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## Osteopontin Isoforms Differentially Promote Arteriogenesis in Response to Ischemia via Macrophage Accumulation and Survival

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### Abstract

**Objective**—Osteopontin (OPN) is critical for ischemia-induced neovascularization. Unlike rodents, humans express 3 OPN isoforms (a, b, and c); however, the roles of these isoforms in post-ischemic neovascularization and cell migration remain undefined. Our objective was to determine if OPN isoforms differentially affect post-ischemic neovascularization and to elucidate the mechanisms underlying these differences.

**Approach and Results**—To investigate if human OPN isoforms exert divergent effects on post-ischemic neovascularization, we utilized OPN<sup>-/-</sup> mice and a loss-of-function/gain-of-function approach *in vivo* and *in vitro*. In this study OPN<sup>-/-</sup> mice underwent hindlimb ischemia surgery and  $1.5 \times 10^6$  lentivirus particles were administered intramuscularly to overexpress OPNa, OPNb or OPNc. OPNa and OPNc significantly improved limb perfusion  $30.4\% \pm 0.8$  and  $70.9\% \pm 6.3$ , respectively, and this translated to improved functional limb use, as measured by voluntary running wheel utilization. OPNa and OPNc treated animals exhibited significant increases in arteriogenesis, defined here as the remodelling of existing arterioles into larger conductance

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Online Supplement: Includes a detailed Materials and Methods section and Supplemental Figures.

### Disclosures

None.

arteries. Macrophages play a prominent role in the arteriogenesis process and OPNa and OPNc treated animals showed significant increases in macrophage accumulation *in vivo*. *In vitro*, OPN isoforms did not affect macrophage polarization, whereas all 3 isoforms increased macrophage survival and decreased macrophage apoptosis. However, OPN isoforms exert differential effects on macrophage migration, where OPNa and OPNc significantly increased macrophage migration with OPNc serving as the most potent isoform for macrophage migration.

**Conclusions**—Human OPN isoforms exert divergent effects on neovascularization through differential effects on arteriogenesis and macrophage accumulation *in vivo* and on macrophage migration and survival, but not polarization, *in vitro*. Altogether, these data support that human OPN isoforms may represent novel therapeutic targets to improve neovascularization and preserve tissue function in patients with obstructive artery diseases.

### Keywords

Peripheral Artery Disease; Human Osteopontin Isoforms; OPNa; OPNb; OPNc; Limb Ischemia; Neovascularization; Collateral Vessel Formation; Arteriogenesis; Macrophage; Migration; Cell Survival; Inflammation

## INTRODUCTION

Coronary artery and peripheral artery disease (CAD and PAD) remain leading causes of mortality in the United States and PAD is increasingly recognized as a growing public health concern and affects >200 million individuals worldwide.<sup>1</sup> CAD and PAD both result in tissue ischemia downstream of vessel occlusions, often the result of atherosclerosis. The body's physiologic response to ischemia is to increase the development of new functional collateral blood vessels to bypass sites of obstruction, restore blood flow and preserve tissue function. To do this, collateral vessels must increase their diameter and number over a very short time period. The formation of collateral vessels is an active, multifactorial, and incompletely understood process involving cell proliferation, inflammatory cell accumulation and vascular remodelling.<sup>2-4</sup> The principle processes that contribute to collateral vessel formation in patients with obstructive arterial disease are defined as follows: 1) vasculogenesis is *de novo* blood vessel formation through the homing and differentiation of endothelial precursor cells to form new capillary plexuses, 2) angiogenesis, which requires sprouting of endothelial cells from existing vessels to form new capillary growth, followed by arterialization with vascular smooth muscle cells (VSMCs), and 3) arteriogenesis, which involves the outward remodelling and enlargement of existing arterioles into larger conductance arteries.<sup>5</sup> However, the ability of patients with PAD to form collateral vessels is often suboptimal or impaired.<sup>6,7</sup> Importantly, a patient's ability to develop collateral blood vessels to bypass sites of occlusion improves patient outcomes and survival.<sup>8</sup>

While many therapeutic strategies focus on promoting angiogenesis, these *de novo* vessels are ultimately more sustainable and functional through arterialization, which requires VSMCs. Furthermore, arteriogenesis can also be achieved via the outward remodelling and enlargement of existing arterioles into larger conductance arteries and this arteriogenesis process is heavily dependent on macrophages.<sup>9,10</sup> Macrophages that infiltrate into the

ischemic region function to promote extracellular matrix (ECM) remodeling and turnover and the production of a variety of growth factors and chemokines that facilitate the arteriogenesis process.<sup>9,11,12</sup> Treatment options for patients with PAD are currently limited to surgical interventions such as angioplasty, bypass, and, in severe cases, limb amputation and the annual costs associated with PAD patient hospitalizations alone are ~\$21 billion.<sup>13,14</sup> Therefore, understanding the mechanisms that promote new collateral vessel formation is necessary to identify targets for the potential development of novel, non-surgical therapeutic approaches to treat patients with PAD.

Osteopontin (OPN) is a secreted matricellular inflammatory cytokine. It is a complex, highly post-translationally modified, secreted protein with 5 O-glycosylated residues, 36 predicted phosphorylation sites, and integrin and CD44 receptor binding sites in exons 6 & 7, respectively.<sup>15–20</sup> We previously demonstrated that OPN is highly upregulated in response to ischemia and is a critical mediator of post-ischemic neovascularization *in vivo*.<sup>21</sup> Multiple cell types upregulate OPN in response to ischemia, including endothelial cells, smooth muscle cells, and macrophages.<sup>21</sup> Additionally, OPN expression is increased in numerous ischemic pathologies including ischemic stroke,<sup>22</sup> myocardial infarction,<sup>23,24</sup> and peripheral artery disease.<sup>25,26</sup> OPN primarily signals through integrin receptors and CD44 and has been linked to cell survival, proliferation, migration and adhesion, all of which are required for collateral vessel formation.<sup>27–30</sup> Importantly, OPN<sup>-/-</sup> animals are defective in collateral vessel formation and macrophage accumulation in response to ischemia.<sup>31</sup> Altogether, these data support that OPN is fundamental to the neovascularization process; however, the mechanisms by which OPN regulates collateral vessel formation remain poorly defined.

Humans express multiple OPN isoforms as the result of alternative splicing of a single mRNA transcript that yields: 1) OPNa, the full-length isoform, 2) OPNb, which lacks exon 5, and 3) OPNc that lacks exon 4 (see NCBI annotation for GeneID 6696).<sup>32,33</sup> Variants of human OPN were first described in 1990<sup>34</sup> and were later identified as OPN isoforms in human glioma cells<sup>33</sup> and have since been shown to be differentially expressed in various tumor subtypes and exhibit isoform-specific functions.<sup>32,35–43</sup> Studies that have investigated the role of OPN in cardiovascular disease (CVD) primarily focus on total OPN expression<sup>44–46</sup> and do not identify expression levels for specific OPN isoforms, with a few studies in patients with calcific aortic valve disease.<sup>47,48</sup> Furthermore, the OPN isoforms expressed in ischemic tissues and the functional roles the OPN isoforms in the process of collateral vessel formation and cell migration, necessary for collateral vessel formation, are unknown. What we do understand about the role of OPN in collateral vessel formation has been established in rodents,<sup>21,31,49,50</sup> which express the equivalent of OPNa. We previously reported that murine OPN is upregulated ~20-fold (mRNA) in response to ischemia<sup>21</sup> and OPN<sup>-/-</sup> mice exhibit an innate inability to collateralize in response to an ischemic insult.<sup>31</sup> Therefore, to experimentally determine if human OPN isoforms have differential effects on collateral vessel formation *in vivo*, we utilized OPN<sup>-/-</sup> mice and a loss-of-function/gain-of-function approach so the only OPN in the system is what we add back in the way of individual human OPN isoforms. Altogether, this approach allowed us to interrogate how each OPN isoform functions independently to influence collateral vessel formation *in vivo*.

The aims of this study were to determine if and how OPN isoforms differentially promote collateral vessel formation and cell migration *in vivo* and *in vitro*. In this study, we demonstrate that human OPN isoforms differentially promote collateral vessel formation *in vivo* and that OPNa (full length) and OPNc (lacks exon 4) are positive regulators of post-ischemic arteriogenesis. The mechanism by which OPNa and OPNc exert their pro-arteriogenic effects *in vivo* are, in part, through their ability to increase macrophage accumulation/migration and macrophage survival. The data presented herein support that specific OPN isoforms may serve as novel therapeutic targets to improve arteriogenesis in patients with obstructive artery disease pathologies, such as PAD.

