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ARTICLE

Osteopontin isoforms differentially promote arteriogenesis in response to ischemia via macrophage accumulation and survival

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Abstract

Osteopontin (OPN) is critical for ischemia-induced neovascularization. Unlike rodents, humans express three 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. To investigate if human OPN isoforms exert divergent effects on post-ischemic neovascularization, we utilized $OPN^{-/-}$ mice and a loss-of-function/gain-of-function approach in vivo and in vitro. In this study $OPN^{-/-}$ mice underwent hindlimb ischemia surgery and 1.5×10^6 lentivirus particles were administered intramuscularly to overexpress OPNa, OPNb, or OPNc. OPNa and OPNc significantly improved limb perfusion 30.4% ± 0.8 and 70.9% ± 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 remodeling of existing arterioles into larger conductance 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 three 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. In conclusion, 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 neovascualrization and preserve tissue function in patients with obstructive artery diseases.

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 [1]. CAD and PAD both result in tissue ischemia downstream of vessel occlusions, often the result of

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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 remodeling [2–4]. The principle processes that contribute to collateral vessel formation in patients with obstructive arterial disease are defined as follows: (1) vasculogenesis, which is the formation of de novo blood vessels 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 capillaries, followed by arterialization with vascular smooth muscle cells (VSMCs), and (3) arteriogenesis, which involves the outward remodeling and enlargement of existing arterioles into larger conductance arteries [5]. However, the ability of patients with PAD to form collateral vessels is often suboptimal or impaired [6, 7]. Importantly, a patient's ability to develop collateral blood vessels to bypass sites of occlusion improves patient outcomes and survival [8].

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 remodeling and enlargement of existing arterioles into larger conductance arteries and this arteriogenesis process is heavily dependent on macrophages [9, 10]. Macrophages infiltrate into the ischemic region function to promote extracellular matrix remodeling and turnover and the production of a variety of growth factors and chemokines that facilitate the arteriogenesis process [9, 11, 12]. 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 [13, 14]. 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 and 7, respectively [15–20]. We previously demonstrated that OPN is highly upregulated in response to ischemia and is a critical mediator of post-ischemic neovascularization in vivo [21]. Multiple cell types upregulate OPN in response to ischemia, including endothelial cells, smooth muscle cells, and macrophages [21]. Additionally, OPN expression is increased in numerous ischemic pathologies including ischemic stroke [22], myocardial infarction [23, 24], and peripheral artery disease [25, 26]. 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 [27–30]. Importantly, $OPN^{-/-}$ animals are defective in collateral vessel formation and macrophage accumulation in response to ischemia [31]. 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 messenger RNA (mRNA) transcript that yields: (1) OPNa, the full-length isoform, (2) OPNb that lacks exon 5, and (3) OPNc that lacks exon 4 (see National Center for Biotechnology Information (NCBI) annotation for GeneID 6696) [32, 33]. Variants of human OPN were first described in 1990 [34] and were later identified as OPN isoforms in human glioma cells [33] and have since been shown to be differentially expressed in various tumor subtypes and exhibit isoform-specific functions [32, 35–43]. Studies that have investigated the role of OPN in cardiovascular disease (CVD) primarily focus on total OPN expression [44–46] and do not identify expression levels for specific OPN isoforms, with a few studies in patients with calcific aortic valve disease [47, 48]. Furthermore, the OPN isoforms expressed in ischemic tissues and the functional roles of 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 [21, 31, 49, 50], which express the equivalent of OPNa. We previously reported that murine OPN is upregulated ~20-fold (mRNA) in response to ischemia [21] and $OPN^{-/-}$ mice exhibit an innate inability to collateralize in response to an ischemic insult [31]. Therefore, to experimentally determine if human OPN isoforms have differential effects on collateral vessel formation in vivo, we utilized $OPN^{-/-}$ mice and a loss-of-function/gain-of-function approach so that 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

