Protective Role of mPGES-1 (Microsomal Prostaglandin E Synthase-1)–Derived PGE_2 (Prostaglandin E2) and the Endothelial EP4 (Prostaglandin E Receptor) in Vascular Responses to Injury

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Objective—Deletion of mPGES-1 (microsomal prostaglandin E synthase-1)—an anti-inflammatory target alternative to COX (cyclooxygenase)-2—attenuates injury-induced neointima formation in mice. This is attributable to the augmented levels of PGI_2 (prostacyclin)—a known restraint of the vascular response to injury, acting via IP (I prostanoid receptor). To examine the role of mPGES-1–derived PGE_2 (prostaglandin E2) in vascular remodeling without the IP.

Approach and Results—Mice deficient in both IP and mPGES-1 (DKO [double knockout] and littermate controls [IP KO (knockout)]) were subjected to angioplasty wire injury. Compared with the deletion of IP alone, coincident deletion of IP and mPGES-1 increased neointima formation, without affecting media area. Early pathological changes include impaired reendothelialization and increased leukocyte invasion in neointima. Endothelial cells (ECs), but not vascular smooth muscle cells, isolated from DKOs exhibited impaired cell proliferation. Activation of EP (prostaglandin E receptor) 4 (and EP2, to a lesser extent), but not of EP1 or EP3, promoted EC proliferation. EP4 antagonism inhibited proliferation of mPGES-1–competent ECs, but not of mPGES-1–deficient ECs, which showed suppressed PGE_2 production. EP4 activation inhibited leukocyte adhesion to ECs in vitro, promoted reendothelialization, and limited neointima formation post-injury in the mouse. Endothelium-restricted deletion of EP4 in mice suppressed reendothelialization, increased neointimal leukocytes, and exacerbated neointimal formation.

Conclusions—Removal of the IP receptors unmasks a protective role of mPGES-1–derived PGE_2 in limiting injury-induced vascular hyperplasia. EP4, in the endothelial compartment, is essential to promote reendothelialization and restrain neointimal formation after injury. Activating EP4 bears therapeutic potential to prevent restenosis after percutaneous coronary intervention (Graphic Abstract). **(*Arterioscler Thromb Vasc Biol*. 2018;38:00-00. DOI: 10.1161/ATVBAHA.118.310713.)**

Key Words: dinoprostone ■ endothelium ■ mice ■ prostaglandin E synthases ■ vascular remodeling

Nonsteroidal anti-inflammatory drugs ameliorate pain, fever, and inflammation by inhibiting COX (cyclooxygenase; 2 major isomers identified as COX-1 and COX-2)—the rate-limiting enzyme in the synthetic cascade of prostanoids.[1] Clinical evidence shows that Nonsteroidal anti-inflammatory drugs selective for COX-2 inhibition increase cardiovascular thrombotic events.[2–4] This is attributable to suppression of COX-2–derived PGI_2 (prostacyclin).[5] mPGES-1 (microsomal prostaglandin E synthase-1)[6] has emerged as a therapeutic target that is downstream of COX-2.[7,8] Deletion of mPGES-1, unlike COX-2 inhibition, does not predispose to thrombosis, because of augmented PGI_2 (redirection of PGH_2—the COX product—to PGI synthase), and retards atherogenesis independent of IP (I prostanoid receptor) deficiency.[9–11] Targeting mPGES-1 may avoid the cardiovascular risk associated with COX-2 inhibitors while preserving their analgesic and anti-inflammatory efficacy.[12] Accumulative evidence suggests that COX pathway components differentially modulate vascular responses to injury.

Celecoxib—a COX-2 selective nonsteroidal anti-inflammatory drug—reduces in-stent late luminal loss in patients with coronary artery disease treated with aspirin

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Nonstandard Abbreviations and Acronyms

cKO	endothelial EP4 conditional-knockout mice
COX	cyclooxygenase
DKO	double knockout
EC	endothelial cell
EP	prostaglandin E receptor
FBS	fetal bovine serum
IL-1β	interleukin 1β
IP	I prostanoid receptor
KO	knockout
MAEC	mouse aorta endothelial cell
mPGES-1	microsomal prostaglandin E synthase-1
PGE	prostaglandin E
PGE2	prostaglandin E2
PGI2	prostacyclin
SMA	smooth muscle actin
SMC	smooth muscle cell
vWF	von Willebrand factor

plus clopidogrel after percutaneous coronary intervention.[13] However, a follow-up trial confirmed an increased thrombotic risk with celecoxib, despite the dual antiplatelet therapy,[14] which limits its clinical application. Tissue-specific deletion of mPGES-1—the underlying alternative target for COX-2—in endothelial cells (ECs) or vascular smooth muscle cells (SMCs) enhances neointima formation, whereas myeloid cell mPGES-1 deletion reduces vascular hyperplasia response to injury.[15] Our previous study shows that deletion of mPGES-1 attenuates injury-induced neointimal formation in mice, with sustained reduction in PGE_2 (prostaglandin E2) and augmentation in PGI_2.[16] PGI_2 signaling via the IP is known to restrain the neointimal formation.[17] In contrast to IP deletion, deletion of the thromboxane A_2 receptor depresses this response.[17] The role of mPGES-1–derived PGE_2 in the vascular remodeling that is independent of the IP signaling remains unknown.

PGE_2 has 4 G protein-coupled receptors, EP (prostaglandin E receptor) 1 through EP4, which mediates differential downstream signaling.[18] EP3 mediates vascular SMC migration facilitating neointimal hyperplasia in mice.[19] EP4 protects against ischemia–reperfusion injury,[20,21] hypertension,[22] or atherosclerosis.[23] EP4 promotes endothelial migration and angiogenesis.[24,25] However, whether EP4 regulates injury-induced neointimal formation is unknown.

Here, we report that removal of the IP receptor uncovers a protective role of mPGES-1–derived PGE_2 in endothelial repair and the vascular response to injury. Furthermore, deletion of endothelial EP4 exacerbates, whereas pharmacological activation of EP4 limits, neointimal hyperplasia after wire injury.

Materials and Methods

Animal Study

Mice deficient in mPGES-1 (gene: *Ptges*)[26] and IP (gene: *Ptgir*)[17] were obtained from Pfizer and the FitzGerald Laboratory at the University of Pennsylvania, respectively. Both strains had been backcrossed to a C57BL/6 background for >10 generations and were used to derive IP/mPGES-1 DKO (double knockout) mice and littermate controls (IP KO [knockout]) by intercrossing *Ptgir*$^{-/-}$ *Ptges*$^{-/-}$ with *Ptgir* $^{-/-}$ *Ptges*$^{+/-}$. DKO mice develop normally without overt abnormalities. Global deletion of EP4 is perinatally lethal because of patent ductus arteriosus.[27] To circumvent this defect, endothelial-specific EP4 (gene: *Ptger4*) KO mice were generated using a tamoxifen-*CreERT2* strategy.[28] Briefly, C57BL/6 *Ptger4*$^{flox/flox}$ mice[29] were intercrossed with *Cdh5* promoter-driven *CreERT2* (*Cdh5 [PAC]-CreERT2*$^{+}$) mice,[30] kindly provided by Ralf Adams. The resulting *Ptger4*$^{f/f}$ *Cdh5-CreERT2*$^{+}$ and *Ptger4*$^{f/f}$ *Cdh5-CreERT2*$^{-}$ mice were then intercrossed to generate the animals used in this study—cKO (endothelial EP4 conditional-knockout mice; *Ptger4*$^{f/f}$ *Cdh5-CreERT2*$^{+}$) and littermate controls (*Ptger4*$^{f/f}$ *Cdh5-CreERT2*$^{-}$). To induce endothelial EP4 deletion, tamoxifen (37.5 mg/mL dissolved in sunflower seed oil) was intraperitoneally injected into the experimental mice and littermate controls at a dose of 150 mg/kg per day for 6 days, which was interrupted for 3 days after the third dose. Gene-modified mice used in this study were sex matched. The pooled data from both sexes were used to show the gene-specific effects in each study, and sex-specific subgroup analyses were provided in Tables I and II in the online-only Data Supplement. Male C57BL/6 mice aged 6 to 8 weeks from the National Institutes for Food and Drug Control (Beijing, China) were used to determine the effect of AE1-329 (gifted by ONO Pharmaceutical Co, Ltd, Osaka, Japan) or misoprostol (410004; purity, 98.9%; National Institutes for Food and Drug Control, Beijing, China) in vascular remodeling. All animal protocols were performed following the guidelines of the Institutional Animal Care and Use Committee, the Experimental Animal Center, Fuwai Hospital, National Center for Cardiovascular Diseases, China.

Femoral Artery Injury Model

Femoral arteries were injured using a protocol as we described previously.[28] Briefly, a groin incision was made on 1 side of the anesthetized mouse. The femoral artery and its small branch between the rectus femoris and vastus medialis muscles, were then carefully exposed and separated from the accompanying nerve and vein via blunt dissection. A 6-0 silk suture was then looped round the proximal femoral artery to stop the blood flow during the surgery. Another 6-0 silk suture was placed under the branch. A transverse arteriotomy was then made in the branch, and a flexible angioplasty wire (0.35 mm diameter; Cook, Inc, IN) was inserted into the femoral artery from the branch for a length no less than 5 mm toward the iliac artery. The wire was left in place for 3 minutes to dilate and denude the artery. Then, the wire was removed, the branch was ligated proximally with the 6-0 silk suture, and the blood flow in the femoral artery was restored by releasing the sutures for blood flow control. The skin incision was then closed with a 5-0 silk suture. Seven or 28 days after injury, the arteries were harvested, embedded in paraffin, and stained with hematoxylin and eosin for determination of the severity of hyperplasia. In detail, cross sections of the arteries were serially obtained for 10 to 13 levels at 150-μm intervals, and the sections with the most severe hyperplasia were used for comparisons. To evaluate reendothelialization, the sections from the arteries 7 days post-injury were immunostained with vWF (von Willebrand factor)—an EC marker—and photographed with a CCD camera coupled to a microscope system (AXI0; Zeiss, Oberkochen, Germany). The segments along the vascular inner wall that were positive in vWF and the peripheral length of the vascular inner wall were measured using Image-Pro Plus 6.0 software (Media Cybernetics, MD). The ratio of the total length of the vWF-positive segments to the peripheral length was calculated to represent the extent of reendothelialization. To evaluate leukocytes infiltration/migration, representative sections from the arteries injured for 7 days were analyzed with hematoxylin and eosin staining. Briefly, after capturing the images, intima leukocytes were objectively determined using a hue, saturation, and intensity-based color selection strategy using Image-Pro Plus 6.0 software (Media Cybernetics, MD).

