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MGP Regulates Perivascular Adipose-Derived Stem Cells Differentiation Toward Smooth Muscle Cells Via BMP2/SMAD Pathway Enhancing Neointimal Formation

Hui Ni[1*], Chang Liu[1*], Yuwen Chen[1], Yunrui Lu[1], Yongli Ji[1], Meixiang Xiang[1], and Yao Xie[1]

Abstract

Perivascular adipose-derived stem cells (PV-ADSCs) could differentiate into smooth muscle cells (SMCs), participating in vascular remodeling. However, its underlying mechanism is not well explored. Our previous single-cell RNA-sequencing dataset identified a unique expression of matrix Gla protein (MGP) in PV-ADSCs compared with subcutaneous ADSCs. MGP involves in regulating SMC behaviors in vascular calcification and atherosclerosis. In this study, we investigated MGP's role in PV-ADSCs differentiation toward SMCs *in vitro* and in vascular remodeling *in vivo*. PV-ADSCs were isolated from perivascular regions of mouse aortas. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR), Western blot, and immunofluorescence confirmed higher MGP expression in PV-ADSCs. The MGP secretion increased along PV-ADSCs differentiation toward SMCs in response to transforming growth factor–beta 1 (TGF-β1). Lentivirus knockdown of MGP markedly promoted the bone morphogenetic protein 2 (BMP2) expression and phosphorylation of SMAD1/5/8 in PV-ADSCs, subsequently inhibiting its differentiation toward SMCs. Such inhibition could be partially reversed by further application of BMP2 inhibitors. On the contrary, exogenous MGP inhibited BMP2 expression and SMAD1/5/8 phosphorylation in PV-ADSCs, thereby promoting its differentiation toward SMCs. Transplantation of cultured PV-ADSCs, which was pretreated by MGP knockdown, in mouse femoral artery guide-wire injury model significantly alleviated neointimal hyperplasia. In conclusion, MGP promoted the differentiation of PV-ADSCs toward SMCs through BMP2/SMAD-mediated signaling pathway. This study offers a supplement to the society of perivascular tissues and PV-ADSCs.

Keywords

MGP, PV-ADSCs, SMCs, cell differentiation, BMP2/SMAD pathway

Introduction

Atherosclerotic cardiovascular diseases (ASCVDs) are the major cause of mortality in the world. Neointimal hyperplasia is the prominent pathological process in ASCVDs, where phenotypic switching of vascular smooth muscle cells (SMCs) and macrophages are initially believed as key participants. Later studies confirmed the importance of vascular adventitial progenitor cells in the differentiation toward vascular lineages and in neointima formation after vascular injury. For example, adventitial $LY6A^+$ cells could migrate to the intima and then differentiate to SMCs, promoting neointimal hyperplasia[1]. Adipose tissues surround the arteries except vasculatures in the central nervous system. Surprisingly, perivascular adipose tissues have long been ignored.

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Adipose tissue is well known for its role in metabolism, inflammation, and adipokine release. Until 2001, the Zuk group first isolated adipose-derived stem cells (ADSCs) from subcutaneous adipose tissue by enzymatic digestion[2,3]. In 2008, mesenchymal stem cells (MSCs) were identified in the perivascular tissue, which could differentiate into multilineages including SMCs and endothelial cells (ECs)[4]. ADSCs from different origins displayed distinctive characteristics. Subcutaneous adipose-derived stem cells (SUB-ADSCs) could give rise to tube-forming ECs in Matrigel and improve the blood perfusion of ischemic limbs in mice[5,6]. Two recent researches confirmed the existence of perivascular adipose-derived stem cells (PV-ADSCs) by immunofluorescence, flow cytometry, and scRNA-sequencing[7,8]. Gu et al. revealed that PV-ADSCs could differentiate into SMCs *in vivo* and enhance neointimal hyperplasia in mouse vein graft model. Pan et al. discovered that PV-ADSCs from older mice could develop more neointima than young PV-ADSCs in guide-wire artery injury model[7]. ADSCs expressed MSC-like markers such as LY6A, PDGFRA, THY1, CD34, and so on. By applying genetic lineage tracing mice, Tang et al. demonstrated that aortic adventitial $LY6A^+/PDGFRA^+$ cells not $LY6A^+/PDGFRB^+$ cells could differentiate into SMCs *in vivo*[9]. However, the mechanism of PV-ADSCs differentiation toward SMCs was unclear.

Matrix Gla protein (MGP) is a vitamin K–dependent secreted protein, which is closely associated with osteogenic differentiation, atherosclerosis, and vascular calcification[10]. Accumulating evidence revealed the importance of MGP in vascular lineages. MGP promoted ECs proliferation, migration, tube formation,[11,12] and regulated SMCs differentiation and maturation. MGP knockout in SMCs significantly decreased ACTA2, TAGLN, and MYH11 expression while it upregulated osteogenic-related protein RUNX2[13]. A recent study showed that MGP was involved in the ADSCs differentiation toward mature adipocytes[14]. Our previous scRNA dataset displayed that PV-ADSCs uniquely expressed MGP. These findings drove us to further investigate MGP's role in PV-ADSCs. MGP is a natural binding inhibitor of bone morphogenetic protein 2 (BMP2), a transforming growth factor–beta (TGF-β) superfamily member. MGP could act on BMP2, well known for regulating SMCs proliferation and phenotypic switching, to prevent vascular calcification[15,16]. BMP2 binds to type I and type II transmembrane serine/threonine kinase receptors, activating SMAD proteins for cell signal transduction to the nucleus[17]. BMP2-mediated signaling pathway could inhibit SMCs contractile marker genes expression[18,19]. Taken together, the above findings highlight the importance of MGP and BMP2 in SMC biology, which may also play crucial roles in PV-ADSCs.

