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Saghar Harirforoosh April 11, 2016

The Role of Human Osteopontin Isoforms in Vascular Smooth Muscle Cell Migration and Cell Signaling

by

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Obstructive arterial disease is characteristic of several cardiovascular disease (CVD) pathologies and ultimately leads to tissue ischemia. The endogenous response to ischemia is to increase collateral formation to restore blood flow and re-establish oxygen delivery to damaged tissue. Collateral formation involves the proliferation and migration of multiple cell types, including vascular smooth muscle cells (VSMCs). Osteopontin (OPN) is a secreted inflammatory protein that is significantly upregulated in response to ischemia. In humans, OPN is alternatively spliced to produce three isoforms: OPNa, OPNb, and OPNc. Several studies show that OPN isoform functions vary based on the tumor subtype, and data from the Lyle lab demonstrate that OPN isoforms diverge in their impacts on functional collateral formation *in vivo*. The migration of VSMCs requires several processes and steps to occur in concert. For example, during the formation of lamellipodia, focal adhesion kinase (FAK) must be activated to aid in the maturation of focal contacts into focal adhesions.

VSMC migration requires lamellipodia and filipodia formation and persistence, cell polarization, and appropriate focal adhesion dynamics. Using several assays to assess cell migration, including a modified Boyden chamber assay, live cell imaging, and scratch wound assay experiments, we establish that OPN is required for proper VSMC migration. $OPN^{-/-}$ VSMCs exhibit impaired migration in response to PDGF, display deficits in lamellipodia formation and persistence, and close a smaller percentage of a wound compared to WT VSMCs. By using live cell imaging with the incorporation of a

scratch wound assay, we were able to further determine that human osteopontin isoforms a and c rescue impaired $OPN^{-/-}$ VSMC migration.

This project investigates the specific mechanisms by which OPN regulates VSMC migration. We establish that human OPN isoforms differentially rescue VSMC migration and identify potential mechanisms that may explain these differences. By understanding the mechanisms by which human OPN isoforms modulate cell migration, we may be able to utilize these isoforms as novel therapeutic targets for cardiovascular disease pathologies.

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List of Symbols and Abbreviations

CAVD	Calcific Aortic Valve Disease
CVD	Cardiovascular Disease
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
hOPN	Human Osteopontin
hOPNa	Human Osteopontin Isoform a
hOPNb	Human Osteopontin Isoform b
hOPNc	Human Osteopontin Isoform c
MMPs	Matrix Metalloproteinases
OPN	Osteopontin
PDGF	Platelet-Derived Growth Factor
VSMCs	Vascular Smooth Muscle Cells

Introduction

1. Introduction

1.1 Cardiovascular Disease

The prevalence of cardiovascular disease (CVD) has failed to diminish throughout the United States and is still the leading cause of death among men and women.[1] On a global scale, cardiovascular disease affects low-, middle- and high-income families and is the number one cause of death.[2] Cardiovascular disease imposes a large financial burden on a national and global scale. The control of CVD on a worldwide scale becomes a large objective in order to diminish the deleterious impact of the disease. Moreover, obstructive arterial disease occurs in many CVD pathologies, including myocardial infarction, stroke and peripheral vascular disease.[3] A coronary blockage ultimately leads to ischemia, and the occurrence of ischemic heart disease has progressively increased.[4] In addition, ischemic heart disease comprises 80% of mortalities that result from cardiovascular disease.

1.2 Ischemia and its Physiological Response

Ischemia is described as an insufficient supply of blood to an organ or part of the body and is caused most commonly by a blocked artery due to atherosclerotic plaque.[5] The physiological response to ischemia is to increase the development of functional collaterals in order to restore blood flow and preserve jeopardized tissue.[6] Collateral growth, the proliferation of a network of arterial connections, can decrease the severity of ischemia in multiple CVD settings. For example, in myocardial ischemia collateral formation helps to restore coronary blood flow and, during periods of ischemia, successfully formed collaterals can prevent the onset of myocardial infarction.[7, 8]

Osteopontin (OPN), a matri-cellular inflammatory protein, has been shown to play a significant role in ischemic tissue.[9-11] It has previously been demonstrated that the serum level of OPN is upregulated after ischemic stroke, and that a knockdown of OPN results in significantly delayed collateral vessel formation and perfusion in an ischemic limb.[9, 12] Additionally, the expression level of OPN increases after myocardial infarction, while the amount of hepatic OPN has been shown to be upregulated in response to hepatic ischemia-reperfusion injury.[10, 11] With the substantial upregulation of OPN in several ischemic conditions and its subsequent impact on vascular growth, OPN may be a critical mediator of neovascularization.

1.3 Role of VSMCs in Collateral Formation, Atherosclerosis, and Arteriogenesis

An increase in migration of vascular smooth muscle cells (VSMCs) is involved in both atherosclerosis and collateral formation.[13] The accumulation of VSMCs is a major component of the propagation of atherosclerosis and is initiated by damage to the endothelium. Migration of vascular smooth muscle cells in these areas propagates the development of neo-intima and plaque, further promoting atherosclerosis development.[14, 15] On the other hand, VSMC proliferation and migration are critical for the processes of collateral formation and arteriogenesis, or the growth of collaterals from pre-existing arteries.[16]

1.4 Proper Collateral Formation Requires Osteopontin

Vasculogenesis, angiogenesis, and arteriogenesis are key processes that contribute to neovascularization. Vascuolgenesis occurs in embryonic development and forms cardiovasculature through the assembly of new vessels from single cell precursors.[17] Angiogenesis is also involved in developmental blood vessel growth, as it

sprouts new blood vessels from pre-existing blood vessels.[18] Arteriogenesis acts to arterialize preformed branched vessels.[19] The onset of arteriogenesis is a response to shear stress, and growth factors are activated that lead to collateral growth.[20] Successful collateralization leads to the offset of arterial occlusion or stenosis, increasing the amount of adequate blood flow and oxygenation to the affected tissue and thereby decreasing mortality.[21] The migration and proliferation of VSMCs is critical and involved in all three types of collateral formation.[16] Therefore, it is fundamental to understand the factors that mediate VSMC migration.

Our interest lies in defining the contribution of OPN to CVD pathologies and understanding how OPN functions to influence the migration and infiltration of various cell types, including VSMCs. OPN has been shown to play a role in VSMC and macrophage cell migration through its ability to serve as an adhesion molecule and chemotactic component for these cell types, as well as others.[9, 22] It was established previously by Duvall, et.al. that OPN is a critical mediator of the collateral formation and bone healing processes, both of which require cell migration.[23] Moreover, the expression of inflammatory proteins, including OPN, is significantly increased with the occurrence of tissue damage and is integral to the process of cell migration, the development of collaterals, and subsequent tissue reperfusion.[10, 23] In fact, the Taylor lab has demonstrated that OPN expression is significantly increased in response to ischemia and that this protein is a critical mediator of collateral formation, where OPN deficient animals have dramatically reduced neovascularization, vessel maturation, and functional perfusion.[19, 23, 24]

1.4.1 Osteopontin

Osteopontin is an extra-and intra-cellular tissue protein, and it can exist as a cytokine in body fluids.[25, 26] This small integrin-binding N-linked glycoprotein is heavily post-translationally modified by serine/threonine phosphorylation, tyrosine sulfation, and glycosylation.[25] An additional post-translational modification of OPN is its cleavage by thrombin and matrix metalloproteinases (MMPs); the protease cleavage sites in OPN act to modify its activity. OPN interacts with both integrin and CD44 receptors.[25] Through its RGD and CD44 domains, OPN binds to these receptors and directly and indirectly activates cellular signaling pathways.[25, 26] OPN is further implicated in cell-cell interactions, inflammation, and cell survival and is expressed in smooth muscle cells, epithelial cells, endothelial cells, and macrophages.[25]

