Loss of osteoglycin promotes angiogenesis in limb ischaemia mouse models via modulation of vascular endothelial growth factor and vascular endothelial growth factor receptor 2 signalling pathway

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Objective
Osteoglycin (OGN) has been noted for its implication in cardiovascular disease in recent studies. However, the relationship between OGN and angiogenesis remains unknown. Therefore, we aimed to investigate the effect of OGN on ischaemia-induced angiogenesis and to address the underlying mechanisms.

Methods and results
The expression of OGN was decreased in a limb ischaemia mouse model. OGN knockout (KO) mice were used to further understand the role of OGN after ischaemia. The perfusion recovery rate after femoral artery ligation was higher in OGN KO mice than in wild-type (WT) mice. The capillary density in the gastrocnemius muscle of the ischaemic limb was also higher in OGN KO mice. Moreover, ex vivo aortic ring explants from OGN KO mice exhibited stronger angiogenic sprouting than those from WT mice. In human umbilical vein endothelial cells (HUVECs), OGN knockdown enhanced endothelial cell (EC) activation, including tube formation, proliferation, and migration. In contrast, OGN overexpression inhibited HUVEC activation. Mechanistic studies revealed that OGN associates with vascular endothelial growth factor receptor 2 (VEGFR2) and negatively regulates the interaction of vascular endothelial growth factor (VEGF) and VEGFR2, thereby negatively modulating the activation of VEGFR2 and its downstream signalling pathways. Consistently, the pro-angiogenic effect of OGN KO was abrogated by VEGFR2 inhibition, supporting the critical role of VEGFR2 signalling in OGN-mediated regulation of angiogenic function.

Conclusions
OGN plays a critical role in negatively regulating ischaemia-induced angiogenesis by inhibiting VEGF–VEGFR2 signalling and thereby attenuating EC tube formation, proliferation, and migration. Thus, OGN may be a novel therapeutic target for ischaemic vascular diseases.

Keywords
Osteoglycin • Angiogenesis • Extracellular matrix • VEGF • VEGFR2

1. Introduction
Angiogenesis, the formation of new capillaries from pre-existing vessels, can be triggered under conditions of ischaemia, hypoxia, or tumour metastasis.1 It is an adaptive response and a well-controlled process regulated by numerous environmental factors. Angiogenesis is often implicated in cardiovascular diseases, including peripheral artery disease (PAD),2 coronary artery disease,3 thoracic aortic aneurysm,4 and...
diseases where ischaemia takes place.\textsuperscript{5} Although angiogenesis has been anticipated as a therapeutic target for ischaemic diseases, the regulatory mechanism has not been clearly elucidated.

Vascular endothelial growth factor (VEGF) and VEGF receptor 2 (VEGFR2) signalling pathway activation is crucial for inducing therapeutic angiogenesis and has been used to treat ischaemic disease.\textsuperscript{6–8} However, the safety and efficiency of VEGF treatment are still under debate. In addition to the VEGF–VEGFR2 signalling pathway, constituents of the extra-cellular matrix (ECM), including collagen, gelatin, and proteoglycans, are also involved in angiogenesis.\textsuperscript{9,10} Consequently, an association between growth factor receptors and the ECM has been implicated in the regulation of angiogenesis.\textsuperscript{3} Different mechanisms have been found involved in the interaction of VEGFR2 and ECM components, and the ECM seems to be a new target for modulating angiogenesis.

Osteoglycin (OGN) is a proteoglycan in the ECM. It is expressed in a variety of organs and exerts actions in bone formation,\textsuperscript{11} collagen fibrillogenesis,\textsuperscript{12} and tumours,\textsuperscript{13} and it is also implicated in connective tissue diseases.\textsuperscript{14} In 2008, OGN was first found to be correlated with left ventricular mass,\textsuperscript{15} which led to the emergence of studies exploring the role of OGN in cardiovascular diseases. These studies revealed that OGN had protective effects against cardiac ageing\textsuperscript{16} and heart failure following myocardial infarction.\textsuperscript{17} However, whether OGN participates in angiogenesis and how it functions as a proteoglycan is still unknown.

