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Prostaglandin D₂ stimulates phenotypic changes in vascular smooth muscle cells

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Abstract

Since chronic inflammation is associated with the pathogenesis of atherosclerosis, inflammatory cytokines might contribute to the phenotypic modulation of vascular smooth muscle cells (VSMCs). Tumor necrosis factor α (TNF α) facilitated the transformation of contractile VSMCs to the synthetic phenotype, as determined by the expression of marker proteins and a collagen gel contraction assay. Western blot analysis and a cyclooxygenase-2 (COX2) promoter assay revealed that TNF α stimulation resulted in the induction of COX2. The overexpression, silencing, or pharmacological inhibition of COX2 significantly affected TNF α -induced phenotypic conversion, and of the tested prostaglandins, only PGD₂ significantly induced phenotypic conversion. ERK was significantly activated by PGD₂ stimulation, and the pharmacological inhibition of ERK blocked the PGD₂-induced phenotypic conversion of VSMCs. However, antagonists or agonists of PGD₂ receptors did not affect VSMC conversion. In contrast, spontaneously dehydrated forms of PGD₂, such as PGJ₂, Δ^{12} -PGJ₂, and 15-d-PGJ₂, strongly induced phenotypic conversion. A reporter gene assay showed that TNF α , PGD₂, and 15-d-PGJ₂ significantly activated the peroxisome proliferator-responsive element (PPRE) promoter. In addition, the overexpression or silencing of peroxisome proliferator-activated receptor δ (PPAR δ) significantly influenced 15-d-PGJ₂-induced phenotypic conversion. Finally, atherosclerotic neointima formation was significantly suppressed in mice lacking TNF α . In addition, mice fed celecoxib exhibited complete inhibition of carotid artery ligation-induced neointima formation. This study shows that PGD₂ regulates the phenotypic conversion of VSMCs by generating an endogenous ligand of PPAR, and that this leads to neointima formation in occlusive arterial disease.

Introduction

Atherosclerosis is a major leading cause of sudden death in western countries¹, and the pathogenesis of atherosclerosis is associated with chronic inflammation resulting from interactions between lipoproteins, immune cells, and surrounding tissues, such as the endothelium and medial layers of the arterial wall. Furthermore, the migration of monocytes into vessel walls represents the initial stage of atherosclerotic

plaque development². Thereafter, these monocytes differentiate into macrophages, which uptake oxidized low-density lipoprotein (ox-LDL) to generate fatty streaks³. In addition to forming fatty streaks, vascular smooth muscle cells (VSMCs) rapidly proliferate and migrate into arterial wall lesions where they synthesize and secrete extracellular matrix proteins to form the fibrous caps of plaques¹.

VSMCs are involved in a variety of vascular physiologies, such as vasoconstriction, vascular tone, blood pressure, and blood flow. In mature blood vessels, VSMC proliferation ceases, and the expression of specialized proteins, such as myosin heavy chain (MHC), myosin light-chain kinase (MLCK), calponin, transgelin 2 (SM22 α), and ion channels, is highly elevated. Unlike

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cardiac or skeletal muscle cells, VSMCs retain their phenotypic plasticity. For example, contractile VSMCs undergo reversible phenotypic changes in response to environmental cues and ultimately gain the ability to proliferate and carry out their contractile function⁴. Such phenotypic conversion and the proliferation of VSMCs are observed in vascular diseases, such as atherosclerosis, arteriosclerosis, and vascular aging⁵. Recently, platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), and laminin were reported to act as environmental cues that regulate VSMC phenotype^{6–8}. However, the mechanism underlying the phenotypic conversion of VSMCs in diseased states has not been elucidated.

Much evidence supports the idea that atherosclerosis is a result of chronic inflammation. Indeed, cells of the innate and adaptive immune systems, such as monocytes, macrophages, dendritic cells (DCs), B and T cells, and mast cells, are found in atherosclerotic plaque^{9,10}. Chronic inflammation is closely associated with lesion progression, which is characterized by VSMC migration across the internal elastic lamina into intimal or subendothelial areas¹. Furthermore, lesion development at this stage is influenced by inflammatory cytokines derived from interactions between monocytes/macrophages and T cells¹¹.

During atherosclerosis progression, inflammatory cytokines, such as interleukin-1 β (IL-1 β) and tumor necrosis factor α (TNF α), are involved in vascular remodeling. For example, IL-1 β deficiency suppresses the progression of atherosclerosis and outward vascular remodeling in a murine model^{12–14}. In particular, vascular remodeling is modulated by TNF α , and vascular wall thicknesses in TNF α /ApoE double-knockout mice have been reported to be thinner during the later stage of atherosclerosis¹⁵. In addition, it has been reported that atherosclerotic lesions are smaller in TNF α /ApoE double-knockout mice^{16,17}. Thus, it appears that TNF α is an important inflammatory cytokine during the progression of atherosclerosis.

It has also been reported that TNF α can induce the expression of cyclooxygenase-2 (COX2) in a variety of cell types, such as renal outer medulla cells, dorsal root ganglion cells, carcinoma cells, endometrial cells, and chondrocytes^{18–22}, and that the induction of COX2 by TNF α plays a key role in VSMC proliferation²³. COX regulates the rate-limiting step of the production of thromboxane A₂ (TXA₂) and prostaglandins, such as prostaglandin D₂ (PGD₂), prostaglandin I₂ (PGI₂), prostaglandin E₂ (PGE₂), and prostaglandin F_{2 α} (PGF_{2 α})²⁴. Furthermore, prostaglandins have diverse effects on VSMCs; for example, they induce contraction, relaxation, and proliferation^{25–28}. In particular, PGD₂ is metabolized to generate additional inflammatory cytokines, such as prostaglandin J₂ (PGJ₂), Δ^{12} -prostaglandin J₂ (Δ^{12} -PGJ₂),

15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15-d-PGJ₂), Δ^{12} -prostaglandin D₂ (Δ^{12} -PGD₂), and 13,14-dihydro-15-keto-PGD₂ (DK-PGD₂)^{29,30}; of these metabolites, PGJ₂ prostaglandins are known to be endogenous ligands of peroxisome proliferator-activated receptor (PPAR)^{31,32}.

PPARs are members of a superfamily of nuclear receptors, which includes PPAR α , PPAR δ , and PPAR γ ³³, and PPARs have been reported to play diverse roles in the vasculature. For instance, it has been reported that the smooth muscle-specific overexpression of PPAR γ represses smooth muscle marker gene expression³⁴, that PPAR δ is upregulated during vascular lesion formation, and that its overexpression enhances VSMC proliferation³⁵. Therefore, the underlying mechanisms of PPARs in the vasculature remain unclear. In the present study, we investigated critical inflammatory cytokines that determine the phenotypic switching of VSMCs and attempted to define the molecular mechanisms underlying inflammatory cytokine-mediated signaling pathways.