1.5 Human OPN Isoforms

While rodents express one OPN isoform, it was recently described that OPN exists as three isoforms in humans.[27] The human OPN isoforms are a direct result of alternative mRNA splicing of a single RNA transcript that results in: 1) OPNa, the full length isoform, 2) OPNb, lacking exon 5, and 3) OPNc, lacking exon 4 (**Figure 1**). What little is understood about OPN isoforms has come from the cancer literature, where investigators show that OPN isoforms may have divergent functions. In prostate cancer, the overexpression of isoforms OPNb and OPNc enhances tumor growth.[28] The splicing isoform OPNa acts to upregulate glucose in breast cancer cells where this glucose is then utilized by OPNc to mediate anoikis resistance.[29] Each human OPN isoform serves various and specific functions in other cancers, such as ovarian and lung cancer.[30, 31] Unpublished data from the Lyle Lab supports that OPN isoforms have differential effects on functional collateral formation. The RGD domain and CD44 binding domains remain

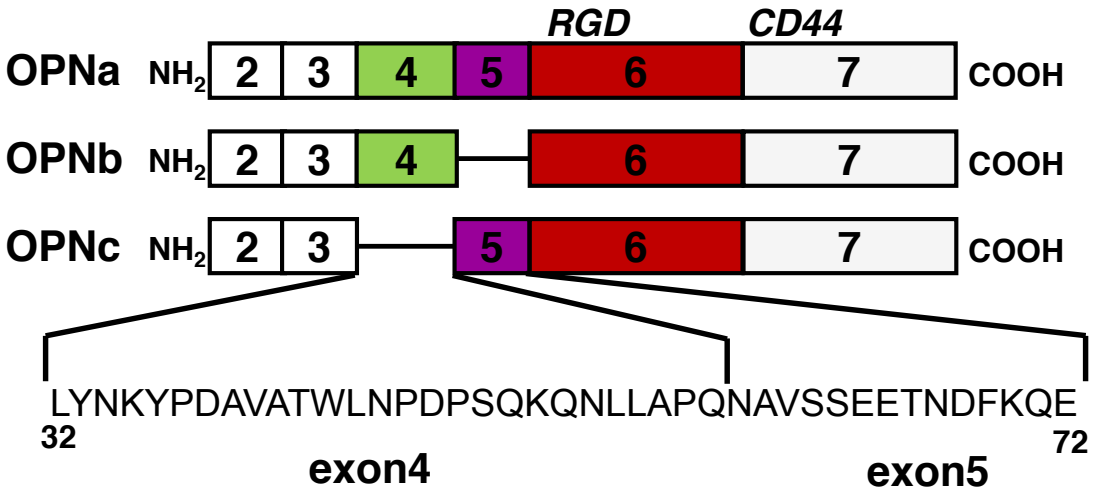

Figure 1. Human osteopontin exists as three isoforms. The human osteopontin mRNA transcript is alternatively spliced to produce OPNa as the full length isoform, OPNb that lacks exon 5, and OPNc that lacks exon 4.

intact in all three isoforms and, presumably, allow binding to integrin and CD44 receptors, respectively. Additional investigations into the amino acid sequences of exons 4 and 5, absent in isoforms c and b, respectively, indicate that differential OPN isoform functions are likely due to key post-translational modifications within these exons that are critical for proper OPN folding and/or signaling.[32]

1.5.1 Expression and Function of Human OPN Isoforms in Pathological Processes

The expression levels of OPN isoforms in tumor subtypes are tissue-specific and their functions may differ.[27] This is demonstrated in OPNa overexpression increasing angiogenesis and vascular endothelial growth factor secretion, while OPNc decreases these same processes in certain types of lung cancer.[31] A recent study also demonstrated that in calcific aortic valve disease (CAVD), OPNa, OPNb, and OPNc are differentially expressed, and different levels of up-regulation of each isoform are seen at various stages throughout the progression of the disease.[33] The Lyle Lab has additionally shown that, in a hind limb model of ischemia, OPN isoforms have differential effects on perfusion recovery in $OPN^{-/-}$ mice. Nonetheless, there is a significant lack of understanding about how each isoform functions to mediate VSMC migration and proliferation or the processes that mediate collateralization.

1.6 Processes of Cell Migration

Several cell types, including fibroblasts, immune cells, epithelial cells, endothelial cells and VSMCs, utilize various actin-based processes for cell migration.[34] Actin polymerization forms a cell protrusion in response to an identified stimulus.[35] The protrusions at the leading edge of the cell are lamellipodia, which are elongated, broad,

and flat extensions. The cell then lays down an integrin-based focal adhesion that interacts with the substrate, while the rear end of the cell releases its adhesions.[36] Filopodium, an additional actin-based structure, extend outside of the leading edge of the cell in a fingerlike manner towards the direction of migration, and these particular protrusions can act as a precursor to a focal adhesion site.[37, 38] In the last phase of cell migration, the remainder of the cell body is pushed forward by contractile stresses that are produced by the interaction of myosin motors on actin filaments.[39] Moreover, focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that is activated during cell migration, binds to focal adhesion proteins, and acts as a signaling protein in focal adhesions.[40] The signaling and localization of FAK has also been shown to be prevalent at lamellipodia.[41] The combination of FAK's roles in focal adhesion and lamellipodium dynamics identifies this signaling protein as an important factor in cell migration mechanisms. In the activation of FAK, the binding of OPN to an integrin receptor results in the phosphorylation of FAK at different tyrosine residues, and secreted OPN, which binds to a CD44 receptor on the cell surface, leads to the downstream activation of FAK.[42, 43] Thus, the phosphorylation of FAK occurs downstream of the activation of both integrin and CD44 receptors, where OPN signals through both of these receptors.[24, 42,43] We intend to identify the specific functional deficits, including lamellipodia development and persistence, filopodia formation, cell adhesions, FAK signaling, or a combination of these processes, that may prohibit proper VSMC migration in the absence of OPN.

Little is known about how the human OPN isoforms differentially influence VSMC migration and is another area of active investigation. A few studies have identified the specific impacts of human OPN isoforms on the proliferation and migration of cancer

cells. Two previous studies have shown that the isoform OPNc and overexpressed isoforms OPNb and OPNc contribute to the invasion, migration, and proliferation of ovarian cancer cells and prostate cancer cells, respectively, through mediation of the PI3K/Akt signaling pathway.[28, 44] However, little is still known about the role of OPN isoforms in cancer cell migration, and there is currently a lack of information about how the human OPN isoforms influence VSMC migration. Greater investigation into the manner by which the isoforms mediate cell migration could allow for the employment of the isoforms as novel therapeutic targets for CVD pathologies.

Methods

2. Methods

2.1 Cell culture

VSMCs isolated from mouse aortas were grown in Dulbecco's Modified Eagle's Media (DMEM; Sigma Aldrich, St. Louis, MO) supplemented with 10% Fetal Bovine Serum (FBS; Invitrogen, Carlsbad, CA), 25 mmol/L HEPES (GE Healthcare Hyclone, South Logan, UT), and 2 mM glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin, all of which were obtained from Invitrogen (Carlsbad, CA). VSMCs were isolated from mouse thoracic aorta by enzymatic digestion, as described previously with a few minor changes as stated herein.[45] Briefly, the aortas were removed, the adventitia, connective tissue, and fat was dissected away and further removed by digestion with collagenase. The aorta was then cut open longitudinally, and the endothelial layer was removed by denudation. The media (minus adventitia and endothelium) was digested with collagenase and elastase to dissociate the media into individual VSMCs. The identity of VSMCs was confirmed by immunostaining with an antibody to smooth muscle α-actin. Cells from passages 4 to 12 were used for all experiments and were cultured in either 10% FBS DMEM, 0.1% FBS DMEM, or serum free DMEM, unless otherwise noted.