One purpose of this study is to clarify whether OGN participates in ischaemia-induced angiogenesis. The expression of OGN was detected, and OGN knockout (KO) mice were used to establish a limb ischaemia mouse model. Another purpose of this study is to investigate how OGN is involved in angiogenesis and to determine the relationship of OGN with VEGF–VEGFR2 signalling.

2. Methods

An expanded methods section with details of reagents and ethical issues are provided as a Supplementary material online.

2.1 Animas model and assessments

OGN KO mice and their wild-type (WT) littermates used in the present study were provided by Professor Song Huai-Dong. All the procedures were approved by the Animal Care Committee of Shanghai Jiaotong University and in compliance with the animal management rules of the Chinese Ministry of Health and NIH guidelines. Mice were anesthetized and subjected to ischaemia of the right hind-limb by right femoral vessel (artery and vein) ligation. Ischaemia and reperfusion of the hind-limb were evaluated by serial laser Doppler imaging and angiogenesis in tissue sections of the gastrocnemius muscle. For VEGFR2 inhibition, a VEGFR2 kinase inhibitor (Millipore 676485) was dissolved in ethanol and stored at 4°C. A dosage of 1 mg/kg in 100 mL ethanol was carefully and slowly injected into the skeletal muscle of mice on the day before surgery, 3 days after surgery, 7 days after surgery, and 11 days after surgery. For the control animals, only ethanol was injected. Mice were anesthetized by isoflurane inhalation during the procedure. On the 14th day after surgery, perfusion was assessed, and samples of skeletal muscle were collected for immunofluorescence staining.

2.2 Immunofluorescence and immunohistochemistry

Immunofluorescence and immunohistochemistry were performed according to a previously established protocol. Heat-mediated antigen retrieval was used to enhance antigen exposure before detection. Antibodies against OGN and CD31 were obtained from Santa Cruz (sc-374463 and sc-1506, respectively) and CD45 antibody was obtained from Abcam (ab10558).

2.3 Western blot

RIPA lysis buffer was used for protein extraction. Protein from either cell lysates or mouse tissues were separated by SDS-PAGE (6–20% gel) and then transferred to Immobilon-P membranes. Western blotting was then performed using antibodies against OGN (sc-374463, sc-47279), VEGF (Millipore 07-1420), VEGFR2 (Cell Signalling Technology 2479S), phospho-VEGFR2 (Cell Signalling Technology 2478), ERK1/2 (Cell Signalling Technology), phospho-ERK1/2 (Cell Signalling Technology 4370), Akt (Cell Signalling Technology 9272), and phospho-Akt (Cell Signalling Technology 4060). Secondary antibodies (Cell Signalling Technology) were used to visualize the immunoblots, and ImageJ was employed to quantitatively analyse the intensity.

2.4 RNA extraction and real-time PCR

Total RNA was isolated according to the standard protocol. Reverse transcription was performed using the Promega Reverse transcription system. Real-time (RT) PCR was performed using Takara SYBR Green Reagents. The primer sequences are listed in the Supplementary material online.

2.5 Cell culture and RNA interference

Human umbilical vein endothelial cells (HUVECs) were used for cell experiments. Primary cells were isolated from fresh umbilical cords, which were donated by six different parturients. All the procedures were approved by the Ethics Committee of Ruijin Hospital and conformed to the declaration of Helsinki. Cords without either damage or infection were used. After cutting both ends of the cord, a cannula at was introduced into each extremity of the vein and tightly maintained with string. A solution of 0.9% saline was used to wash the cord vein until the effluent buffer was slightly pink. HUVECs were isolated by 2.5% collagenase (Sigma) and subsequently cultured in endothelial cell (EC) medium and ECGS supplements obtained from ScienCell.

Small interfering RNA (siRNA) was transfected using Lipofectamine. Cells were cultured in serum-free OPTI-MEM medium for approximately 6 h. Then, siRNA was mixed with Lipofectamine and added to the cells with fresh OPTI-MEM. After 6 h, the OPTI-MEM containing siRNA and Lipofectamine was replaced with complete medium with 5% FBS. The negative control (NC) (sc-37007) and OGN (sc-61267) siRNAs were used at a concentration of 50 nM.