Our investigation demonstrated that MGP secretion and expression markedly changed during PV-ADSCs differentiation toward SMCs in response to TGF-β1. By applying MGP lentivirus inhibition and exogenous MGP, we confirmed MGP's participation in PV-ADSCs differentiation. Mechanistically, MGP inhibited BMP2 expression, thus inhibiting SMAD1/5/8 phosphorylation. In mouse femoral artery injury model, transplantation of cultured PV-ADSCs with MGP knockdown significantly alleviated neointima formation. These results implied that MGP may potentially serve as a therapeutic target for neointimal hyperplasia.

Materials and Methods

Mice

C57BL/6 mice aged 8 to 12 weeks were purchased from Shanghai SLAC Laboratory Animal Corporation (Shanghai, China). All animal experiments were approved by the Animal Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine.

PV-ADSCs Isolation and Culture

Briefly, perivascular adipose tissue was dissected from aorta and rinsed with phosphate buffered saline (PBS) for three times. Then, 1-mm^3 pieces of adipose tissue were digested with 2 mg/ml collagenase type I (Invitrogen, Carlsbad, CA, USA) at 37°C water bath for 30 min. The pellet was resuspended in α-MEM (α-minimal essential medium; Gibco, Rockville, MD, USA) with 20% FBS (fetal bovine serum; Gibco), 10 ng/ml recombinant human leukemia inhibitory factor (Sigma, St. Louis, MO, USA), 5 ng/ml bFGF (basic fibroblast growth factor; R&D systems, Minneapolis, MN, USA), 2 mmol/l L-glutamine (Gibco), and 1% penicillin/streptomycin (Gibco) at 37°C with 5% CO_2. Medium was changed every second day. PV-ADSCs at passages 3 to 4 were used for subsequent experiments.

Phenotyping of Cultured PV-ADSCs

PV-ADSCs at passage 3 were resuspended in flow cytometric staining buffer containing the following antibodies: anti-LY6A-PerCP (Abcam, Cambridge, MA, USA), anti-CD29-PE (BD Biosciences, Franklin Lakes, NJ, USA), anti-CD105-FITC (Abcam), anti-CD34-FITC (Invitrogen), anti-THY1-FITC (Invitrogen), anti-CD31-PE (BD Biosciences), and anti-CD45-FITC (MultiSciences, Hangzhou, China). LIVE/DEAD™ Fixable Dead Cell Stain Kits (Invitrogen) were applied to distinguish viable cells from dead cells. Cells were analyzed with Beckman Cytoflex.

Differentiation of PV-ADSCs

StemPro® Adipogenesis Differentiation Kit (Gibco) and StemPro® Osteogenesis Differentiation Kit (Gibco) were used for adipogenic and osteogenic differentiation of PV-ADSCs, respectively, by following kits' standard protocols. After 21-day culture, PV-ADSCs were fixed with 4% paraformaldehyde, followed by Oil Red O and Alizarin Red staining. For SMCs differentiation, cultured PV-ADSCs

were changed to medium with 2% FBS and 5 ng/ml TGF-β1 (R&D systems) for indicated time.

ScRNA-Sequencing Data Analysis

The data analyzed in this study were from our previous ADSCs' scRNA database. Subpopulation analysis and differential gene analysis were processed by R package Seurat (version 4.0). scRNA data have been previously uploaded to GEO database reference as GSE172336 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE172336).

Treatment of PV-ADSCs With MGP/BMP2 and Lentivirus Infection

PV-ADSCs underwent starvation for 12 to 24 h and then treated with SMCs differentiation medium supplemented with different concentrations of MGP/BMP2 protein (Peprotech, Cranbury, NJ, USA). Medium was changed every second day. Lentivirus targeting mouse *Mgp* (*Mgp* shRNA) and scramble shRNA were produced by GeneChem (Shanghai, China). *Mgp* shRNA sequence: AGT AGC ATT ACT GAA GTA T, scramble shRNA sequence: TTC TCC GAA CGT GTC ACG T. The viruses mixed with Polybrene were used to infect PV-ADSCs for 48 to 72 h and then changed to complete medium.

Enzyme-Linked Immunosorbent Assay

PV-ADSCs were seeded in 12-well plate and then treated with 5 ng/ml TGF-β1. Cells cultured without TGF-β1 were served as control. After 3 and 5 days, cell supernatant was collected, respectively, and MGP concentration was measured by using mouse MGP ELISA kit (FineTest, Wuhan, China) as per manufacturer's instructions.

Immunofluorescent Staining

Paraffin sections were first processed to deparaffinage and antigen retrieval before staining. Cells on the coverslips were fixed with 4% paraformaldehyde. The slides were then blocked with 10% donkey serum in PBS for 1 h, followed by incubation with PLIN (1:200, Abcam), LY6A (1:200, Invitrogen), MGP (1:200, Proteintech, Rosemont, IL, USA), ACTA2 (1:200, Abcam), CNN1 (1:200, Abcam), and TAGLN (1:200, Abcam). After incubation, slide staining was performed with fluorochrome-conjugated secondary antibodies. DAPI was used for nuclei staining.

