Estrogen-Stimulated Endothelial Repair Requires Osteopontin

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Objective—Estradiol (E2) is known to accelerate reendothelialization and thus prevent intimal thickening and in-stent restenosis after angioplasty. Transplantation experiments with ERα−/− mice have previously shown that E2 acts through local and bone marrow compartments to enhance endothelial healing. However, the downstream mechanisms induced by E2 to mediate endothelial repair are still poorly understood.

Methods and Results—We show here that after endovascular carotid artery injury, E2-enhanced endothelial repair is lost in osteopontin-deficient mice (OPN−/−). Transplantation of OPN−/− bone marrow into wild-type lethally irradiated mice, and vice versa, suggested that osteopontin plays a crucial role in both the local and the bone marrow actions of E2. In the vascular compartment, using transgenic mice expressing doxycyclin regulatable-osteopontin, we show that endothelial cell specific osteopontin overexpression mimics E2-enhanced endothelial cell migration and proliferation in the regenerating endothelium. In the bone marrow cell compartment, we demonstrate that E2 enhances bone marrow–derived mononuclear cell adhesion to regenerating endothelium in vivo, and that this effect is dependent on osteopontin.

Conclusions—We demonstrate here that E2 acceleration of the endothelial repair requires osteopontin, both for bone marrow–derived cell recruitment and for endothelial cell migration and proliferation. (Arterioscler Thromb Vasc Biol. 2008;28:000-000)

Key Words: endothelium ■ endothelial cells ■ hormone ■ bone marrow cells ■ wound healing ■ osteopontin

Maintaining the integrity and function of the endothelial barrier is critical to vessel physiology. Indeed, endothelial damages lead to activation of thrombosis processes, namely after percutaneous transluminal coronary angioplasty often associated with stent fixing. It is now accepted that improving the reendothelialization process limits stent-induced thrombosis formation.

Endothelial repair clearly involves migration and proliferation of endothelial cells (EC), which are in interaction with extracellular matrix proteins,1,2 as well as recruitment of bone marrow (BM)–derived cells including endothelial progenitor cells (EPC).3 At the molecular level, growth factors such as VEGF,4 FGF2,5 and compounds such as statins,6 prostacyclins,6 and estrogens7 accelerate endothelial repair. As previously demonstrated by us and others, 17β-estradiol (E2) accelerates endothelial healing via the estrogen receptor ER-α but not ER-β8 and through the involvement of endothelial NO synthase (eNOS)9,10 and basic FGF.11 This effect of E2 is mediated by BM cells, because it is lost in wild-type mice grafted with ERα-deficient BM cells.11 Moreover, E2 allows mobilization of EPCs12 and increases their number in the regenerating area of wounded arteries.9 On the other hand, E2 favors the proliferation and migration of ECs13 and prevents them from undergoing apoptosis.14 Despite these various effects, the molecular and cellular mechanisms that might account for the stimulating effects of E2 on endothelial repair remain unclear.7,15

Since the 1990s, estrogens have been known to regulate the synthesis of osteopontin (OPN).16 A first in vivo report demonstrated that in absence of OPN, mice are resistant to ovariectomy-induced bone resorption.17 Inhibition of OPN expression by estrogens was also found in vascular smooth muscle cells (SMCs) in vitro and in vivo.18 Conversely, estrogens are known to induce OPN expression in osteoblasts,19 in the kidney,20 and in the uterus luminal epithelium.21 Estrogen responsive elements were actually identified in the OPN promoter.22,23 Estrogen regulation of OPN promoter in ECs or EPCs is not known. OPN functions both as...
a cell attachment protein and as a chemokine, delivering signals to the cells via a number of receptors including several integrins and CD44. Moreover, an intracellular form of OPN is also involved in cell adhesion and migration. As a cell attachment protein, OPN allows adhesion of numerous cell types including ECs. As a signaling molecule, OPN is able to modify gene expression and promote migration of various cell types including ECs, osteoclasts, monocyes, and SMCs. Moreover, OPN inhibits apoptosis in ECs and is involved in angiogenesis and postischemic neovascularization. In contrast to uninjured blood vessels where very little osteopontin is expressed, abundant levels of osteopontin were found in the remodeling media after balloon injury and in atherosclerotic plaques. OPN was also found at the regenerating endothelium wound edge and VEGF, which mediates acceleration of the reendothelialization, increased OPN expression in ECs, suggesting an involvement of OPN in endothelial repair. However, a recent report showed that in vitro, OPN overexpression dramatically impaired reendothelialization by inhibiting EC motility. Thus the role of OPN in vivo must be clarified.