Materials and methods

Animals

Mice lacking TNF α (TNF α ^{-/-}, B6.129S-Tnf^{tm1Gkl}/J mice) and ApoE (ApoE^{-/-}, C57B6.129P2-ApoE^{tm1Unc}/J mice) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). TNF α ^{-/-} mice were crossed with ApoE-deficient mice (ApoE^{-/-}) to generate mice heterozygous at both loci. TNF α ^{+/-}ApoE^{-/-} mice were intercrossed to produce TNF α ^{+/+}ApoE^{-/-} and TNF α ^{-/-}ApoE^{-/-} littermates. Animals were housed under specific pathogen-free conditions. All animal procedures were performed in accordance with our institutional guidelines for animal research and were approved by our institutional animal care and use committee (PNU-2016-1195). The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Cell preparation and cell culture

VSMCs were isolated from 4-week-old male Sprague-Dawley rats by using a tissue explantation method. Briefly, rats were euthanized by intravenous ketamine (100 mg/kg) injection and perfused with phosphate-buffered saline (PBS) for 5 min. The thoracic aorta was aseptically isolated, and the surrounding fat and connective tissues were discarded. Vessels were cut longitudinally, and the lumen sides were scraped with a razor blade to remove the intima. Vessels were cut into 3–5-mm lengths and explanted the lumen side down onto collagen-coated culture dishes. Seven days after explantation, the tissue fragments were discarded, and sprouted VSMCs (referred to as P0-stage VSMCs) were collected.

Preparation of contractile phenotype VSMCs and phenotypic conversion

To prepare contractile VSMCs, P0-stage VSMCs were differentiated as described previously⁸. The differentiation of VSMCs was verified by western blotting for SMC marker proteins, namely, MHC, MLCK, SM22 α , calponin, and smooth muscle actin (SMA). Contractile VSMCs were cultured with medium containing the indicated inflammatory cytokines for 4 days. To examine the facilitation of phenotypic conversion by PPAR δ overexpression, cells were incubated with the indicated cytokines for 2 days.

Immunocytochemistry

For immunocytochemistry, cells were washed with ice-cold PBS and fixed with 4% paraformaldehyde for 10 min. The cells were permeabilized with 0.2% Triton X-100, incubated with the indicated primary antibodies for 1 h, and then treated with Cy3- or Alexa Fluor 488-conjugated secondary antibodies for 30 min. The samples were mounted with anti-fading reagent (2% n-propyl gallate in 80% glycerol/PBS solution), and images were obtained by using a confocal microscope (FV1000-ZDC, Olympus, Tokyo Japan).

Western blotting

Cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10% polyacrylamide gels under reducing conditions. The proteins were transferred to nitrocellulose membranes, which were immunoblotted by using the indicated primary antibodies and IRDye-conjugated secondary antibodies (Li-COR Biosciences, Lincoln, NE, USA). The western blots were developed by using the Odyssey system (Li-COR Biosciences).

Collagen gel contraction assay

VSMCs were isolated by trypsin digestion and resuspended in serum-free DMEM (1×10^6 cells/ml). The cell suspensions were mixed on ice with collagen gel solution (8 mg/ml collagen type I in $2 \times$ PBS, pH 8.0) to give 5×10^5 cells/ml and 4 mg/ml collagen gel solution. One hundred microliters of VSMC–collagen gel mixture was added to 12-well plates, which were then incubated for 30 min at 37 °C to enable polymerization. The gels were then floated in serum-free DMEM for 5 h, and angiotensin II (AngII, 1 μ M) was added to initiate contraction, while images were captured by using a digital charge-coupled device camera. Collagen gel contraction was defined as a decrease in the gel area as determined by Scion Image software (compliments of Scion Corporation, Frederick, MD; <http://www.scioncorp.com>). Relative gel areas were obtained by dividing the areas by the initial areas.

Plasmids and promoter assay

pGL3-SMA and pGL3-SM22 α were kindly provided by Dr. Gary K. Owens (University of Virginia). pGL3-myocardin was prepared as described previously⁸. pGL3-PPRE and pCDNA3.1-PPAR δ were provided by Dr. Jang Hyun Choi (UNIST, Ulsan, Korea). Human COX2 cDNA was subcloned into the *Bam*HI/*Eco*RI site of the pMIGR2 vector. To assess promoter activity, we used a dual-luciferase reporter assay system. VSMCs were plated in 12-well plates and cotransfected with the luciferase reporter constructs and the renilla luciferase plasmids by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Each well contained 0.88 μ g of luciferase reporter plasmid, 0.8 μ g of expression vector, and 80 ng of renilla luciferase plasmid. The medium was replaced with fresh medium 7 h post transfection, and the cells were lysed and assayed for luciferase activity 24 h post transfection. The protein extracts (20 μ l) were analyzed by using a Glo-maxTM 20/20 luminometer (Promega, WI, USA).

Short-hairpin RNA and constructs

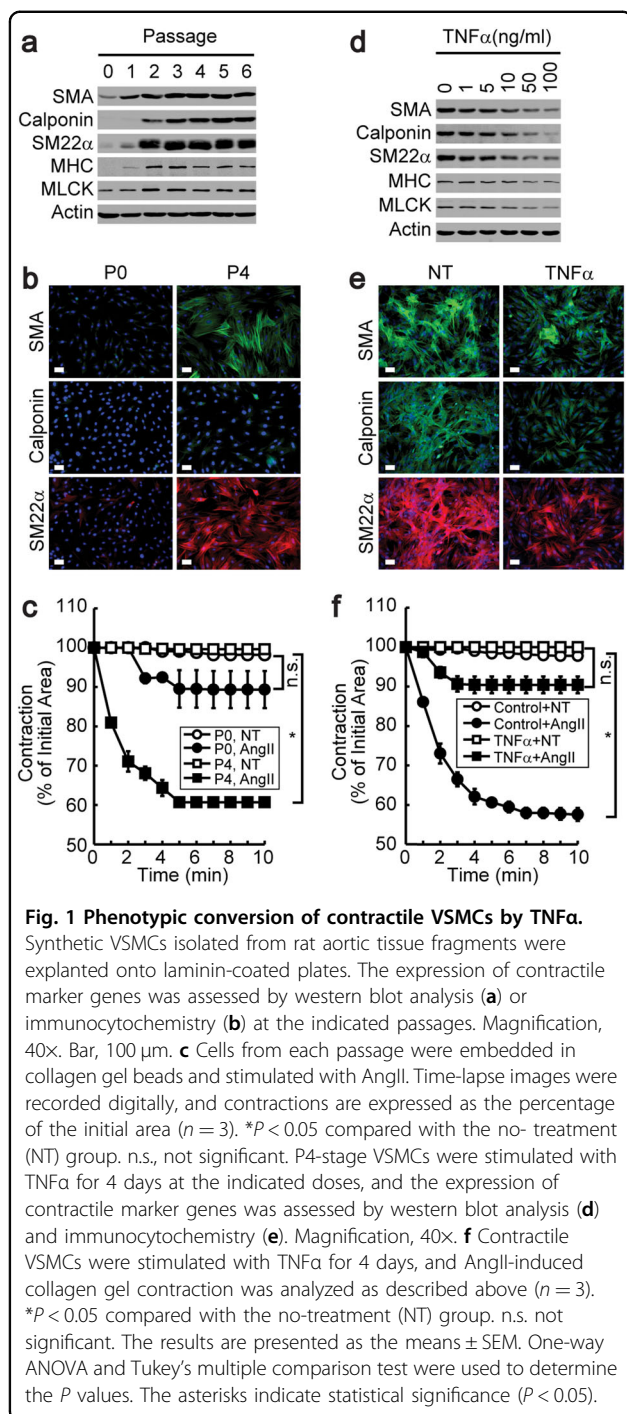
To silence COX2 and PPAR δ , shCOX2 (5'-CCG GTA AGA CAG ATC AGA AGC GAG GAC TCG AGT CCT CGC TTC TGA TCT GTC TTT TTT TG-3') and shPPAR δ (5'-CCG GTG CAA GCC CTT CAG TGA CAT CAC TCG AGT GAT GTC ACT GAA GGG CTT GCT TTT TG-3') oligonucleotides with an *Age*I site at the 5'-end site and an *Eco*RI site at the 3' end were designed, and then sense and antisense oligonucleotides were synthesized (XENOTECH, Daejeon, Korea). Both complementary oligonucleotides were mixed, heated at 98 °C for 5 min, and cooled to room temperature. The annealed nucleotides were subcloned into the *Age*I/*Eco*RI sites of the pLKO.1 lentiviral vector.