2.6 Plasmid construction and transfection

Human OGN cDNA was chemically synthesized. The cDNA fragments were cloned into the pcDNA3.1 plasmid. Constructs were verified by sequencing. Transient transfection of HUVECs with either control pcDNA3.1 or pcDNA3.1-OGN plasmids were conducted using Lipofectamine reagent (Invitrogen).

2.7 In vitro tube formation, proliferation, and migration assays

HUVECs were transfected with siRNA for 48 h and then seeded into 96-well plates on Matrigel (BD: 356234). Tube formation was observed every hour after cell seeding. Representative photos were taken 4 h after seeding, and randomly selected microscopic fields were selected for quantity. Tube length was calculated by using Image-Pro Plus software. The proliferation rates of HUVECs after siRNA interference were measured by EdU staining (RiboBio C10310-3). Transfected cells were starved without serum for 12 h, after which the medium was replenished with FBS and EdU staining was observed.
expression was decreased early after onset of ischaemia and was maintained at this lower level after reperfusion.

3.2 OGN deficiency promoted blood flow recovery after ischaemia in vivo and aortic ring angiogenesis ex vivo
To explore the role of OGN in ischaemia-induced angiogenesis, OGN KO mice were used. The baseline blood flow, vasculature, and ECM of OGN KO mouse did not differ from WT control mice (Supplementary material online, Figure 1). In our mouse model of limb ischaemia, we found that the KO group had a much faster blood flow recovery than the WT group (Figure 2A). The blood flow recovery of WT mice was 24, 8 ± 5.4%, 44.6 ± 9.4%, and 63.5 ± 15.7% at Day 4, Day 7, and Day 14 after limb ischaemia, respectively, while that of the KO mice was 46.5 ± 6.9%, 71.5 ± 17.0%, and 83.0 ± 9.2%, respectively (Figure 2B). Histology, angiogenesis, inflammatory cell infiltration and the levels of inflammatory cytokines were analysed seven days after ischaemia. The data showed that the capillary densities measured by CD31-positive cells in the gastrocnemius were significantly higher in KO mice compared with WT mice (Figure 2C). However, no difference was observed in the histology, inflammatory cell infiltration and levels of inflammatory cytokines between these two groups (Supplementary material online, Figure 2).

To evaluate the contribution of OGN in vascular cells, the ex vivo aortic ring sprouting assay was performed. We found that aortic rings from KO mice had stronger angiogenic growth than aortic rings from WT mice as quantified by the sprouting numbers (Figure 2D). In addition, we examined corneal angiogenesis and found that alkali-induced corneal neovascularization as measured by CD31-positive cells was more robust in KO mice than WT mice (Supplementary material online, Figure 3). We further evaluated the effect of gain-of-OGN function on angiogenesis. First, we examined corneal vessels in mice with the systemic delivery of an OGN-expressing adenovirus. Overexpression of OGN revealed a much smaller number of vessels in the cornea 10 days after alkali injury (Supplementary material online, Figure 4A). Secondly, aortic sprouting was attenuated in aortic rings treated with OGN adenovirus ex vivo. Thirdly, recombinant OGN also inhibited aortic sprouting of aortic rings (Supplementary material online, Figure 4B and C). These data together indicate that OGN deficiency enhanced angiogenesis while OGN overexpression inhibited angiogenesis.

3.3 OGN negatively regulated EC tube formation, proliferation, and migration in vitro
Because ECs play critical roles in angiogenesis, we determined the role of OGN in tube formation, proliferation, and migration, all of which are important events in the angiogenic process. We used primary cultured HUVECs, in which we detected significant expression levels of OGN (Supplementary material online, Table 1). Knockdown of OGN with siRNA increased tube formation in Matrigel (Figure 3A and Supplementary material online, Figure 5). Additionally, HUVEC proliferation as measured by the 5-ethyl-2′-deoxyuridine (EdU) cell proliferation assay was increased (Figure 3B). In addition, HUVECs treated with OGN siRNA displayed an increased capacity of migrating in the wound-healing assay (Figure 3C). Moreover, the EC functions of tube formation, proliferation, and migration were also assessed in HUVECs transfected with a plasmid overexpressing OGN. The data showed that tube formation was inhibited in cells overexpressing OGN (Figure 3D), and fewer EdU-positive cells were detected in cells overexpressing OGN (Figure 3E).
Migration speed in the wound healing assay was impeded when OGN was increased (Figure 3F). Thus, these data indicate that altering OGN expression influenced the tube formation, proliferation, and migration of ECs, which are essential for angiogenesis.