Quantitative Reverse Transcription–Polymerase Chain Reaction Analysis

Total RNA was extracted by Trizol (Invitrogen). RNA reverse transcription was performed with PrimeScript RT Master Mix kits (TaKaRa Biotechnology Co., Ltd., Dalian, China) and quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was achieved by TB Green Premix Ex Taq kits (TaKaRa Biotechnology Co., Ltd.) on Roche Lightcycle 480 machine. *Actb* was chose as internal control. All primer sequences are listed in Supplemental Table S1.

Western Blot

Protein lysate mixed with 1× SDS loading buffer was loaded on SDS-PAGE gel for electrophoresis and then transferred to the polyvinylidene fluoride (PVDF) membrane. After blocked with 5% to 10% milk in PBS-Tween or TBS-Tween, membranes were incubated with primary antibodies including MGP (1:1,000, Proteintech), ACTA2 (1:2,000, Abcam), CNN1 (1:2,000, Abcam), TAGLN (1:2,000, Abcam), BMP2 (1:1,000, Santa Cruz, Dallas, TX, USA), P-SMAD1/5/8 (1:1,000, Cell Signaling Technology, Boston, MA, USA), and SMAD1 (1:1,000, Cell Signaling Technology) at 4°C overnight. The membranes were washed three times and subsequently incubated with secondary antibodies. Following incubation with enhanced chemiluminescence (ECL) detection solutions, the membranes were placed in the ImageLab machine for exposure.

Femoral Artery Injury and Perivascular Transplantation of PV-ADSCs

Detailed procedures were described in previous studies[7,20]. Briefly, 8- to 12-weeks-old mice were anesthetized, and femoral artery injury was achieved by passing a guide wire back and forth in femoral artery to denudate endothelium. After vascular injury, PV-ADSCs were resuspended in Matrigel and delivered to the adventitial side of femoral artery. Four weeks after injury, femoral arteries were collected for further experiments, for example, hematoxylin–eosin staining. Image J software was used for quantification.

Statistical Analysis

All statistical analyses in this study were performed by using GraphPad Prism 8.0. Unpaired *t* test was used to compare the statistical differences between two experimental groups. One-way and two-way analysis of variance (ANOVA) were used to compare the statistical differences among more than two experimental groups. Data were represented as mean ± SD. $P < 0.05$ was considered statistically significant.

Results

Identification of PV-ADSCs

PV-ADSCs were isolated and cultured as described previously. Flow cytometry showed that PV-ADSCs highly expressed MSC markers such as LY6A, ITGB1, THY1,