Lentiviral knockdown

For gene silencing, HEK293-FT packaging cells (Invitrogen) were grown to ~70% confluence in six-well plates. The cells were triple transfected with 6 μ g of the pLKO.1 lentiviral construct, 1 μ g of Δ 8.9, and 1 μ g of pVSV-G by using a calcium phosphate method. The medium was replaced with fresh medium 8 h post transfection. Lentiviral supernatants were harvested 24 h post transfection and passed through 0.45- μ m filters. Cell-free viral culture supernatants were used to infect contractile VSMCs in the presence of 8 μ g/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA). Additional rounds of infection were performed 48 and 72 h post transfection. Infected cells were isolated by selection by using 10 μ g/ml puromycin for 2 days.

Carotid artery ligation, induction of atherosclerosis, and immunohistochemistry

To induce neointima formation, the left common carotid artery of male mice (6 weeks old) was ligated



proximal to the bifurcation, and 4 weeks later, both the left and right common carotid arteries were isolated. To treat the mice with celecoxib, the mice were fed a D12450B diet containing 1500 ppm of celecoxib (Central Lab. Animal Inc., Seoul) for 2 weeks before ligation and fed a diet containing celecoxib for an additional 4 weeks. Atherosclerosis was induced by feeding male mice

(8 weeks old) a high-fat western-type diet containing 1.25% cholesterol (Research Diets, D12108) for 15 weeks. After killing, the mice were perfused with PBS, and the isolated carotid arteries and aortas were fixed in 4% paraformaldehyde at 4 $^{\circ}$ C overnight and embedded in paraffin for immunohistochemistry. Five-micrometer sections of each block were either stained with H&E or the indicated primary antibodies. Staining was visualized by using a MIRAX MIDI Versatile Digital Slide Scanner (Carl Zeiss, Jena, Germany) or by confocal microscopy (Olympus). For oil red O staining, aortic tissues were stained with oil red O solution (0.5% in isopropyl alcohol) for 2 h and washed with distilled water four times. For quantification, oil red O was extracted with isopropyl alcohol (1 ml), and the absorbance was measured at 518 nm.

Statistical analysis

The data were plotted and analyzed by using GraphPad Prism. Unpaired Student's t test (two tails) was used to determine the significance of intergroup differences. Multiple sets of data were analyzed by analysis of variance (one-way ANOVA) and Tukey's multiple comparison test. The results are expressed as the means \pm SEMs, and P values less than 0.05 were considered significant.