### 3.4 OGN regulated the VEGF–VEGFR2 signalling pathway

Because VEGF–VEGFR2 is critical in angiogenesis, we examined whether OGN affects the VEGF–VEGFR2 pathway. We first detected the expression of VEGF and VEGFR2 levels and found that OGN knockdown did not alter either VEGF or VEGFR2 protein levels (Supplementary material online, Figure 5E). Next, we examined whether activation of the VEGF signalling pathway was modulated by OGN suppression. Upon VEGF stimulation, phosphorylation of VEGFR2 was observed at 5 min and declined after 15 min in HUVECs treated with NC siRNA. However, HUVECs transfected with OGN siRNA revealed stronger and longer instances of VEGFR2 phosphorylation. Similarly, AKT and ERK1/2 activation in response to VEGF was also enhanced and lasted longer in OGN-knockdown HUVECs (Figure 4A). On the other hand, overexpression of OGN suppressed the activation of VEGF-stimulated VEGFR2, AKT, and ERK (Figure 4B). The quantitative results are presented in Figure 4C–H. These data suggested that OGN negatively regulates VEGF–VEGFR2 signalling.

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**Figure 1** OGN expression was down-regulated after ischaemia. (A) Representative western blot shows that protein levels of OGN were down-regulated after ischaemia. Tubulin was used as an internal reference. (B) Quantitative analysis of the western blot is presented as the ratio of OGN/tubulin. Data are shown as a dot plot (n = 9 for each time point). **P < 0.01 vs. control by one-way ANOVA followed by Tukey’s test. (C) Relative OGN mRNA levels were detected by RT–PCR using tubulin as an internal reference. The results were normalized to the value of the control. **P < 0.01 vs. control by one-way ANOVA followed by Tukey’s test. (D) Representative immunofluorescence pictures of OGN expression in intermuscular ECs of the mouse limb. OGN was stained red; ECs were stained with anti-CD31 (green); and nuclei were stained with DAPI (blue). Scale bar indicates 50 μm. The magnified image with the three merged channels (green, red, and blue) shows colocalization of OGN and CD31 in physiological conditions without ischaemia. In ischaemic conditions (seven days after femoral artery ligation), OGN fluorescence was much weaker than control on CD31-positive cells. OGN, osteoglycin; ECs, endothelial cells.
Figure 2. Improved blood flow recovery and EC sprouting in OGN KO mice. (A) Temporal blood flow reperfusion measured using a laser Doppler imaging system. (B) Line chart indicates the ratio of the ischaemic side to the non-ischaemic side at different time points after injury of the WT group (n = 12) and KO group (n = 16). Data are presented as the mean ± SD. *, #, and @ indicate P-values of KO < 0.05 vs. WT by repeated ANOVA followed by Bonferroni tests. (C) Capillary density was assessed by immunohistochemical staining of CD31 (left) at Day 7 after ischaemia. Scale bar represents 20 μm. Scatter plot on the right shows data from 27 fields of three different mice per group (nine fields per mouse). **P < 0.01 vs. WT by nested ANOVA test. (D) Aortic rings from WT and OGN KO mice embedded on collagen and cultured with VEGF (30 ng/mL) supplement. Scale bar represents 50 μm. Scatter plot on the right shows the number of sprouts per aortic ring after seven days of culture. The sample number of the WT group is 12 aortic rings isolated from four mice while the sample number of the KO group is 18 aortic rings from six KO mice. *P < 0.01 vs. WT by nested ANOVA test. OGN, osteoglycin; KO, knockout; WT, wild-type; VEGF, vascular endothelial growth factor.
3.5 OGN is associated with VEGFR2 and negatively modulated VEGF–VEGFR2 interactions

To investigate how OGN regulates the VEGFR2 signalling pathway, we performed a co-immunoprecipitation (co-IP) assay between OGN and VEGFR2. We found that IP of OGN pulled down VEGFR2 (Figure 5A) and IP of VEGFR2 pulled down OGN (Figure 5C) in cultured HUVECs. We also performed co-IP using samples of mouse skeletal muscles and obtained similar findings (Figure 5B and D). Moreover, in OGN-deficient ECs from OGN KO mice, exogenous OGN could also interact with VEGFR2 in ECs (Supplementary material online, Figure 6). These results strongly suggested a physical association between OGN and VEGFR2.