Cell Study

Endothelial Cells

Mouse aorta ECs (MAECs) were isolated as described previously.[28,31] Briefly, aortas were harvested and cut into 1 to 2 mm^2 sections. The aortic segments were attached to a petri dish by their luminal side and were then cultured in a DMEM medium containing 20% fetal bovine serum (FBS) and 100 µg/mL EC growth supplement for 5 to 7 days, to allow the outgrowth of ECs. Then, the ECs were passaged and cultured. MAECs in passages 2 to 6 were used in this study. EC proliferation was compared in MAECs isolated from IP KO or DKO, in passage 2. Primary human microvascular ECs were brought from ScienCell (6000; Carlsbad, CA) and cultured in the same medium as that for MAEC culture.

Smooth Muscle Cells

Mouse aorta SMCs were isolated from IP KOs and DKOs. Briefly, aortas were isolated, scratched for 3× on the inner surfaces, and cut into 1 to 2 mm^2 sections. The aortic segments were then attached to a petri dish by their luminal side, covered by a cover slid, and cultured in a DMEM medium containing 10% FBS for 5 to 7 days. The mouse aorta SMCs were then passaged and cultured. Proliferation of mouse aorta SMCs in passage 2 was compared.

Cell Proliferation

Cell growth was determined with a cell counting kit-8 (40203ES60; Yeasen, Shanghai, China), following the manufacturer's instructions, as described previously.[28] The assay allows multiple detection without obvious cell toxicity. Briefly, the cells were seeded onto a 96-well flat-bottomed plate. After the cells were attached, ECs were cultured in the medium containing 3% FBS for 6 to 8 hours. Afterward, the culture medium was replaced with a 3% FBS medium-cell counting kit-8 mixture (10:1 in volume). Thereafter, the cells were cultured in the mixture for no longer than 4 hours; absorbance at 450 nm was determined as the baseline. Then the cells were cultured in fresh 3% FBS medium with the indicated reagents for another 48 hours. The culture medium was then replaced by the medium-cell counting kit-8 mixture, and the culture continued for the same time as above for determination of the absorbance at 450 nm. The change in absorbance between the 2 measurements was used to define the cell growth. To determine mouse aorta SMC proliferation, cells were prestarved in FBS-free serum for 24 hours, and then cultured in a medium containing 1% FBS, with 2 sequential incubations with cell counting kit-8. The reagents and their concentrations used in the proliferation studies were as follows: AE1-329 (0.1–1 µmol/L; gifted by ONO Pharmaceutical Co, Ltd, Osaka, Japan), butaprost (1 µmol/L; 13740; Cayman Chemical, MI), sulprostone (1 µmol/L; 14765; Cayman), GW627368X (0.1–1 µmol/L; HY-16963; MedChemExpress, NJ), L-798106 (1 µmol/L; 11129; Cayman), iloprost (1 µmol/L; 18215; Cayman), Cay10441 (10 µmol/L; 10005186; Cayman), PF-04418948 (1 µmol/L; S7211; Selleck), ONO-8130 (1 µmol/L; 19118; Cayman), SQ22536 (200 µmol/L; S8283; Selleck), H89 2HCl (10 µmol/L; S1582; Selleck), ESI-09 (10 µmol/L; 19130; Cayman), db-cAMP (3–100 µmol/L; D0260; Sigma, Darmstadt, Germany), and misoprostol (10 µmol/L; 410004; National Institutes for Food and Drug Control, Beijing, China).

Endothelium–Leukocyte Adhesion Study

For endothelium–leukocyte adhesion assay, MAECs were seeded into the 96-well plate, prestarved in the DMEM containing 3% FBS for 6 to 8 hours, and incubated with tested reagents for 2 hours. Leukocytes were gathered from mouse peritoneal. Briefly, 4% Brewer-modified thioglycollate medium (211716; BD Biosciences, NJ) was injected into the mouse peritoneal (1 mL per mouse). Four to 5 hours later, the peritoneal leukocytes were washed out with 0.1% bovine serum albumin, centrifuged, and resuspended in the 1640 medium containing 10% FBS. When the preparation of ECs was completed, the culture medium with indicated drugs was replaced by the 1640 medium containing leukocytes ($3×10^4$ per well). ECs and leukocytes were then cocultured for 30 minutes. Later, the cells were rinsed once with 1640 medium containing rhodamine 6G (200 µg/mL; 252433; Sigma, Darmstadt, Germany), followed by 3 washes with fresh 1640 medium. The fluorescent signals were finally detected by a microplate reader (excitation wavelength, 560 nm;

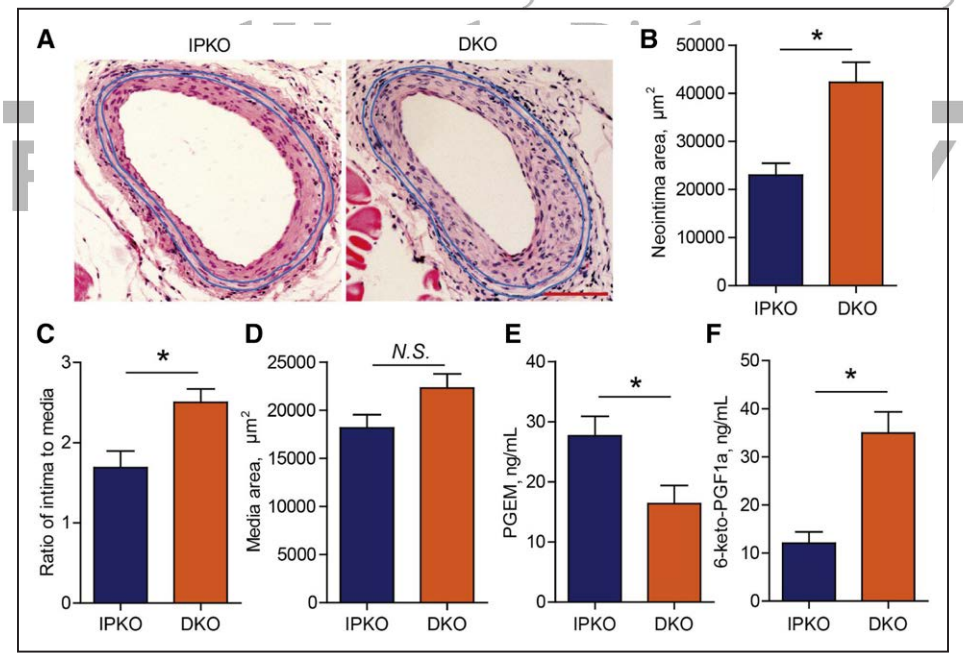

Figure 1. IP (I prostanoid receptor) deletion reveals a protective role of mPGES-1 (microsomal prostaglandin E synthase-1)–derived PGE_2 (prostaglandin E2) against wire injury-induced neointimal formation. DKO (double knockout; *Ptgir*$^{-/-}$ *Ptges*$^{-/-}$) and littermate IPKO (I prostanoid receptor knockout; *Ptgir*$^{-/-}$) mice were subjected to wire injury at femoral arteries, and the vessels were harvested at 28 d after injury and quantified for neointima formation. Representative images of hematoxylin and eosin staining are shown (**A**; bar=100 µm). Neointimal area (**B**), ratio of intima to media (**C**), and media thickness (**D**) were determined (n=14 IPKO and 16 DKO). PGE_2 (**E**) and PGI_2 (prostacyclin; **F**) metabolites in urine were determined by HPLC-MS/MS as detailed in the Methods (n=8). Student unpaired *t* test. N.S. indicates nonsignificant. *P<0.05.