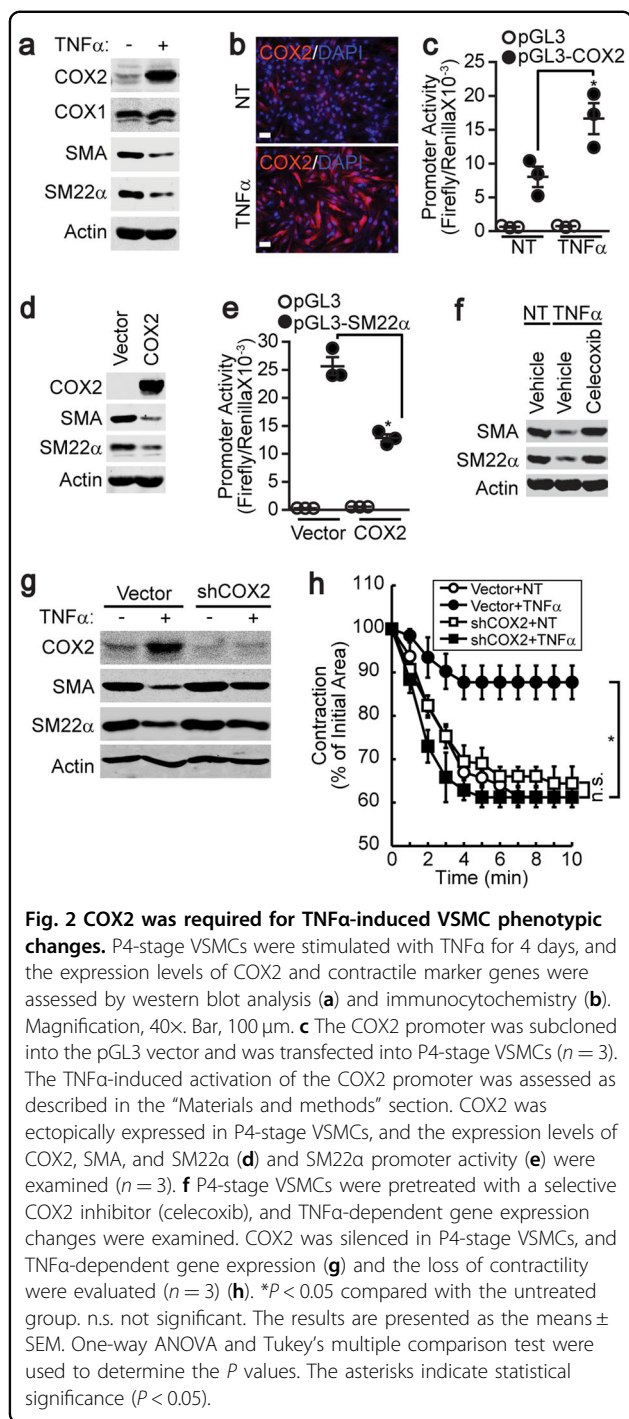
Results

Phenotypic changes in VSMCs induced by TNF α

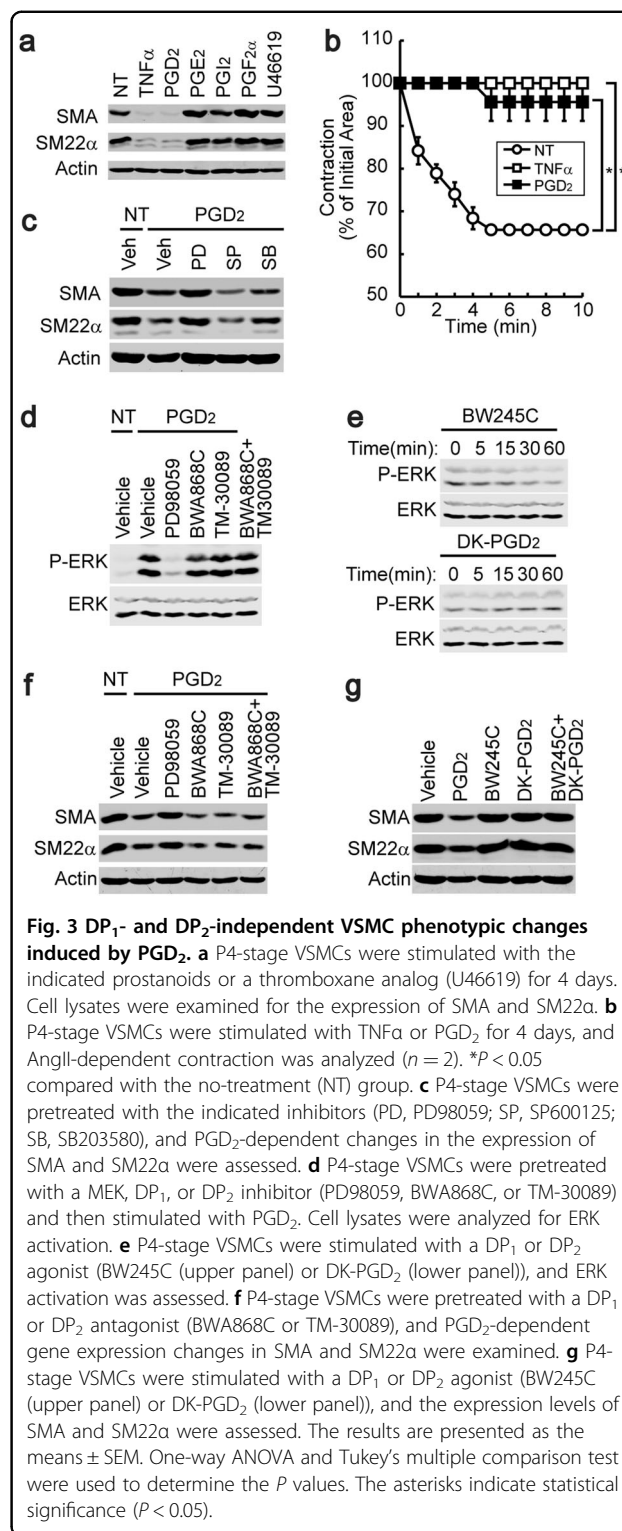
Since VSMC sprouting after the explantation of aortic tissue fragments on collagen-coated plates is indicative of the synthetic phenotype, we employed a previously described differentiation protocol⁸. Plating VSMCs on laminin-coated plates initiated differentiation, as shown in Fig. 1a, b. P0-stage VSMCs did not respond to AngII stimulation, whereas P4-stage VSMCs rapidly contracted in response to AngII stimulation (Fig. 1c). However, the differentiation of established cell lines, such as A10 cells, did not result in contraction in response to AngII stimulation (Supplementary Fig. S1). The treatment of contractile VSMCs (P4 stage) with TNF α significantly reduced the expression of SMC marker proteins (Fig. 1d, e) and the promoter activity of myocardin, SMA, and SM22 α (Supplementary Fig. S2). Furthermore, TNF α treatment resulted in the loss of AngII-dependent contraction (Fig. 1f).

COX2 was required for TNF α -induced phenotypic changes in VSMCs

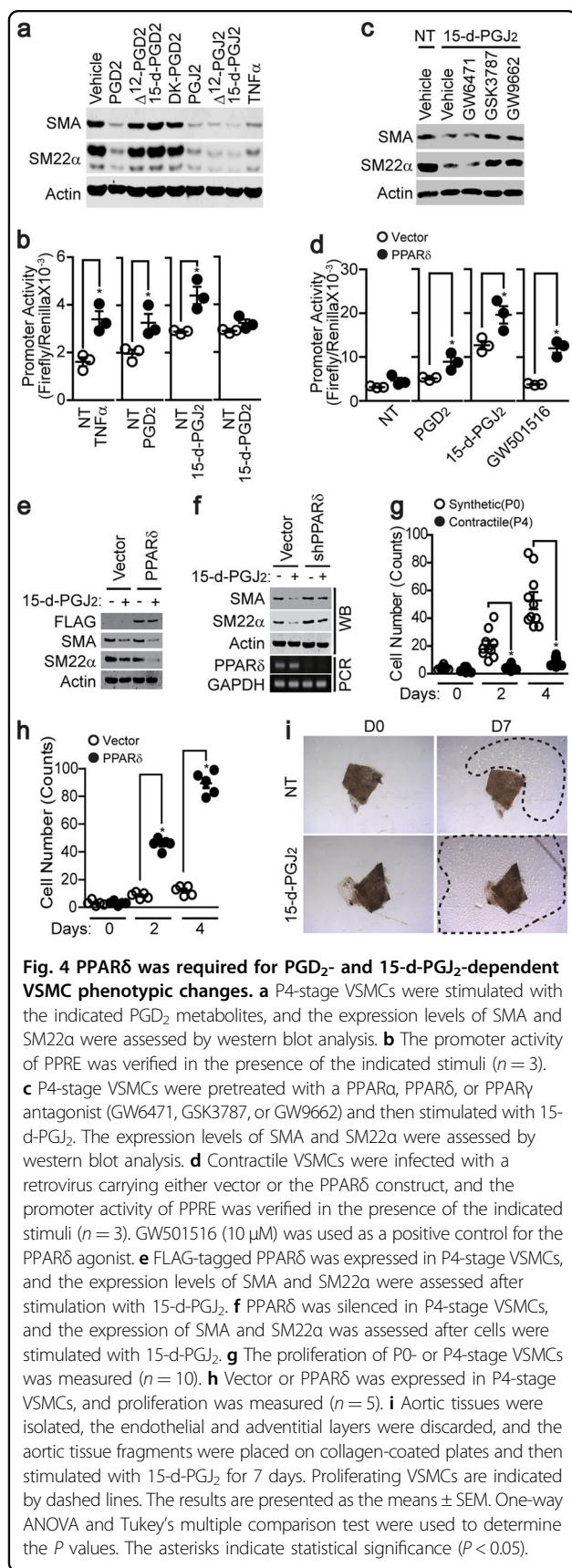
COX2 expression was significantly reduced during the differentiation of VSMCs from the synthetic to the contractile phenotype (Supplementary Fig. S3a), whereas the stimulation of contractile VSMCs with TNF α resulted in the induction of COX2 (Fig. 2a, b). In addition, the stimulation of VSMCs with TNF α strongly enhanced the promoter activity of COX2 (Fig. 2c). Furthermore, ectopic



COX2 expression significantly reduced the expression of marker proteins, such as SMA and SM22 α , and the promoter activity of SM22 α (Fig. 2d, e). In addition, the pharmacological inhibition of COX2 by celecoxib (10 μ M) markedly inhibited the TNF α -induced phenotypic conversion of contractile VSMCs (Fig. 2f). The silencing of COX2 blunted the TNF α -induced phenotypic conversion



of contractile VSMCs and preserved the expression of contractile phenotype marker proteins and AngII-dependent contraction (Fig. 2g, h, Supplementary Fig. S3b).