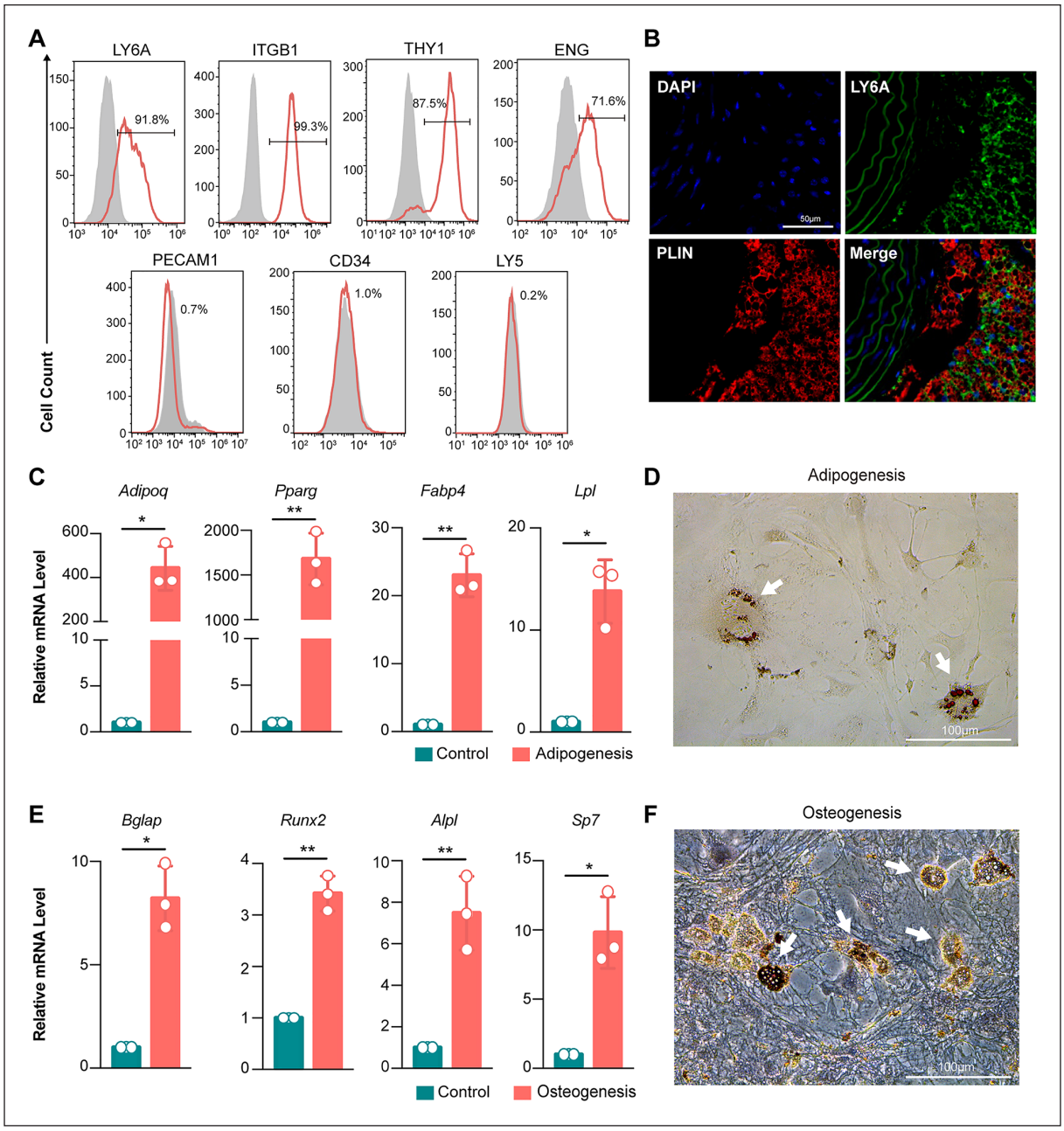

Figure 1. Identification of PV-ADSCs. (A) Flow cytometry of cultured PV-ADSCs. (B) Immunofluorescent staining of PV-ADSCs in the perivascular adipose tissue. Scale bar, 50 μm. (C) Quantitative polymerase chain reaction of adipocyte markers in response to adipogenic differentiation medium for 21 days ($n = 3$). (D) Oil Red O staining of differentiated PV-ADSCs toward adipocytes. Scale bar, 100 μm. (E) Quantitative polymerase chain reaction of osteocyte markers in response to osteogenic differentiation medium for 21 days ($n = 3$). (F) Alizarin Red staining of differentiated PV-ADSCs toward osteocytes. Scale bar, 100 μm. PV-ADSC: perivascular adipose-derived stem cells. *$P < 0.05$; **$P < 0.01$.

and ENG, while they hardly expressed hematopoietic marker LY5 and endothelial marker PECAM1 (Fig. 1A). Immunofluorescent staining displayed the existence of $LY6A^+$ cells not only in the adventitial regions but also in perivascular adipose tissue (Fig. 1B). When treated with adipogenic and osteogenic differentiation medium, significant changes in adipocyte markers (Fig. 1C) and osteocyte markers (Fig. 1E) were observed. Oil Red O (Fig. 1D) and Alizarin Red staining (Fig. 1F) further confirmed PV-ADSCs adipogenic and osteogenic differentiating capacities. The above results identified the existence and MSCs' properties of PV-ADSCs.

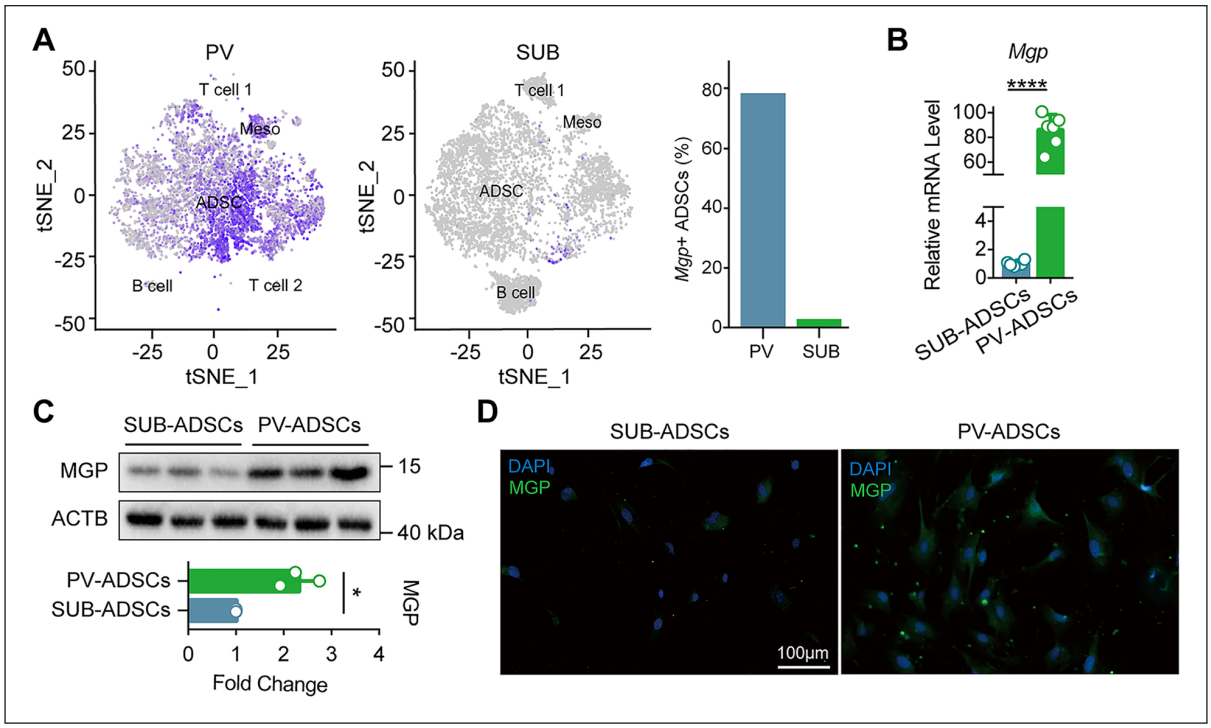

Figure 2. ScRNA dataset identified a unique expression of *Mgp* in PV-ADSCs. (A) T-SNE map displayed *Mgp* expression of SUB-ADSCs and PV-ADSCs. (B–D) MGP expression by quantitative polymerase chain reaction (B; $n = 6$), Western blot (C; $n = 3$) and immunofluorescent staining (D) in *in vitro* cultured SUB-ADSCs and PV-ADSCs. Scale bar, 100 μm. ScRNA: single-cell RNA-sequencing; PV-ADSC: perivascular adipose-derived stem cell; SUB-ADSC: subcutaneous adipose-derived stem cell; MGP: matrix Gla protein; T-SNE: t-distributed stochastic neighbor embedding. *$P < 0.05$; ****$P < 0.0001$.