2.2 Antibodies

Commercial antibodies were used for immunoblotting: phospho-FAK (Tyr397) and β-Actin (Cell Signaling Technology, Danvers, MA). Rabbit antibody was purchased from Cell Signaling Technology (Danvers, MA), and the Goat Anti-Rabbit IgG (H+L)-HRP Conjugate was obtained from Bio-Rad (Hercules, CA).

2.3 Boyden Chamber Assay

VSMCs were quiesced for 24 hours in serum-free DMEM before 50,000 cells were added to the upper chamber of a transwell plate with 8-μm pores (Costar, Washington, DC). Serum free DMEM (Sigma Aldrich, St. Louis, MO) containing no stimulus or 10 ng/mL platelet-derived growth factor (PDGF; R&D Systems, Minneapolis, MN) was added to the lower chamber. After a duration of four hours in conditions of 5% CO_2 at 37°C, cells that had not migrated were removed, and cells that had migrated were stained with 4', 6-diamidino-2-phenylindole (DAPI; 1 μg/mL; Sigma, Saint Louis, MO) for 10 minutes. Membranes were removed from the transwell and mounted on glass slides using Fluoromount (Fisher Scientific, Pittsburgh, PA). Images were then taken on a Zeiss Axioskop Microscope using an AxioCam CCD high resolution 1.4 megapixel digital/color camera and Zeiss AxioVision, v. 4.8.2 software. Five to eight images from random fields were obtained for each condition, and the number of cells in each image were quantified using ImageJ.

2.4 Scratch Wound Assay

WT and $OPN^{-/-}$ VSMCs were plated at a density of 250,000 cells/cm^2 in 6-well plates (Corning Incorporated, Durham, NC) and grown to 95% confluence. Once 95% confluence was reached, cells were quiesced for 24 hours in 0.1% FBS DMEM. A scratch wound was then made within each well using a p200 pipette tip to create a cross in the middle of the well. Floating cells were removed by washing the cell surface three times with 0.1% serum-containing DMEM. Images were acquired at five different points of the wound for each condition before stimulation (baseline) with 10 ng/mL of PDGF. Images were acquired again at 18 and 24 hours after PDGF stimulation. All images were acquired with an Olympus IX71 Inverted Fluorescence

Microscope using Plan-Fluorite 10x PH1 air objective lens (numerical aperture 0.30 NA), Olympus DP71 single-CCD color camera, and F/DP71 Digital Camera Software. Images were quantified by Elizabeth Iffrig. To calculate the extent of wound closure, images were imported to Matlab (Mathworks, Natick, MA) and converted to a grey-scale image. For each side of the scratch wound, manual contours were defined and then refined by utilizing an interpolated fit for the line in addition to threshold to identify the closest cell edge. Once edges of the wound were determined, approximately 1000 equally spaced lines connecting the two edges and oriented perpendicularly the averaged centerline of the wound were sampled for each image. The average of the distances of each line segment was taken for comparison among each condition and distances were reported in mm.

2.5. Live Cell Imaging with and without Scratch Wound Assay

Live Cell Imaging without Scratch Wound Assay: WT and $OPN^{-/-}$ MASMs were plated at a density of 25,000 cells/cm^2 in 4 chamber glass bottom dishes (Ibidi, Martinsried, Germany) and grown to 95% confluence. Once a confluence of 95% was determined, cells were quiesced for 24 hours in 0.1% FBS DMEM. WT and $OPN^{-/-}$ cells were plated in duplicate, and PDFG (10 ng/mL) was used as a cell stimulus. Images were acquired at four different positions of each well for 10-15 minutes (baseline), prior to stimulation with PDGF (10 ng/mL). After the addition of PDGF, imaging was continued at the same positions for a period of two hours. All live-cell images were acquired with a Leica True Confocal Scanning-Spectral Photometric 5 using 20x air objective lens (numerical aperture 0.70 NA), LAS MicroLab Software, and “Mark and

Find" and "Adaptive Focus" within the Leica software. Imaging files were made into an .avi file and were processed on the Leica software.

Live Cell Imaging with Scratch Wound Assay: WT and OPN$^{-/-}$ MASMs were plated at a density of 25,000 cells/cm^2 in 8 chamber glass bottom dishes (Ibidi, Martinsried, Germany) and grown to 95% confluence. Once a confluence of 95% was determined, cells were quiesced for 24 hours in 0.1% FBS DMEM. A scratch wound was then made within each well using a p10 pipette tip to create a vertical line in the middle of the well. Floating cells were removed by washing the cell surface three times with 0.1% serum-containing DMEM. Images were acquired at one location of the wound for each condition for 15 minutes (baseline) before stimulation with PDGF (10 ng/mL) or human OPNa (hOPNa), human OPNb (hOPNb), and human OPNc (hOPNc) (50 ng/mL). Once PDGF or hOPNa, hOPNb, and hOPNc were added to the appropriate wells, imaging was continued at the same positions and images were acquired every minute for a period of two hours. All live-cell images were acquired with a Leica True Confocal Scanning-Spectral Photometric 5 using 20x air objective lens (numerical aperture 0.70 NA) or 63x oil objective lens (numerical aperture 1.4-0.6 NA), LAS MicroLab Software, and "Mark and Find" and "Adaptive Focus" within the Leica software. Imaging files were converted to an .avi format and were processed on the Leica software. ImageJ software was used to quantify cell velocity (μm/min) and generate kymographs of lamellipodia for each condition. Measurements of length (μm) of protrusions and retractions were made from the kymographs and were inserted into a perl script that was provided by Dr. Holly Williams of the Griendling Lab.

2.6. Recombinant hOPN Protein

Human recombinant protein for the three osteopontin isoforms used (a, b, and c) was purchased through Origene (Rockville, MD). Origene reports that the recombinant OPN isoform proteins were generated using HEK293 cells. The recombinant protein expressed by the cells was then captured using an anti-DDK affinity column, followed by conventional chromatography steps. The recombinant protein was resolved in buffer containing 25 mM Tris.HCl, pH 7.3, 100 mM glycine, 10% glycerol. Recombinant proteins were shipped on dry ice and, upon arrival, aliquoted and stored at -80 until use to maintain any and all post-translational modifications made to the recombinant protein. Cells were stimulated with a hOPNa, hOPNb, or hOPNc at a final concentration of 50 ng/mL for live cell imaging and at the following concentrations for the FAK dose response: 0.1 ng/mL, 1.0 ng/mL, 10 ng/mL and 50 ng/mL.

2.7 Cell Stimulation for Dose Response

Dose Response: $OPN^{-/-}$ MASMs were cultured in 10% FBS DMEM, and cells were split and plated at a density of 75,000 cells/cm^2 in 6-well plates and grown to 50-60% confluence before being quiesced for 48 hours in 0.1% FBS DMEM. 0.1% FBS DMEM was changed every 24 hours during the quiescing period. On the day of stimulation, fresh 0.1% FBS DMEM was added to each well two hours prior to stimulation. Cells were stimulated with the human OPN isoforms at concentrations of 0.1 ng/mL, 1.0 ng/mL, 10.0 ng/mL, and 50.0 ng/mL for 15 minutes.

For the dose response experiment, cells were lysed and scraped, as described previously, in Hunter's Buffer supplemented with protease inhibitors: 0.5 mmol/L PMSF, 0.5 μg/mL Leupeptin, and 10 μg/mL Aprotinin, and all media was transferred to a labeled 1.5 mL Eppendorf tube.[46] Cells were lysed on ice for 20 minutes and cell

samples were sonicated on ice at 4 watts for 6 x 1-s pulses to further disrupt the cell membrane using a 130 Watt Ultrasonic Processor (Sonics & Materials, Inc., Newton, CT). To determine protein concentration, a Bradford assay was performed on whole cell lysates. Samples were processed and used for immunoblotting for FAK phosphorylation.