PGD $_2$ regulated the phenotypic changes in VSMCs independently of the DP $_1$ and DP $_2$ receptor signaling pathways

Since COX2 plays a key role in the rate-limiting step of prostaglandin synthesis, we examined the effect of prostaglandins on the expression of SMC marker proteins. The stimulation of contractile VSMCs with PGD $_2$ (10 μ M) significantly downregulated the expression of SMC marker proteins, whereas other prostaglandins had no effect (Fig. 3a, Supplementary Fig. S4a, b). In addition, the stimulation of contractile VSMCs with PGD $_2$ abrogated AngII-dependent contraction (Fig. 3b). The stimulation of contractile VSMCs with PGD $_2$ significantly activated the ERK signaling pathway in a time-dependent manner (Supplementary Fig. S4c), and the inhibition of ERK blunted PGD $_2$ -induced VSMC phenotypic changes (Fig. 3c). DP $_1$ and DP $_2$, which couple to G $_s$ and G $_i$, respectively, regulate cAMP production and are known to be PGD $_2$ receptors³⁶. However, the treatment of contractile VSMCs with PGD $_2$, a DP $_1$ agonist (BW245C, 10 μ M), or a DP $_2$ agonist (DK-PGD $_2$, 10 μ M) did not change cAMP production (Supplementary Fig. S5a–d). In addition, the expression of DP $_1$ and DP $_2$ was not observed in either synthetic or contractile VSMCs (Supplementary Fig. S5e). PGD $_2$ did not induce calcium mobilization, which is mediated by the activation of G $_q$ and phospholipase C- β (Supplementary Fig. S5f–h). Since the ERK signaling pathway is important for the PGD $_2$ -dependent suppression of SMC marker gene expression, we examined whether the PGD $_2$ receptors DP $_1$ and DP $_2$ are involved in the activation of the ERK signaling pathway. As shown in Fig. 3d, the inhibition of MEK by PD98059 (10 μ M) completely blocked the PGD $_2$ -induced activation of ERK. However, the inhibition of DP $_1$ (BWA868C, 10 μ M) or DP $_2$ (TM-30089, 10 μ M) or the simultaneous inhibition of DP $_1$ and DP $_2$ did not block the PGD $_2$ -induced activation of ERK. In addition, a selective agonist of DP $_1$ (BW245C, 10 μ M) or DP $_2$ (DK-PGD $_2$, 10 μ M) did not induce the activation of ERK (Fig. 3e). Furthermore, neither agonists nor antagonists of DP $_1$ and DP $_2$ affected the expression of SMC marker proteins (Fig. 3f, g).

The PGJ $_2$ series of PGD $_2$ metabolites affected VSMC phenotypes

PGD $_2$ generates inflammatory metabolites, such as Δ^{12} -PGD $_2$ and 15-d-PGJ $_2$ ^{29,30}. Since our results showed that DP $_1$ and/or DP $_2$ receptors did not affect VSMC phenotypic changes, we hypothesized that PGD $_2$ metabolites are involved in the phenotypic modulation of VSMCs. As shown in Fig. 4a, the PGJ $_2$ series of PGD $_2$ metabolites significantly induced VSMC phenotypic changes, whereas metabolites of other series had no effect. In addition, PGJ $_2$ series metabolites significantly induced ERK activation, whereas other series of metabolites did not

(Supplementary Fig. S6). Since PGD_2 and PGJ_2 are endogenous ligands of $\text{PPAR}^{31,32}$, we explored the effect of PGD_2 metabolites on PPRE promoter activity. As shown in Fig. 4b, $\text{TNF}\alpha$, PGD_2 , and 15-d- PGJ_2 significantly elevated PPRE promoter activity, whereas 15-d- PGD_2 , which did not induce VSMC phenotypic changes, had no effect. In addition, VSMCs mainly expressed $\text{PPAR}\delta$ and $\text{PPAR}\gamma$ (Supplementary Fig. S7a, b); the selective inhibition of $\text{PPAR}\delta$ (GSK3787, 10 μM) and $\text{PPAR}\gamma$ (GW9662, 10 μM) suppressed 15-d- PGJ_2 -induced phenotypic changes in contractile VSMCs, whereas the inhibition of $\text{PPAR}\alpha$ (GW6471, 10 μM) had no effect (Fig. 4c). Since 15-d- PGJ_2 significantly activated the $\text{PPAR}\delta$ -dependent activation of the PPRE promoter (Fig. 4d), we investigated the effect of 15-d- PGJ_2 in the presence or absence of $\text{PPAR}\delta$. The overexpression of $\text{PPAR}\delta$ significantly enhanced contractile VSMC phenotypic changes in the presence of 15-d- PGJ_2 (Fig. 4e). Furthermore, the silencing of $\text{PPAR}\delta$ inhibited 15-d- PGJ_2 -induced contractile VSMC phenotypic changes (Fig. 4f). The proliferation of contractile VSMCs was significantly lower than that of synthetic VSMCs (Fig. 4g), whereas the proliferation of contractile VSMCs was enhanced by the overexpression of $\text{PPAR}\delta$ (Fig. 4h). In addition, the stimulation of abdominal aortic fragments with 15-d- PGJ_2 facilitated VSMC sprouting ex vivo (Fig. 4i).

Atherosclerotic neointima formation was attenuated in mice lacking $\text{TNF}\alpha$

To assess the role of $\text{TNF}\alpha$ in the regulation of the VSMC phenotype in vivo, mice lacking both ApoE and $\text{TNF}\alpha$ ($\text{ApoE}^{-/-}\text{TNF}\alpha^{-/-}$) were fed a high-fat western diet, and neointima formation was examined. As shown in Fig. 5a, mice fed a high-fat diet exhibited significantly induced COX2 expression in the inflamed medial layers and plaque lesions, whereas COX2 expression was abolished in mice lacking $\text{TNF}\alpha$. Macrophages were observed only in plaque lesions in both genotypes. Neointima formation was significantly lower in $\text{ApoE}^{-/-}\text{TNF}\alpha^{-/-}$ mice than in $\text{ApoE}^{-/-}\text{TNF}\alpha^{+/+}$ mice (Fig. 5b). To avoid inadvertent errors caused by the locations of the sections, we also examined neointima formation in the aortic sinuses. As shown in Fig. 5c, neointima formation in mice lacking $\text{TNF}\alpha$ was significantly reduced.

Carotid artery ligation-induced neointima formation was attenuated in mice lacking $\text{TNF}\alpha$ or fed celecoxib

We next adapted the carotid artery ligation model to determine whether $\text{TNF}\alpha$ and COX2 are required for neointima formation. In control mice, the ligation of the left common carotid artery distal to the aortic arch near the bifurcation markedly induced neointima formation, whereas neointima formation was significantly lower in mice lacking $\text{TNF}\alpha$ (Fig. 6a). In addition, mice fed the