## MATERIALS and METHODS

A detailed Materials and Methods section is available in the Online Supplement.

### Animals

OPN<sup>-/-</sup> (stock #004936) and C57Bl/6J mice (stock #000664) were purchased from Jackson Laboratories (Maine, USA). All animals used were male and 8 to 10 weeks old. Animals were housed and cared for according to the Emory University Institutional Animal Care and Use Committee (IACUC) and all studies were approved by the Emory University IACUC.

### Hindlimb Ischemia Surgery

To assess how each human OPN isoform affects neovascularization, OPN<sup>-/-</sup> and C57Bl/6 (WT) mice underwent hindlimb ischemia (HLI) surgery, as reported previously.<sup>21,31</sup> Briefly, animals were subjected to ligation and excision of the left superficial femoral artery to induce hindlimb ischemia. For animals that received treatment with human OPNa, human OPNb, or human OPNc, 1.5×10<sup>6</sup> lentivirus particles expressing GFP (negative control) or c-terminally Myc-tagged human OPNa, OPNb or OPNc were delivered at the time of HLI surgery in a final volume of 20 µl across 4 sites (5 µl/site) of the adductor muscle by intramuscular (IM) injection (see Online Figure 1A) using a 25 µl micro-syringe and a custom made 27 gauge, 0.5 inch, small hub needle with a 30° bevel tip (cat# 7643-01; Hamilton, Reno, NV).

### LASER Doppler Perfusion Imaging

LASER Doppler perfusion imaging (LDPI) was used to assess perfusion at 4, 7 and 14 days post-surgery for each treatment group, as described previously.<sup>21</sup> Briefly, mice were anesthetized by inhalation of 2% isoflurane and were scanned using the LDPI system (PIM II Laser Doppler Perfusion Imager). Perfusion of the ischemic limb (IL) and non-ischemic limb (NIL) was assessed. Perfusion of the IL was quantified and normalized to the animal's contralateral NIL.

### Quantitative Assessment of Functional Limb Use by Voluntary Running Wheel

Seven days post-HLI, animals were individually housed single activity wheel cages (Model# 80820; Lafayette Instruments, Lafayette, IN), as described previously.<sup>51</sup> For the duration of the functional limb assessment, animals were given free access to food, water, and activity wheel cages were housed in a behavioural suite with controlled temperature and regular

light/dark cycles. After acclimation for 1 night, the distance run (meters) on the activity wheels by each animal was recorded daily for 7 days and nights.

### Lentivirus

The lentivirus vector used was a dual promoter vector from Gentarget, Inc. (San Diego, CA). The expression of human osteopontin isoform a, b, or c, each Myc- and DDK-tagged at the C-terminus, was driven by a constitutively active cytomegalovirus (suCMV) promoter. A constitutively active respiratory syncytial virus (RSV) promoter drove expression of the antibiotic-fluorescent fusion dual marker green fluorescent protein (GFP)-Blasticidin. We previously demonstrated that multiple cell types express OPN in response to ischemia;<sup>21</sup> therefore, a lentivirus (LV) without a tissue specific promoter was utilized to overexpress each OPN isoform. Lentivirus was provided by Gentarget at a final titre of  $\sim 1 \times 10^8$  infectious units per mL in sterile 1X PBS, 10X polybrene. For *in vivo* use, lentiviruses were diluted in sterile saline to deliver  $1.5 \times 10^6$  lentivirus particles by intramuscular injection to overexpress GFP (negative control) or OPNa, OPNb or OPNc. Lentivirus treatment occurred at the time of HLI surgery, as described in the “Hindlimb Ischemia Surgery” method section.

### Antibodies

The Myc-tag rabbit monoclonal antibody from Cell Signaling Technology, Inc. (clone 71D10, cat# 2278; Danvers, MA) was used for immunofluorescence staining of Myc-tagged OPN *in vivo* and was used in the HRP-conjugate form (cat# 14038) as the ELISA detection antibody; the anti-human Osteopontin antibody used as the ELISA capture antibody was purchased from IBL International (cat# JP10011) and binds to a region within the N-terminus and common to all 3 isoforms (Minneapolis, MN); the CD68 and  $\alpha$  smooth muscle actin ( $\alpha$ -SMA) antibodies used for immunofluorescence staining were from Abcam (cat# ab-125212; Cambridge, MA), and Sigma Aldrich (cat# A5691; Saint Louis, MO), respectively; the Annexin V antibody used for apoptosis via fluorescence-activated cell sorting (FACS) analysis was from BD Biosciences (cat# 556547; San Jose, CA).

### Histology and Immunofluorescence

Mice were sacrificed and tissues were perfused with saline, followed by perfusion with 10% buffered formalin. Bone was demineralized in formic acid based solution (Cal-Ex II, Fisher Scientific, Pittsburgh, PA) for 48 hours. Tissue sections from paraffin embedded proximal hindlimbs were paraffin embedded and cut into 5  $\mu$ m sections. Sections were stained with lectin (cat# FL-1201; Vector Labs; Burlingame, CA) or antibodies against Myc-tagged OPN or  $\alpha$  smooth muscle actin ( $\alpha$ -SMA). Staining details are in the Online Supplement. Myc and lectin stained images were acquired using the 20X Plan-Neo air objective on a Zeiss Axioskop microscope equipped with an AxioCam camera. For quantification of lectin positive vessels, 6 images per section and 2 sections per animal were quantified. Images for CD68 and Sma $\alpha$ A stained sections were acquired using a Hamamatsu Nanozoomer slide scanner. For quantification of Sma $\alpha$ A positive vessels, the number and size of Sma $\alpha$ A positive vessels was quantified across the entire muscle section and averaged for 2 sections per animal. All treatments group consisted of 6–8 animals.

## ELISA

Adductor muscle tissues were harvested 7 days post-HLI and protein was extracted using Hunter's lysis buffer.<sup>52</sup> Briefly, tissues were homogenized with a glass mortar and pestle and tissue lysates were used for protein analysis by ELISA, as described in the Online Supplement. The plate was read on a BioTek Synergy H1 microplate reader at 450 nm. OPN isoform amounts were normalized to protein concentration, measured by the Bradford Assay (cat# 500-0006; Bio-Rad).

## Cell Culture

Peritoneal macrophages were isolated from OPN<sup>-/-</sup> and C57BL/6 (WT) mice as described in detail in the Online Supplement. Macrophages were plated in RPMI (cat# 22400-105; ThermoFisher Scientific) supplemented with 10% fetal bovine serum (cat# F6178; FBS; Sigma Aldrich), 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin (all from ThermoFisher Scientific) and allowed to attach for 2–3 hours prior to changing the media to fresh 10% FBS-RPMI for all experiments. For macrophage survival studies, cells acclimated in 10% FBS-RPMI overnight before being changed to serum free-RPMI the next day. For macrophage polarization studies, 3 hours after plating cells were stimulated with 10% FBS-RPMI with 100 ng/mL purified recombinant human OPNa, OPNb, or OPNc (cat# TP304803, TP305118, and TP31059; Origene, Rockville, MD), or were stimulated with 20 ng/mL of either interferon gamma (INF $\gamma$ ) or interleukin (IL)-4 for 48 hours (cat# 554587 and 550067; BD Biosciences) as positive controls for macrophage polarization. For macrophage migration studies, cells were cultured in 10% FBS-RPMI for 2 days and then quiesced for 24 hours in 0.1% FBS-RPMI prior to use in the migration assays.

## Migration Assay

Macrophage migration was measured using a modified Boyden Chamber assay, as described previously,<sup>31,53,54</sup> with the following modifications. Briefly,  $5 \times 10^4$  macrophages were seeded in a transwell plate containing 6.5 mm inserts with 8-µm pores (cat# 29442-120; Costar) coated with 50 µg/mL fibronectin (cat# CB40008; Corning, Corning, NY). Cells were allowed to migrate for 2 hours in response to the following stimuli diluted in 0.1% FBS-DMEM: 100 ng/mL monocyte chemoattractant protein (MCP)-1, 50 ng/mL of OPNa, OPNb, or OPNc recombinant protein (Origene). Non-migrated cells were removed from the upper surface and migrated cells were fixed and stained with DAPI (1 µg/mL). The number of migrated cells was quantified from 6–8 random fields from 4 independent experiments and images were acquired using a Zeiss Axioskop microscope. The number of migrated cells was quantified using ImageJ 1.38 software.