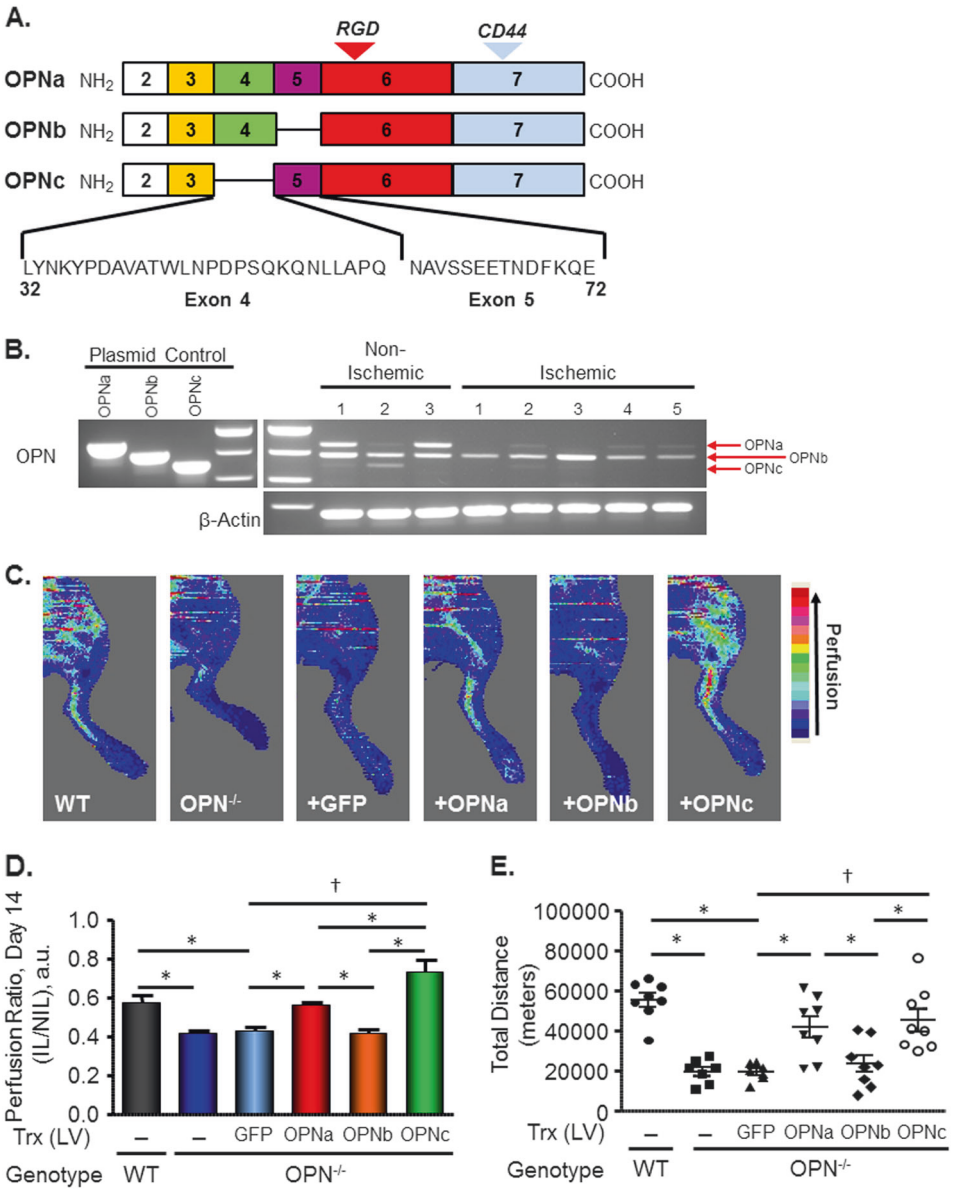

Fig. 1 OPN isoforms differentially affect perfusion recovery in vivo and functional limb use. **a** Illustration of OPN isoform primary domain structure. Each block corresponds to an exon (numbered). OPNa is full length (top), OPNb lacks exon 5 (middle), and OPNc lacks exon 4 (bottom). Expanded amino acid sequences of exons 4 and 5, absent in OPNc and OPNb, respectively, are included. **b** OPN primers were used to measure OPNa (277 bp), OPNb (235 bp), and OPNc (196 bp) 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$^{-/-}$, or OPN$^{-/-}$ 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; $^{*}p < 0.05$, $^{\dagger}p < 0.001$ 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) was plotted for all groups as a measure of limb function; $^{*}p < 0.05$, $^{\dagger}p < 0.001$ vs. GFP; $n = 9$–10

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

$OPN^{-/-}$ (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, $OPN^{-/-}$ and C57Bl/6 (wild-type (WT)) mice underwent hindlimb ischemia (HLI) surgery, as reported previously [21, 31]. 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, 1.5×10^6 lentivirus particles expressing green fluorescent protein (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 injection (see online Fig. 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 [21]. 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