Here, we have investigated the mechanisms involved in E2-stimulated endothelial repair and evaluated the implication of OPN in this process. We demonstrate that OPN is required for E2 to accelerate endothelial repair through cooperation between two cell compartments, ECs and BM-derived cells.

Materials and Methods

Mice

Mice were maintained in a conventional animal facility on a 12-hour light/12-hour dark cycle. Food and water were available ad libitum. All procedures were carried out in compliance with the principles and guidelines established by the National Institute of Medical Research (INSERM) and approved by the Institutional Animal Care and Use Committee. Animals were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg).

OPN deficient mice (OPN−/−) are global knockout. Genotyping was performed as previously described. Tie2-rOPN mice (Tet off) were obtained by mating Tie2-tTA mice with TRE-rOPN mice carrying mouse recombinant OPN (rOPN) under the control of the doxycycline/tetracycline regulatory element (TRE; please see supplemental materials). No intimal thickening was observed after this endothelial repair after experimental endothelial injury of the mouse carotid artery.

Endothelial repair was evaluated by staining the denuded areas with Evans blue dye (Merck; please see supplemental materials). Percentage between blue stained area (unrepaired area) and injured carotid artery area was calculated.

Proliferating EC was evaluated by immunodetection of incorporated 5-bromo-2-deoxyuridine (please see supplemental materials). Proliferation index was calculated by dividing the number of proliferating ECs in ARA or RA by the respective surfaces of these areas.

Immunohistochemistry

OPN protein was detected using a goat polyclonal anti-OPN antibody (Sigma). ECs were identified using anti-CD31 antibody (BD Pharmigen), and nuclei were stained by the DAPI. The control antibody is a goat non immune IgG (Sigma).

In Vivo BM MNC Adhesion Test

The BM was flushed out of the cleaned bone (femur and tibia) with PBS. Mononuclear cells (MNCs) were isolated on a Ficoll gradient (Ficoll-Paque Plus, GE Healthcare, 1100 rpm, 30 minutes) and resuspended in EGM2 Bulletkit medium (Clonetics).

Two hours after carotid injury, mice received a retro-orbital injection of 4×10^9 PKH26-GL (Sigma) labeled BM MNC suspended in 250 μL of 150 mmol/L NaCl (please see supplemental materials). The recipient mouse carotids were harvested 24 hours later. The fluorescent BM MNCs adhering to the wounded area of the carotid artery were counted using fluorescent microscopy.

Statistical Analysis

Results are expressed as means±SEM. Data were analyzed using Kruskal-Wallis 1-way ANOVA with NCSS statistical software. When the test indicated that at least 2 medians are different (probability value <0.05), a 2-sample t test (Mann–Whitney U) was performed to compare medians of each group to each other. A probability value <0.05 indicated significant difference between 2 groups. It was indicated by *. Nonsignificant difference was indicated by NS.

Results

Endothelial OPN Contributes to Endothelial Repair

Previous conflicting in vitro reports show either that OPN is chemotactic for ECs or in contrast that it inhibits in vitro reendothelialization in a model of cultured EC layer injury. To clarify this point, we have generated Tie2-rOPN transgenic mice designed to overexpress recombinant OPN in ECs in a doxycycline-regulatable fashion (Tet-Off strategy). These mice allowed to evaluate the role of EC OPN expression during in vivo EC migration occurring during the endothelial repair after experimental endothelial injury of the mouse carotid artery.