2.8. Immunoblotting

For WB analysis, proteins were separated using SDS-PAGE and transferred to nitrocellulose membranes, blocked, and incubated with appropriate primary antibodies. Primary antibody pFAK (Y397), at a 1:1000 dilution, was used for immunoblotting and detected by ECL (GE Healthcare, Buckingham, UK).

2.9. Statistical Analysis

The results are expressed as mean ± SEM, as derived from at least three independent experiments. Statistical significance for quantitative results was assessed using ANOVA, followed by the appropriate post hoc analysis. Differences were considered statistically significant at $p<0.05$.

Results

3. Results

3.1 OPN$^{-/-}$ VSMCs Exhibit Impaired Migration

VSMCs participate in the neovascularization process through their ability to proliferate and migrate.[13] Osteopontin has been shown previously to play a role in the migration of other cell types, including endothelial and cancer cells, and OPN acts as a tumor-promoting factor with its overexpression in breast, lung, and stomach cancers.[22, 47] We have shown previously that OPN$^{-/-}$ mice exhibit a significantly impaired ability to generate new vessels, suggesting that OPN may be necessary for the migration of other cell types, such as VSMCs.[23] To determine if OPN is necessary for VSMC migration, we used a modified Boyden chamber system. As shown in **Figure 2**, VSMCs lacking OPN expression exhibit a significantly impaired ability to properly migrate in response to PDGF (10 ng/mL, 4 hours). While these data clearly show that OPN is necessary for proper VSMC migration, we cannot determine from this assay type where the functional deficits lie. Initial live cell migration results showed a possible impairment in sustained lamellipodia formation in OPN$^{-/-}$ VSMCS, but the live cell imaging and Boyden chamber assay experiments lacked a proper degree of directionality. Therefore, we decided to optimize a method for migration that would give cells directionality and allow us to identify specific functional deficits.

To give the VSMCs a directional signal in conjunction with a migratory stimulus (PDGF), we utilized a scratch wound assay. This assay would allow us to further test the effects of the absence of OPN on the migration of MASMs with directionality, since the cells migrate toward the empty space left by the scratch. For these experiments, cells were grown to a proper confluence of 90-95%, quiesced, and a scratch wound

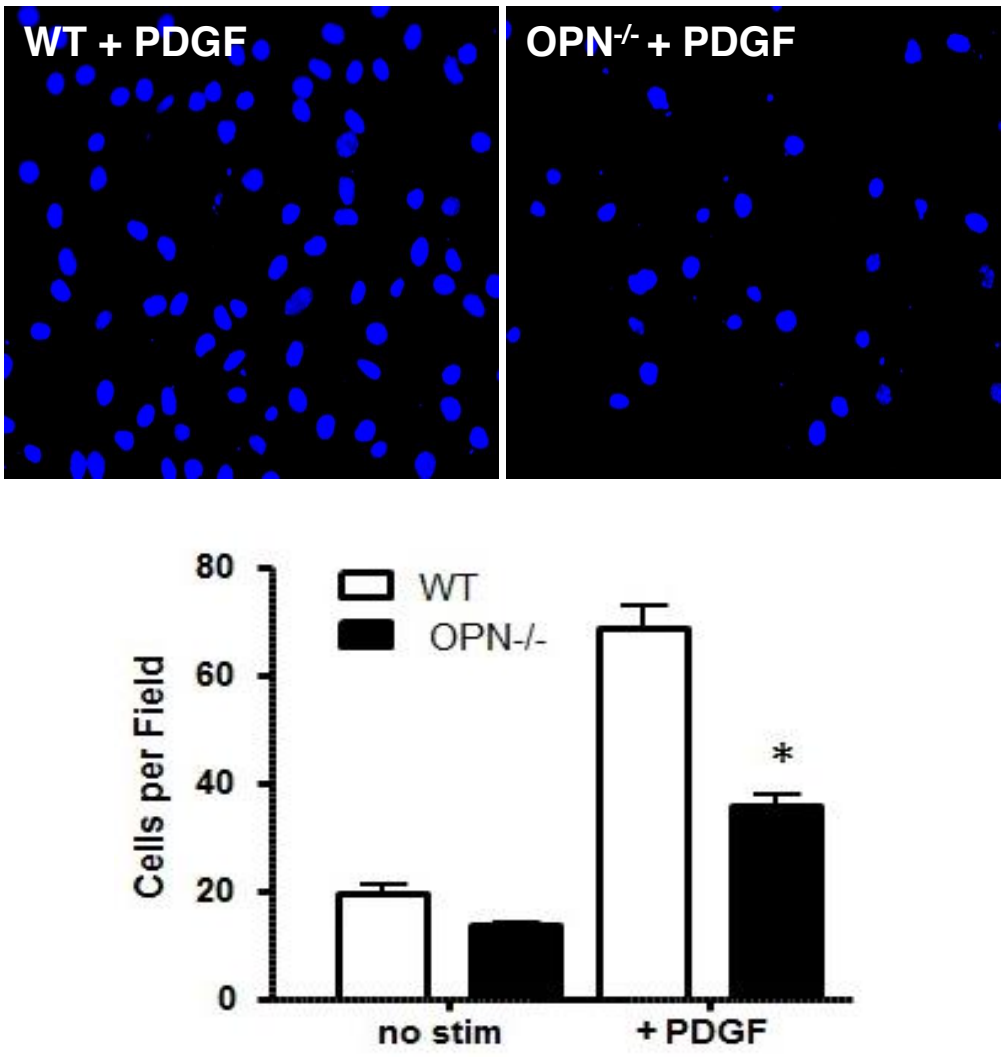

Figure 2. OPN$^{-/-}$ VSMCs exhibit impaired PDGF-induced migration compared to WT VSMCs. Migration of WT and OPN$^{-/-}$ VSMCs was analyzed with and without PDGF stimulation. Cells were quiesced for 24 hours before addition to the upper chamber of a transwell plate. DMEM +/- 10 ng/mL PDGF was added to the lower chamber. Cells that migrated after 4 hours were stained with 4', 6-diamidino-2-phenylindole (DAPI; 1 µg/mL). 6-8 images were taken on a Zeiss Axioskop microscope for each experiment and quantified and averaged in ImageJ for 3 independent experiments. n=3, *p<0.001.

was performed. Images were acquired at baseline and at 18 and 24 hours post-stimulation with 10 ng/mL of PDGF. We observed that $OPN^{-/-}$ VSMCs display impaired migration in comparison to WT VSMCs through the quantification of the distance from edge to edge (mm) of each wound (**Figure 3**). In baseline imaging without PDGF, there was a similar distance of the wound between WT and $OPN^{-/-}$ VSMCs. However, once PDGF was added, the distance of the wound for WT cells, but not $OPN^{-/-}$ cells, became significantly smaller after 24 hours (from a distance of 0.796 to 0.425 mm), indicating a 53 percent wound closure. Additionally, with n=3, there is a trend that cells with a lack of OPN do not close the wound as efficiently as WT cells, suggesting a potential deficit in cell polarization or some other process necessary for directional migration. Having established that the scratch wound method showed similar impaired migration in $OPN^{-/-}$ VSMCs and allowed for the cells to sense a direction, we revisited the use of live cell imaging and incorporated the use of the scratch wound method.

3.2 $OPN^{-/-}$ VSMCs May Exhibit Impaired Lamellipodia Protrusion and Persistence

Initial attempts to look at the functional deficits by live cell imaging were flawed because of a lack of a direction for the movement of the VSMCs. The cells utilized in preliminary live cell imaging produced protrusions, but VSMCs primarily remained in one area of the plate (**Figure 4**). We had hoped to establish why $OPN^{-/-}$ VSMCs migrate poorly and to determine where the functional deficits lie in $OPN^{-/-}$ cells that prevent proper migration, by using live cell imaging. And, while we were able to observe trends in lamellipodia protrusion and persistence, the cells used with this method lacked directionality.