At 7 days post-HLI, animals were individually housed in activity wheel cages, as described previously [51]. During functional limb assessement, animals were given free access to food, water, and activity wheel cages were housed in a behavioral 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 promoter. A constitutively active respiratory syncytial virus promoter drove expression of the antibiotic-fluorescent fusion dual marker GFP–Blasticidin. We previously demonstrated that multiple cell types express OPN in response to ischemia [21]; therefore, a lentivirus (LV) without a tissue-specific promoter was utilized to overexpress each OPN isoform. Lentivirus was provided by Gentarget at a final titer of ~1×10^8 infectious units per mL in sterile 1× phosphate-buffered saline (PBS) and 10× polybrene. For in vivo use, lentiviruses were diluted in sterile saline to deliver 1.5×10^6 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 horseradish peroxidase-conjugate form (cat# 14038) as the enzyme-linked immunosorbent assay (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 three isoforms (Minneapolis, MN); the CD68 and α-smooth muscle actin (α-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 killed 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 h. Tissue sections from paraffin embedded proximal hindlimbs were paraffin

embedded and cut into 5 µm sections. Sections were stained with lectin (cat# FL-1201; Vector Labs; Burlingame, CA) or antibodies against Myc-tagged OPN or α-SMA. Staining details are in the Online Supplement. Myc- and lectin-stained images were acquired using the 20× 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 SmαA-stained sections were acquired using a Hamamatzu Nanozoomer slide scanner. For quantification of SMαA-positive vessels, the number and size of SMαA-positive vessels was quantified across the entire muscle section and averaged for two sections per animal. All treatment groups consisted of 6–8 animals.

ELISA

Adductor muscle tissues were harvested 7 days post HLI and protein was extracted using Hunter's lysis buffer [52]. 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 $OPN^{-/-}$ 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 (FBS; 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 h prior to changing the media to fresh 10% FBS–RPMI for all experiments. For macrophage survival studies, cells were acclimated in 10% FBS–RPMI overnight before being changed to serum free-RPMI the next day. For macrophage polarization studies, 3 h 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 (INFγ) or interleukin (IL)-4 for 48 h (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 h 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 [31, 53, 54], with the following modifications. Briefly, 5×10^4 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 h in response to the following stimuli diluted in 0.1% FBS–Dulbecco's modified Eagle's medium: 100 ng/mL monocyte chemoattractant protein-1 (MCP-1), and 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 4′,6-diamidino-2-phenylindol (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 h 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 [21]. CD68 mRNA levels were measured from the ischemic and non-ischemic hindlimb muscle tissues by amplification of complementary DNA (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 percent change relative to LV-GFP control animals is reported. The human OPN primers were designed for the detection of all three OPN isoforms via reverse transcription polymerase chain reaction (RT-PCR) using primers designed against exon 2 (forward 5′-CATCACCTGTGCC ATACCAG-3′) and exon 6 (reverse 5′- GTCAAT GGAGTCCTGGCTGT-3′) to amplify OPNa (277 bp), OPNb (235 bp), and OPNc (196 bp).