In Tie2-rOPN mice, immunohistological analyses showed that OPN protein is expressed in all the ECs of the healthy carotid artery while it is not detected in healthy carotid artery of wild-type mice (supplemental Figure II). In these mice, the endothelial repair was accelerated compared to that in Tie2-rOPN mice in which the transgene expression was inhibited by doxycyclin (Figure 1). These data demonstrate that in vivo, OPN overexpression in ECs induced an acceleration of the endothelial repair and thus suggest that in vivo, OPN increases EC migration.
E2-Accelerated Reendothelialization Requires OPN

As previously reported, E2 treatment of ovariectomized wild-type mice induced a significant increase of the repaired endothelial area measured 3 and 5 days after injury compared to placebo-treated mice (supplemental Figure IIIA; Figure 2A). Interestingly, the E2 effect was lost in osteopontin-deficient (OPN\(^{-/-}\)) mice. Because the endothelial repair was identical in OPN\(^{-/-}\) and OPN\(^{+/+}\) placebo-treated mice (Figure 2A), this experiment demonstrated that OPN is required for the E2-mediated acceleration of endothelial repair.

When OPN expression was rescued in EC of OPN\(^{-/-}\) mice expressing rOPN (Tie2-rOPN x OPN\(^{-/-}\)/H11002 mice in absence of doxycyclin), endothelial healing was accelerated compared to control doxycyclin-watersed mice (Figure 1). Endothelial OPN overexpression, alone, was thus sufficient to induce an acceleration of the endothelial repair. This led us to postulate that OPN is a crucial downstream mediator of E2 effects on endothelial healing.

To characterize events involved in E2-mediated endothelial repair, proliferating ECs were identified and counted. The number of proliferating ECs in the injured carotid 5 days after injury was significantly increased in E2-treated compared to placebo-treated OPN\(^{-/-}\) mice (Figure 2B). Proliferating ECs were found not only in the repaired endothelium area (RA) but also in a retrograde area (ARA) located in the healthy endothelium upstream of the lesion site. Respective surfaces of these 2 areas were strongly increased in E2-treated OPN\(^{+/+}\) mice (Figure 2C; supplemental Figure IIIB and IIIC). In the RA, the E2-mediated increase in proliferating ECs was proportional to the increase of its surface. The proliferating EC index was thus not significantly different with placebo or E2 treatment (Figure 2D). In contrast, in the ARA, the proliferation index was significantly increased in E2-treated OPN\(^{+/+}\) mice (Figure 2D).

In OPN\(^{-/-}\) mice, E2 was unable to increase the number of proliferating ECs, or to increase the ARA size, which were similar to those in OPN\(^{+/+}\) mice with placebo treatment (Figure 2B and 2C).

These results thus demonstrate that the E2-mediated processes leading to the acceleration of endothelial repair absolutely require OPN.

Origin of OPN Required for the Effect of E2 on Endothelial Healing

The OPN required for the E2-induced endothelial repair increase could come from either vascular or nonvascular cells. OPN was not detected in any layer of the healthy carotid artery of wild-type mice (supplemental Figure II). In contrast, 3 days after endothelium removal, OPN protein was detected in SMCs of the media in the vicinity of the carotid artery bifurcation and in inflammatory cells present in the adventitia, mainly at the level of areas that remained deendothelialized (Figure 3, right panel). Interestingly, OPN was also detected in ECs of the endothelial repaired area but not in ECs of the uninjured endothelial area far from the limit of injury (Figure 3).

Because we previously had shown BM-derived cells to be involved in the E2 effect on reendothelialization,\(^{11}\) we have studied OPN expression in cultured BM-derived cells and demonstrate here that these cells not only expressed the OPN protein (supplemental Figure IVA) but also that the level of OPN expression was dependent on E2 activity (supplemental Figure IVB).