ScRNA Identified a Unique Expression of Mgp *in PV-ADSCs*

Perivascular and subcutaneous adipose tissue altered neointimal hyperplasia reversely after vascular injury[21]. Such opposition might be partially attributed to the distinctive characteristics between SUB-ADSCs and PV-ADSCs. By analyzing scRNA dataset, *Mgp* was highly expressed in PV-ADSCs compared with SUB-ADSCs (Fig. 2A). *In vitro*, qRT-PCR (Fig. 2B), Western blot (Fig. 2C), and immunofluorescence (Fig. 2D) confirmed such projection from scRNA analysis.

MGP Expression Significantly Changed During PV-ADSCs Differentiation Toward SMCs in Vitro

Recent studies have confirmed differentiation of PV-ADSCs toward SMCs. To explore whether MGP participates in PV-ADSCs differentiation toward SMCs, we treated PV-ADSCs with 5 ng/ml TGF-β1 for inducing SMCs differentiation. SMC markers including ACTA2, TAGLN, and CNN1 markedly increased at both mRNA and protein level (Fig. 3A–D) in response to TGF-β1. On the contrary, MSC markers such as *Ly6a*, *Thy1*, and *Kit* decreased significantly (Fig. 3E). Altogether, PV-ADSCs could differentiate toward SMCs *in vitro*. Along SMCs differentiation at different time points, qRT-PCR demonstrated an elevated *Mgp* expression (Fig. 4A), but a decreased intracellular MGP expression as confirmed by Western blot (Fig. 4B) or immunofluorescence (Fig. 4C). MGP is a secreted protein and such results further drove us to detect supernatant MGP expression. Western blot confirmed that extracellular MGP significantly increased after TGF-β1 treatment at days 3 and 5 (Fig. 4D). Consistently, ELISA assays showed a higher concentration of MGP in the supernatant of TGF-β1 treated group at days 3 and 5 (Fig. 4E). The above results indicated that PV-ADSCs might secrete more MGP during the differentiation toward SMCs.

MGP Regulated PV-ADSCs Differentiation Toward SMCs

To clarify whether MGP regulated SMCs differentiation, MGP was inhibited in PV-ADSCs by using lentivirus encoding *Mgp* shRNA. The efficiency of MGP inhibition in PV-ADSCs was verified by both Western blot (Fig. 5A) and qRT-PCR (Fig. 5B). When treated with MGP inhibitory virus, the expression of SMC markers in PV-ADSCs greatly declined after TGF-β1 stimulation (Fig. 5C). Moreover, treatment of exogenous MGP significantly promoted PV-ADSCs differentiation toward SMCs (Fig. 5D). The above findings showed the involvement of MGP in regulating the differentiation of PV-ADSCs toward SMCs.

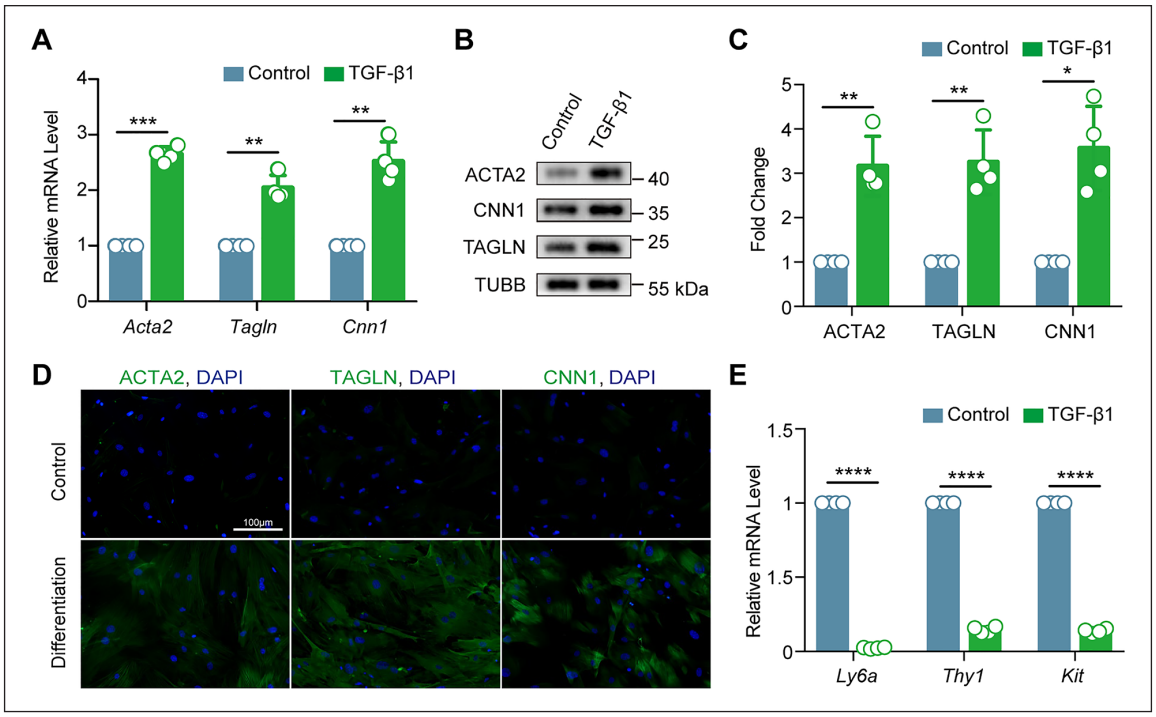

Figure 3. PV-ADSCs differentiated toward SMCs *in vitro*. (A–D) Expression of SMC markers by quantitative polymerase chain reaction (A; $n = 4$), Western blot (B and C; $n = 4$), and immunofluorescent staining (D) in PV-ADSCs treated with 5 ng/ml TGF-β1 for 5 days. Scale bar, 100 μm. (E) Quantitative polymerase chain reaction of MSC markers after 5 ng/ml TGF-β1 treatment for 5 days ($n = 4$). PV-ADSC: perivascular adipose-derived stem cell; SMC: smooth muscle cell; TGF-β1: transforming growth factor–beta 1; MSC: mesenchymal stem cell. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