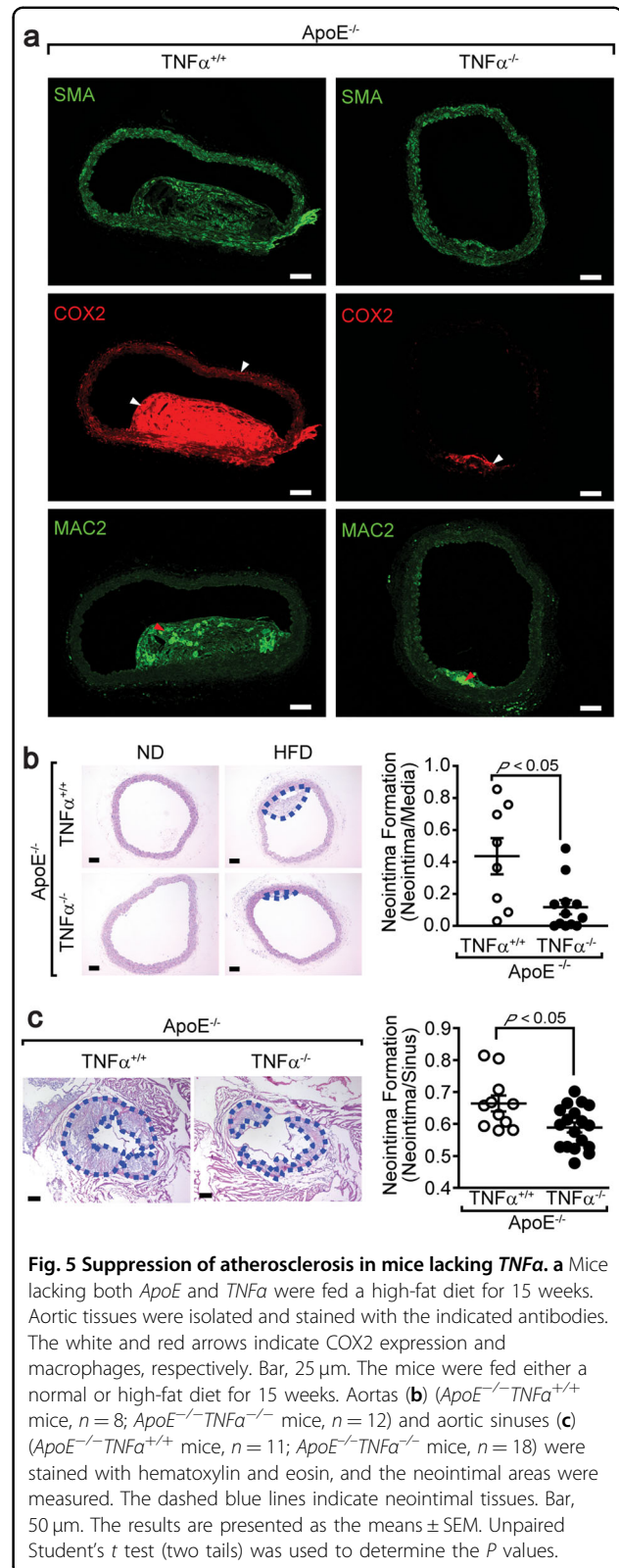
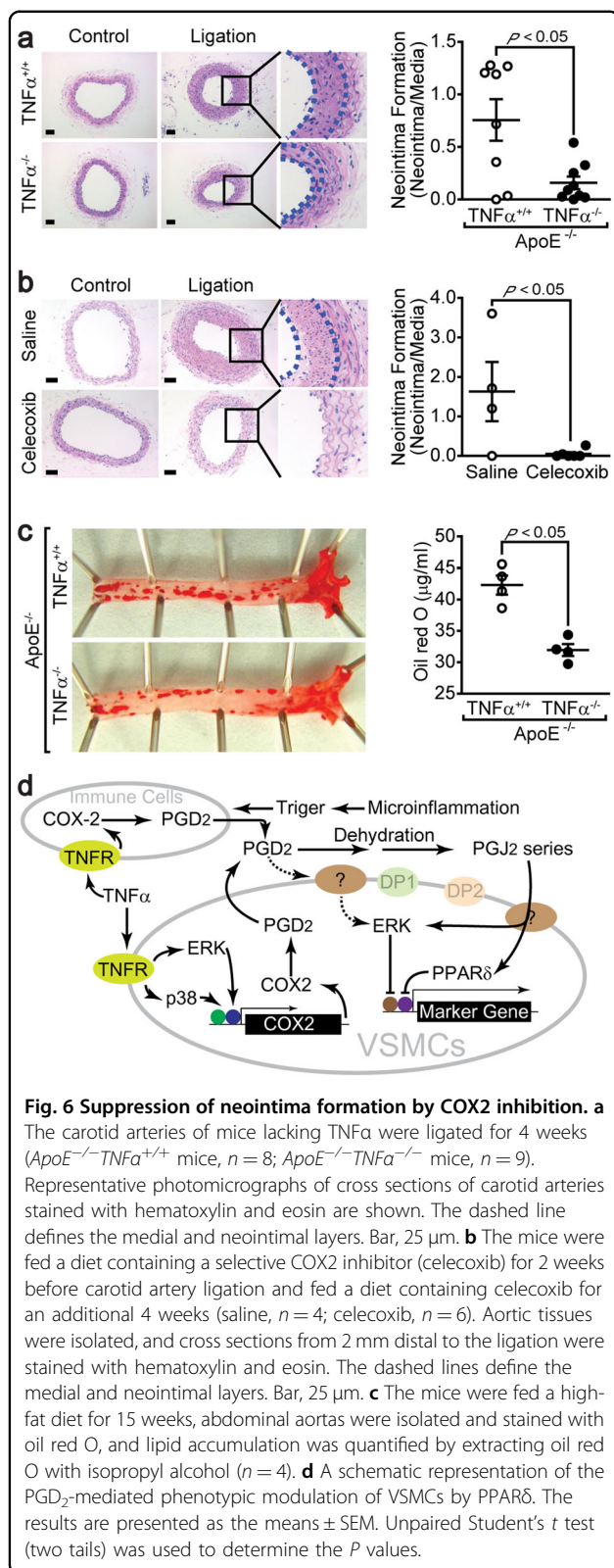


Fig. 5 Suppression of atherosclerosis in mice lacking $\text{TNF}\alpha$. **a** Mice lacking both ApoE and $\text{TNF}\alpha$ were fed a high-fat diet for 15 weeks. Aortic tissues were isolated and stained with the indicated antibodies. The white and red arrows indicate COX2 expression and macrophages, respectively. Bar, 25 μm . The mice were fed either a normal or high-fat diet for 15 weeks. Aortas (**b**) ($\text{ApoE}^{-/-}\text{TNF}\alpha^{+/+}$ mice, $n = 8$; $\text{ApoE}^{-/-}\text{TNF}\alpha^{-/-}$ mice, $n = 12$) and aortic sinuses (**c**) ($\text{ApoE}^{-/-}\text{TNF}\alpha^{+/+}$ mice, $n = 11$; $\text{ApoE}^{-/-}\text{TNF}\alpha^{-/-}$ mice, $n = 18$) were stained with hematoxylin and eosin, and the neointimal areas were measured. The dashed blue lines indicate neointimal tissues. Bar, 50 μm . The results are presented as the means \pm SEM. Unpaired Student's t test (two tails) was used to determine the P values.



selective COX2 inhibitor celecoxib exhibited completely blocked carotid artery ligation-induced neointima formation (Fig. 6b). Overall, plaque formation was

significantly decreased in mice lacking TNF α , as assessed by oil red O staining (Fig. 6c).

Discussion

The present study demonstrates that TNF α regulates the dedifferentiation of contractile VSMCs to the synthetic phenotype via the induction of COX2 and the production of prostaglandins, such as PGD₂ and PGJ₂. In addition, it shows that the loss of TNF α and the inhibition of COX2 suppresses the induction of intimal thickening in mice. Consequently, we propose that the PGD₂-mediated activation of PPAR δ is a potential target for the inhibition of neointima formation.

It has been reported that vascular remodeling is closely associated with chronic inflammation^{9,37}. Therefore, it is possible that inflammatory cytokines play pivotal roles in vascular remodeling, particularly in the phenotypic switching of VSMCs. In particular, TNF α has been implicated in the pathogenesis of atherosclerosis; for example, the disruption of TNF α has been reported to delay the progression of atherosclerosis and to reduce lesion sizes^{15,17}. The production of TNF α by cells of the hematopoietic lineage appears to be important because the transplantation of bone marrow from mice lacking TNF α has been found to reduce the severity of atherosclerosis¹⁶. Likewise, our results show that atherosclerotic lesion size was significantly reduced in mice lacking TNF α (Fig. 5). One of the major roles of TNF α seems to be involved in VSMC phenotypic changes. For instance, the stimulation of contractile VSMCs suppresses the expression of smooth muscle marker genes and results in the loss of contractile function in smooth muscle cells (Fig. 1). The consequence of TNF α -induced VSMC phenotypic changes is VSMC hyperproliferation since synthetic VSMCs grow faster than contractile VSMCs in vitro (Fig. 4g)⁸. Hence, we suggest that TNF α stimulates phenotypic conversion in smooth muscle, thereby enhancing VSMC proliferation and causing neointima formation.