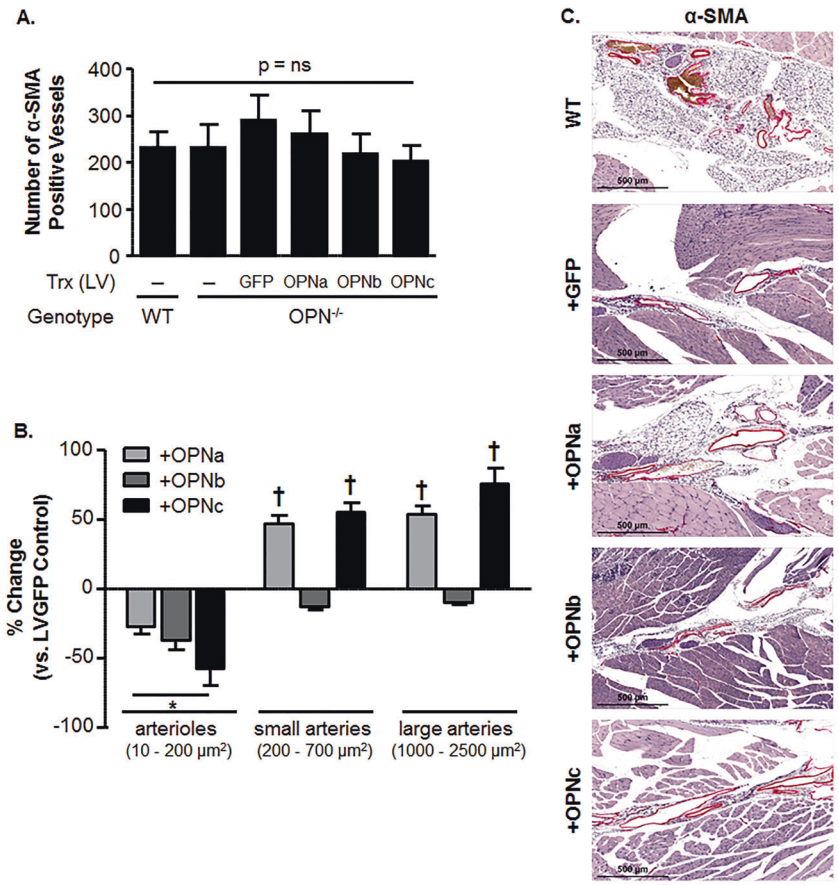

Fig. 2 OPNa and OPNc increase arteriogenesis in vivo. To determine if OPN isoforms differentially affect arteriogenesis, tissue sections from animals 14 days post HLI treated (trx) with lentivirus (LV) to overexpress OPN isoform a, b, or c were stained with α-smooth muscle actin (α-SMA). α-SMA-positive vessel numbers and sizes were quantified as a readout for arteriogenesis. **a** The number of α-SMA-positive vessels was counted across treatment groups and plotted (p = ns). **b** α-SMA-positive vessel sizes were measured in WT or OPN$^{-/-}$ mice treated (trx) with lentivirus (LV) to overexpress GFP, OPNa, OPNb, or OPNc. The number of vessels measured within the arteriole (10–200 μm^2), small artery (200–700 μm^2), and large artery (1000–2500 μm^2) size ranges were compared across all animal groups. Data are expressed as percent 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 α-SMA are shown. α-SMA stain is red and counterstain is violet. Scale bars = 500 μm

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 s, 56 °C annealing for 15 s, and extension at 72 °C for 28 s, followed by a final extension at 72 °C for 10 min. 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 s, 60 °C annealing for 17 s, and extension at 72 °C for 28 s, followed by a final extension at 72 °C for 10 min. 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). Inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNFα), arginase 1 (Arg1), IL-10, and transforming growth factor-β (TGFβ) 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 ± S.E.M. from at least three independent experiments. Statistical significance for quantitative results was assessed using analysis of variance, followed by either 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 three 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, LDPI was used to measure perfusion as an indicator of collateral vessel formation in mice treated with 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, at 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 Fig. 1e (and Online Fig. 2b), OPN$^{-/-}$ 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 Fig. 1b, c). 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