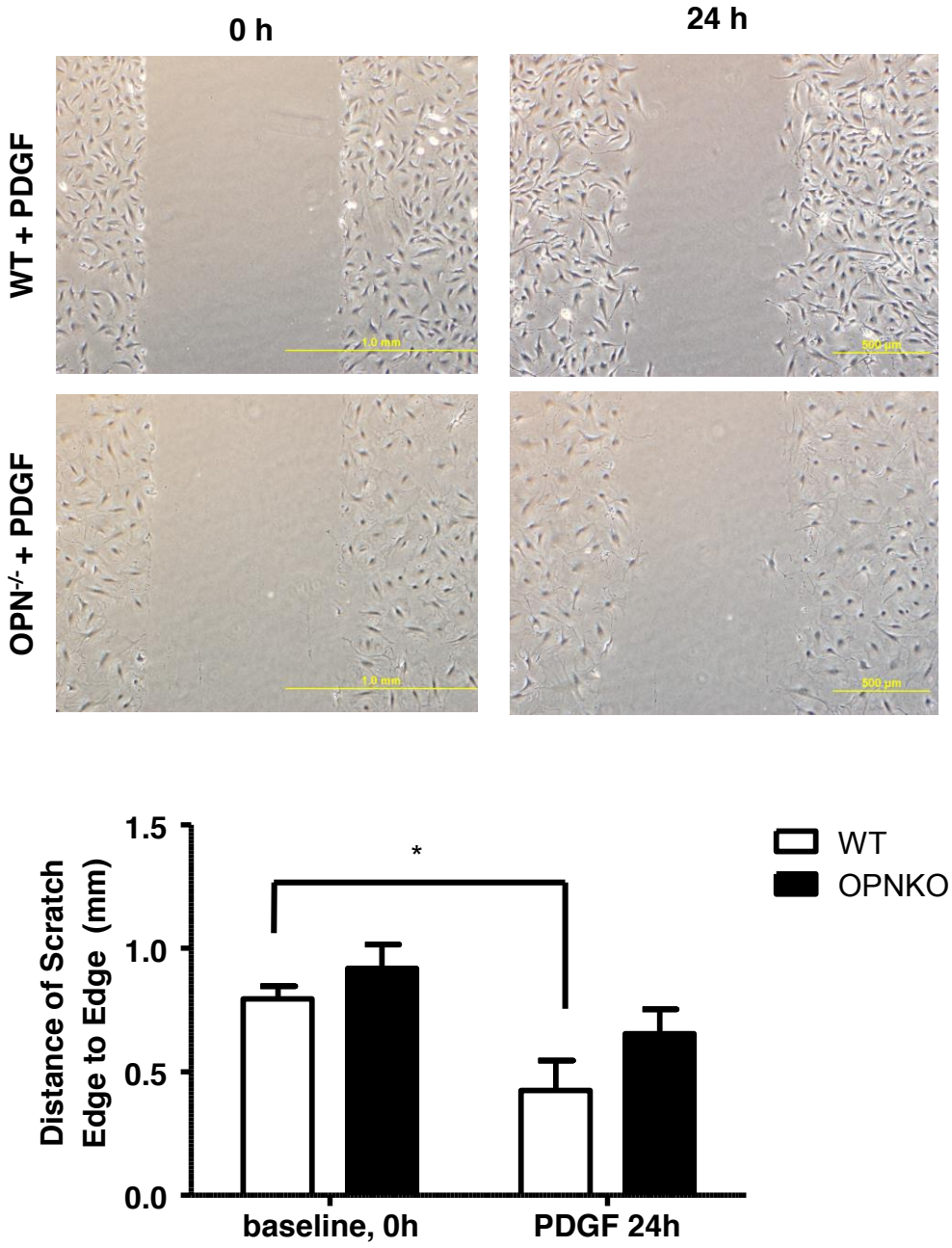

Figure 3. OPN$^{-/-}$ VSMCs display impaired migration compared to WT VSMCs with PDGF stimulation using a scratch wound assay. WT and OPN$^{-/-}$ VSMCs were plated and serum deprived for 24 hours prior to making a scratch wound. Images in five sections of the wound were taken to establish the 0h baseline. PDGF was then added at a final concentration of 10 ng/mL. Images were acquired at baseline, 18 hours, and 24 hours. Representative images from 0h and 24h are shown. The images were quantified using Matlab, and the distance from edge to edge of the scratch was calculated for each image. n=3, *p<0.05.

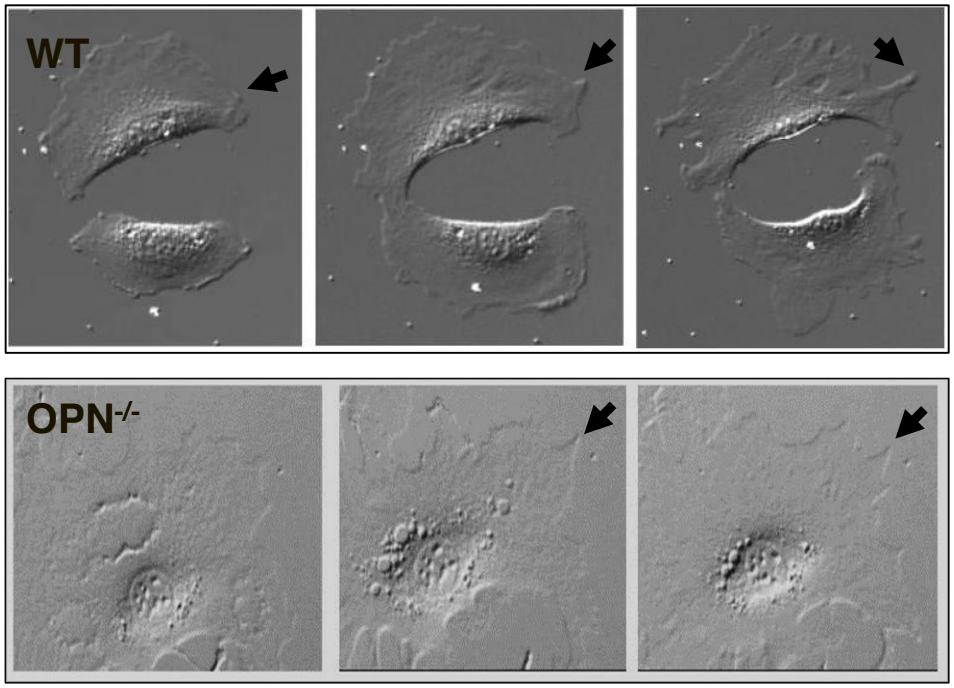

Figure 4. WT MASMs, in response to PDGF, exhibit normal migration and OPN$^{-/-}$ MASMs demonstrate functional deficits in migration. WT and OPN$^{-/-}$ VSMCs were prepared to 95% confluence and serum deprived for 24 hours prior to acquiring images on a Leica True Confocal Scanning-Spectral Photometric 5. Images were acquired every minute for 10-15 minutes (baseline). PDGF (10 ng/mL) was added and imaging continued for two hours. The left-hand image represents the beginning of the two hours, the center image shows imaging taken at 60 minutes of the two hours, and the right-hand image is at the end of the two-hour period.

Therefore, we revised our method for live cell imaging and incorporated the scratch wound assay used above to establish directionality. Theoretically, this would allow us to be able to better quantify specific functional deficits in the cell migration process at the leading edges of the cells that were not able to be captured by the previous methods used. Migration, in particular, is dependent upon a number of factors including the initial protrusion of the plasma membrane, known as a lamellipodium, and the enlargement of that initial extension into a fillopodia. These protrusions are stabilized through the formation of adhesive complexes called focal complexes. As a cell begins to migrate, these focal complexes at the front of the cell strengthen into larger, more organized focal adhesions that serve as a point of traction over which the cell body moves. For the cell to make forward progression, it must release its rear adhesions to allow a net forward displacement.