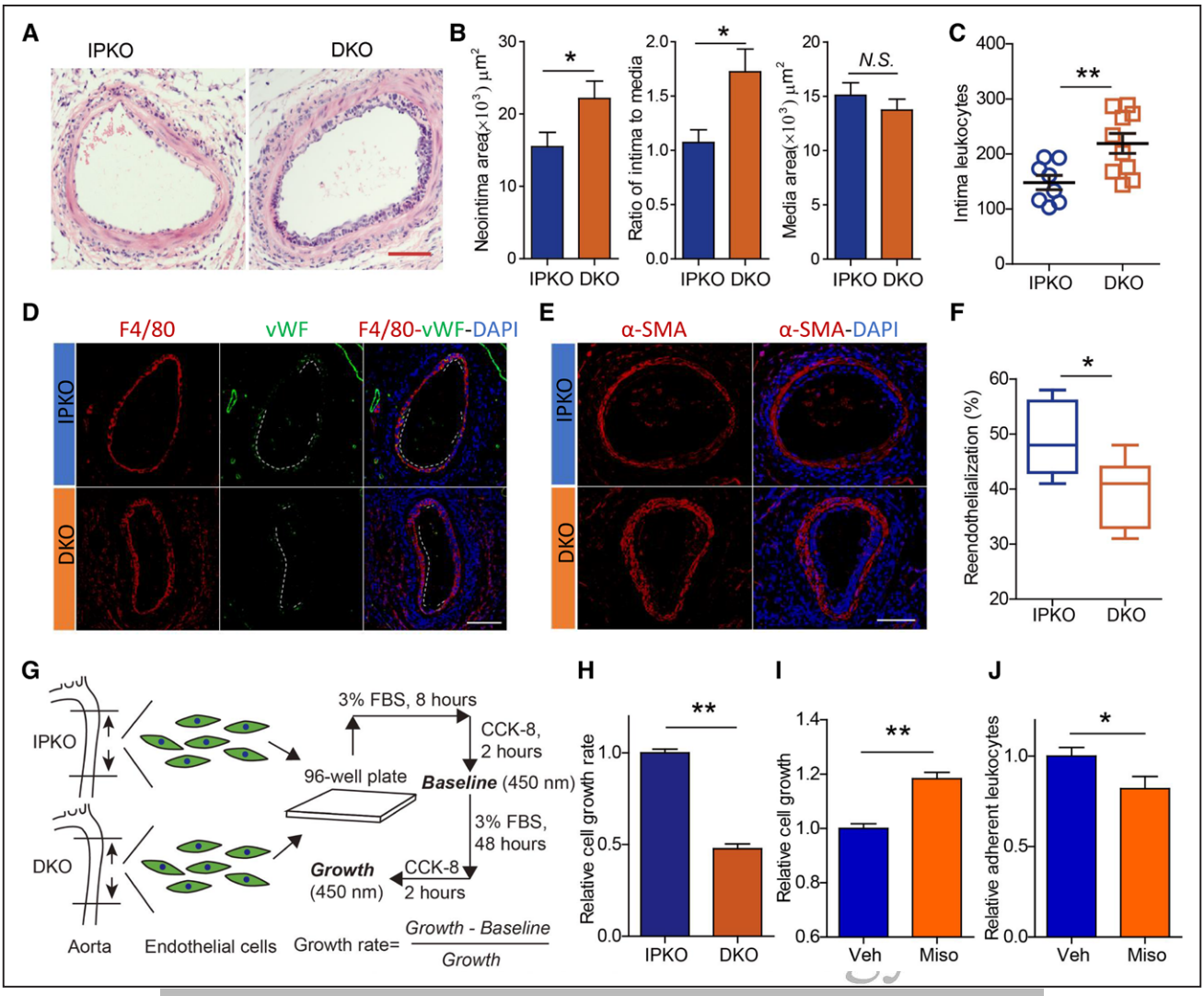

Figure 2. Deletion of mPGES-1 (microsomal prostaglandin E synthase-1) suppresses reendothelialization after endothelial denudation injury in IP (I prostanoid receptor)-deficient mice. Injured femoral arteries of DKO (double knockout) and IPKO (I prostanoid receptor knockout) were harvested at 7 d after surgery. Neointima formation and leukocyte infiltration were evaluated on hematoxylin and eosin (H&E) stained sections, with representative H&E images shown (**A**; bar=100 μm). Neointimal area, ratio of intima to media, and media thickness were quantified (**B**; n=14 IPKO and 16 DKO). The number of neointima leukocytes was quantified by analyzing the H&E stained tissue sections with IPP software (**C**; n=8 IPKO and 10 DKO). Injured vessels were immunostained for F4/80 (a marker of macrophage, red) and vWF (von Willebrand factor; an endothelial cell [EC] marker, green; **D**; bar=100 μm) and for α-SMA (α-smooth muscle actin; a smooth muscle cell marker; **E**; bar=100 μm). DAPI stains nuclei in blue. The number of vWF-positive cells was quantified to determine reendothelialization (**F**; n=7). ECs were isolated from the descending aortae of IPKO and DKO mice, and their cell proliferation was analyzed in vitro (**G**) and compared (**H**). Calculation of the EC proliferation rate was based on the difference in cell number before vs after treatment with 3% fetal bovine serum (FBS) for 48 h. Misoprostol—a PGE (prostaglandin E) analogue at 10 μmol/L—promoted endothelial proliferation (**I**; n=9 from 2 independent experiments) and inhibited endothelium–leukocyte adhesion (**J**; n=9 from 3 independent experiments). Student unpaired *t* test. CCK-8 indicates cell counting kit-8; and N.S, nonsignificant. **P*<0.05, ***P*<0.01.

emitter wavelength, 560 nm; Infinite M200; Tecan, Hombrechtikon, Switzerland). The reagents and their concentrations used in this study were as follows: misoprostol (10 μmol/L; 410004; National Institutes for Food and Drug Control, Beijing, China), AE1-329 (1 μmol/L; gifted by ONO Pharmaceutical Co, Ltd, Osaka, Japan), GW627368X (1 μmol/L; HY-16963; MedChemExpress, NJ), and db-cAMP (30 μmol/L; D0260; Sigma, Darmstadt, Germany).

Immunofluorescence Staining

Immunofluorescence staining was performed following the same protocol as we described previously.[28] Briefly, the paraffin sections (5 μm) were deparaffinized, rehydrated, and subjected to antigen retrieval using an EDTA antigen-retrieval water (PH 9.0; ZSGB-BIO, Beijing, China). After 1-hour incubation with normal goat serum at room temperature, the samples were incubated with primary antibodies overnight at 4°C and subsequently stained with Alexa Fluor-488–coupled or Alexa Fluor-594–coupled secondary antibodies for 3 hours at room temperature. The sections were then mounted with a VectaShield medium containing DAPI to stain nuclei and imaged using a Zeiss microscope system (AXI0; Zeiss) or a laser-scanning confocal microscope system (SP8; Leica). For determination of reendothelialization, images were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc, Rockville, MD). The primary antibodies and dilution fold used in the study were polyclonal anti-EP4 antibody (2.5 μg/mL; 101775; Cayman Chemical, MI), rabbit polyclonal anti-vWF antibody (1 μg/mL; F3520; Sigma, Darmstadt, Germany), sheep polyclonal anti-vWF antibody (10 μg/mL; Ab11713;