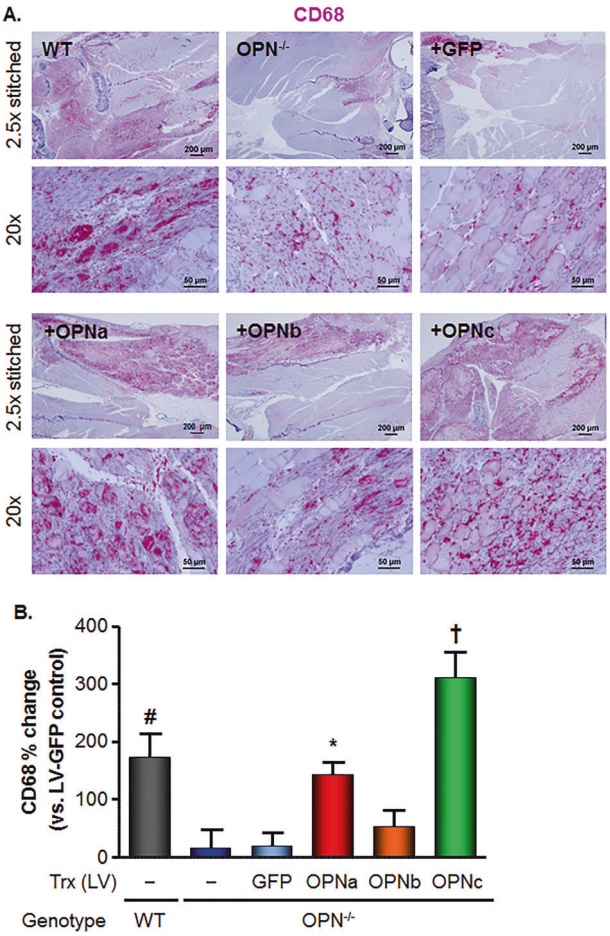

Fig. 3 OPN isoforms differentially influence macrophage accumulation in vivo. **a** To determine if OPN isoforms differentially promote macrophage accumulation, histology sections from WT, OPN$^{-/-}$, and OPN isoform a-, b-, or c-treated animals were stained for CD68 (macrophage marker; pink stain) 5 days post HLI. The 20× representative images show detail. The 2.5× images stitched together across the hindlimb demonstrate tissue-wide differences. Scale bars for 2.5× stitched = 200 µm. Scale bars for 20× = 50 µm. **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$^{-/-}$, LV-GFP; $^{*}p < 0.05$ vs. OPN$^{-/-}$, LV-GFP, and LV-OPNb, $^{\dagger}p < 0.001$ vs. OPN$^{-/-}$, LV-GFP, LV-OPNa, and LV-OPNb; $n = 4$

vessel counts were quantified as a readout for angiogenesis, whereas α-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, b), OPN isoforms did differentially affect arteriogenesis. OPN isoforms did not affect the total number of α-SMA-positive vessels (Fig. 2a, representative images in Fig. 2c); however, OPNa and OPNc both significantly increased the overall sizes of the α-SMA-positive vessels, decreasing the number of vessels that fall within the arteriole size bins (10–200 µm^2 in size), while significantly increasing the number of vessels that fall within the small

artery (200–700 μm^2) and large artery (1000–2500 μm^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 quantitative RT-PCR (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$^{-/-}$ peritoneal macrophages were stimulated with 100 ng/mL of OPN a, b, or c for 48 h or with INFγ 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, TNFα, Arg, IL-10, and TGFβ (Fig. 4a–e). No significant differences in macrophage polarization markers were detected in response to OPN isoform stimulation, unlike the increases measured in response to INFγ 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$^{-/-}$ macrophages exhibit increased apoptosis compared to WT cells by assessing annexin V and PI staining by FACS analysis as early and late markers of apoptosis, respectively. WT and OPN$^{-/-}$ macrophages exhibited no differences in annexin V or PI staining with serum withdrawal (Fig. 5a, b). 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 Fig. 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 affect macrophage migration, we used a modified Boyden chamber assay. WT or OPN$^{-/-}$ macrophages were stimulated with 50 ng/mL of OPN isoform a, b, or c, an established EC_{50} (half-maximal effective concentration) dose of OPN isoforms for migration and in line with reported pathophysiologic levels of OPN (Online Fig. 5a, b) [45], and allowed to migrate for 2 h. OPN$^{-/-}$ macrophages exhibited impaired migration in response to MCP-1 compared to WT macrophages (Fig. 6a), which is consistent with what has been reported previously [31]. Interestingly, OPNa and OPNc significantly rescued impaired migration in OPN$^{-/-}$ 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 Fig. 6c.

Discussion

The data presented in this study are the first to establish that OPN isoforms differentially promote functional post-