Although the mechanisms underlying the TNF α -mediated phenotypic conversion of VSMCs remain unclear, recent evidence supports the role of COX2 induction during vascular remodeling. TNF α can induce the expression of COX2 in various cell types^{18–22}, and likewise, we found that TNF α stimulates the promoter activity of COX2 and thereby enhances COX2 expression in contractile VSMCs (Fig. 2a–c). Several lines of evidence support the role of COX2 induction by TNF α in contractile VSMC phenotypic changes. First, the silencing or overexpression of COX2 significantly affected smooth muscle marker gene expression and contractile function in VSMCs (Fig. 2d, e, g, h, Supplementary Fig. S3b). Second, the inhibition of COX enzymatic activity suppressed TNF α -induced contractile VSMC phenotypic changes and carotid artery ligation-induced neointima

formation (Figs. 2f, 6b). Third, it has been reported that the induction of COX2 by TNF α is required for VSMC proliferation²³. Therefore, these results suggest that the TNF α -dependent induction of COX2 and COX2 enzymatic activity are required for VSMC phenotypic conversion.

Prostanoids, which consist of prostaglandins and thromboxanes, are produced by COX family members, and our results show that PGD₂ (a prostaglandin produced by COX2) seems to be a major factor in VSMC phenotypic changes (Fig. 3a, Supplementary Fig. S4a, b). Likewise, it has been reported that lipocalin-type prostaglandin D synthase serum levels are correlated with coronary artery disease severity³⁸. Two PGD₂ receptors, namely, DP₁ and DP₂, have been identified thus far³⁹, but neither of these two receptors seems to be involved in PGD₂-mediated VSMC phenotypic changes. For instance, in the present study, neither the inhibition nor activation of DP₁ and/or DP₂ affected VSMC phenotypic change (Fig. 3f, g). In line with this, no significant difference in atherosclerosis is found in mice lacking the DP₁ receptor⁴⁰. In addition, the expression of DP₁ and DP₂ was not observed in VSMCs, and the stimulation of VSMCs with PGD₂ did not alter the levels of cAMP, which is a second messenger of the DP₁ and DP₂ receptor signaling pathways (Supplementary Fig. S5). The ERK signaling pathway plays an important role in PGD₂-induced VSMC phenotypic changes. For example, the stimulation of contractile VSMCs with PGD₂ strongly activated ERK, and the inhibition of ERK signaling completely blocked PGD₂-induced VSMC phenotypic changes (Fig. 3c, d), indicating that PGD₂ induces VSMC phenotypic changes by a DP₁- or DP₂-independent mechanism.

There are two mechanistic possibilities for PGD₂-induced VSMC phenotypic changes: (1) a novel receptor that activates the ERK signaling pathway and (2) the metabolic conversion of PGD₂. PGD₂ is metabolized to lipid mediators, such as prostaglandin J₂ (PGJ₂), Δ^{12} -prostaglandin J₂ (Δ^{12} -PGJ₂), 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15-d-PGJ₂), Δ^{12} -prostaglandin D₂ (Δ^{12} -PGD₂), and 13,14-dihydro-15-keto-PGD₂ (DK-PGD₂)^{29,30}. Indeed, in the present study, PGJ-type PGD₂ metabolites significantly induced VSMC phenotypic changes (Fig. 4a). PGJs have been reported to be endogenous ligands of PPAR δ ³², and our results also showed that PGJs significantly activated PPRE promoter activity (Fig. 4b), which was also significantly blunted by the inhibition of ERK. It is also noteworthy that PGJs significantly enhanced ERK activity (Supplementary Fig. S6). Furthermore, several of our observations support the role of PPAR δ in VSMC phenotypic changes. (1) A selective PPAR δ antagonist blunted VSMC phenotypic changes induced by 15-d-PGJ₂ (Fig. 4c). (2) The ectopic regulation of PPAR δ expression significantly affected phenotypic

changes in VSMCs (Fig. 4e, f). (3) The overexpression of PPAR δ enhanced VSMC proliferation (Fig. 4h). These observations encourage us to suggest that ERK-dependent PPAR δ activation is involved in the regulation of VSMC phenotypes.

Since VSMCs also exhibited a decreased expression of PPAR γ (Supplementary Fig. S7b), it is possible that PGD₂-dependent phenotypic changes in VSMCs are mediated by PPAR γ . Indeed, the PPAR γ isoform showed a similar effect in terms of phenotypic changes in VSMCs (Supplementary Fig. S8). The role of PPAR γ in the development of cardiovascular disease is somewhat controversial. For example, the forced expression or disruption of PPAR γ has incompatible effects in terms of VSMC proliferation, cardiovascular disease progression, and the mediation of pro- or anti-inflammatory responses^{34,41}. Currently, we do not have any direct evidence defining the exact role of PPAR γ in cardiovascular disease, but strongly believe that the role of PPAR γ is determined by cellular or disease contexts that might be influenced by many factors.

In the present study, we provide evidence that the COX2-dependent generation of PGD₂ followed by PPAR δ activation plays an important role in pathological changes in VSMC phenotypes (Fig. 6d). In this regard, our findings suggest that selective modulation of the biological activity of PGD₂ might be therapeutically useful in the context of cardiovascular disease.