We further tested the involvement of OPN from either BM-derived or vascular cells in E2 effect. We therefore generated hematopoietic chimeric mice by grafting OPN\(^{-/-}\)/H11002 BM to lethally irradiated OPN\(^{+/+}\)/H11001 mice and vice versa. One
month after grafting, endothelial repair was assessed 3 days after endothelial injury. In control mice (OPN+/+ BM+/+ OPN+/+ mouse), E2 still induced an acceleration of endothelial repair, showing that neither the irradiation nor the BM transplantation altered the E2 effect (Figure 4). When OPN+/+ BM was grafted in OPN−/− mice, E2 was unable to accelerate the endothelial repair, suggesting that OPN from BM-derived cells is not sufficient to mediate the E2 effect. Similarly, when OPN−/− BM was grafted in OPN+/+ mice, E2 was also unable to induce an acceleration of the endothelial repair (Figure 4). These results suggest that OPN expression in both BM-derived cells and the remaining vascular cells is required to bring about the E2 effect on reendothelialization.

**OPN Is Involved in the E2-Induced Increase of BM-Derived Cell Adhesion on the Regenerating Endothelial Area**

Although OPN is required in E2-mediated reendothelialization, and E2 supplementation increases EPC number, results of supplemental Figure V indicated that the loss of the E2 effect on reendothelialization in OPN−/− mice was not the result of an alteration of the mobilization of this BM-derived cell population.

According to the adhesive property of OPN,37 we hypothesized that the OPN contribution to reendothelialization could be mediated through its capacity to enhance the adhesion of BM-derived cells to the injured carotid artery in the presence of E2. We therefore investigated the ability of PKH26-GL-labeled BM MNCs from OPN+/+ or OPN−/− mice to adhere to the injured carotid artery of OPN+/+ or OPN−/− ovariectomized mice treated with E2 or placebo. We showed first that OPN−/− BM MNCs adhered to a lesser extent than OPN+/+ BM MNCs from OPN+/+ or OPN−/− mice to adhere to the injured carotid artery of OPN+/+ or OPN−/− ovariectomized mice treated with E2 or placebo. We showed first that OPN−/− BM MNCs adhered to a lesser extent than OPN+/+ BM MNCs from OPN+/+ or OPN−/− mice to adhere to the injured carotid artery of OPN+/+ or OPN−/− ovariectomized mice treated with E2 or placebo. 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the E2 effect and demonstrated that BM MNC adhesion was enhanced by E2 supplementation only when both MNCs and vascular cells expressed OPN (Figure 5). Altogether these data demonstrate that OPN is involved in BM-derived cell adhesion in vivo and is required for increased adherence induced by E2 treatment.

Discussion

We report here for the first time that E2 requires the multifunctional protein OPN to mediate acceleration of endothelial repair after injury. Moreover, we show that this is a complex process requiring vascular as well as BM-derived events because OPN from both bone marrow and nonbone marrow origins is involved in this process.

Reendothelialization is mainly the result of migration and proliferation of ECs located at the edges of the lesion site in the rat aorta.1 In the mouse carotid, the reendothelialization process occurs mainly from the proximal edge of the injured endothelium, in the direction of the blood flow.2 Previous reports suggested that, in vitro, E2 was able to increase EC migration and proliferation.13 We demonstrate here that, in vivo, E2 is able to increase the EC proliferation index not only in the regenerating area (RA) but also in a healthy endothelial area located upstream of the lesion (ARA), thus suggesting that the increased rate of endothelial repair resulted from an enhanced EC migration toward the acellular area.2

In this article we demonstrate that E2-mediated enhancement of EC migration and proliferation is dependent on OPN. Indeed, E2 was unable to induce these effects in OPN−/− mice. Thus, OPN could be considered as a downstream component of the E2 pathway in EC.