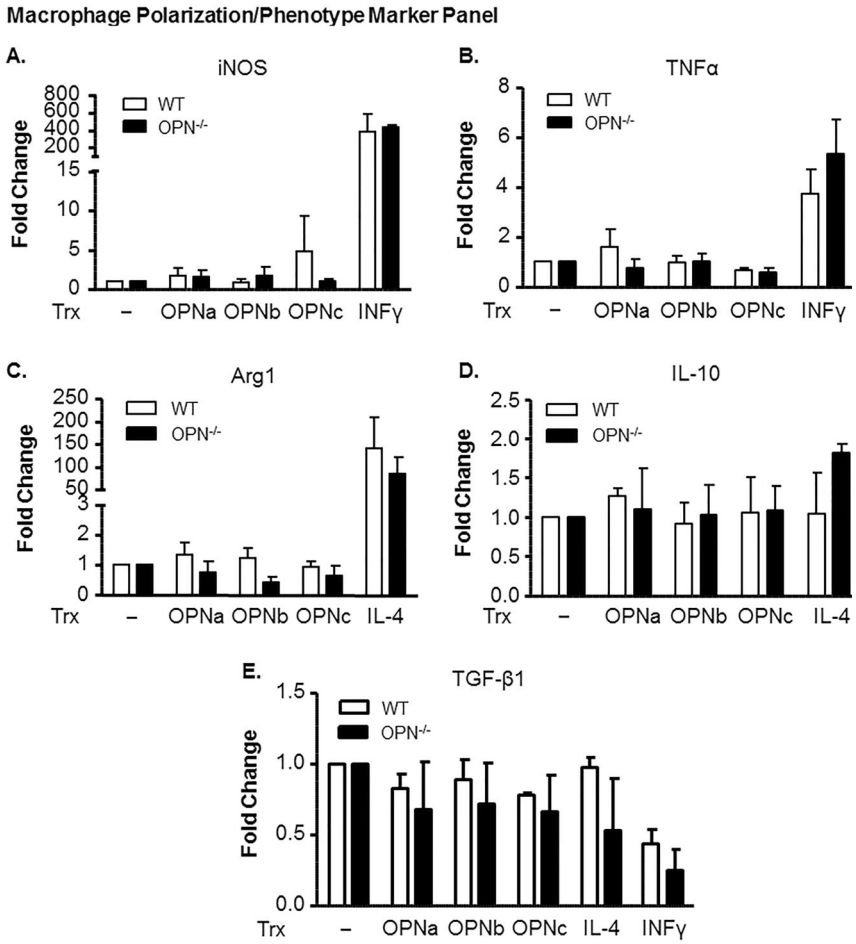

Fig. 4 Human OPN isoforms do not affect macrophage polarization. To determine if OPN isoforms influence macrophage phenotype/polarization, OPN$^{-/-}$ peritoneal macrophages were cultured in vitro for 48 h, followed by stimulation with 100 ng/mL of OPN a, b, or c for 48 h or with 20 ng/mL of INFγ or 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** TNFα, **c** Arginase (Arg1), **d** IL-10, and **e** TGFβ. Stimulation with INFγ and IL-4 served as positive controls. No significant differences were detected in response to OPN isoforms ($n = 3$)

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 who 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 [55, 56]. It has been shown previously that there is a substantial decrease in migration of OPN$^{-/-}$ monocytes/macrophages, which ultimately leads to impaired collateral vessel formation in OPN$^{-/-}$ mice [31], 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 three 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 different effects on neovascularization is, in part, through divergent effects on post-ischemic macrophage accumulation. Subsequently, after macrophage migration into the ischemic region