## Flow Cytometry

Macrophage survival was evaluated using FITC-Annexin V Apoptosis Detection Kit I (cat# 556547; BD) by flow cytometry analysis. Peritoneal macrophages were cultured as described in the "Cell Culture" section and serum-deprived to initiate apoptosis. Macrophages were stimulated with 100 ng/µl of each human OPN isoform (recombinant protein, Origene) for 24 hours before harvest, washed in cold PBS, and stained with FITC-



Annexin V, propidium iodide (PI), or both and analyzed by flow cytometry (CytOFLEX, Beckman). Analysis was done using the CytExpert 1.2.11.0 Software.

### RNA Isolation, RT-PCR and Quantitative RT-PCR

Total RNA was extracted from muscle tissues using the RNeasy kit (Qiagen, Valencia, CA), as described previously.<sup>21</sup> CD68 mRNA levels were measured from the ischemic and non-ischemic hindlimb muscle tissues by amplification of cDNA using the thermocycler (Applied Biosystems), SYBR green dye, and quantitect primer sets from QIAGEN (Valencia, CA). Ischemic limb mRNA expression levels were normalized to mRNA expression levels in the contralateral non-ischemic limb and % change relative to LV-GFP control animals is reported. The human OPN primers were designed for the detection of all 3 OPN isoforms via RT-PCR using primers designed against exon 3 (forward 5'-CATCACCTGTGCCATACCAG-3') and exon 6 (reverse 5'-GTCAATGGAGTCCTGGCTGT-3') to amplify OPNa (277bp), OPNb (235bp), and OPNc (196bp). Conditions for the semi-quantitative RT-PCR reaction were as follows: initial denaturation at 93°C for 2 min, 35 cycles of 93°C denaturation for 15 sec, 56°C annealing for 15 sec, and extension at 72°C for 28 sec, followed by a final extension at 72°C for 10 minutes. The OPN isoforms are then resolved by gel electrophoresis in a 2% ethidium bromide gel. Beta actin was used as a loading control (forward 5'-CATCACCTGTGCCATACCAG-3') and (reverse 5'-GTCAATGGAGTCCTGGCTGT-3') with the following reaction conditions: initial denaturation at 93°C for 2 min, 35 cycles of 93°C denaturation for 15 sec, 60°C annealing for 17 sec, and extension at 72°C for 28 sec, followed by a final extension at 72°C for 10 minutes. Total RNA was extracted from cultured macrophages using the Zymo Quick RNA Miniprep kit (Zymo Research, Irvine, CA) following the company's protocol and total RNA was extracted from skeletal muscle tissues using the RNeasy kit (Qiagen, Valencia, CA). iNOS, TNF $\alpha$ , Arg1, IL-10, and TGF $\beta$  and 18S rRNA were measured from peritoneal macrophages by amplification of cDNA using the thermocycler (Applied Biosystems), SYBR green dye, and quantitect primer sets from QIAGEN (Valencia, CA). For all targets reported, copy numbers were normalized to 18S rRNA.

### Statistical Analysis

Results are expressed as mean  $\pm$  S.E.M. from at least three independent experiments. Statistical significance for quantitative results was assessed using analysis of variance (ANOVA), followed by either a Tukey or Bonferroni Multiple Comparison post-hoc test. A value of  $p < 0.05$  was considered statistically significant.

## RESULTS

### OPN isoforms differentially affect functional collateral vessel formation *in vivo*

Humans express 3 OPN isoforms as a result of alternative splicing (Fig. 1A). To investigate if OPN isoform expression levels differ in PAD, mRNA was extracted from de-identified skeletal muscle tissues from non-ischemic and critical limb ischemia (CLI) patients. Isoform expression was analyzed by semi-quantitative RT-PCR. Results suggest that OPNb is the main isoform expressed in CLI compared to non-ischemic samples (Fig. 1B). To investigate

the effects of OPN isoforms on collateral vessel formation, Laser Doppler perfusion imaging (LDPI) was used to measure perfusion as an indicator of collateral vessel formation in mice treated with lentivirus (LV) particles expressing GFP, OPNa, OPNb or OPNc. Perfusion was assessed at 3, 7, and 14 days after surgery and representative perfusion images from day 14 are shown (Fig. 1C; **full time course**, Online Fig. 2A). Quantitative evaluation of day 14 perfusion showed that OPNb had no effect on perfusion, whereas OPNa and OPNc significantly increased perfusion compared to GFP control and OPNb treated animals (Fig. 1D). These data demonstrate that OPNa and OPNc increase collateral vessel formation *in vivo*. To determine if the OPN isoform-dependent increases in perfusion translate to increases in functional limb use, 7 days post-HLI animals were individually housed, given free access to running wheels, and allowed to run for 7 days and nights. As shown Figure 1E (and Online Fig. 2B), OPN<sup>-/-</sup> mice treated with GFP run significantly less compared to WT mice. In contrast, OPNa and OPNc treated animals ran significantly more than GFP control and OPNb treated animals. Altogether, the perfusion and running wheel data establish that OPNa and OPNc significantly increase functional collateral vessel formation *in vivo*. To validate LV-OPN isoform treated animals express equal amounts of OPN isoform protein *in vivo*, we used an ELISA to measure OPN isoform protein expression levels. We found no differences in OPN isoform expression in LV-OPN treated animals (Online Figs. 1B and 1C). Therefore, we conclude the differences in functional collateral vessel formation in response to OPN isoforms are due to different OPN isoform-mediated effects.

### **OPNa and OPNc promote arteriogenesis *in vivo***

The processes of vasculogenesis, angiogenesis, and arteriogenesis all contribute to collateral vessel formation in patients with obstructive arterial disease. To determine if OPN isoforms differentially affect these processes, we used immunohistochemistry and stained tissue sections from animals treated with LV-OPN a, b, or c. Lectin-positive vessel counts were quantified as a readout for angiogenesis, whereas  $\alpha$  smooth muscle actin ( $\alpha$ -SMA) positive vessel counts and vessel sizes were quantified as a readout for arteriogenesis. While OPN isoforms did not differentially affect angiogenesis *in vivo* (Online Fig. 3A, 3B), OPN isoforms did differentially affect arteriogenesis. OPN isoforms did not affect the total number of  $\alpha$ -SMA positive vessels (Fig. 2A, **representative images in 2C**); however, OPNa and OPNc both significantly increased the overall sizes of the  $\alpha$ -SMA positive vessels, decreasing the number of vessels that fall within the arteriole size bins (10 – 200  $\mu\text{m}^2$  in size), while significantly increasing the number of vessels that fall within the small artery (200 – 700  $\mu\text{m}^2$ ) and large artery (1000 – 2500  $\mu\text{m}^2$ ) sizes compared to OPNb treated animals (Fig. 2B). Outward remodeling of existing arterioles into larger arteries to increase blood flow without increasing vessel numbers is a hallmark of arteriogenesis. Thus, these data altogether suggest that OPNa and OPNc increase arteriogenesis by promoting the remodeling and enlargement of existing arterioles into larger conductance arteries.

### **Osteopontin isoforms differentially promote macrophage accumulation *in vivo***

The process of arteriogenesis is heavily dependent on macrophages due to their ability to promote remodeling through the secretion of proteinases that alter the extracellular matrix and through their production of a variety of growth factors and chemokines. To determine how OPN isoforms affect macrophage accumulation *in vivo*, we used



immunohistochemistry and qRT-PCR. To determine if mice treated with LV to express OPN isoform a, b, or c showed differences in macrophages accumulation post-HLI, we stained for the macrophage cell surface marker CD68 (Fig. 3A; H&E in Online Fig. 4) and quantitatively assessed CD68 by qRT-PCR (Fig. 3B). CD68 staining of mice treated with OPNa and OPNc showed increased macrophage accumulation (Fig. 3A, pink stain). Measurement of CD68 by qRT-PCR confirmed that CD68 expression was significantly higher in OPNa and OPNc treated mice, supporting higher macrophage accumulation. CD68 expression was highest in OPNc treated mice compared to all other treatment groups (Fig. 3B). These data led us to hypothesize that OPN isoforms increase functional collateral vessel formation through divergent effects on arteriogenesis and ischemia-dependent macrophage accumulation *in vivo*. However, to determine if OPN isoforms directly affect macrophage function, additional *in vitro* assays were performed.