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Conflict of interests

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References

1. Glass, C. K. & Witztum, J. L. Atherosclerosis. the road ahead. *Cell* **104**, 503–516 (2001).

2. Ross, R. Rous-Whipple Award Lecture. Atherosclerosis: a defense mechanism gone awry. *Am. J. Pathol.* **143**, 987–1002 (1993).
3. Navab, M. et al. The Yin and Yang of oxidation in the development of the fatty streak. A review based on the 1994 George Lyman Duff Memorial Lecture. *Arterioscler. Thromb. Vasc. Biol.* **16**, 831–842 (1996).
4. Owens, G. K. Regulation of differentiation of vascular smooth muscle cells. *Physiol. Rev.* **75**, 487–517 (1995).
5. Hao, H., Gabbiani, G. & Bochaton-Piallat, M. L. Arterial smooth muscle cell heterogeneity: implications for atherosclerosis and restenosis development. *Arterioscler. Thromb. Vasc. Biol.* **23**, 1510–1520 (2003).
6. Hayashi, K. et al. Insulin receptor substrate-1/SHP-2 interaction, a phenotype-dependent switching machinery of insulin-like growth factor-I signaling in vascular smooth muscle cells. *J. Biol. Chem.* **279**, 40807–40818 (2004).
7. Yoshida, T., Gan, Q., Shang, Y. & Owens, G. K. Platelet-derived growth factor-BB represses smooth muscle cell marker genes via changes in binding of MKL factors and histone deacetylases to their promoters. *Am. J. Physiol.* **292**, C886–C895 (2007).
8. Yun, S. J. et al. Akt1 isoform modulates phenotypic conversion of vascular smooth muscle cells. *Biochim. Biophys. Acta* **1842**, 2184–2192 (2014).
9. Andersson, J., Libby, P. & Hansson, G. K. Adaptive immunity and atherosclerosis. *Clin. Immunol.* **134**, 33–46 (2010).
10. Lundberg, A. M. & Hansson, G. K. Innate immune signals in atherosclerosis. *Clin. Immunol.* **134**, 5–24 (2010).
11. Hansson, G. K. Cell-mediated immunity in atherosclerosis. *Curr. Opin. Lipidol.* **8**, 301–311 (1997).
12. Alexander, M. R. et al. Genetic inactivation of IL-1 signaling enhances atherosclerotic plaque instability and reduces outward vessel remodeling in advanced atherosclerosis in mice. *J. Clin. Invest.* **122**, 70–79 (2012).
13. Alexander, M. R., Murgai, M., Moehle, C. W. & Owens, G. K. Interleukin-1beta modulates smooth muscle cell phenotype to a distinct inflammatory state relative to PDGF-DD via NF-kappaB-dependent mechanisms. *Physiol. Genomics* **44**, 417–429 (2012).
14. Kirii, H. et al. Lack of interleukin-1beta decreases the severity of atherosclerosis in ApoE-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **23**, 656–660 (2003).
15. Kober, F. et al. MRI follow-up of TNF-dependent differential progression of atherosclerotic wall-thickening in mouse aortic arch from early to advanced stages. *Atherosclerosis* **195**, e93–e99 (2007).
16. Branen, L. et al. Inhibition of tumor necrosis factor-alpha reduces atherosclerosis in apolipoprotein E knockout mice. *Arterioscler. Thromb. Vasc. Biol.* **24**, 2137–2142 (2004).
17. Ohta, H. et al. Disruption of tumor necrosis factor-alpha gene diminishes the development of atherosclerosis in ApoE-deficient mice. *Atherosclerosis* **180**, 11–17 (2005).
18. Battula, S., Hao, S., Pedraza, P. L., Stier, C. T. & Ferreri, N. R. Tumor necrosis factor-alpha induces renal cyclooxygenase-2 expression in response to hypercalcemia. *Prostaglandins Other Lipid Mediat.* **99**, 45–50 (2012).
19. Fehrenbacher, J. C., Burkey, T. H., Nicol, G. D. & Vasko, M. R. Tumor necrosis factor alpha and interleukin-1beta stimulate the expression of cyclooxygenase II but do not alter prostaglandin E2 receptor mRNA levels in cultured dorsal root ganglia cells. *Pain* **113**, 113–122 (2005).
20. Itatsu, K. et al. Cyclooxygenase-2 is involved in the up-regulation of matrix metalloproteinase-9 in cholangiocarcinoma induced by tumor necrosis factor-alpha. *Am. J. Pathol.* **174**, 829–841 (2009).
21. Kim, Y. A. et al. Tumor necrosis factor-alpha-induced cyclooxygenase-2 over-expression in eutopic endometrium of women with endometriosis by stromal cell culture through nuclear factor-kappaB activation. *J. Reprod. Med.* **54**, 625–630 (2009).
22. Morisset, S., Patry, C., Lora, M. & de Brum-Fernandes, A. J. Regulation of cyclooxygenase-2 expression in bovine chondrocytes in culture by interleukin 1alpha, tumor necrosis factor-alpha, glucocorticoids, and 17beta-estradiol. *J. Rheumatol.* **25**, 1146–1153 (1998).
23. Young, W., Mahboubi, K., Haider, A., Li, I. & Ferreri, N. R. Cyclooxygenase-2 is required for tumor necrosis factor-alpha- and angiotensin II-mediated proliferation of vascular smooth muscle cells. *Circ. Res.* **86**, 906–914 (2000).
24. Bozza, P. T., Bakker-Abreu, I., Navarro-Xavier, R. A. & Bandeira-Melo, C. Lipid body function in eicosanoid synthesis: an update. *Prostaglandins Leukot. Essent. Fat. Acids* **85**, 205–213 (2011).
25. Ragolia, L., Palaia, T., Paric, E. & Maesaka, J. K. Prostaglandin D2 synthase inhibits the exaggerated growth phenotype of spontaneously hypertensive rat vascular smooth muscle cells. *J. Biol. Chem.* **278**, 22175–22181 (2003).
26. Ren, J., Karpinski, E. & Benishin, C. G. Prostaglandin E2 contracts vascular smooth muscle and inhibits potassium currents in vascular smooth muscle cells of rat tail artery. *J. Pharmacol. Exp. Ther.* **275**, 710–719 (1995).
27. Rice, K. M. et al. PGF2alpha-associated vascular smooth muscle hypertrophy is ROS dependent and involves the activation of mTOR, p70S6k, and PTEN. *Prostaglandins Other Lipid Mediat.* **85**, 49–57 (2008).
28. Williams, S. P., Dorn, G. W. 2nd & Rapoport, R. M. Prostaglandin I2 mediates contraction and relaxation of vascular smooth muscle. *Am. J. Physiol.* **267**, H796–H803 (1994).
29. Fitzpatrick, F. A. & Wynalda, M. A. Albumin-catalyzed metabolism of prostaglandin D2. Identification of products formed in vitro. *J. Biol. Chem.* **258**, 11713–11718 (1983).
30. Kikawa, Y., Narumiya, S., Fukushima, M., Wakatsuka, H. & Hayaishi, O. 9-Deoxy-delta 9, delta 12-13,14-dihydroprostaglandin D2, a metabolite of prostaglandin D2 formed in human plasma. *Proc. Natl Acad. Sci. USA* **81**, 1317–1321 (1984).
31. Forman, B. M. et al. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell* **83**, 803–812 (1995).
32. Reddy, A. T., Lakshmi, S. P., Banno, A. & Reddy, R. C. Identification and molecular characterization of peroxisome proliferator-activated receptor delta as a novel target for covalent modification by 15-deoxy-delta(12,14)-prostaglandin J2. *ACS Chem. Biol.* **13**, 3269–3278 (2018).
33. Kliewer, S. A., Umesonu, K., Noonan, D. J., Heyman, R. A. & Evans, R. M. Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. *Nature* **358**, 771–774 (1992).
34. Kleinhenz, J. M. et al. Smooth muscle-targeted overexpression of peroxisome proliferator activated receptor-gamma disrupts vascular wall structure and function. *PLoS ONE* **10**, e0139756 (2015).
35. Zhang, J. et al. Peroxisome proliferator-activated receptor delta is up-regulated during vascular lesion formation and promotes post-confluent cell proliferation in vascular smooth muscle cells. *J. Biol. Chem.* **277**, 11505–11512 (2002).
36. Matsuoka, T. & Narumiya, S. Prostaglandin receptor signaling in disease. *Sci. World J.* **7**, 1329–1347 (2007).
37. Libby, P. Inflammation in atherosclerosis. *Nature* **420**, 868–874 (2002).
38. Inoue, T. et al. Lipocalin-type prostaglandin D synthase is a powerful biomarker for severity of stable coronary artery disease. *Atherosclerosis* **201**, 385–391 (2008).
39. Pettipher, R. The roles of the prostaglandin D(2) receptors DP(1) and CRTH2 in promoting allergic responses. *Br. J. Pharmacol.* **153**, S191–S199 (2008).
40. Strack, A. M. et al. Nicotinic acid and DP1 blockade: studies in mouse models of atherosclerosis. *J. Lipid Res.* **54**, 177–188 (2013).
41. Hamblin, M. et al. Vascular smooth muscle cell peroxisome proliferator-activated receptor-gamma deletion promotes abdominal aortic aneurysms. *J. Vasc. Surg.* **52**, 984–993 (2010).