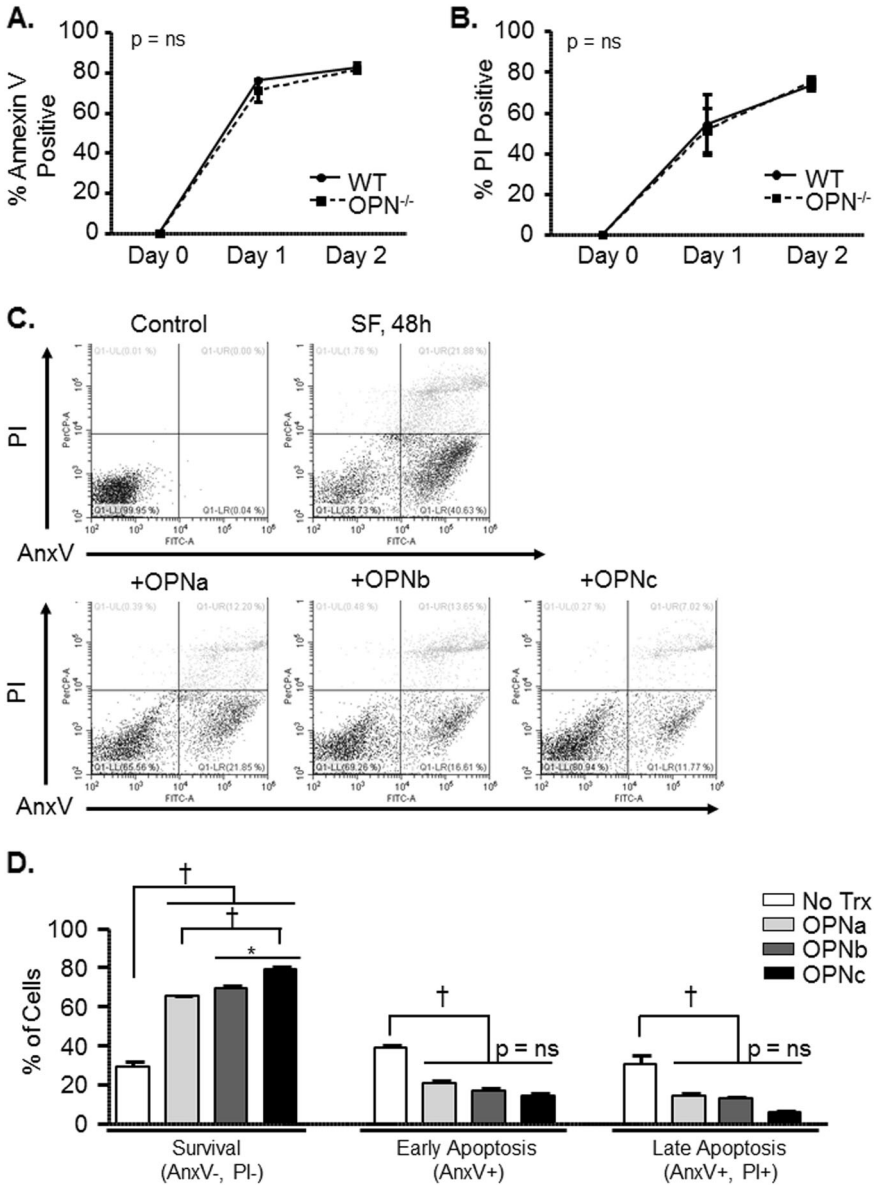

Fig. 5 Human OPN isoforms increase macrophage survival and decrease macrophage apoptosis. To determine if OPN isoforms affect macrophage survival and apoptosis, WT and $OPN^{-/-}$ peritoneal macrophages were harvested and plated. Media were changed to serum free media ± 100 ng/mL of OPN a, b, or c for 48 h before staining with annexin V (AnxV, early apoptosis marker) and/or propidium iodide (PI, late apoptosis marker) and analyzing by flow cytometry. **a**, **b** Macrophage apoptosis, measured by AnxV and PI staining, did not differ between $OPN^{-/-}$ and WT upon serum withdrawal. **c** Representative scatter plots for OPN isoform effects on macrophage survival are shown. Viable cells = $AnxV^{-}/PI^{-}$, early apoptosis = $AnxV^{+}/PI^{-}$, late apoptosis = $AnxV^{+}/PI^{+}$, and necrosis = $AnxV^{-}/PI^{+}$. 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 ($n = 4$, $^{*}p < 0.05$, $^{\dagger}p < 0.001$)

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 study 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.

$OPN^{-/-}$ mice exhibit impaired collateral vessel formation in response to an ischemic insult [31], 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 [21]. Additionally, the use of $OPN^{-/-}$ mice allowed us to utilize a loss-of-function/gain-of-function approach for our studies. To add the human OPN isoforms to the $OPN^{-/-}$ 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