### Osteopontin isoforms differentially affect macrophage migration and survival *in vitro*

To determine the mechanism by which OPN isoforms differentially promote collateral vessel formation and macrophage accumulation increases *in vivo*, we investigated if OPN isoforms have independent effects on macrophage migration, survival, and/or polarization. To determine if OPN isoforms differentially affect macrophage polarization, OPN<sup>-/-</sup> peritoneal macrophages were stimulated with 100 ng/mL of OPN a, b, or c for 48 hours or with INF $\gamma$  or IL-4 as positive controls. qRT-PCR was used to quantify mRNA expression levels for a panel of macrophage polarization/phenotype markers including: iNOS, TNF $\alpha$ , Arginase (Arg), IL-10, and TGF $\beta$  (Fig. 4, A–E). No significant differences in macrophage polarization markers were detected in response to OPN isoform stimulation, unlike the increases measured in response to INF $\gamma$  and IL-4 (Fig. 4), suggesting that OPN isoforms do not influence macrophage phenotype.

To determine if OPN isoforms have distinct effects on macrophage survival *in vitro*, we first determined if OPN<sup>-/-</sup> macrophages exhibit increased apoptosis compared to WT cells by assessing annexin V and propidium iodide (PI) staining by FACS analysis as early and late markers of apoptosis, respectively. WT and OPN<sup>-/-</sup> macrophages exhibited no differences in annexin V or PI staining with serum withdrawal (Fig. 5A, 5B). To determine if OPN isoforms have divergent effects on macrophage survival, macrophages were serum deprived and stimulated with OPN a, b, or c and positive staining for annexin V and PI were assessed by FACS. Representative scatter plots are shown in Figure 5C. All OPN isoforms significantly increased macrophage survival and decreased annexin V and PI staining compared to no treatment (Fig. 5D). However, we found that the pro-survival, anti-apoptosis effects of OPNc were significantly increased over other OPN isoforms (Fig. 5D), as assessed by flow cytometry, indicating OPNc is the most potent stimulus for macrophage survival.

Macrophage migration is a necessary component of the arteriogenesis process. To investigate if OPN isoforms differentially effect macrophage migration, we used a modified Boyden chamber assay. WT or OPN<sup>-/-</sup> macrophages were stimulated with 50 ng/mL of OPN isoform a, b, or c, an established EC50 dose of OPN isoforms for migration and in line with reported pathophysiologic levels of OPN (Online Fig. 5A and 5B),<sup>45</sup> and allowed to migrate for 2 hours. OPN<sup>-/-</sup> macrophages exhibited impaired migration in response to

monocyte chemoattractant protein 1 (MCP-1) compared to WT macrophages (Fig. 6A), which is consistent with what has been reported previously.<sup>31</sup> Interestingly, OPNa and OPNc significantly rescued impaired migration in OPN<sup>-/-</sup> macrophages compared to OPNb and MCP-1 (Fig. 6B). Knockdown of OPN, which induces a loss of macrophage migration, is significantly restored with OPNa and OPNc treatment *in vitro*, where OPNc is the most potent migratory stimulus. However, the effects of OPN isoforms on a fundamental migration processes remains to be investigated. Altogether, these data support that the mechanisms by which OPNa and OPNc exert their pro-arteriogenic effects *in vivo* are, in part, through their ability to increase macrophage accumulation/migration and macrophage survival, supporting the pathway proposed in Figure 6C.

## DISCUSSION

The data presented in this manuscript are the first to establish that OPN isoforms differentially promote functional post-ischemic neovascularization *in vivo* through increased arteriogenesis. Furthermore, we demonstrate that OPN isoforms have different effects on macrophage cell migration *in vivo* and *in vitro*, suggesting that specific OPN isoforms may serve as novel therapeutic targets to improve functional collateral vessel formation in patients with obstructive artery disease pathologies like CAD and PAD. In this study, we demonstrate that OPNa and OPNc specifically increase perfusion by promoting arteriogenesis, or the enlargement of existing arterioles into larger conductance arteries, compared to control and OPNb treated animals. Based on these data, one would hypothesize that patients that express less OPNa and OPNc would exhibit less arteriogenesis. In a small number of PAD patients with critical limb ischemia, we find that the primary OPN isoform expressed is OPNb and that these patients express little OPNa and OPNc. However, while these data point to isoform expression differences in PAD, a controlled study in a larger PAD patient cohort is required to fully establish how PAD affects OPN isoform expression levels.

The principle cell types involved in the arteriogenesis process are vascular smooth muscle cells and macrophages.<sup>55,56</sup> It has been shown previously that there is a substantial decrease in migration of OPN<sup>-/-</sup> monocytes/macrophages, which ultimately leads to impaired collateral vessel formation in OPN<sup>-/-</sup> mice,<sup>31</sup> and we also find that OPN is critical for monocyte/macrophage migration and collateral formation. The *in vivo* studies presented here not only show that isoforms have differential effects on arteriogenesis, but demonstrate that one mechanism by which OPNa and OPNc exert their pro-arteriogenic effects *in vivo* are, in part, through their ability to increase macrophage accumulation in response to ischemia. Our *in vitro* findings confirm that OPNa and OPNc serve as pro-migratory stimuli for macrophages and that OPNc is the most potent macrophage migratory stimulus *in vitro*. Additionally, we establish that all 3 OPN isoforms are able to significantly increase macrophage survival *in vitro*, with OPNc being the more potent pro-survival, anti-apoptosis isoform. Together, these findings provide evidence that the mechanism by which OPN isoforms exert divergent effects on neovascularization is, in part, through divergent effects on post-ischemic macrophage accumulation. Subsequently, after macrophage migration into the ischemic region increases in response to OPNa and OPNc, the isoforms are then able to exert pro-survival effects and, thus, enhance arteriogenesis and functional limb use. The findings presented in this manuscript are the first to report that specific human OPN

isoforms play a critical role in post-ischemic collateral vessel formation and provides an insight into the mechanisms by which OPN isoforms have differential effects on post-ischemic neovascularization.

OPN<sup>-/-</sup> mice exhibit impaired collateral vessel formation in response to an ischemic insult,<sup>31</sup> making them an ideal model system to experimentally determine if individual human OPN isoforms can rescue collateral vessel formation *in vivo* and if the mechanisms by which they do so are different. Furthermore, with no endogenous murine OPN expressed, the only OPN in the system is what we add back and the human OPN isoforms do not have to compete with an ischemia-induced 20 fold increase in murine OPN expression that typically occurs *in vivo*.<sup>21</sup> Additionally, the use of OPN<sup>-/-</sup> mice allowed us to utilize a loss-of-function/gain-of-function approach for our studies. To add the human OPN isoforms to the OPN<sup>-/-</sup> mice, we utilized a lentivirus designed to overexpress either Myc-tagged osteopontin isoform a, b, or c. Importantly, we were able to express comparable levels of each isoform in each mouse, allowing us to discern what functional effects belong to each independent isoform without being due to differences in OPN isoform protein expression levels. One caveat to this experimental design is that multiple cell types are transduced with the lentivirus, thus we do not know the relative contribution of each cell type and their influence on these outcomes. However, without humanized OPN isoform mice available, this remains one of the best *in vivo* approaches at this time to investigate the role of each specific OPN isoform in the neovascularization process. While there are some limitations to expressing human OPN isoforms in a rodent system, the conservation of key binding domains and receptors across species makes this less of an overall concern.

While one of the strengths of the design of this study was using OPN<sup>-/-</sup> animals and lentivirus to add back a single OPN isoform, allowing us to identify isoform-specific effects, this limits our ability to fully understand the complexities of OPN isoform interplay. Furthermore, this manuscript focuses on OPNa, OPNb, and OPNc isoforms, while NCBI reports 5 splice variants including OPN4 and OPN5; however, if OPN4 and OPN5 translate to protein has yet to be determined. Our experimental system allows us to determine how each independent OPN isoform functions to promote neovascularization; however, it does not lend any insight into how the presence of one OPN isoform may positively or negatively influence the function of another OPN isoform and this is an area of ongoing investigation. These types of studies will be necessary to fully understand OPN isoform function since we, and others, find that typically there are at least two OPN isoforms expressed, whether it be in various cancer subtypes or in PAD patient samples.<sup>32,35,36,38,39,42,43,57</sup> Therefore, additional investigations to determine how isoforms interact and how one isoform affects the activity of another when co-expressed are needed.