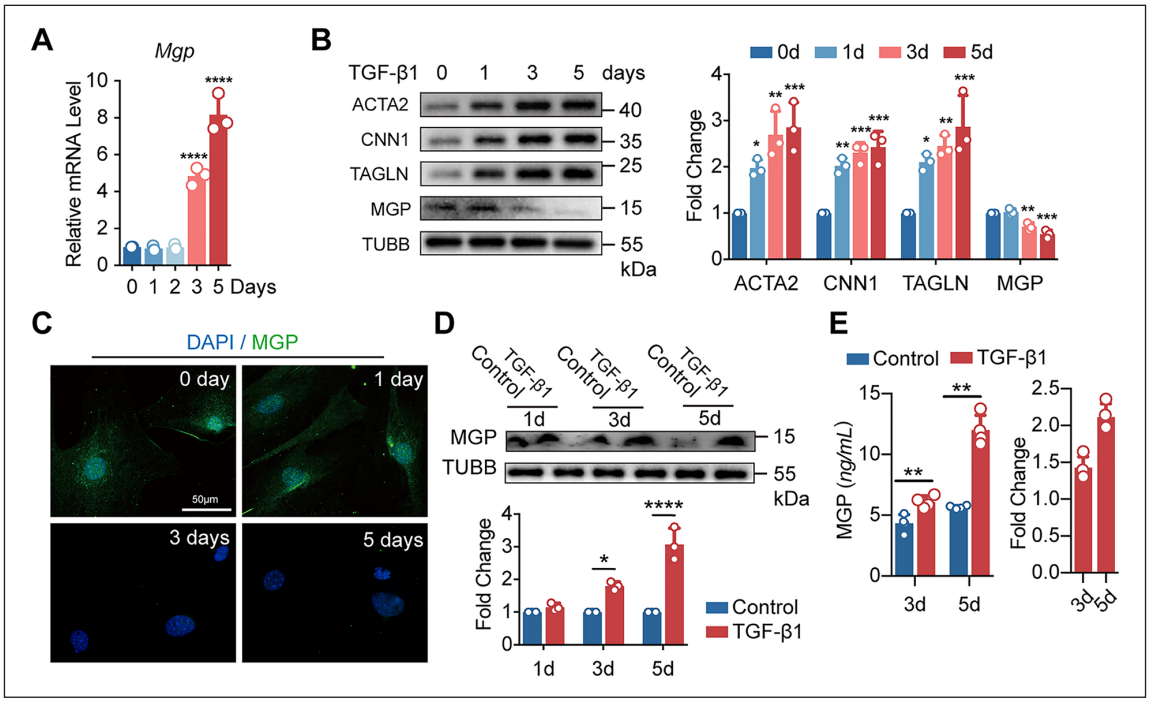

Figure 4. Increased secretion of MGP during PV-ADSCs differentiation toward SMCs. (A–C) Quantitative polymerase chain reaction (A; $n = 3$), Western blot (B; $n = 3$), and immunofluorescent staining (C) for MGP expression in PV-ADSCs treated with 5 ng/ml TGF-β1 for different time. Scale bar, 50 μm. (D–E) Western blot (D; $n = 3$) and ELISA (E; $n = 4$) for MGP expression in the supernatant of PV-ADSCs after TGF-β1 treatment for 3 and 5 days. MGP: matrix Gla protein; PV-ADSC: perivascular adipose-derived stem cell; SMC: smooth muscle cell; TGF-β1: transforming growth factor–beta 1. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