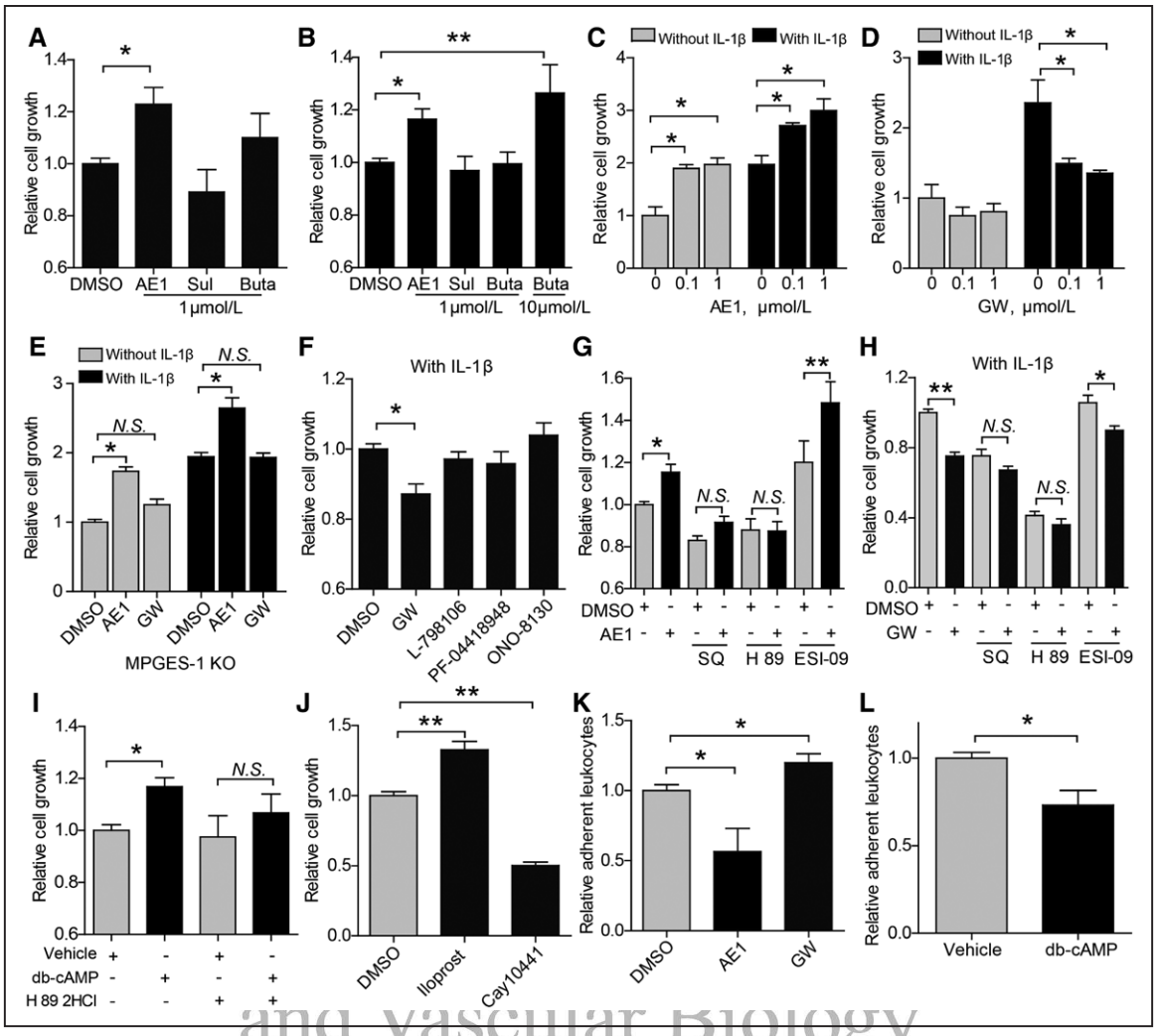

Figure 3. PGE_2 (prostaglandin E2) promoted in vitro endothelial cell proliferation via EP (prostaglandin E receptor) 4/cAMP/PKA signaling. Mouse aortic endothelial cells (MAECs) were used for the study of cell proliferation in vitro. Cells from DKO (double knockout; **A**) and wild-type (WT) mice (**B**) were stimulated with agonists for PGE_2 receptors (AE1-329 [AE1]—an EP4 agonist, sulprostone [Sul]—an EP1/3 agonist, and butaprost [Buta]—an EP2 agonist), and relative proliferation is shown. WT endothelial cells (ECs) were treated with AE1 of different concentrations, with or without IL (interleukin)-1β (10 ng/mL), and proliferation was determined (**C**). GW627368X (GW—an EP4 antagonist) suppressed proliferation of IL-1β–stimulated MAECs (**D**). Effects of AE1 or GW on the proliferation of *Ptges*$^{-/-}$ ECs, stimulated with IL-1β (10 ng/mL) or not, were shown (**E**). Under IL-1β (10 ng/mL) stimulation, GW (an EP4 antagonist) but not L-798106 (an EP3 antagonist), PF-04418948 (an EP2 antagonist), or ONO-8130 (an EP1 antagonist), all at a concentration of 1 μmol/L, inhibited endothelial cell proliferation (**F**). The proproliferative effect of AE1 (**G**) and the antiproliferative effect of GW (**H**) was prevented by SQ (SQ22536—an adenylate cyclase inhibitor; 200 μmol/L) or H 89 (H 89 2HCl—a PKA inhibitor; 10 μmol/L) but not by ESI-09 (an EPAC inhibitor; 10 μmol/L). Db-cAMP (**I**; a cell-permeable cAMP analogue, 30 μmol/L) promoted endothelial proliferation, and this was also blunted by H89 2HCl (a PKA inhibitor). Endothelial proliferation was stimulated by iloprost (an IP [I prostanoid receptor] agonist; 1 μmol/L) and inhibited by Cay10441 (an IP antagonist; 10 μmol/L; **J**). The adhesion of leukocytes to ECs was inhibited by the EP4 agonist, AE1-329, and was promoted by GW—an EP4 antagonist (**K**). Db-cAMP treatment reduced leukocyte adhesion to ECs (**L**). All results are from at least 3 independent data sets. One-way ANOVA was used for data comparisons with Bonferroni (**A**, **G**–**K**), Dunnett (**B**), or Tukey (**C**–**F**) post hoc test. Student unpaired *t* test was used in L. N.S. indicates nonsignificant. **P*<0.05, ***P*<0.01.

Abcam, Cambridge, United Kingdom), monoclonal anti-α-SMA (smooth muscle actin) antibody (5 μg/mL; A5228; Sigma, Darmstadt, Germany), and polyclonal anti-F4/80 antibody (2 μg/mL; Ab16911; Abcam, Cambridge, United Kingdom).

Western Blot Analysis

For Western blot analysis, the cells were lysed in a RIPA buffer that contained protease inhibitors (4693116001; Roche, Basel, Switzerland). After centrifugation (15800 g, 10 minutes), cell lysates were mixed with a loading buffer, fractionated with 10% SDS-PAGE, and transferred onto polyvinylidene fluoride membranes. The membranes were then probed with the primary polyclonal antibodies against EP4 (2.5 μg/mL; 101775; Cayman, MI) overnight at 4°C, followed by incubation with goat anti-rabbit secondary antibody for 1 hour at room temperature. Finally, the membranes were incubated with ECL luminous liquid (P1020; PPLYGEN, Beijing, China), and signals from immunoreactive bands were visualized using a FluorChem System (ProteinSimple, CA).

Prostanoid Determination

PGE_2 and PGI_2 levels were determined via measuring their major urinary metabolites, tetranor-PGEM and 2,3-dinor-6-keto-PGF1α by liquid chromatography–tandem mass spectrometry, as described previously.[32]

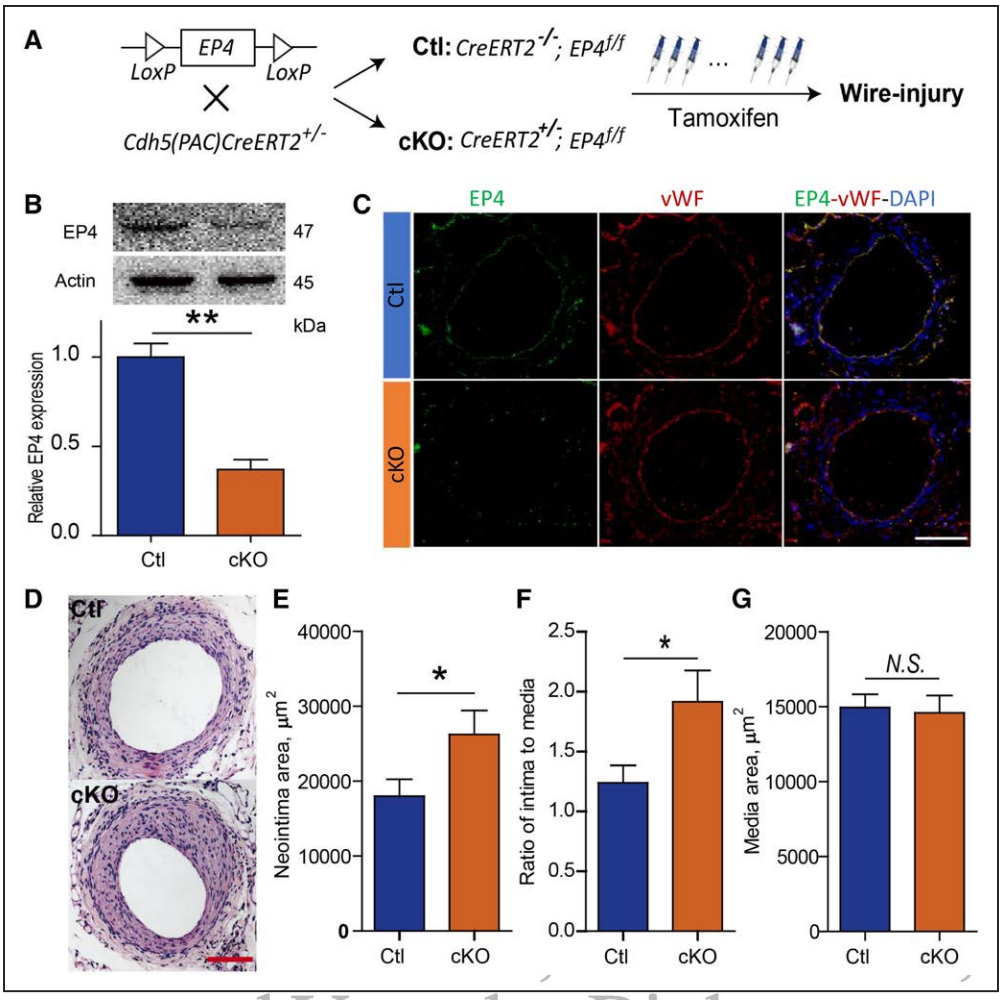

Figure 4. Induced deletion of endothelial EP (prostaglandin E receptor) 4 promotes neointimal formation. Postnatal deletion of endothelial *EP4* gene was induced in mice by tamoxifen treatment (**A**). Expression of EP4 in the primary endothelial cells isolated from cKO (endothelial EP4 conditional knockout) and Ctl (littermate control) mice was detected with Western blot (**B**), and its expression in femoral arteries was determined by immunofluorescent staining (**C**; bar=100 μm). Representative images are shown (EP4, green; vWF [von Willebrand factor], red). The injured vessels, harvested at 28 d after surgery, were stained with hematoxylin and eosin, and representative images are shown (**D**; bar=100 μm). Neointimal area (**E**), ratio of intima to media (**F**), and medial thickness (**G**) were quantified. n=14 Ctl, 10 cKO. Student unpaired *t* test. N.S. indicates nonsignificant. **P*<0.05, ***P*<0.01.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc, San Diego, CA). When only 2 groups were involved, Student 2-tailed unpaired *t* test was used for comparison. Comparisons of multiple groups were made using a 1-way ANOVA analysis. Tukey or Bonferroni post hoc tests were performed for the data with equal variances, whereas Dunnett post hoc tests were used for data with unequal variances. And Bartlett tests were used for analyzing the variances of data. Results are expressed as mean±SEM. Differences were considered statistically significant at $P<0.05$.

Results

mPGES-1–Derived PGE_2 Protects Against Wire Injury-Induced Neointimal Formation in IP-Deficient Mice

Deletion of mPGES-1 in mice enhances PGI_2 production, which leads to the attenuated vascular remodeling.[16] To elucidate a role of mPGES-1–derived PGE_2 in vascular remodeling, we crossed mPGES-1 KOs with IP KOs to generate DKO mice and littermate IP KO mice as control. The mice were subjected to wire injury (endothelial denudation) of the femoral artery for 28 days. On this IP-deficient background, mPGES-1 deletion increased neointima area by ≈84% and also increased the ratio of intimal-to-medial area, with media thickness unaltered (Figure 1A through 1D). This reveals a protective role of mPGES-1–derived PGE_2 in the vascular response to injury. Urinary metabolite of PGE_2 was reduced in DKO mice, whereas PGI_2 metabolite was enhanced (Figure 1E and 1F).