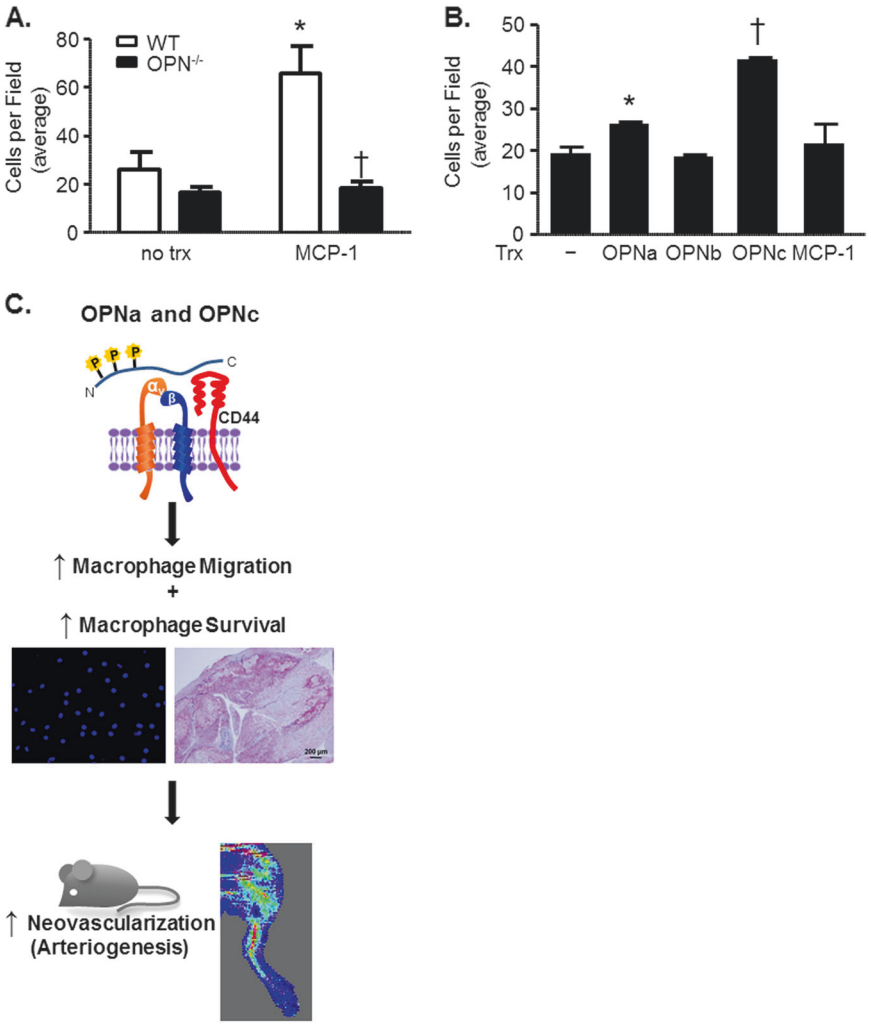

Fig. 6 Effects of MCP-1 and OPN isoforms on macrophage migration in vitro. Peritoneal macrophages were used as an in vitro cell model to measure isoform effects on macrophage migration using a modified Boyden chamber assay. **a** Monocyte chemoattractant protein-1 (MCP-1), a known stimulus for macrophage migration, was used to first determine if $OPN^{-/-}$ and WT macrophages exhibit differences in MCP-1-mediated migration after 2 h ($n = 4$, $^{*}p = 0.0027$ vs. WT− no trx, $^{\dagger}p < 0.001$ vs. WT+ MCP-1). **b** To assess if OPN isoforms differentially promote macrophage migration, $OPN^{-/-}$ macrophages were stimulated with 50 ng/mL of recombinant OPN a, b, or c and macrophage migration was quantified after 2 h ($n = 4$, $^{*}p < 0.05$ vs. no trx and OPNb, $^{\dagger}p < 0.001$ 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

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 $OPN^{-/-}$ 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 study 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 [32, 35, 36, 38, 39, 42, 43, 57]. 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 [58]. 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 Fig. 1a, OPNc (transcript variant 3) lacks exon 4, which contains several glutamine residues subject to transglutamination [59, 60]. Consequently, unlike OPNa and OPNb,

OPNc is likely unable to be cross-linked to form polymeric complexes [61, 62], 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, but this has yet to be determined through X-ray crystallography or nuclear magnetic resonance and may prove difficult given the flexible structure of OPN [63]. 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 [64]. 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 [65, 66]. Therefore, to investigate how each human OPN isoform influences macrophage biology, we utilized an in vitro approach and isolated $OPN^{-/-}$ 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) [15, 16, 20], which are necessary for function [15–17, 20, 67, 68].

Macrophages are critical to arteriogenesis [31, 69], 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 [70, 71], we found that $OPN^{-/-}$ and WT primary macrophages were not different in their ability to polarize when stimulated with INF-γ or IL-4. Importantly, stimulation of $OPN^{-/-}$ 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 [72], and thus promotes cancer cell metastasis due to its anti-apoptotic characteristics [73]. 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 $OPN^{-/-}$ mice normally migrate towards colony stimulating factor-1 (CSF-1), but not towards MCP-1, which signals through G protein-coupled receptors (GPCRs) [74]. Since GPCR-mediated migration is dependent upon the level of cell surface CD44 receptors, we hypothesize that the macrophages from $OPN^{-/-}$ 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 [75] demonstrated that human primary macrophages stimulated with recombinant OPNa have an increase in pro-inflammatory response in an RGD-dependent manner and that all three isoforms equally increase macrophage phagocytosis. However, the study utilized human OPN isoforms (OPN a, 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 [76]. Additionally, they utilized a very high dose of OPNa (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 [77]. 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.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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