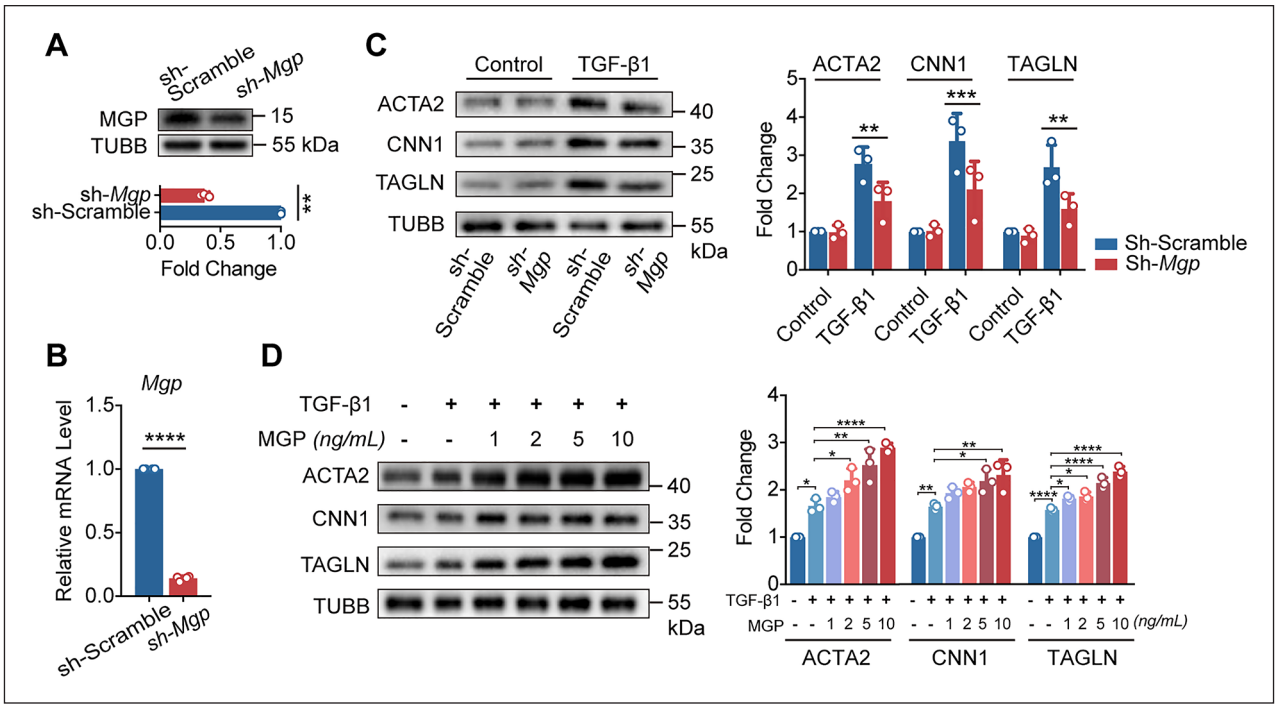

Figure 5. MGP regulated PV-ADSCs differentiation toward SMCs. (A and B) MGP expression in PV-ADSCs after shRNA *Mgp* infection by Western blot (A; $n = 3$) and quantitative polymerase chain reaction (B; $n = 4$). (C) Expression of SMC markers after *Mgp* knockdown in PV-ADSCs treated with or without TGF-β1 ($n = 3$). (D) Western blot of SMC markers in PV-ADSCs treated with TGF-β1 mixed with different concentration of MGP recombinant protein ($n = 3$). MGP: matrix Gla protein; PV-ADSC: perivascular adipose-derived stem cell; SMC: smooth muscle cell; TGF-β1: transforming growth factor–beta 1. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

MGP Promoted PV-ADSCs Differentiation Toward SMCs Via BMP2/SMAD Pathway

MGP/BMP2 signaling pathway regulated the process of vascular calcification and cartilage/bone formation. BMP2, as a member of the TGF-β superfamily, was reported to promote SMCs migration and transdifferentiation[13,22]. To uncover whether MGP/BMP2 signaling pathway was involved in SMCs differentiation, *Bmp2* mRNA and BMP2 protein after MGP knockdown were measured. MGP knockdown enhanced BMP2 expression, verified by qRT-PCR (Fig. 6A) and Western blot (Fig. 6B). Meanwhile, P-SMAD1/5/8 expression, which was canonical downstream of TGF-β superfamily, was also upregulated after MGP knockdown (Fig. 6B). On the contrary, BMP2 and P-SMAD1/5/8 expression significantly decreased after MGP treatment at a dose-dependent manner (Fig. 6C). To further confirm the above hypothesis, BMP2 pathway inhibitor noggin was applied. SMC markers including ACTA2, TAGLN, and CNN1 were significantly suppressed after MGP knockdown, such effect could be attenuated by the treatment of noggin (Fig. 6D). Furthermore, exogenous BMP2 inhibited SMCs differentiation of PV-ADSCs at a dose-dependent manner (Fig. 6E). To conclude, MGP regulated SMCs differentiation through BMP2/SMAD pathway.

Knockdown of MGP in PV-ADSCs Alleviated Neointima Formation

Recent research reported that PV-ADSCs could differentiate toward SMCs *in vivo* and promoted neointimal hyperplasia post vascular injury[8]. Given the role of MGP in SMCs differentiation, we transplanted sh-*Mgp* PV-ADSCs at the adventitial side of injured femoral arteries. Notably, transplantation of sh-*Mgp* PV-ADSCs showed less neointimal area and lower neointima/media ratio than that of sh-Scramble PV-ADSCs (Fig. 7A, B), implying that MGP could regulate PV-ADSCs behaviors *in vivo*. Immunofluorescent staining further confirmed the composition of neointima was mainly $ACTA2^{+}$ SMCs not lymphocytes (Supplemental Fig. S1).

Discussion

Our study elucidated the role and mechanism of MGP in differentiation of PV-ADSCs toward SMCs. This article suggests that MGP and PV-ADSCs may become potential therapeutic targets in vascular remodeling.