To be able to visualize and quantify these processes in real time, we utilized the live cell imaging and scratch wound assays together. WT and $OPN^{-/-}$ cells were grown to sub-confluence, and a scratch wound was made in the monolayer. Baseline images were acquired for a period of 15 minutes for each cell type at positions chosen with "Mark and Find." Cells were then stimulated with PDGF, and the same positions were imaged every 10-15 minutes for the next two-hours. Each of these images was assembled into a movie file and, through careful examination, we observed a relatively consistent impact of OPN deficiency on the cells' ability to migrate that seemed to be due to a lack of consistent lamellipodia formation and persistence (**Figure 5**). To quantify this, we measured and analyzed the length and number of lamellipodial protrusions and retractions through the generation of kymographs in ImageJ software.

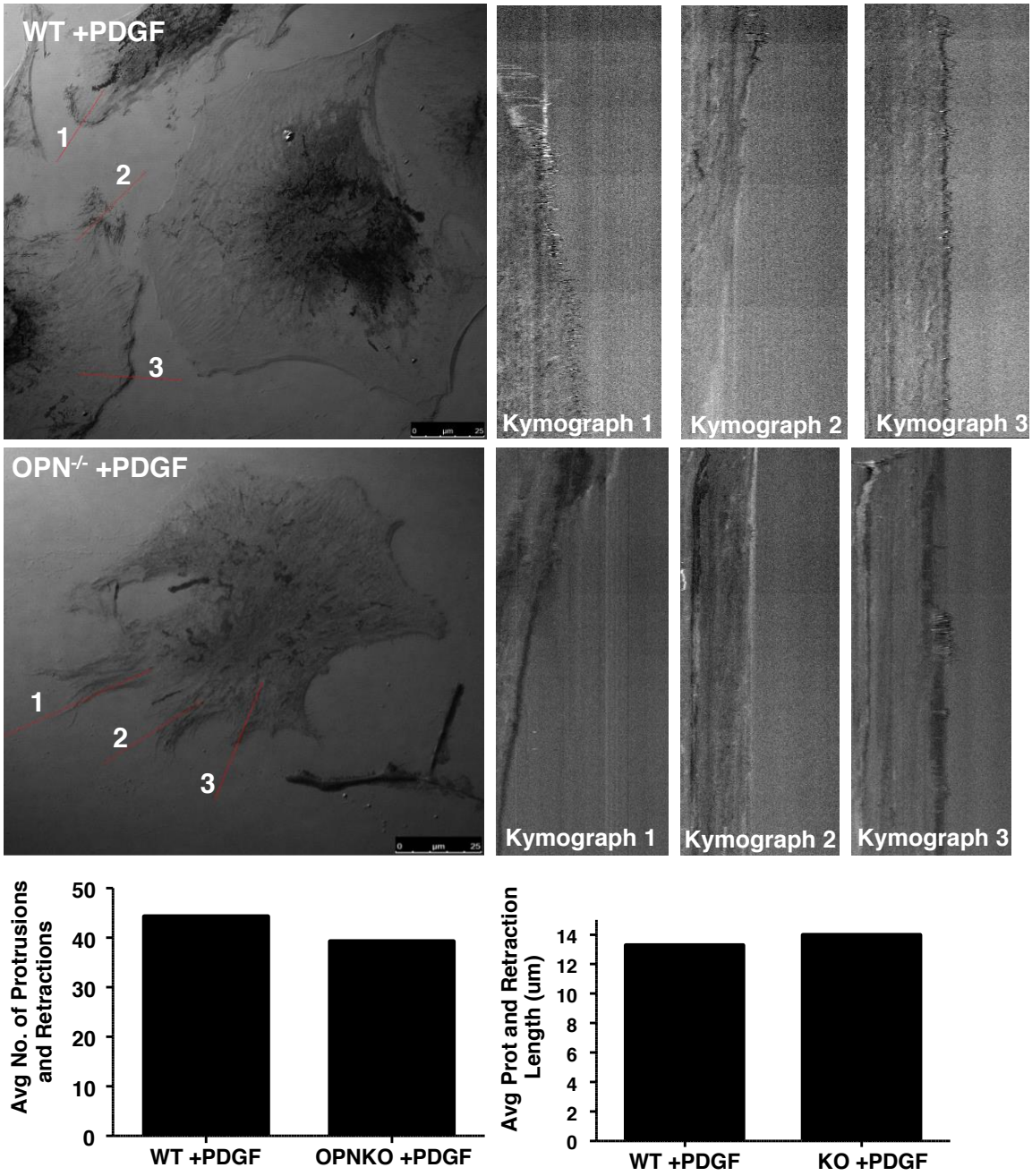

Figure 5. OPN$^{-/-}$ VSMCs may exhibit impaired lamellipodia protrusion and persistence in comparison to WT VSMCs. WT and OPN$^{-/-}$ VSMCs were plated in an 8-well chamber plate and serum deprived for 24 hours prior to making a scratch wound. Imaging was acquired on a Leica True Confocal Scanning-Spectral Photometric 5. Images were taken every 10 seconds for 15 minutes to establish the baseline. PDGF (10 ng/mL) was added to stimulate cells, and imaging continued for two hours. Kymographs of lamellipodia were generated from each corresponding numbered line with ImageJ in order to quantify the length and number of protrusions retractions the cells generated over two hours. n=1.

In our preliminary findings, we found the number of protrusions and retractions to be slightly higher in WT VSMCs compared to OPN$^{-/-}$ VSMCs, but there was no difference in the length of these protrusions or retractions between the two cell types.

3.3 OPN$^{-/-}$ VSMCs Migration is Rescued by Human OPN Isoforms a and c

Humans, unlike rodents, express three OPN isoforms as a result of alternative mRNA splicing. However, nothing is known about how these different OPN isoforms influence VSMC migration. Unpublished data from the Lyle Lab showed that OPN isoforms a, b, and c exhibit differential effects on neo-vascularization in vivo, a process that involves VSMC proliferation and migration. To determine if OPN isoform a, b, and/or c could rescue OPN$^{-/-}$ VSMC migration, we again used the Leica microscope and live cell imaging with a scratch wound assay set up as described above. Images were again captured at set positions chosen with “Mark and Find” to serve as the baseline. WT and OPN$^{-/-}$ MASMs were then stimulated with either PDGF (10 ng/mL, as a positive control) or 50 ng/mL of each hOPN isoform (a, b, or c) in the form of recombinant protein. We continued to image each set position chosen with “Mark and Find” every minute for an additional two hours. On the basis of cell velocity (μm/min), we found that isoforms hOPNa and hOPNc restored the migration of OPN$^{-/-}$ VSMCs in comparison to both the baseline migration of OPN$^{-/-}$ VSMCs that were absent of treatment with the isoforms and the migration of OPN$^{-/-}$ VSMCs with PDGF (**Figure 6**). To identify the mechanisms underlying these differential effects of the hOPN isoforms on OPN$^{-/-}$ VSMCs, we then examined a component of cell signaling in these cells.