We next examined the effect of VEGF on the OGN–VEGFR2 interaction. A 5-min treatment with VEGF did not change the expression of either OGN or VEGFR2 (Figure 5F). However, the association of OGN and VEGFR2 as detected by co-IP was attenuated by VEGF stimulation (Figure 5E). Furthermore, we examined the effect of OGN on the interaction between VEGF and VEGFR2. We found that knockdown of OGN did not increase VEGF expression but enhanced the association of VEGF–VEGFR2 with or without VEGF treatment (Figure 5G and H). These data suggested that by associating with VEGFR2, OGN competed with VEGF to interact with VEGFR2, thus serving as an inhibitory role of VEGF–VEGFR2 signalling.

3.6 Inhibition of VEGFR2 abrogated the effects of OGN KO in vivo

To determine the role of VEGF2 in OGN-mediated effects in vivo, a VEGFR2 inhibitor was used. Mice were injected with the VEGFR2 inhibitor, and blood flow was assessed 14 days after surgery. As shown in Figure 6A, the VEGFR2 inhibitor impeded the perfusion recovery in both OGN WT and KO mice. The perfusion recovery of WT and KO mice after VEGFR2 inhibition was no longer significantly different. The quantitative result is presented in Figure 6C. Similarly, there was no difference of CD31-positive cells in these two groups (Figure 6B), and the quantitative result is presented in Figure 6D. These data indicate that the pro-angiogenic effect of OGN KO was abrogated by the VEGFR2 inhibitor and suggest that VEGFR2 is a key mediator in OGN-regulated angiogenesis.

4. Discussion

In the present study, we provided novel insight into the role of OGN in ischaemia-induced angiogenesis using both in vitro and in vivo approaches. We found that the loss of OGN is protective against ischaemia through the activation of an angiogenic signalling pathway. Another key discovery was used to determine significance. **P < 0.01 vs. control. (E) Overexpression of OGN inhibited HUVEC proliferation as measured by EdU staining (left). Quantitative results are (right) shown as percentage of positive cells (green/blue) from four different batches of HUVECs. **P < 0.01 vs. NC by t-test. Scale bar represents 100 μm. (F) OGN impeded migration in the wound-healing migration assay (left). Scatter plot on the right indicated the average migration speed 24 h after scraping from four independent cultures of HUVECs. **P < 0.01 vs. control by t-test. Scale bar represents 50 μm. OGN, osteoglycin; HUVECs, human umbilical vein endothelial cells; NC, negative control; siRNA, small interfering ribonucleic acid.
The interaction of the ECM and VEGFR2 has long been a focus in angiogenesis. Harbouring 7 leucine-rich repeats in its structure, OGN belongs to the small leucine-rich proteoglycan (SLRP) family, which is characterized by relatively small protein cores and tandem leucine-rich repeats. In recent years, the importance of the SLRP family in angiogenesis has been reported. For example, decorin, one of the most intensively studied members of the SLRP family, has also been found to interact with VEGFR2 via its core peptides LRR5 and thus attenuates cell proliferation and migration induced by VEGF. Moreover, Thomas Neill et al. also found that decorin has a high binding affinity to VEGFR2 in a region between Ig domains 3–5, which partially overlap with VEGF-A. Interestingly, although both decorin and OGN belong to the same family and can interact with the same receptor, the amino acid sequence in decorin that binds to VEGFR2 is not present in OGN. Therefore, we performed a molecular docking analysis of OGN to VEGFR2 to understand how OGN associates with VEGFR2. As shown in Supplementary material online, OGN was docked into the binding pocket of the VEGF2. The detailed analysis shows that Tyr196, Glu176, and Glu108 of OGN form hydrogen bonds with residues Glu140, Tyr194, and Tyr190 of VEGF2, respectively, which is the main binding interaction between OGN and VEGF2. This model of molecular simulation supports the direct interaction between OGN and VEGF2 and provides valuable information for further understanding how OGN functions.