To explore the underlying mechanism, vessels were harvested at 7 days after surgery for histology examination. Again, the neointimal area and the ratio of intima to media, but not the medial area, were significantly enhanced in DKO (Figure 2A and 2B). Intima leukocytes, mainly macrophages (positive for F4/80 staining), were increased in the DKOs (Figure 2C and 2D; Figure I in the online-only Data Supplement). Expression of α-SMA was mainly detected in SMCs in the media, but rarely detected in the neointima area regardless genotypes (Figure 2E). When vWF (an endothelial

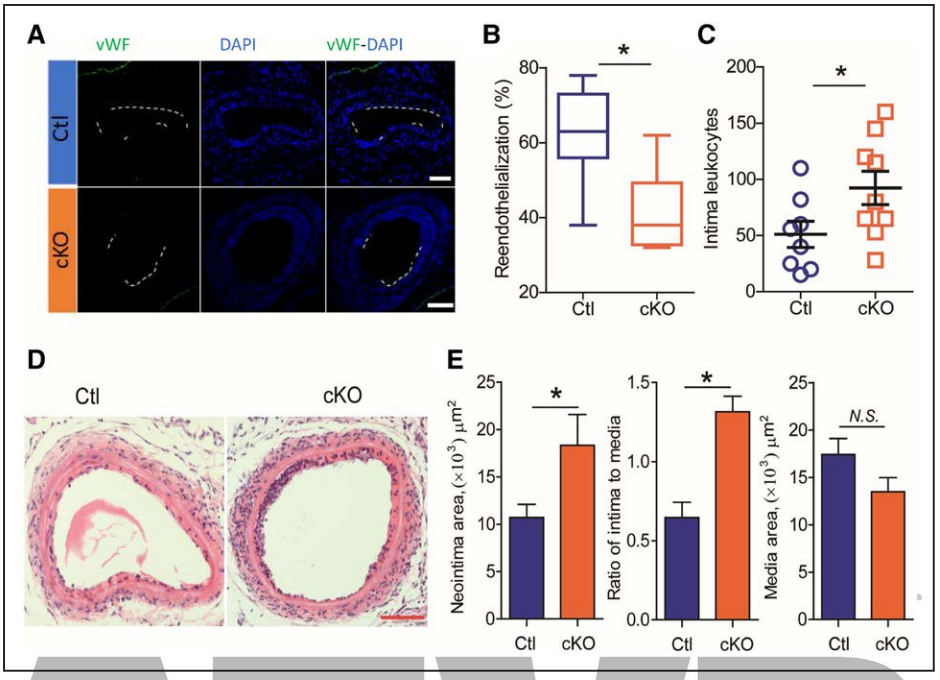

Figure 5. Induced deletion of endothelial EP (prostaglandin E receptor) 4 impaired reendothelialization. Injured femoral arteries of Ctl (littermate control) and cKO (endothelial EP4 conditional knockout) mice were harvested at 7 d after wire injury. Endothelial cells were immunostained for vWF (von Willebrand factor; green) and quantified for vascular coverage (reendothelialization; **A** and **B**, respectively; n=7 Ctl and 6 cKO). Neointimal formation and leukocyte infiltration were evaluated with hematoxylin and eosin (H&E) staining. The number of neointimal leukocytes was quantified (**C**; n=8 Ctl and 9 cKO). Representative H&E images are shown (**D**; bar=100 μm). Neointimal area, ratio of intima to media, and media thickness were statistically quantified (**E**; n=8). DAPI stains nuclei in blue. Student unpaired *t* test. N.S. indicates nonsignificant. *P<0.05.

marker) was stained, a striking reduction in the number of ECs was observed in the DKOs (Figure 2D and 2F), reflecting suppressed reendothelialization after the wire denudation injury.

To pursue a potential impact on endothelial repair, ECs were isolated from IP KOs and DKOs, and their capacity for proliferation was evaluated in vitro (Figure 2G). The EC proliferation was significantly impaired in the DKOs (Figure 2H), revealing a proliferative effect of PGE_2 signaling in ECs. Further, treatment of ECs with misoprostol—a PGE (prostaglandin E) analogue—promoted EC proliferation in vitro (Figure 2I) and also decreased leukocyte adhesion to endothelial monolayer in vitro (Figure 2J).

Primary aortic SMCs were also isolated from IP KOs and DKOs. No difference in cell proliferation was detected between the 2 groups (Figure II in the online-only Data Supplement).

EP4 Activation Promotes EC Proliferation and Reduces Endothelium–Leukocyte Adhesion

Receptors that might mediate the effect of PGE_2 on EC proliferation were then studied in vitro. ECs from DKO mice were treated with agonists[33,34] for the EP1/3 (sulprostone, EC50=0.42 nmol/L for EP3, also a weak agonist for EP1), EP2 (butaprost, EC50=32 nmol/L), or EP4 (AE1-329, EC50=3.1 nmol/L) receptors, all at a concentration of 1 μmol/L. AE1-329, but not sulprostone or butaprost, promoted proliferation in the DKO ECs (Figure 3A). Similar effects were observed with wild-type ECs (Figure 3B). We further tested each drug at mutiple concentrations for cell proliferating activity (Figure III in the online-only Data Supplement). AE1-329 dose dependently promoted EC proliferation, whereas butaprost showed a proproliferative effect at a higher concentration, that is, 10 μmol/L. AE1-329 enhanced EC proliferation in either absence or presence of IL (interleukin)-1β—a stimulus to PGE_2 production (Figure 3C; Figure IVA in the online-only Data Supplement). Conversely, GW627368X (a selective EP4 antagonist) markedly inhibited EC proliferation under IL-1β stimulation (Figure 3D). When mPGES-1 KO ECs were used, where PGE_2 production was depressed (Figure IVB in the online-only Data Supplement), the effect of EP4 agonism, but not that of EP4 antagonism, remained significant (Figure 3E). Thus, endogenous PGE_2 derived from EC mPGES-1 plays an active role in promoting proliferation of the IL-1β–stimulated ECs, which can be blocked by antagonism of EP4 but not of other EPs (Figure 3F). This further confirms the proliferative effect of EP4 activation in ECs (Figure 3A and 3B).

To elucidate EP4 downstream signaling, SQ22536 (an AC [adenylyl cyclase] inhibitor), H 89 2HCl (a PKA inhibitor), and ESI-09 (an EPAC inhibitor) were used. Treatment with SQ22536 or H 89 2HCl, but not with ESI-09, abrogated the proproliferation effect of AE1-329 (Figure 3G) and the antiproliferation effect of GW627368X (Figure 3H), indicating that cAMP-PKA axis underlies the enhanced EC proliferation driven by EP4 activation. Consistent with this finding, Db-cAMP (a cell-permeable cAMP analogue) and forskolin (a potent AC activator) both promoted EC proliferation, and such proliferative effects were blunted by PKA inhibition with H89 2HCl or PKI (Figure 3I; Figures V and VI in the online-only

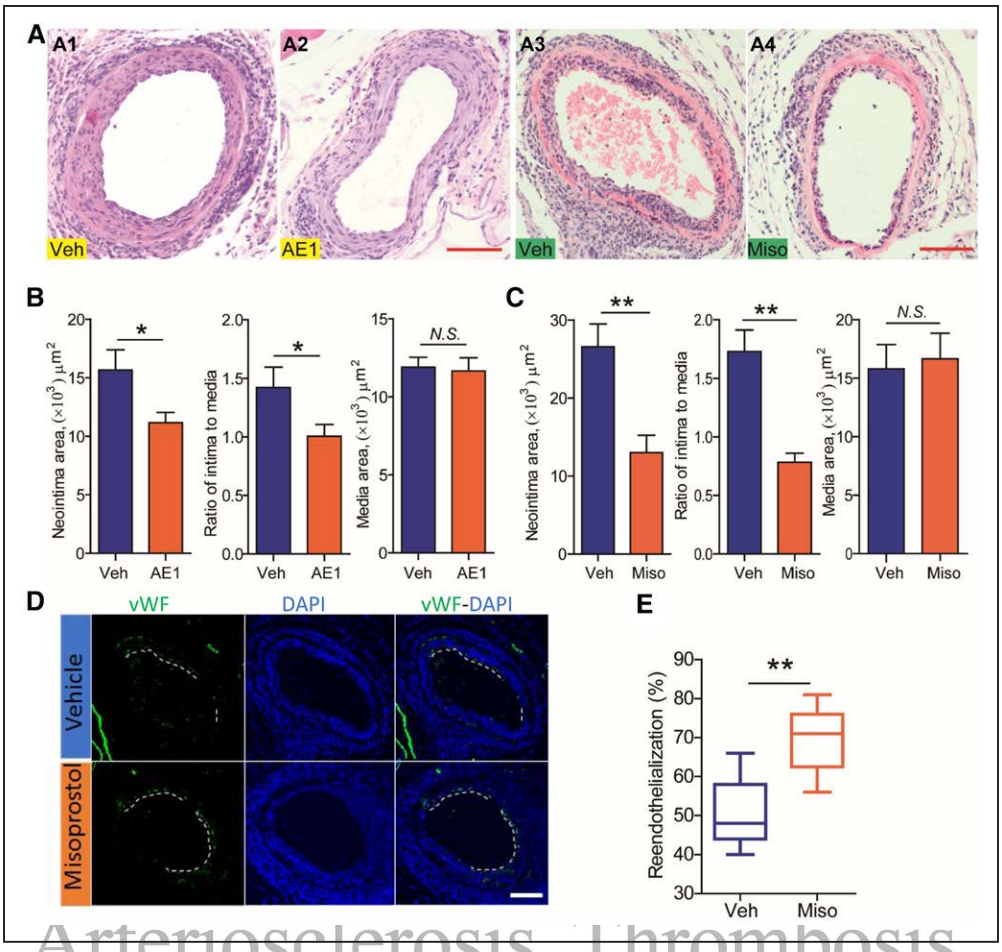

Figure 6. Pharmacological activation of EP (prostaglandin E receptor) 4 promoted endothelial repair and protected against neointimal formation. After vascular injury, C57BL/6 mice were sinjected with vehicle (Veh) or with AE1-329 (AE1—an EP4 selective agonist) at a dose of 0.3 mg/kg per day for 28 d (n=12). Injured vessels were stained with hematoxylin and eosin (H&E), with representative images shown (**A**; A1 and A2). Neointimal area, ratio of intima to media, and media thickness were quantified (**B**). Another batch of mice, after vascular injury, were treated via i.p. injection with vehicle (Veh) or with misoprostol (Miso—a PGE [prostaglandin E] analogue) at a dose of 100 μg/kg, TID, for a total of 7 d (n=7 Veh and 5 Miso). Injured vessels were stained with H&E, with representative images shown (**A**; A3 and A4). Neointimal area, ratio of intima to media, and media thickness were quantified (**C**). Reendothelialization was examined by immunostaining of vWF (von Willebrand factor) and quantified (**D** and **E**) as detailed previously. Student unpaired *t* test. N.S. indicates nonsignificant. **P*<0.05, ***P*<0.01.