Since 1995, OPN was recognized as a chemotactic factor for ECs.26 A controversy regarding the role of OPN originated from the work of Leali et al,23 which showed that OPN overexpression inhibited in vitro reendothelialization. Our data suggest that OPN is not required for E2-independent in vivo reendothelialization, because the velocity of endothelial repair was identical in OPN+/+ and OPN−/− mice under placebo treatment but is required for E2-mediated increase of endothelial repair. The fact that OPN deficiency did not affect endothelial repair suggested that an E2-independent compensatory mechanism could occur in OPN−/− mice. When OPN was constitutively expressed in an EC-specific manner in the TET-inducible transgenic mouse model, reendothelialization was accelerated, suggesting that in vivo, OPN overexpression indeed stimulates endothelial repair. This result underlines the difference between in vivo and in vitro studies probably linked to the differences in EC phenotype, in the extracellular matrix layer and potentially at the level of OPN expression in ECs.

Bone marrow transplantation experiments demonstrating that OPN, whether from the bone marrow or the local compartments, is unable to assume alone the E2-mediated improvement of the endothelial repair, suggested an autocrine role for OPN. Because the intracellular form of OPN has been previously involved in osteoblast and fibroblast migration,25 we can also hypothesize that in our in vivo model, OPN would act intracellularly in ECs rather than through an extracellular action involving integrin engagement.33

Neointima formation after acute endovascular injury is inhibited by E2 supplementation and different mechanisms have been proposed including inhibition of SMC migration and proliferation, and stimulation of reendothelialization.12,38 Interestingly, it was suggested by an in vitro study that E2 inhibits OPN expression in SMCs, which is required for adventitial fibroblast migration involved in intimal thickening.18 In addition, our data show that E2 mediates the acceleration of endothelial repair through an OPN-dependent pathway suggesting dual opposite roles for OPN in SMCs and ECs, which both contribute to neo-intima inhibition.

Different lines of evidence show that E2 increased the number of EPCs in BM and blood but also in the regenerating endothelium.5,12 We show here that E2 not only increases BM mobilization but also enhances their homing to the injured area, and that OPN is required in this last effect. Mechanisms involved in the homing are complex, but adhesion molecules previously known to be involved in phases of rolling and firm adhesion of leukocytes appear as key regulators. Here we suggest that OPN is also a key factor of the BM cell homing. How OPN is involved remains unclear, but this effect is probably linked to the OPN ability to bind on one side to integrin and CD44 receptors and on the other side to extracellular matrix components, notably to collagen.

Altogether, these results suggest a possible cascade of events occurring during E2-mediated acceleration of endothelial repair (Figure 6). In a first step, E2 increases the pool of BM-derived cells according to a mechanism involving FGF211 but not OPN. Then, E2 induces the production of OPN by BM-derived cells, allowing them to more efficiently adhere to endothelial regenerating areas. The role of adherent...
BM-derived cells is still unknown, but they could contribute to an OPN-dependent E2-mediated stimulation of EC migration from the adjacent healthy zone. Finally, we can speculate that consequently to the EC migration from the healthy adjacent endothelium (ARA) toward the deendothelialized area, the cell density decreases in ARA, leading to an EC proliferation to refill an adequate cell density.

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Disclosures

None.

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Online data supplements

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Expanded Materials and methods