The source of OGN detected in limb vasculature is likely contributed by multiple cell types, including ECs, SMCs, and others. We have shown expression of OGN in CD31-positive EC cells by immunostaining (Figure 1D) and in cultured HUVECs by RT–PCR, western blotting, and ELISA (Supplementary material online, Figure 5 and Table 1). We detected OGN in the supernatant of cultured HUVECs (1887 ± 569 pg/mL, n = 6 cultures); by comparison, the levels of OGN detected in the supernatants from human vascular smooth muscle cells were higher (4396 and 5470 pg/mL in two independent experiments). However, a few previous studies failed to detect OGN in EC by either in situ hybridization.
Figure 5 OGN associated with VEGFR2 and modulated VEGF–VEGFR2 interaction. (A) Co-IP assay of OGN associating with VEGFR2 using anti-OGN antibody as the IP antibody and IgG as the control. IB showed a specific VEGFR2 band in the anti-OGN lane while no band was observed in the IgG lane when co-IP was performed using lysate from HUVECs. The present blots are examples of four independent experiments. The three other blots are incorporated in Supplementary material online, Figure 10A–C. (B) Co-IP assay of OGN associating with VEGFR2 using protein extracted from mouse skeletal muscle using anti-OGN antibody as the IP antibody and IgG as the control. The present blots are examples of four independent experiments. The three other blots are incorporated in Supplementary material online, Figure 10D–F. (C) Co-IP assay of VEGFR2 associating with OGN using anti-VEGFR2 antibody as the IP antibody and IgG as the control. OGN was detectable in the anti-OGN lane while no band was observed in the IgG lane when co-IP was performed using lysate from HUVECs. The present blots are examples of four independent experiments. The three other blots are incorporated in Supplementary material online, Figure 10G–I. (D) Co-IP assay of VEGFR2 associating with OGN using protein extracted from mouse skeletal muscle. The present blots are examples of four independent experiments. The three other blots are incorporated in Supplementary material online, Figure 10J–L. (E) Co-IP assay of OGN associating with VEGFR2 after different doses of VEGF treatment. IB showed that after incubation with VEGF for 5 min, the amount of VEGFR2 that interacted with OGN was decreased. The present blots are examples of four independent experiments. The three other blots are incorporated in Supplementary material online, Figure 11A, C, and E. (F) Western blot of cell lysates was used as the input for (E), showing that short incubation of VEGF treatment did not change the expression of OGN or VEGFR2. The present blots are examples of four independent experiments. The three other blots are incorporated in Supplementary material online, Figure 11B, D, and F. (G) Co-IP assay of VEGFR2 associating with either OGN or VEGF. OGN knockdown led to more VEGF interacting with VEGFR2. In HUVECs transfected with either NC or OGN siRNA, VEGF treatment (100 ng/mL 5 min) resulted in more VEGF but less OGN interacting with VEGFR2. The present blots are examples of four independent experiments. The three other blots are incorporated in Supplementary material online, Figure 11G, I, and K. (H) Western blot shows that OGN was down-regulated after OGN siRNA transfection. However, OGN knockdown did not change the protein expression of VEGFR2 or VEGF, and VEGF treatment did not alter OGN or VEGFR2 expression. The present blots are examples of four independent experiments. The three other blots are incorporated in Supplementary material online, Figure 11H, J, and L. OGN, osteoglycin; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; co-IP, co-immunoprecipitation; IB, immunoblotting; HUVECs, human umbilical vein endothelial cells; NC, negative control; siRNA, small interfering ribonucleic acid.
Inhibition of VEGFR2 abrogated the effects of OGN KO. (A) Blood flow reperfusion was measured with a laser Doppler imaging system. After 14 days of ischaemia, blood flow in KO mice exhibited better recovery than in WT mice. After injection of the VEGFR2 inhibitor, the advantage of the OGN KO was fully abrogated. Both WT and KO mice had compromised reperfusion after VEGFR2 inhibition. N = 8 for each group. (B) Immunofluorescence shows that CD31 was present 14 days after ischaemia. In mice subjected to VEGFR2 inhibition, fewer CD31-positive cells were present in post-ischaemic skeletal muscle. Scale bar represents 20 μm. (C) Quantification of (A) is demonstrated in a scatter plot. The ratio of blood flow of the ischaemic side to the non-ischaemic side was used to evaluate reperfusion. **P < 0.01 vs. WT, ###P < 0.01 vs. KO by one-way ANOVA followed by Dunn’s test. No significant difference between WT with VEGFR2 inhibition and KO with VEGFR2 inhibition was found. (D) Quantitative analysis of CD31-positive cells in post-ischaemic skeletal muscle. The 24 individual fields from 8 mice (3 fields for each mouse) were plotted as a group. **P < 0.01 vs. WT, ###P < 0.01 vs. KO by nested ANOVA test. No significant difference between WT with VEGFR2 inhibition and KO with VEGFR2 inhibition has been found. (E) Schematic of the role of OGN in angiogenesis. Under normal conditions, the ECM proteoglycan OGN, but not VEGF, interacts with the extracellular domains of VEGFR2 and inhibits its signal transduction. Under ischaemic conditions, OGN is down-regulated, and VEGFR2 is mostly occupied by VEGF, which results in cell proliferation, migration, tube formation, and angiogenesis. OGN, osteoglycin; VEGFR2, vascular endothelial growth factor receptor 2; VI, VEGFR2 inhibitor; WT, wild-type; KO, knock out.
or immunostaining.2,3,24 The discrepancy might be due to the different sensitivities of the detection methods used in current study and previous studies from a decade ago. In addition to ECs, we found that SMCs also highly express OGN, which is consistent with previous reports of detecting OGN in the vasculature.23,24 Thus, OGN, when localized in proximity with CD31-positive EC cells (Figure 1D), might be derived from more than one cell type. Regardless of the source of OGN, we believe that extracellular OGN plays a critical role in regulating VEGF–VEGFR2 signalling in ECs and subsequently modulates the angiogenic process.