Data Supplement). PGI_2 is known to elevate cAMP signaling via IP receptor.[1] Indeed, iloprost (1 μmol/L)—an IP agonist—promoted endothelial proliferation, whereas Cay10441 (10 μmol/L)—an IP antagonist—inhibited this response (Figure 3J), indicating a proliferative effect of PGI_2 on ECs.

The effect of EP4 on endothelium–leukocyte adhesion was also studied in vitro. Incubation of a monolayer of ECs with AE1-329—the EP4 selective agonist—significantly suppressed endothelium—leukocyte adhesion, whereas inhibition of endothelial EP4 by GW627368X enhanced the leukocyte adhesion to ECs (Figure 3K). This is consistent with a cAMP-mediated effect because Db-cAMP (a cell-permeable cAMP analogue, 30 μmol/L) treatment similarly reduced the leukocyte adhesion (Figure 3L).

Endothelium-Restricted Deletion of EP4 Impairs Reendothelization and Exacerbates Neointimal Formation

The role of endothelial EP4 in vascular remodeling was then examined in mice deficient in this receptor only in ECs (Figure 4A) as confirmed by significant suppression of EP4 protein expression in ECs (Figure 4B and 4C). The mice were subjected to the wire injury and studied 28 days later. The neointimal area and the ratio of intimal-to-medial area were both enhanced in the mice lacking endothelial EP4, while the medial area was unchanged (Figure 4D through 4G). Seven days after vascular injury, reendothelialization was significantly suppressed in mice lacking EP4 only in ECs (Figure 5A and 5B). This coincided with an increase in the number of intimal leukocytes and enhanced neointimal formation in cKOs (Figure 5C through 5E).

EP4 Activation Protects Against Wire Injury-Induced Neointimal Formation

Systemic administration of AE1-329—the EP4 agonist—also ameliorated neointimal formation without affecting media thickness (Figure 6A and 6B). Misoprostol, which promotes endothelial proliferation in vitro (Figure 2I), attenuated neointimal formation (Figure 6A and 6C) and promoted reendothelialization at 7 days after vascular injury (Figure 6D and

6E) in vivo. Misoprostol also decreased leukocyte infiltration (Figure VII in the online-only Data Supplement).

EP4 Critically Involves in Human EC Proliferation

Activation of EP4 by AE1-329, but not of other EPs, promoted proliferation of human primary ECs (Figure 7A). Conversely, among antagonists of the 4 EPs, only EP4 blockade by GW627368X inhibited the EC proliferation (Figure 7B).

Discussion

Here, we report a novel mechanism that regulates vascular responses to injury in mice: PGE_2 acts via endothelial EP4 to promote reendothelialization and restrain neointimal formation after wire-induced vascular injury. A similar mechanism promotes proliferation of human ECs in vitro. These observations raise the possibility that EP4 agonists may have value as adjunctive therapy in patients undergoing percutaneous revascularization.

We have previously reported that augmented biosynthesis of PGI_2 explains the reduced neointimal response to injury in mPGES-1 KO, raising the possibility that inhibitors of this enzyme will have a more favorable cardiovascular profile than nonsteroidal anti-inflammatory drugs targeting COX-2 that depress PGI_2 biosynthesis.[16] Here, removal of the PGI_2 receptor reveals a second therapeutic opportunity because mPGES-1–derived PGE_2 can limit neointimal formation after vascular injury (Figures 1 and 2). Indeed, we show in mice that pharmacological activation of EP4 confers protection against the injury-induced intima hyperplasia (Figure 6). Mechanistically, these beneficial effects are at least mediated through a support of EC repair by mPGES-1–derived PGE_2 and EP4 (Figures 2 and 6). We also observed an increased leukocyte infiltration (Figure 2C). This is consistent with a suppressing effect of EP4 activation on leukocyte adhesion (Figures 2J and 3K). Previous studies[22,35–39] indicate that the PGE_2/EP4 axis inhibits mobilization of inflammatory cells and their response to inflammation. These studies highlight a possible contribution of the PGE_2/EP4 axis on bone marrow microenvironment and on inflammatory cells per se in mediating the increased leukocyte infiltration to the injured vessels observed in the present study. Here, we discovered that EP4 in the endothelial compartment also mediates endothelial–leukocyte adhesion (Figure 5). There was no discernible difference in the neointimal SMCs (Figure 2E) or in the proliferation of vascular SMCs isolated from the mPGES-1–deficient mice (Figure II in the online-only Data Supplement). In cultured ECs, activation of EP4 (and perhaps EP2 but not EP1 and EP3), via cAMP-PKA pathway, promotes endothelial proliferation in vitro (Figure 3), consistent with the observed suppression of neointimal formation after injury by such ligands in vivo. In line with this mechanistic hypothesis, deletion of the endothelial EP4 impaired reendothelialization and exacerbated neointimal formation after injury (Figures 4 and 5). Furthermore, EP4 activation promoted reendothelialization and inhibited neointima formation in mice (Figure 6). Therefore, EC EP4-mediated endothelial repair underscores a key mechanism that restrains the vascular hyperplasia after denudation injury. Promotion of endothelialization by activating endothelial EP4 represents a novel strategy to limit restenosis after percutaneous coronary intervention.[40] The mechanism of mPGES-1–derived PGE_2 in vascular remodeling is schematically illustrated in Figure VIII in the online-only Data Supplement.

Development of mPGES-1 inhibitors has now reached the clinical stage. Interestingly, an augmented production of PGI_2 was observed with suppression of mPGES-1–derived PGE_2.[7] This is consistent with our initial observation with mPGES-1 KO mice.[11] Potential elevation of prostaglandin products because of substrate rediversion may depend on specific tissue/cell or disease pathology.[10] Their significance in mediating the cardiovascular profile of mPGES-1 inhibition relative to COX-2 inhibition warrants future study. Our study here suggests that augmented PGI_2 may balance a vascular risk of suppressing PGE_2–EP4 axis when mPGES-1 inhibition is pursued under pathological insult.

In conclusion, we have previously provided evidence that inhibition of mPGES-1 may offer an approach adjunctive to percutaneous coronary intervention to limit restenosis because of its capacity to augment PGI_2. Here, we provide evidence that PGE_2 formation might be targeted to achieve the same objective, here by activating the EP4 receptor in ECs. Given that restenosis remains a restraint on the clinical effectiveness of percutaneous coronary intervention,[40,41] the provision of preclinical data consistent with 2 distinct strategies by which this might be mitigated is timely.

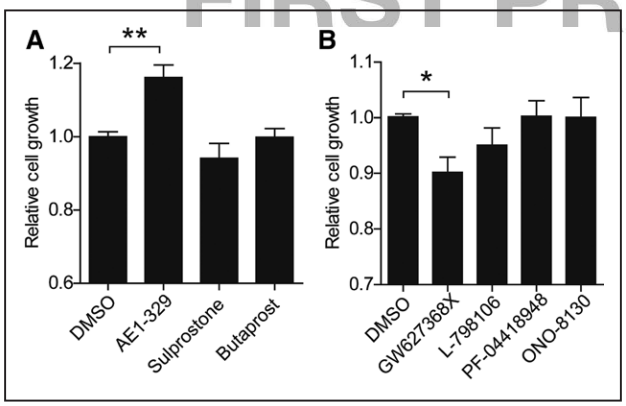

Figure 7. EP (prostaglandin E receptor) 4 signaling participated in the proliferation of human endothelial cells. Human microvascular endothelial cells were treated with 1 μmol/L of agonists (**A**) or antagonist (**B**) of the receptors of PGE_2 (prostaglandin E2), and relative proliferation was determined. Agonists: AE1-329 (EP4), sulprostone (EP1/3), and butaprost (EP2). Antagonists: GW627368X (EP4), L-798106 (EP3), PF-04418948 (EP2), and ONO-8130 (EP1). Both results were from 3 independent data sets. 1-way ANOVA with Dunnett post hoc test was used for data comparisons. *P<0.05, **P<0.01.

Study Limitations

The mechanistic exploration of this study was mainly focused on endothelial proliferation/repair by mPGES-1–derived PGE_2 and EP receptors. Whether impaired reendothelialization process might allow enhanced exposure to certain thrombotic components that may also contribute to the vascular responses warrants future study. This study was conducted with mice of C57BL/6 background. Confirming this study with other mouse strains or other species more relevant to

human pathophysiology would help increase the translational potential of the mechanism discovered here.

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Disclosures

Patent application (CN201710293360.9) is pending.

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Highlights

- Deletion of mPGES-1 (microsomal prostaglandin E synthase-1), on a background of IP (I prostanoid receptor) depletion, exacerbates wire injury-induced neointima formation, with early impairment of reendothelialization.
- Activation of EP (prostaglandin E receptor) 4 or EP2 promotes endothelial cell proliferation, and endothelium-restricted deletion of EP4 impairs reendothelialization and increases neointima formation.
- EP4 agonism promotes reendothelialization and attenuates neointima formation, raising a possibility that EP4 agonism may prevent restenosis after percutaneous coronary intervention.

Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

Protective Role of mPGES-1 (Microsomal Prostaglandin E Synthase-1)–Derived PGE_2 (Prostaglandin E2) and the Endothelial EP4 (Prostaglandin E Receptor) in Vascular Responses to Injury

Huifeng Hao, Sheng Hu, Qing Wan, Chuansheng Xu, Hong Chen, Liyuan Zhu, Zhenyu Xu, Jian Meng, Richard M. Breyer, Nailin Li, De-Pei Liu, Garret A. FitzGerald and Miao Wang

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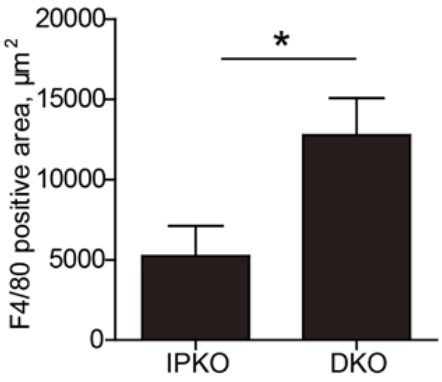
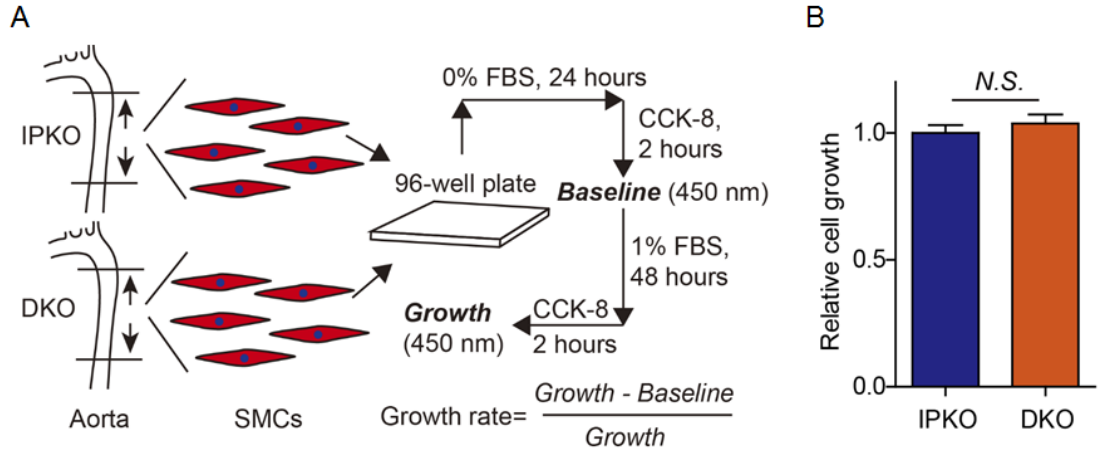
Supplemental Material

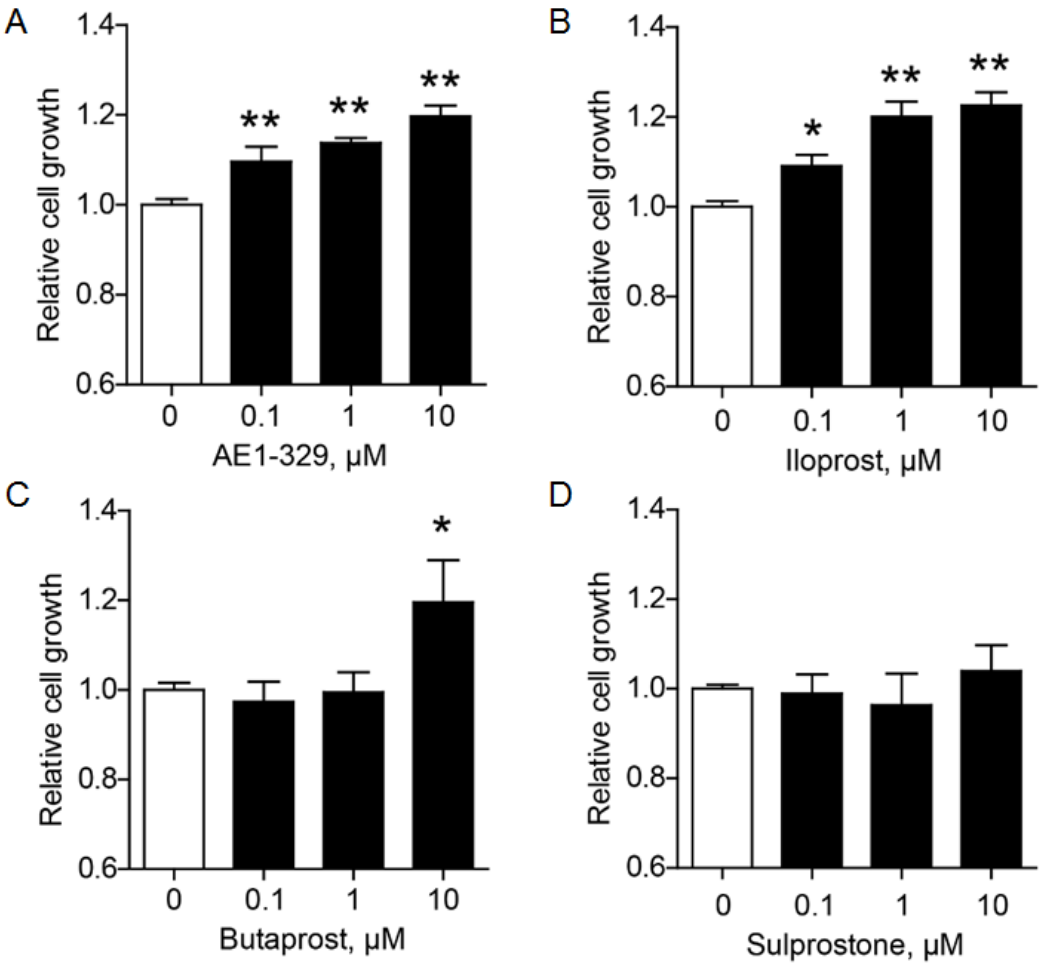
Supplemental Figures and Figure Legends



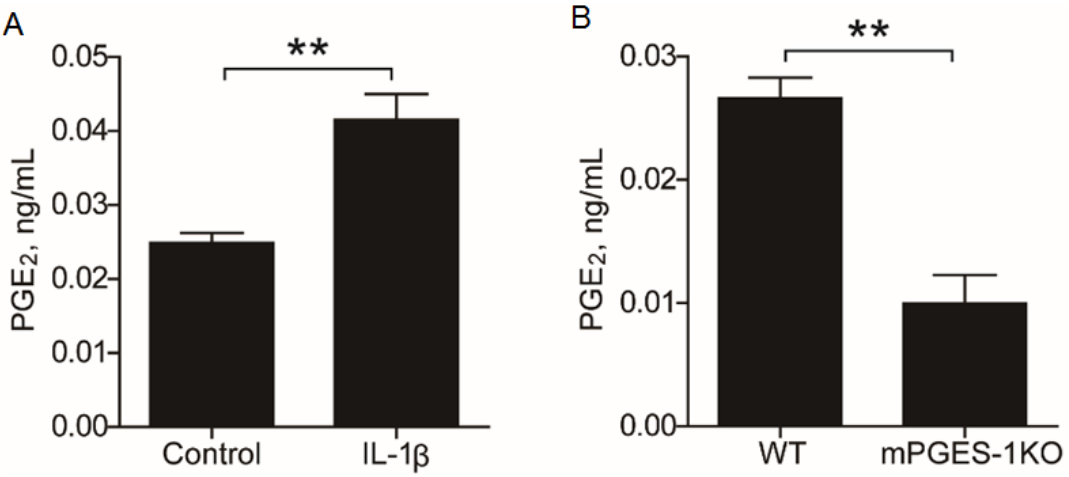
Supplemental Figure Ⅰ. Quantification of F4/80 positive area in the vessels from IP KO and DKO mice 7 days after injury. n=4 IPKO, 5 DKO; *P<0.05; Student's unpaired t-test.



Supplemental Figure Ⅱ. Determination of proliferation of the aortic smooth muscle cells isolated from IPKO and DKO mice.
Smooth muscle cells (SMCs) were isolated from the descending aortae of IPKO and DKO mice, and their cell proliferation was analyzed in vitro (A) and compared (B). n=8 wells from 2 independent experiments.

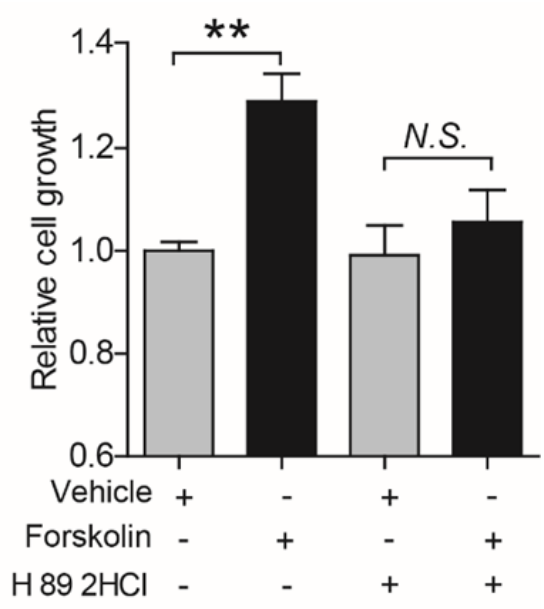


Supplemental Figure Ⅲ. The concentration-response of AE1-329 (A), iloprost (B), butaprost (C) and sulprostone (D) on endothelial proliferation. n=9 wells from three independent experiments; *p<0.05, **p<0.01, One-way ANOVA with Turkey's post tests.

