the murine tissue. So, if the problem with the α -SMA on the positive controls was that the primary antibody concentrations were too low, not very potent, or that the peroxidase enzyme was acting weakly, this might not be a concern when staining BVMs because there would be higher primary antibody-to-cell and peroxidase-to-AB enzyme ratios. However, prior to optimizing the α -SMA protocol for analyzing HUVSMCs in the BVM system, these problems with the positive controls should be identified and addressed.

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