OPN isoforms have been shown to be differentially expressed in multiple cancer subtypes and this has been reviewed previously.<sup>58</sup> While studies have looked at OPN isoform expression levels, little is understood about why OPN isoforms often have differential effects. In this study, we demonstrate that OPN isoforms have divergent effects on post-ischemic neovascularization, where OPNc is the most potent stimulus of functional arteriogenesis *in vivo* and macrophage migration and survival *in vitro*. As pictured in Figure 1A, OPN-c (transcript variant 3) lacks exon 4, which contains several glutamine residues

subject to transglutamination.<sup>59,60</sup> Consequently, unlike OPNa and OPNb, OPNc is likely unable to be cross-linked to form polymeric complexes,<sup>61,62</sup> which may in turn affect downstream functions. OPNc also lacks several phosphorylation sites, suggesting that differences in function may also stem from differences in post-translational modifications. Functional differences between OPN isoforms may also be attributable to differences in three dimensional structure, however this has yet to be determined through x-ray crystallography or NMR and may prove difficult given the flexible structure of OPN.<sup>63</sup> One group recently performed an *in silico* structure prediction of osteopontin-c, which showed that the RGD integrin binding domain of OPNc may be more exposed compared to OPNa and OPNb.<sup>64</sup> However, validation studies are needed to confirm these predictions and how they translate to function.

Monocytes typically express low levels of OPN, but as they differentiate into macrophages, OPN is dramatically upregulated and constitutes one of the major macrophage products.<sup>65,66</sup> Therefore, to investigate how each human OPN isoform influences macrophage biology, we utilized an *in vitro* approach and isolated OPN<sup>-/-</sup> peritoneal macrophages and stimulated them with recombinant OPN protein generated in human cell lines for these studies. The use of recombinant OPN protein generated in human cell lines, in lieu of bacteria, allows for appropriate post-translational modifications to be made to OPN (36 predicted phosphorylation sites and 5 glycosylation sites),<sup>15,16,20</sup> which are necessary for function.<sup>15-17,20,67,68</sup>

Macrophages are critical to arteriogenesis,<sup>31,69</sup> leading us to investigate if OPN isoforms have divergent effects on macrophage function. Despite conflicting reports in the literature about whether OPN is necessary for macrophage polarization,<sup>70,71</sup> we found that OPN<sup>-/-</sup> and WT primary macrophages were not different in their ability to polarize when stimulated with INF- $\gamma$  or IL-4. Importantly, stimulation of OPN<sup>-/-</sup> macrophages with OPN isoforms did not alter expression of any of the macrophage polarization/phenotype markers that were interrogated (Fig. 4), suggesting that isoforms do not differentially influence macrophage polarization. OPN has been shown to promote cell survival, in part by inhibiting apoptosis,<sup>72</sup> and thus promotes cancer cell metastasis due to its anti-apoptotic characteristics.<sup>73</sup> However, this study is the first to show that OPN isoform differentially increase macrophage survival. Although there was no effect of endogenous OPN on macrophage survival, stimulation with all three OPN isoforms significantly increased macrophage survival compared to controls. OPNc increased macrophage survival greater than the other OPN isoforms; however, if this statistically significant increase translates to a biological significance remains unknown. In addition to macrophage survival, we have also demonstrated that all three OPN isoforms inhibit both early and late apoptosis, but the detailed mechanisms remain to be investigated. In contrast to macrophage polarization, we found clear differential effects of OPN isoforms on macrophage migration *in vitro*. OPNc significantly promotes macrophage migration *in vitro* compared to all other treatment conditions, including treatment with the full length human OPNa isoform. While it could be argued that these dramatic differential effects of human OPN isoforms is due to the use of murine macrophages for migration studies, the fact that the functional regions of the OPN protein are highly conserved across diverse species suggests otherwise. Moreover, to eliminate this possibility, we have included a group of macrophages in WT mice in order to demonstrate endogenous OPN effects on macrophage

migration and other macrophage functions, which are similar to OPNc-treated macrophages. These *in vitro* migration data suggest that *in vivo* differences are likely due to isoform-specific effects on macrophage recruitment; however, we cannot rule out that there may also be changes in macrophage extravasation and this requires further investigation. It should be noted that macrophages from OPN<sup>-/-</sup> mice normally migrate towards CSF-1, but not towards MCP-1, which signals through G-protein coupled receptors (GPCRs).<sup>74</sup> Since GPCR-mediated migration is dependent upon the level of cell surface CD44 receptors, we hypothesize that the macrophages from OPN<sup>-/-</sup> mice may express low levels of CD44 and thus do not migrate towards MCP-1, unlike their WT counterparts.

With respect to inflammatory responses to isoforms, recent studies show different effects depending on the model system being utilized and the source of recombinant OPN isoform protein. A recent paper<sup>75</sup> demonstrated that human primary macrophages stimulated with recombinant OPNa have an increase in pro-inflammatory response in an RGD-dependent manner and that all 3 isoforms equally increase macrophage phagocytosis. However, the study utilized human OPN isoforms (OPNa, -b and -c) expressed as glutathione S-transferase (GST) fusion proteins in *Escherichia coli*, affinity purified and cleaved from GST and would, therefore, not be properly post-translationally modified.<sup>76</sup> Additionally, they utilized a very high dose of OPN-a (1 µg/mL), whereas we utilized a pathophysiological dose that was within the range of total OPN expressed in patients with CVD and/or Type 1 Diabetes.<sup>77</sup> It is now understood that when OPN is expressed in bacteria, the proper post-translational modifications do not occur and this can, in fact, affect functional outcomes.

In conclusion, we demonstrate for the first time that OPN isoforms differentially affect ischemic collateral vessel formation and propose that this is, in part, through differential effects of OPN isoforms of macrophage migration and survival, but not polarization. While additional OPN isoform-dependent functions remain to be investigated in various CVD settings, the identification of OPN isoform-specific differences in the neovascularization process suggests new potential functions for OPN isoforms. Furthermore, our data suggest that specific OPN isoforms may serve as novel therapeutic targets to improve arteriogenesis in patients with obstructive artery disease pathologies, such as CAD and PAD.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Non-Standard Abbreviations and Acronyms

CAD                      Coronary Artery Disease

<b>CVD</b>	Cardiovascular Disease
<b>ECM</b>	Extracellular Matrix
<b>GFP</b>	Green Fluorescent Protein
<b>HLI</b>	Hindlimb Ischemia
<b>IF</b>	Immunofluorescence
<b>IL</b>	Ischemic Limb
<b>IM</b>	Intramuscular
<b>LDPI</b>	LASER Doppler Perfusion Imaging
<b>LV</b>	Lentivirus
<b>NIL</b>	Non-ischemic Limb
<b>OPN</b>	Osteopontin
<b>PAD</b>	Peripheral Artery Disease
<b>PI</b>	Propidium Iodide
<b>RGD</b>	Arginine-Glycine-Aspartate
<b><math>\alpha</math>-SMA</b>	$\alpha$ Smooth Muscle Actin
<b>VSMC</b>	Vascular Smooth Muscle Cells