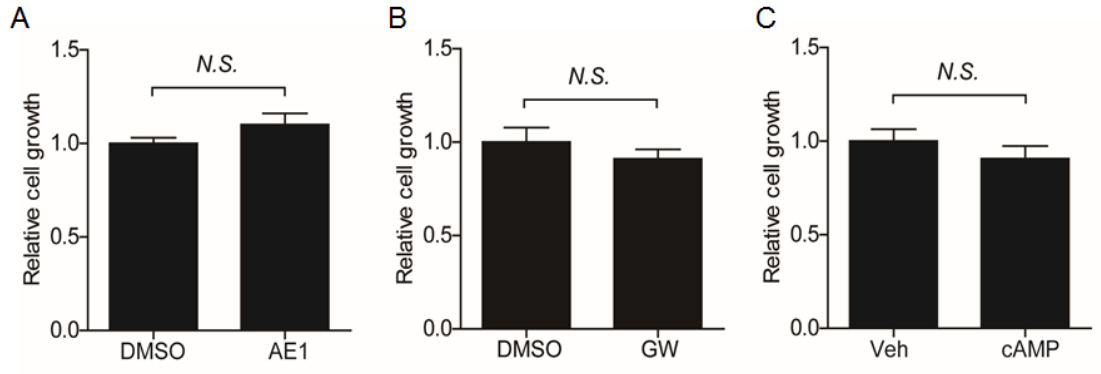


Supplemental Figure Ⅳ. Supernatant PGE_2 levels of cultured mouse aortic endothelial cells (MAECs) determined by HPLC-MS/MS method.

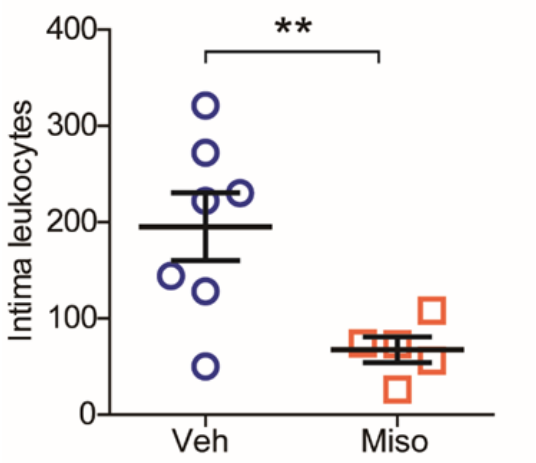
MAECs were treated with vehicle (Control) or IL-1β (10 ng/mL) for 12 hours, PGE_2 levels were shown (**A**). Supernatant levels of PGE_2 were significantly depressed in mPGES-1 deficient (mPGES-1KO) MAECs, compared with that in wildtype (WT) cells (**B**). n=4, **P<0.01; Student's unpaired t-test.



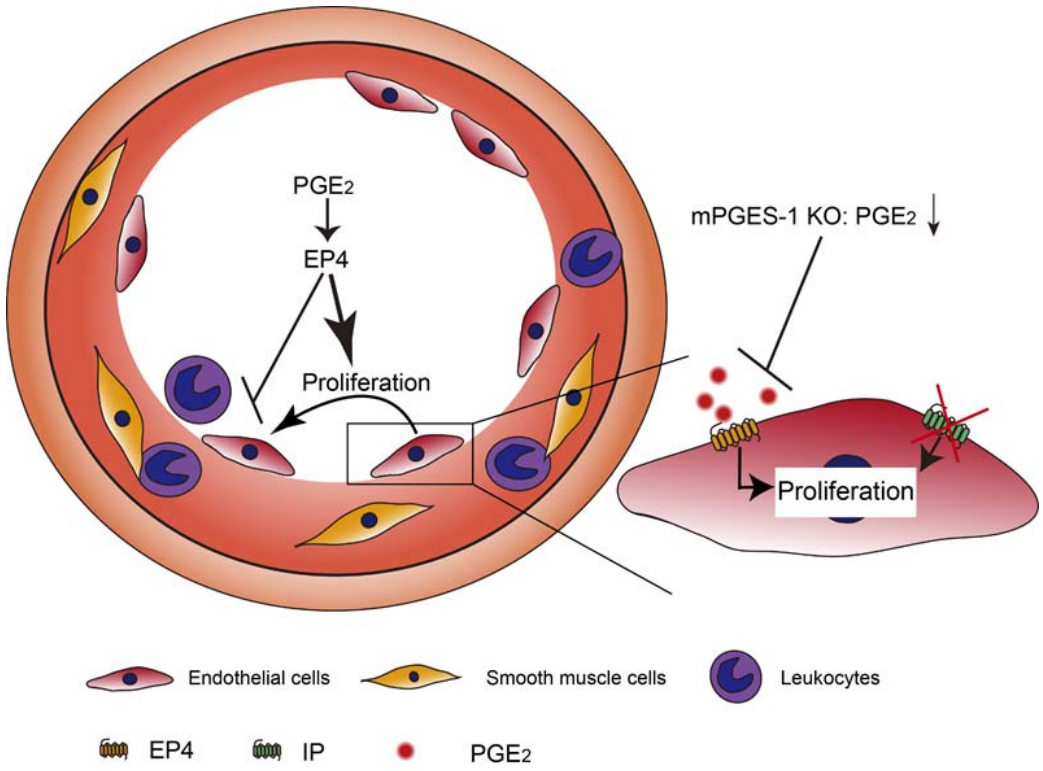
Supplemental Figure Ⅴ. Forskolin (an adenylate cyclase activator, 3 μmol/L) promoted endothelial proliferation, which were blunted by H89 2HCl (a PKA inhibitor). n=7; **P<0.01; One-way ANOVA with Bonferroni's post-test.



Supplemental Figure Ⅵ. AE1-329, GW627368X and db-cAMP did not affect EC proliferation under condition of PKA inhibition.
When MAECs were treated with PKI (a PKA inhibitor, 10 μmol/L), EP4 agonist (AE1-329, abbreviated as AE1, 1 μmol/L), EP4 antagonist (GW627368X, abbreviated as GW, 1 μmol/L), or db-cAMP (a cell permeable cAMP analogue, 30 μmol/L) failed to affect cell proliferation. Each result was from three independent data sets.



Supplemental Figure Ⅶ. Misoprostol administration (100 μg/Kg, i.p., three times a day) decreased the number of leukocytes infiltrated to intima, as examined at 7 days after the vascular injury. **P<0.01; Student's unpaired t-test.



Supplemental Figure Ⅷ. Schematic illustration of the mechanism of mPGES-1 derived PGE_2 in the vascular response to injury.

Knockout of mPGES-1 decreases PGE_2, and increases PGI_2. In IP deficient mice, a protective role of mPGES-1 derived PGE_2 was discovered. PGE_2, via EP4 receptor on endothelial cells, stimulates endothelial proliferation, suppresses leukocytes adhering to endothelial cells, and protected the injured vessels against development of neointimal hyperplasia. Arrow indicates stimulating effect, while line with a blunt end indicates inhibitory effect.

Supplemental Tables

Supplemental Table Ⅰ. Data analysis with gender information in the IPKOs and DKOs.

Gender	Male		Female		Gender mixed	
Genotype	IPKO	DKO	IPKO	DKO	IPKO	DKO
Intima area (28 days)	24.1±2.7 (n=10)	44.6±6.4 (n=10)	20.3±5.9 (n=4)	38.5±3.3 (n=6)	23±2.5 (n=14)	42.3±4.2 (n=16)
Ratio of I to M (28 days)	1.85±0.2 (n=10)	2.39±0.2 (n=10)	1.15±2.7 (n=4)	2.68±0.3 (n=6)	1.69±0.2 (n=14)	2.5±0.17 (n=16)
Intima area (7 days)	16.3±2.5 (n=11)	22.4±3.2 (n=12)	12.5±0.5 (n=3)	21.4±0.9 (n=4)	15.5±2.0 (n=14)	22.1±2.4 (n=16)
Ratio of I to M (7 days)	1.05±0.2 (n=11)	1.68±0.3 (n=12)	1.14±0.1 (n=3)	1.84±0.2 (n=4)	1.12±0.1 (n=14)	1.72±0.2 (n=16)
Intima leukocytes	140±17 (n=5)	193±26 (n=5)	161±23 (n=3)	246±21 (n=5)	148±13 (n=8)	219±18 (n=10)
Reendotheliali zation (%)	49.0±3.0 (n=6)	38.0±2.7 (n=5)	47.7 (n=1)	41.9±6.6 (n=2)	48.8±2.5 (n=7)	39.2±2.5 (n=7)

Note: Area ($\times 10^3$ μm^2).

Supplemental Table Ⅱ. Data analysis with gender information in the EP4 Ctls and EP4 cKOs.

Gender	Male		Female		Gender mixed	
Genotype	EP4 Ctl	EP4 cKO	EP4 Ctl	EP4 cKO	EP4 Ctl	EP4 cKO
Intima area (28 days)	19.3±4.1 (n=6)	30.5±6.7 (n=4)	17.1±2.6 (n=8)	23.4±3.0 (n=6)	18±2.2 (n=14)	26.2±3.2 (n=10)
Ratio of I to M (28 days)	1.20±0.3 (n=6)	2.14±0.3 (n=4)	1.26±0.2 (n=8)	1.76±0.4 (n=6)	1.24±0.1 (n=14)	1.91±0.3 (n=10)
Intima area (7 days)	11.6±1.7 (n=5)	20.6±4.0 (n=5)	8.1±2.4 (n=2)	11.7±0.7 (n=2)	10.7±1.4 (n=7)	18.3±3.2 (n=7)
Ratio of I to M (7 days)	0.66±0.2 (n=5)	1.37±0.2 (n=5)	0.61±0.2 (n=2)	1.15±0.1 (n=2)	0.65±0.1 (n=7)	1.31±0.1 (n=7)
Intima leukocytes	57±15 (n=6)	105±17 (n=6)	33±8 (n=2)	41±13 (n=2)	51±12 (n=8)	89±16 (n=8)
Reendotheliali zation (%)	62.3±5.7 (n=6)	42.1±7.0 (n=5)	59.6 (n=1)	39.6±5.2 (n=2)	61.9±4.8 (n=7)	41.3±4.6 (n=7)

Note: Area ($\times 10^3$ μm^2).