TRE-OPN transgenic mouse construction

The mouse OPN cDNA (bases 9 to 955, accession number BC057858) was obtained by PCR amplification using primers with Sal I and Pst I restriction enzyme extensions (5’-GCAGCTTGGTTTGCAGTCTTCTG-3’ and 5’-ACGCCTCGACTTAAGCGTAATCTGGAGACATCGTATGGTAGTTGACCTCAGAAGA TGAAC-3’). The cDNA was introduced into the Sal I and Pst I restriction sites of the multiple cloning site of the bidirectional Tet-responsive promoter of pBI-G Tet plasmid (Clontech). The transgenic mouse line was produced by microinjection of this construct, which allows the coexpression of the target gene and the β-galactosidase gene (LacZ). Functionality and efficiency of the 6 founders were assessed by crossing these mice with MHC-tTA mice (FVB/N-Tg(MHCAtTA)6Smbf/J, Jackson Laboratory) and by analysis of OPN expression (mRNA and protein). The founder exhibiting the stronger doxycyclin-regulated OPN and β-galactosidase expression was used to obtain Tie2-rOPN. Primers for Tie2-rOPN mice genotyping were: 5’-GCTGCTTAATGAGGTCGG-3’ and 5’-CTCTGCACCTTGGTGATGCTCCATAC-3’ for Tie2; and 5’-GATGAGTTCTGGACAACACCAC-3’ for rOPN. Beta-galactosidase was detected with the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, 2.4 mM, Euromedex).
**Carotid artery deendothelialization**

Endovascular carotid injury was performed mechanically by a modification of the protocol described elsewhere. The right common carotid artery was exposed from the bifurcation point, via an anterior incision of the neck. The external carotid was bound distally and looped proximally with an 8-0 silk suture. Silk ligatures were placed around the common carotid to temporally restrict blood flow in the area of surgical manipulation. Ligature on the common carotid was precisely positioned against the sterno-cleido-mastoid muscle. The external carotid was then incised and the endovascular injury performed using a 0.25 mm diameter swab built by winding and sticking an 8-0 silk suture around 0.16 mm diameter blunted guide wire. The swab was introduced, advanced through the common carotid artery and withdrawn 3 times. The swab removed endothelial cells with only a moderate vessel wall enlargement. A 4 mm length denudation from bifurcation of the common carotid artery was performed using the silk ligature on the common carotid at the injury limit. The size of the injury is very reproducible. It corresponds to 49.7±0.8% of the total common carotid. The device was removed and the external carotid bound proximally. Blood flow was then restored. Skin incision was closed and animals were allowed to wake up under warm conditions.

**Evaluation of the endothelial repair**

Endothelial repair was evaluated by staining the denuded areas with Evans blue dye (Merck) according to a previously described technique. Briefly, just before euthanasia, mice were anaesthetized; the thorax was open and 500 µL Evans blue (1% diluted in saline) was injected into the left ventricle of the heart. Two minutes later, to limit potential dye diffusion through the endothelium, the right atrium was incised and blood and dye were washed out with 10 ml phosphate buffered saline. Finally, the carotids were fixed by perfusion with 4% paraformaldehyde (pH 7.0) for 1 minute. Carotid arteries were opened longitudinally, flattened (“en face” preparations) and placed between slides. Total and stained carotid artery areas were evaluated after image digitalization. Ratio between blue stained area (unrepaired area) and injured carotid artery area was calculated.

**In vivo BM MNC adhesion test**

To limit the discrepancy of BM MNC number delivered in the different mice, only one cell suspension was prepared and homogenized by mild shaking between injections. Moreover, efficiency and reproducibility of the BM MNC administration was verified by evaluating the number of labeled cells respectively in blood, BM and spleen (not shown). In blood, no
labeled BM MNC remained after 24 hours. In BM and spleen, the number of labeled cells was not significantly different between experimental groups.

**Evaluation of OPN mRNA level expression by real time quantitative RT-qPCR.**

Messenger RNA purification was performed with the Nucleospin RNA II kit (Macherey Nagel). cDNAs were subjected to real-time PCR (qPCR) in an Opticon 2 thermocycler (MJ Research) using “iQ SYBR Green Supermix” (Ab-Gene). The following primers were used: total OPN: 5’-CAGTCGATGTCCCTGACGG-3’ and 5’-GTTGCTGTCTGATCAGAGG-3’, β-actin primers: 5’-GGAGGAAGAGGATGCCCCA-3’ and 5’-GAAGCTGTGCTATGTTGCTCTA-3’.