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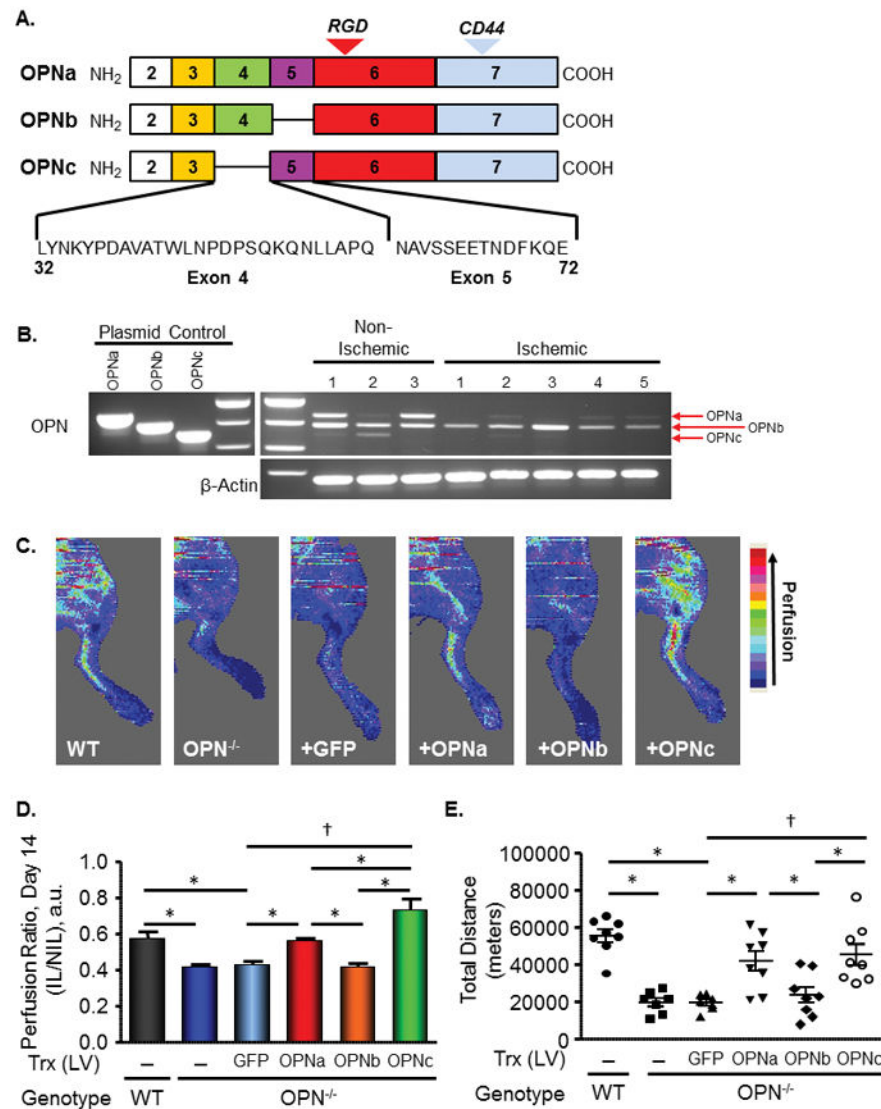


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**Figure 1. OPN isoforms differentially affect perfusion recovery *in vivo* and functional limb use**  
**A.** Illustration of OPN isoform primary domain structure. Each block corresponds to an exon (numbered). OPNa is full length (top), OPNb lacks exon 5 (middle), and OPNc lacks exon 4 (bottom). Expanded amino acid sequences of exons 4 and 5, absent in OPNc and OPNb, respectively, are included. **B.** OPN primers were used to measure OPNa (277bp), OPNb (235bp), and OPNc (196bp) mRNA levels in tissue samples from non-ischemic tissues and tissues from PAD patients with critical limb ischemia by RT-PCR (n=3–5). Isoform plasmid DNA controls and beta actin for loading are both shown. **C.** To investigate if OPN isoforms differentially affect collateral vessel formation *in vivo*, the hindlimb ischemia was performed on the following groups: WT, OPN<sup>-/-</sup>, or OPN<sup>-/-</sup> mice treated (trx) with lentivirus (LV) to overexpress GFP, OPNa, OPNb, or OPNc. Perfusion was measured by Laser Doppler perfusion imaging (LDPI). Representative LDPI images 14 days post-HLI are shown. **D.** Ischemic limb (IL) perfusion was quantified and normalized to the contralateral non-ischemic limb (NIL) and compared across groups. \* p<0.05, † p<0.001 vs. GFP; d14, n=6.

**E.** To determine if the OPN isoform effects on perfusion translate to increased functional limb use, animals were given free access to a running wheel (d7 post-HLI) and allowed to run for 7 days. Total distance run (meters) by was plotted for all groups as a measure of limb function. \* $p < 0.05$ , †  $p < 0.001$  vs. GFP;  $n = 9-10$ .

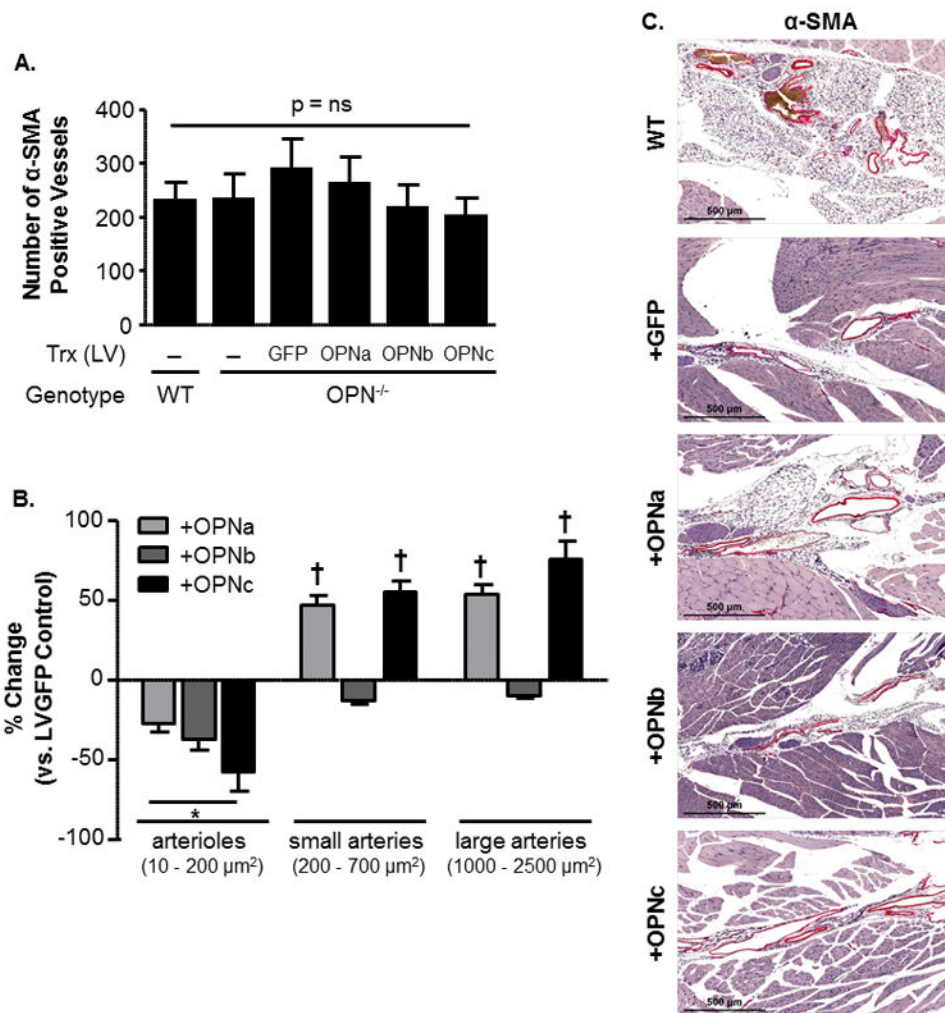
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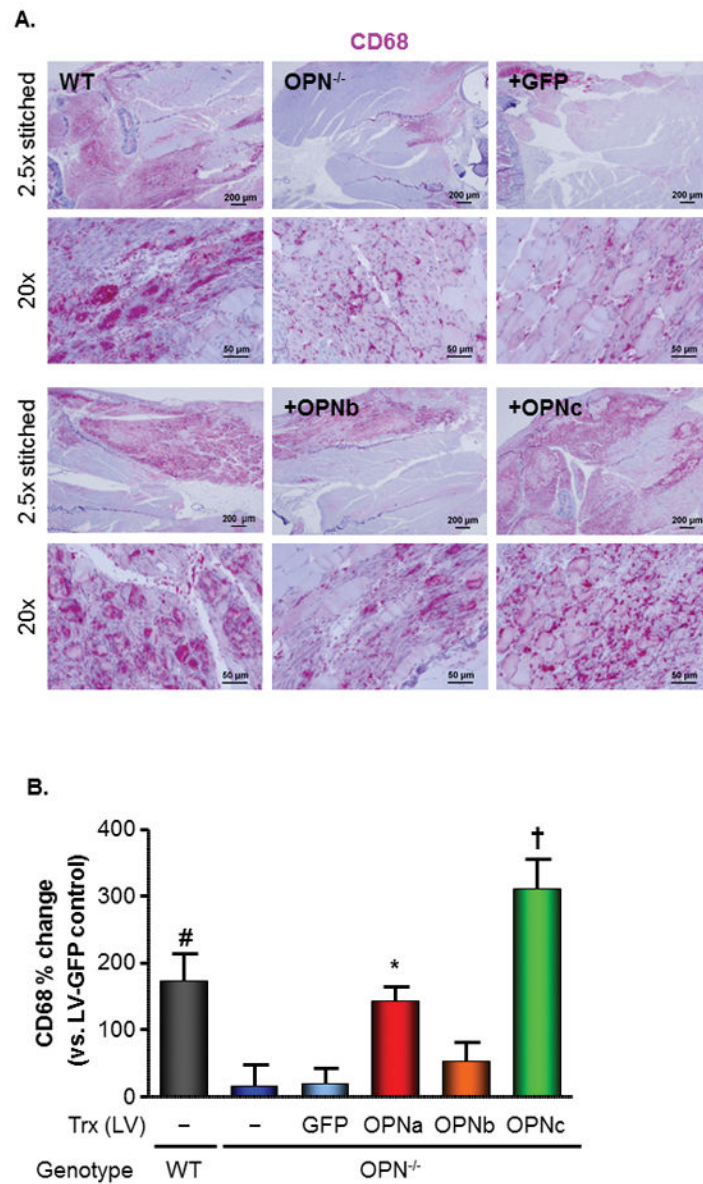
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### Figure 2. OPNa and OPNc increase arteriogenesis *in vivo*