In order for cells to migrate forward in a direction towards a stimulus, the cells must form protrusions. These extensions of the cell membrane are lamellipodia, and

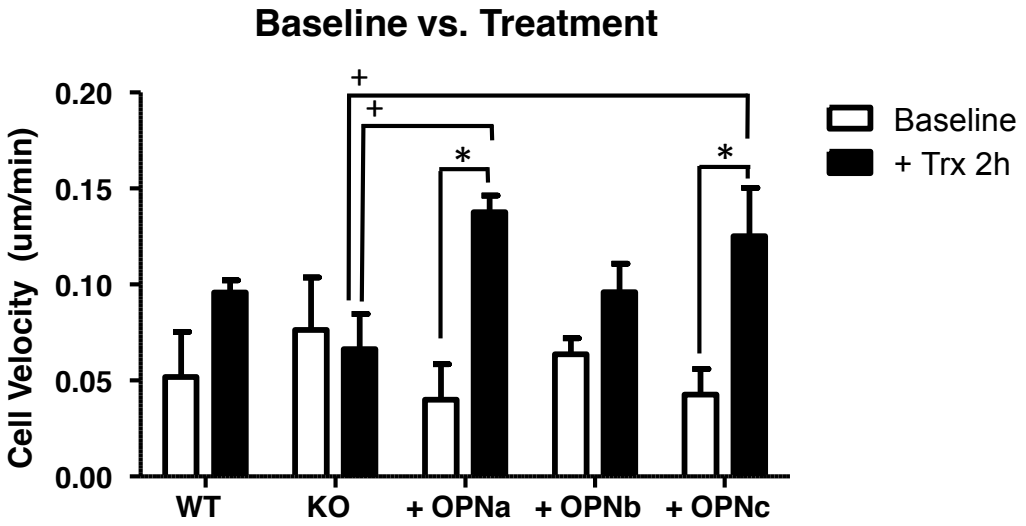

Figure 6. OPN$^{-/-}$ VSMCs migration is rescued by human OPN isoforms a and c. WT and OPN$^{-/-}$ VSMCs were plated in an 8-well chamber plate and serum deprived for 24 hours prior to making a scratch wound. Imaging was acquired on a Leica True Confocal Scanning-Spectral Photometric 5. Images were taken every minute for 15 minutes to establish the baseline. PDGF (10 ng/mL) and isoforms, hOPNa, hOPNb, and hOPNc (50 ng/mL), were added to stimulate cells, and imaging continued for two hours. Cell velocity (μm/min) was calculated using "Manual Tracking" in ImageJ. n=3-4, *$p<0.01$, $^{+}p<0.05$.

they assemble at the leading edge of the cell.[35] Due to the necessity of an interface between the internal complexes of the cell and the extracellular matrix, the cells require an anchor that will allow for the persistence of the lamellipodia and for a consequent shift of the cell body.[36] The anchors for these migrating cells are focal contacts that mature to focal adhesions. For this maturation process to occur, cells require activation of focal adhesion kinase (FAK), which is specifically phosphorylated at Y397.[40] Without the phosphorylation at this site and subsequent activation of FAK, cells will be compromised in their ability to form lamellipodia that persist throughout the progression of migration.

3.4 Effects of Human OPN Isoforms on FAK Phosphorylation

The initial protrusion of the lamellipodia can only persist if the protrusion is stabilized through the formation of focal contacts and the maturation of these complexes into focal adhesions. Therefore, we hypothesized that the impaired lamellipodial persistence was due to a lack of focal contact formation and/or maturation. We next examined what happens to activation of FAK, as specifically assayed by phosphorylation of Y397, which is a necessary event for focal contact and adhesion formation.

Prior to looking at FAK signaling, we first performed a dose response experiment to attempt to identify the proper concentration of OPN isoforms to use for our cell signaling experiments. For the dose response experiments, $OPN^{-/-}$ MASMs were grown to proper sub-confluence and quiesced for 48 hours before stimulation with recombinant hOPN isoform a, b, or c at the following concentrations: 0.1, 1.0, 10.0, and 50.0 ng/mL. We observed that the cells showed a large degree of variation in FAK phosphorylation

at Y397 in response to the isoforms within and between concentration (**Figure 7**), making it difficult to select a proper dose for the remaining signaling experiments. However, a recent paper published in early 2016 shows that in Type 1 Diabetes patients with CVD complications, OPN serum levels range from 30-200 ng/mL.[48] In addition, a dose response experiment on macrophage migration (unpublished data from the Lyle lab) demonstrates improved migration at an ED50 of 50 ng/mL. Taking these results and the latest literature into consideration, we will utilize a concentration of 50 ng/mL, which falls within the known range of 30-200 ng/mL in CVD patients, for future FAK phosphorylation and signaling experiments and VSMC migration studies. Based on the data presented, it is difficult to conclude if the effects of the human OPN isoforms on lamellipodia extension and persistence are, indeed, due to changes in focal contact formation and/or maturation and these endpoints will be further explored in the future.

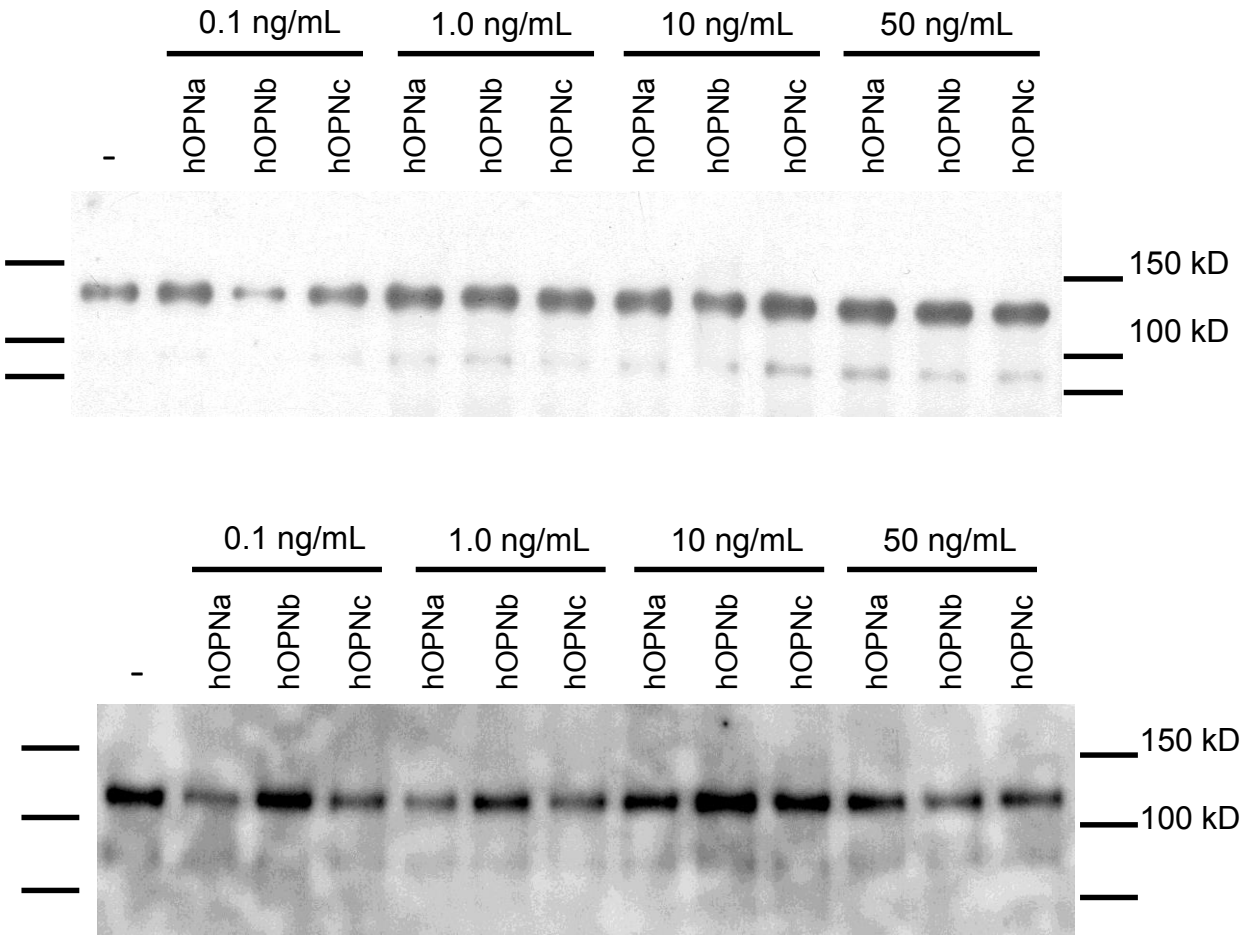

Figure 7. hOPN Isoforms differentially influence FAK phosphorylation in OPN$^{-/-}$ VSMCs in a dose response. The phosphorylation of FAK at Y397 was assessed in WT and OPN$^{-/-}$ MASMS by western blot. A dose response was conducted in which OPN$^{-/-}$ MASMs were stimulated with 0.1, 1.0, 10.0, and 50.0 ng/mL of hOPNa, hOPNb, and hOPNc for 15 minutes in 6-well plates. Protein was harvested to assess Y397 FAK phosphorylation (n=2).