**Evaluation of OPN expression in BM MNC by immunohistochemistry.**

BM MNC were plated onto collagen I-coated wells at 10⁶ cells/cm² in EGM2 BulletKit medium (Clonetics) during 5 day before immunohistochemical analysis. OPN protein was detected using a goat polyclonal anti-OPN antibody (Sigma) and a peroxidase labeled antigoat secondary antibody (Sigma). OPN immunocomplexes were revealed by diaminobenzidine. The control antibody is a goat non immune IgG (Sigma).

**EC proliferation**

Sixteen hours before euthanasia, mice received an intraperitoneal injection of bromodeoxyuridine (BrdU, 30 mg/kg, Sigma). Proliferating cells were detected with a rat anti-BrdU antibody (Oxford Biotechnology Ltd) and EC with a rat monoclonal anti-CD31 antibody (BD Pharmingen), and revealed with the corresponding Alexa 488 conjugated secondary antibodies. Nuclei were labeled with propidium iodide (2 µg/mL, Sigma). Proliferating EC were counted under fluorescent microscope (magnification x40).

**Endothelial progenitor cells numbering**

Peripheral blood and BM from mice were collected after 3 weeks of either placebo or E₂ treatment to follow a parallel protocol to that of carotid injury/reendothelialization. EPC number was evaluated by Fluorescence-activated cell sorter analysis (FACS) analysis. After lysis of red blood cells and Fc blockade (Fc-Block, BD Pharmingen), the viable lymphocyte population was labeled with fluorescein isothiocyanate conjugated anti-Sca-1 antibody (clone E13-161.7, BD Pharmingen) and anti-VEGF-R2 antibody (clone A3, Santa Cruz Biotechnology) revealed with the corresponding phycoerythrin-labeled secondary antibody
(Sigma). Isotype-identical antibodies served as controls (Becton, Dickinson and Company). Single- and two-color flow cytometric analyses were performed using a Becton Dickinson FACScan equipped with an argon laser. Data were evaluated with Cellquest software.
Supplemental figures

Figure I: Beta galactosidase expression in double transgenic Tie2-OPN embryo, carotid artery and adult aorta.

β-galactosidase was detected in E11.5 embryo vessels (A). In uninjured carotid artery (B) and aorta (C) of adult Tie2-rOPN, β-galactosidase was detected only in sparse EC. Five days after carotid artery injury (B) β-galactosidase was detected in about 30% of the EC of the repairing endothelial area.

(A) LacZ whole mount stained E11.5 embryo show that EC of embryo vessels expressed β-galactosidase. Representative “en face” pictures of longitudinally opened and X-gal stained control (top picture) or injured (middle picture) carotid arteries from Tie2-rOPN mice 5 days after endovascular injury. The bottom picture shows a magnification of the indicated zone of the injured carotid artery (middle picture). Scale bars: 100 μm. (C) Representative “en face” pictures of longitudinally opened and X-gal stained aortas from Tie2-rOPN or control mice. Scale bar: 50 μm.
Figure II: Distribution of the OPN protein in healthy carotid arteries of wild type or Tie2-rOPN mice.

Healthy carotids were harvested from wild type (WT) or transgenic mice Tie2-rOPN (Tg) and OPN protein (red) was detected by immunohistochemistry with anti-OPN antibody. Endothelial cells were identified with anti-CD31 antibody (green) and nuclei (blue) were stained with DAPI. Scale bar = 20 µm.

OPN is expressed only in EC of the transgenic Tie2-rOPN.
Figure III: A retrograde area located at the edge of healthy endothelium is committed to reendothelialization.