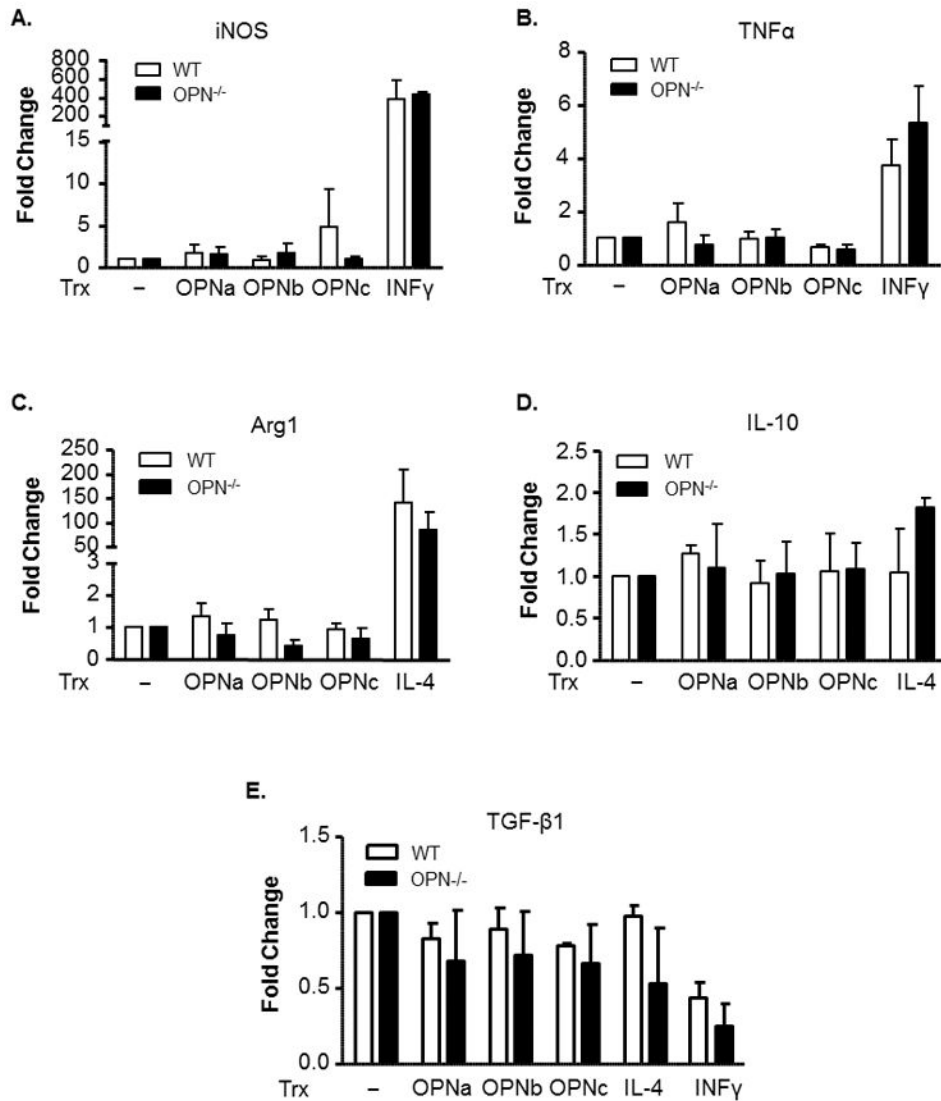
To determine if OPN isoforms differentially affect arteriogenesis, tissue sections from animals 14 days post-HLI treated (trx) with lentivirus (LV) to overexpress OPN isoform a, b, or c were stained with  $\alpha$  smooth muscle actin ( $\alpha$ -SMA).  $\alpha$ -SMA positive vessel numbers and sizes were quantified as a readout for arteriogenesis. **A.** The number of  $\alpha$ -SMA positive vessels was counted across treatment groups and plotted (*p* = ns). **B.**  $\alpha$ -SMA positive vessel sizes were measured in WT or OPN<sup>-/-</sup> mice treated (trx) with lentivirus (LV) to overexpress GFP, OPNa, OPNb, or OPNc. The number of vessels measured within the arteriole (10 – 200  $\mu\text{m}^2$ ), small artery (200 – 700  $\mu\text{m}^2$ ) and large artery (1000 – 2500  $\mu\text{m}^2$ ) size ranges were compared across all animal groups. Data are expressed as % change compared to +LV-GFP (control). \**p* < 0.05 vs. OPNa, † *p* < 0.001 vs. OPNb; *n* = 8–10. **C.** Representative histology images from 14 days post-HLI stained with  $\alpha$ -SMA are shown.  $\alpha$ -SMA stain is red and counterstain is violet. Scale bars = 500  $\mu\text{m}$ .



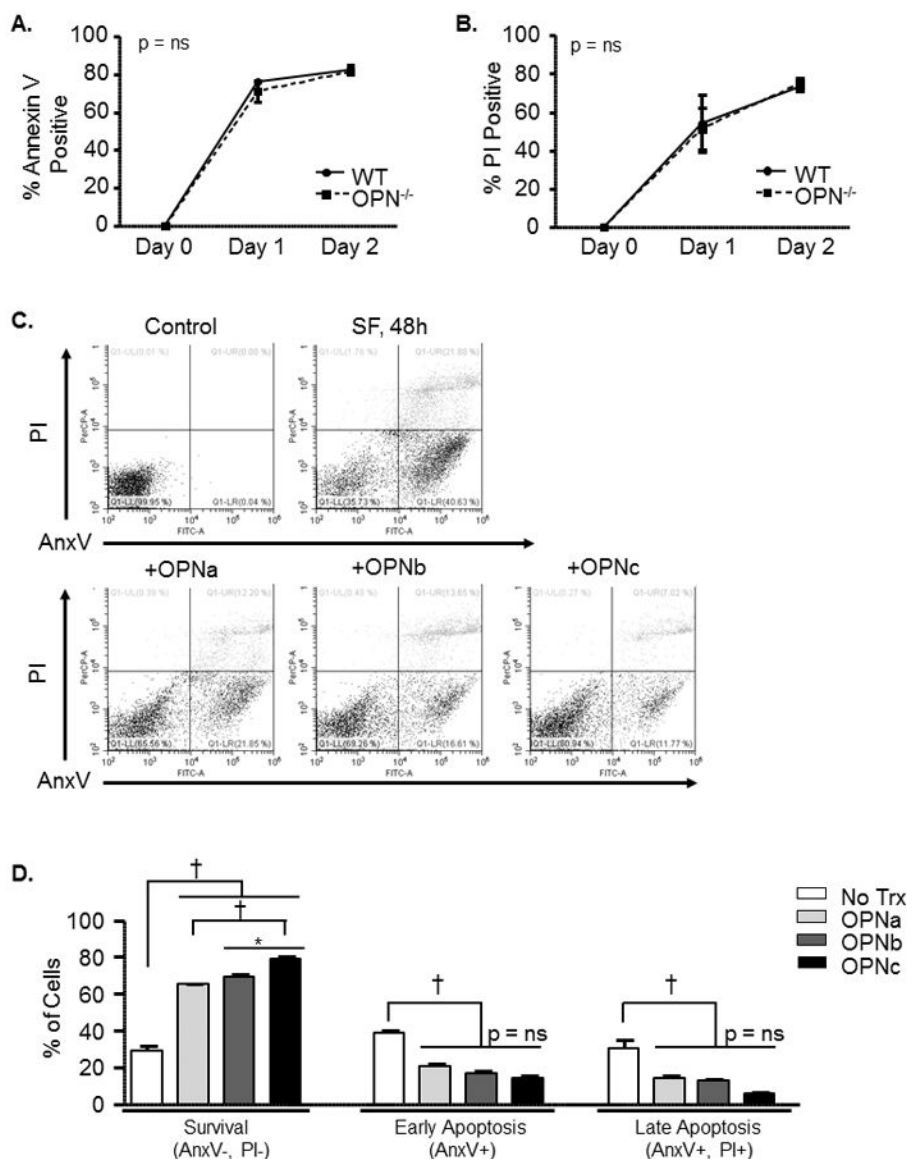
**Figure 3. OPN isoforms differentially influence macrophage accumulation *in vivo***