Discussion

4. Discussion

Osteopontin is a key, matricellular inflammatory protein fundamental to the migration of vascular smooth muscle cells, collateral formation, and tissue reperfusion in relation to cardiovascular disease pathologies.[9] Three isoforms of OPN exist and each is involved in various pathological states in a distinct manner.[27] However, the expression of the isoforms in the presence of CVD and, conversely, how each isoform acts to mediate VSMC migration and collateralization remains largely unknown. The aim of this study was to determine the functional deficits that underlie impaired VSMC migration in the absence of OPN, and to identify the differential effects of the hOPN isoforms on VSMC migration. Through acquiring greater knowledge of how hOPN isoforms control migratory processes, application of hOPN isoforms as therapeutic agents in cardiovascular disease may be utilized in the future. In this study, we provided evidence that the absence of OPN acts to impair VSMC migration in wound closure (**Figure 3**). We show that irregularities in lamellipodia formation and persistence, potentially due to focal adhesion formation or maturation, may play a role in the normal migratory function of these cells (**Figures 4, 5**).

We initially examined the differences in migration of VSMCs, particularly MASMs, with and without OPN utilizing a modified Boyden chamber assay (**Figure 2**). Our results indicated that $OPN^{-/-}$ VSMCs have impaired PDGF-induced migration in comparison to WT VSMCs. Further investigation using a scratch wound assay, which provides a directional stimulus for the cells, shows an increased size of the wound in $OPN^{-/-}$ compared to WT VSMCs, demonstrating decreased wound closure in $OPN^{-/-}$ VSMCs (**Figure 3**). We established with the combination of the methods of a Boyden

chamber assay and a scratch wound assay that a deficiency in OPN leads to impaired migration in VSMCs.

The proliferation and migration of VSMCs is necessary in the processes of collateral formation and arteriogenesis, and OPN is concurrently involved in mediating VSMC migration and collateral formation.[8, 13, 16, 23] Recent studies conducted by the Taylor Lab have also shown that OPN expression is significantly increased in response to ischemia.[23, 24] Furthermore, we demonstrate that OPN is required for proper VSMC migration, but with our previous methods of a Boyden chamber assay and a scratch wound assay, we lacked the ability to understand the specific deficits that cause this impairment in the VSMCs. Whether there are particular components of the migratory process that may differ in cells without OPN compared to WT cells is a point of further investigation in this study.

The scratch wound assay we employed provided the VSMCs directionality that would allow us to identify specific functional deficits that impede $OPN^{-/-}$ cells that we could combine with preliminary live cell imaging we conducted on WT and $OPN^{-/-}$ cells. We saw that an OPN deficit conceivably acts to inhibit focal adhesion formation or development, lamellipodia continuance vital in migration, or a combination of these processes (**Figure 4**). In VSMCs, lamellipodia formation and persistence are critical for cell migration, and focal adhesions allow for the continuation and progression of VSMC movement.[35, 36] We then utilized the established directionality that a scratch wound assay provides in combination with live cell imaging of WT and $OPN^{-/-}$ cells. We observed that $OPN^{-/-}$ VSMCs generate lamellipodia, but we speculate that either these protrusions do not persist or focal adhesions are not established for proper cell

migration (**Figure 5**). The analysis of lamellipodia in WT and OPN$^{-/-}$ VSMCs indicate that the number of protrusions and retractions may differ between the cells and show that the factor of correct lamellipodia production and its consistency could serve as one mechanism that impedes the migration of VSMCs to close a wound. Additional studies will need to be conduced in order to more comprehensively identify the functional deficits in OPN$^{-/-}$ VSMCs.

Previous studies have determined that lamellipodia formation and its continuation are critical steps in the cell migration process.[35, 36] The lamellipodia can persist and allow for the movement of the cell body only with the stability that focal contacts, which mature into focal adhesions, provide these protrusions.[36, 40] Despite the fact that we observed differences in OPN$^{-/-}$ VSMCs in the degree of lamellipodia formation and persistence compared to WT VSMCs in PDGF-induced migration, we lack an understanding into the several other factors that could contribute to the impairment in the migration of OPN$^{-/-}$ cells. We additionally investigated whether cell signaling, specifically phosphorylation at the Y397 site of FAK, could be a mechanism to explain the deficit of focal adhesion constancy, but the interactions of OPN with the cell surface receptors of integrin and CD44 could also be a factor into the deficiency in migration.[41,49]

We again utilized live cell imaging of a scratch wound assay and added each human OPN isoform, OPNa, OPNb, or OPNc, to OPN$^{-/-}$ VSMCs to identify whether the isoforms rescue impaired migration in these cells. From this method, we identified that treatment with hOPNa and hOPNc rescued the migration of OPN$^{-/-}$ VSMCs (**Figure 6**). In comparison to baseline cell velocity in OPN$^{-/-}$ VSMCs, the addition of the hOPNa and

hOPNc to these cells significantly improved their ability to migrate. Moreover, the incorporation of the isoforms (50.0 ng/mL) to OPN$^{-/-}$ VSMCs did not initially seem to lead to observed differences in terms of the amount of phosphorylation of FAK at the site of Y397 (**Figures 7**). However, due to the high degree of variability observed, these experiments will need to be repeated to determine if each OPN isoform may in fact decrease or increase the amount of phospho-FAK in OPN$^{-/-}$ VSMCs. Additional analysis of live cell images at 63x may also help to identify additional targets downstream of OPN-mediated VSMC migration.

The bulk of the understanding behind OPN isoforms comes from the cancer literature, in which several studies note divergences in the functions and expression levels of the isoforms in cancer subtypes.[28-31] Unpublished data from the Lyle lab concurrently shows that the isoforms have differential effects on collateral formation in response to ischemia *in vivo*. The RGD and CD44 binding domains remain intact in all three isoforms and allow for the potential to bind to integrin and CD44 cell surface receptors, thus integral post-translational modifications of the isoforms may play a significant role in the folding of OPN and how these isoforms function in various pathological processes.[25] Our studies signify that the isoforms may hold the ability to restore specific impairments in OPN$^{-/-}$ VSMCs that prohibit proper cell migration, but they may not show differential effects of each isoform in the activation of FAK and, conversely, on focal adhesion assembly. Whether the isoforms affect downstream cell signaling targets other than FAK or whether the post-translational modifications affect additional cell processes, such as filopodia formation, are points of further investigation

to fully characterize the specific manner that human OPN isoforms affect VSMC migration.

In conclusion, the data presented herein support that OPN is required for vascular smooth muscle cell migration. A lack of OPN in VSMCs gives way to functional deficits in migration compared to WT cells. This migration deficit may stem from a malfunction in the frequency of lamellipodia formation and persistence, focal adhesion development, or a combination of these or other migratory processes. A more expansive understanding of the role of hOPN isoforms in VSMC migration and the deficits that underlie impeded migration is critical in the utilization of OPN isoforms as therapeutic agents in cardiovascular disease.

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