(A) Carotid injury was performed in OPN^{+/+} or OPN^{-/-} ovariectomized mice supplemented with either E_{2} or placebo (Pl). Endothelial repair was followed by Evans blue staining: (A) representative images of Evans blue stained carotids at day 0, day 3 and day 5 after injury. (B) Distribution of the proliferating EC on the injured carotid artery after endovascular injury of OPN^{+/+} (up panel) and OPN^{-/-} (bottom panel) ovariectomized mice supplemented with either E_{2} or placebo. EC proliferation was followed by BrdU incorporation. Thick arrows indicate injury limit immediately after injury. ARA: Activated Retrograde Area, RA: Reendothelialized Area. (n=6 in each group).

(C) Carotid electric injury was applied to the distal part of the left common carotid artery with a bipolar microregulator. With this technique, all three vessel layers (endothelium, media and adventitia) are destroyed, allowing a precise visualization of the initial limit between the
injured and healthy areas. Carotids where harvested from placebo or E2-treated ovariectomized OPN+/+ mice 3 days after electric injury. EC nuclei were stained with propidium iodide (blue) and proliferating EC were immunolabeled with an anti-BrdU antibody (red). Samples were analyzed by confocal microscopy to focus on EC. Scale bars: 100 µm.

E2 induced the enlargement of both the reendothelialized area and the committed retrograde area in both carotid injury models.
Figure IV: OPN expression in BM-MNC

(A) Representative photos of OPN immunodetection in 5 days cultured BM-MNC. Scale bar = 100 µm. (B) OPN mRNA level in 10 days cultured BM-MNC stimulated by $10^{-9}$ mol/L E$_2$ in the presence or absence of E$_2$ receptor inhibitor (ICI 182780) for 16 hours was quantified by RT-qPCR. (N=3 in each group, *p<0.05). OPN is expressed in cultured BM-MNC and E$_2$ stimulated this expression.
Figure V: OPN is not involved in E2-mediated EPC increase

Quantitative FACS analysis of Sca-1+/VEGF-R2+ cells in BM or peripheral blood of OPN+/+ or OPN−/− ovariectomized mice supplemented with either E2 or placebo (pl). (* p<0.05, n=7 in each group). As previously demonstrated, E2 supplementation increased the number of EPC in BM and blood of ovariectomized OPN+/+ mice.

E2 supplementation increases EPC number in blood, in BM and in the repairing endothelium, thus contributing to reendothelialization. Although OPN is required in E2-mediated reendothelialization, E2 supplementation induced the same increase in Sca-1+/VEGF-R2+ cells in OPN+/− and OPN+/+ mice both in the peripheral blood and in the BM, indicating that the loss of the E2 effect on reendothelialization in OPN−/− mice was not the result of an alteration of the mobilization of this BM-derived cell population.
Supplemental discussion

The Tet inducible transgenic mouse model used in this study allows Tie2-directed gene expression. The TIE2 gene, coding for the angiopoietin I receptors, is strongly expressed during development and remains detectable in adult tissues. TIE2 is weakly expressed in EC of regenerating endothelium \(^4\) but it becomes superinduced during skin wound healing and tumor angiogenesis \(^5\). Although Tie2-promoter/enhancer elements used in the construct were able to reliably direct gene expression by EC both during embryogenesis and adulthood \(^5\), the level of transgene expression in the double transgenic system Tie2-tTA x TRE-target gene is strongly dependent on the TRE-target gene mouse line \(^6, 7\). Tie2-tTA x TRE-OPN double transgenic offspring mice appeared as a low EC expressing β-galactosidase reporter gene system that was superinduced in regenerating endothelium. However, OPN expression was detected in all the EC of the healthy carotid artery of Tie2-rOPN transgenic mice as compared to that in the wild type mice. This difference could be related to a low sensitivity of β-galactosidase activity measurement or a difference between the rOPN and β-galactosidase-driven promoter efficiency.

References


cytoskeletal reorganization by a sFRP-1 and Frizzled 4 and 7 dependent pathway; role in neo vessel formation. *The American journal of pathology.* 2007;In press.