**A.** To determine if OPN isoforms differentially promote macrophage accumulation, histology sections from WT, OPN<sup>-/-</sup>, and OPN isoform a, b, or c treated animals were stained for CD68 (macrophage marker; pink stain) 5 days post-HLI. 20x representative images show detail. 2.5x images stitched together across the hindlimb demonstrate tissue-wide differences. Scale bars for 2.5x stitched = 200  $\mu$ m. Scale bars for 20x = 50  $\mu$ m. **B.** To quantitatively assess macrophage accumulation in response to OPN isoforms, qRT-PCR on mRNA isolated from the muscles of the IL was used to quantify CD68 expression. CD68 expression was compared across all treatment groups at day 7; # p<0.05 vs. OPN<sup>-/-</sup>, LV-GFP; \* p<0.05 vs. OPN<sup>-/-</sup>, LV-GFP and LV-OPNb, † p<0.001 vs. OPN<sup>-/-</sup>, LV-GFP, LV-OPNa, and LV-OPNb; n=4.

## Macrophage Polarization/Phenotype Marker Panel

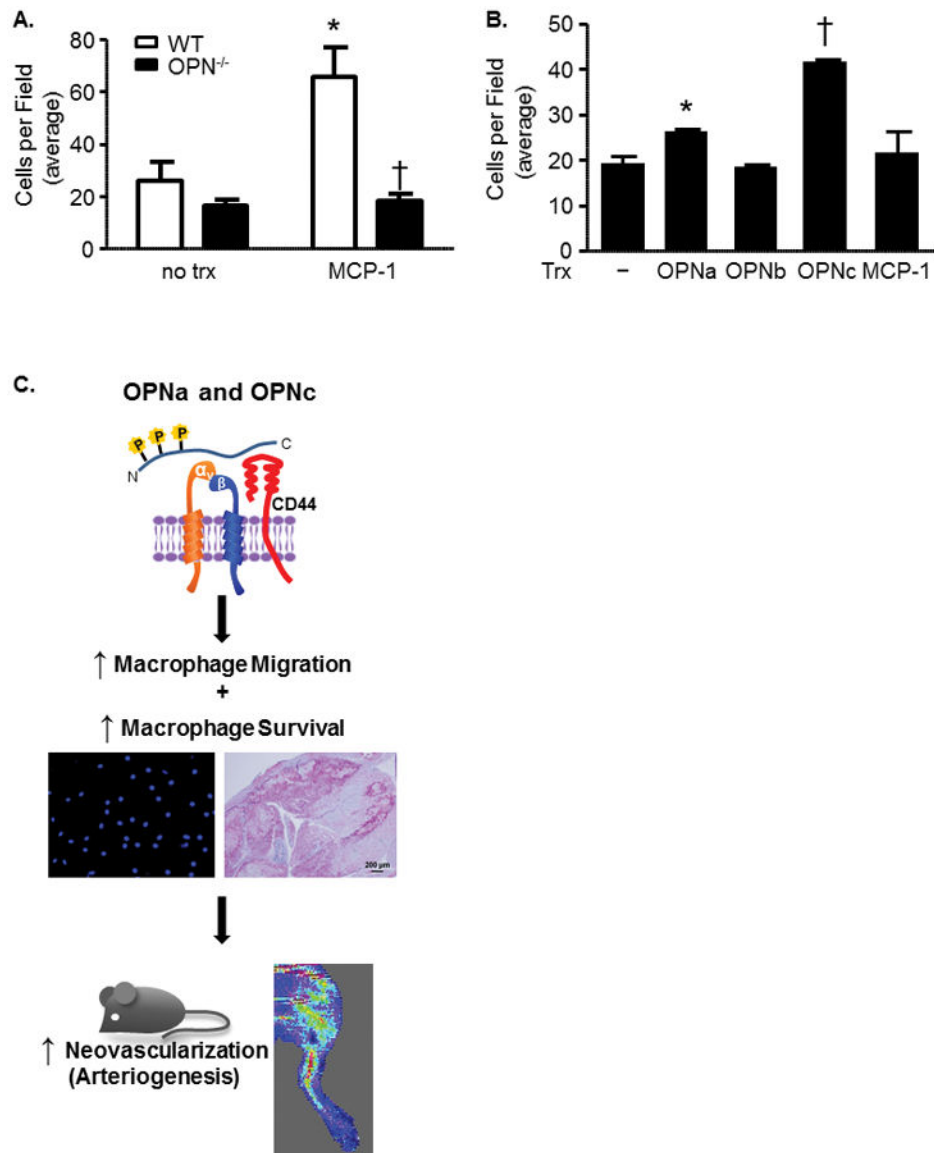
**Figure 4. Human OPN isoforms do not affect macrophage polarization**

To determine if OPN isoforms influence macrophage phenotype/polarization, OPN<sup>-/-</sup> peritoneal macrophages were cultured *in vitro* for 48 hours, followed by stimulation with 100 ng/mL of OPN a, b, or c for 48 hours or with 20 ng/mL of INF $\gamma$  or IL-4 (controls) before analyzing for macrophage polarization/phenotype marker expression. mRNA expression levels for the following markers were all measured by qRT-PCR. **A.** iNOS, **B.** TNF $\alpha$ , **C.** Arginase (Arg1), **D.** IL-10, and **E.** TGF $\beta$ . Stimulation with INF $\gamma$  and IL-4 served as positive controls. No significant differences were detected in response to OPN isoforms (n=3).



**Figure 5. Human OPN isoforms increase macrophage survival and decrease macrophage apoptosis**

To determine if OPN isoforms affect macrophage survival and apoptosis, WT and OPN<sup>-/-</sup> peritoneal macrophages were harvested and plated. Media was changed to serum free media ± 100 ng/mL of OPN a, b, or c for 48 hours before staining with annexin V (AnxV, early apoptosis marker) and/or propidium iodide (PI, late apoptosis marker) and analyzing by flow cytometry. **A and B.** Macrophage apoptosis, measured by AnxV and PI staining, did not differ between OPN<sup>-/-</sup> and WT upon serum withdrawal. **C.** Representative scatter plots for OPN isoform effects on macrophage survival are shown. Viable cells = AnxV<sup>-</sup>/PI<sup>-</sup>, early apoptosis = AnxV<sup>+</sup>/PI<sup>-</sup>, late apoptosis = AnxV<sup>+</sup>/PI<sup>+</sup>, and necrosis = AnxV<sup>-</sup>/PI<sup>+</sup>. Percentages are shown in each quadrant. **D.** Quantification of FACS data to measure macrophage viability, apoptosis, and necrosis with no treatment (no trx), or treatment with 100 ng/mL of OPN a, b, or c. (n=4, \* p<0.05, † p<0.001).



**Figure 6. Effects of MCP-1 and OPN isoforms on macrophage migration *in vitro***  
 Peritoneal macrophages were used as an *in vitro* cell model to measure isoform effects on macrophage migration using a modified Boyden chamber assay. **A.** Monocyte chemoattractant protein-1 (MCP-1), a known stimulus for macrophage migration, was used to first determine if OPN<sup>-/-</sup> and WT macrophages exhibit differences in MCP-1-mediated migration after 2 hours. (n=4, \* p=0.0027 vs. WT - no trx, † p<0.001 vs. WT+MCP-1). **B.** To assess if OPN isoforms differentially promote macrophage migration, OPN<sup>-/-</sup> macrophages were stimulated with 50 ng/mL of recombinant OPN a, b, or c and macrophage migration was quantified after 2 hours. (n=4, \* p<0.05 vs. no trx and OPNb, † p<0.001 vs. no trx, OPNa, and OPNb). **C.** Proposed mechanism by which OPN isoforms a and c increase arteriogenesis *in vivo* by increasing macrophage migration and survival.