Inflammation has also been implicated in angiogenesis.1 We therefore performed immunostaining of CD45 and found no difference between the two groups (Supplementary material online, Figure 2B). Furthermore, the expression of inflammatory cytokines such as TNF-α, IL-1β, IL-6, and MCP-1 in ischaemic gastrocnemius muscles from KO mice did not significantly differ from those of WT mice (Supplementary material online, Figure 2D–G). In the ex vivo culture model, more resident ECs were sprouting out in OGN-null aortic rings than WT rings, but there was no recruitment of inflammatory cells (Figure 2D). These observations suggest that OGN does not regulate the inflammatory response.

Arteriogenesis, which is the enlargement of collateral vessels after ischaemia, is also an undeniable factor that influences blood flow recovery.25 We were uncertain whether enhanced neovascularization could still be observed in KO mice without the participation of arteriogenesis. As the cornea is avascular, an alkali injury-induced corneal neovascularization model was used to rule out the possibility of involvement of collateral circulation.26,27 We found that KO mice had more robust angiogenesis with more CD31 after alkali injury (Supplementary material online, Figure 3). These data make it reasonable to speculate that the difference in blood flow recoveries may be largely attributable to the function of ECs.

Our study also has some clinical perspectives. The phenomenon of OGN down-regulation in an angiogenic region is also observed in the skeletal muscle of PAD patients (Supplementary material online, Figure 8), which is in accordance with the results of our animal experiments. It is known that capillary density is lower in the limbs of PAD patients suffering from chronic and severe ischaemia, and angiogenic regions are actually insufficient and desired in PAD patients in the later stages of disease.28,29 Therefore, it is rational to suggest that down-regulating the expression of OGN in PAD patients to induce therapeutic angiogenesis may be a new target for PAD treatment.

In conclusion, our results suggested that the down-regulation of OGN has beneficial effects on endothelial sprouting and that as a proteoglycan of the ECM, OGN modulates the VEGF–VEGFR2 interaction and consequently influences angiogenesis (Figure 6E). Thus, OGN may become a novel therapeutic target for ischaemic vascular diseases.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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**References**


Corrigendum

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The authors of the above paper wish to inform readers that there was a spelling error in author, Heleen M. M. Van Beusekom’s name as given. The paper has now been corrected online.