ADSCs have been widely studied over last decade. The characteristics of ADSCs could be distinctive upon their origins, including SUB-ADSCs, visceral ADSCs, cardiac ADSCs, perivascular ADSCs, and so on. Among them, the

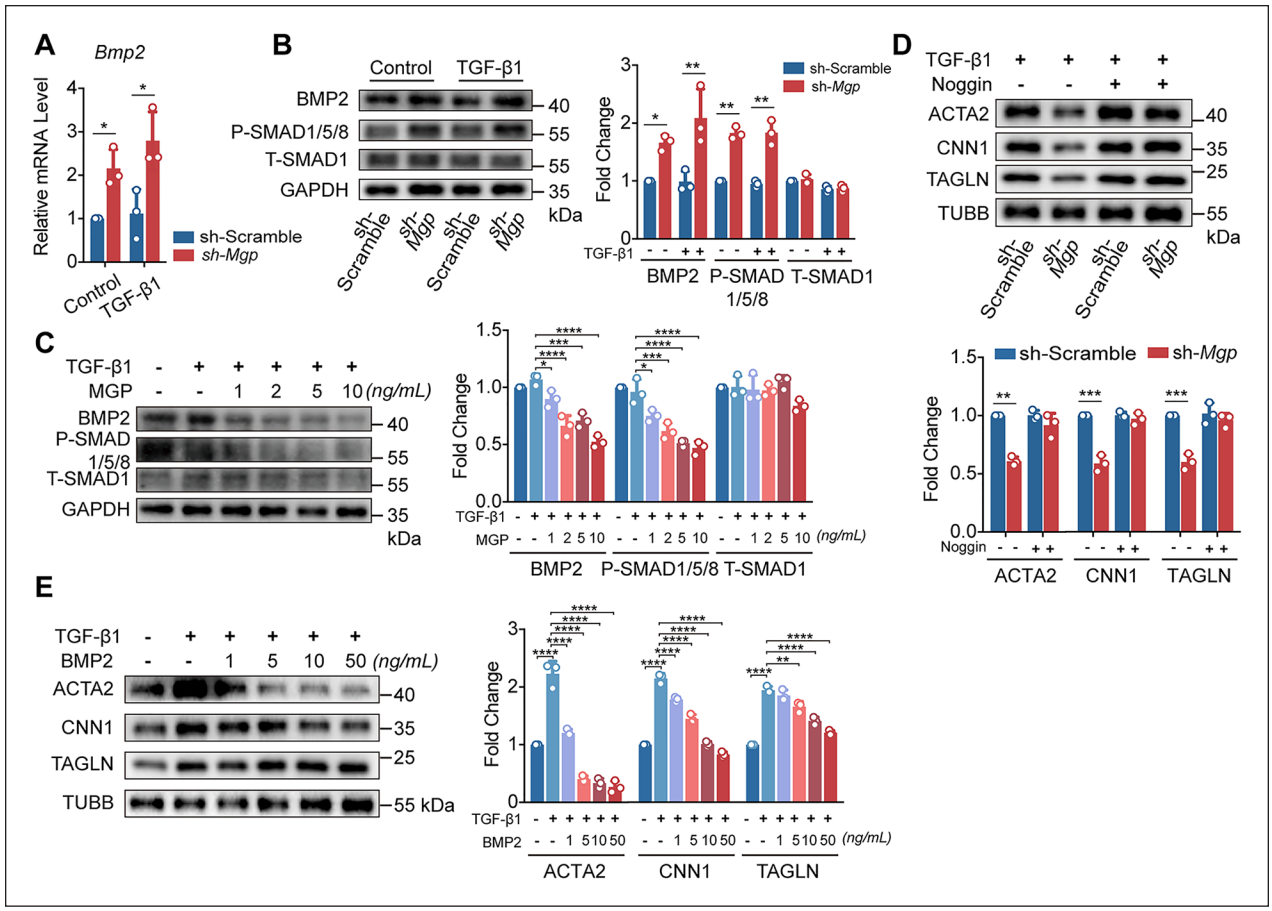

Figure 6. MGP promoted PV-ADSCs differentiation toward SMCs via BMP2/SMAD pathway. (A) Quantitative polymerase chain reaction of *Bmp2* in PV-ADSCs after *Mgp* knockdown ($n = 3$). (B and C) Western blot of BMP2/SMAD pathway protein after *Mgp* knockdown (B; $n = 3$) or exogenous MGP recombinant protein addition (C; $n = 3$). (D) Western blot of SMC markers in PV-ADSCs treated with Noggin and/or *Mgp* shRNA with TGF-β1 ($n = 3$). (E) Western blot of SMC markers in PV-ADSCs treated by TGF-β1 with BMP2 recombinant protein ($n = 3$). MGP: matrix Gla protein; PV-ADSC: perivascular adipose-derived stem cell; SMC: smooth muscle cell; BMP2: bone morphogenetic protein 2; TGF-β1: transforming growth factor–beta 1. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

former two subtypes of ADSCs were paid heavy attention due to their easy access while little effort was carried out on PV-ADSCs. Recently, PV-ADSCs were identified in perivascular adipose tissue by the two independent investigators[7,8]. Analogous to their findings, our PV-ADSCs expressed similar markers and multiple-lineage differentiation ability. To better illustrate the unique characteristics of PV-ADSCs, we reanalyzed scRNA dataset for the comparison between SUB-ADSCs and PV-ADSCs[23]. Our results confirmed the unique expression of *Mgp* in PV-ADSCs. MGP was involved in osteogenic differentiation and vascular calcification in the study of vascular SMCs[13]. Recent researches revealed the roles of MGP in the differentiation of stem cells toward ECs and adipocytes[14,24]. Given its effects on both SMCs and stem cells, as well as its high expression in PV-ADSCs, we hence further investigated its roles in PV-ADSCs. During the differentiation of PV-ADSCs toward SMCs, MGP expression significantly changed. Further knockdown and exogenous overexpression experiments indicated that MGP regulated PV-ADSCs differentiation toward SMCs.

With respect to the underlying mechanism, accumulating evidence has proved the link between MGP and BMP2 in vascular SMCs. It was reported that MGP promoted the maturation and differentiation of vascular SMCs via inhibiting BMP2 pathway[13]. Our study proposed the significance of BMP2/SMAD in regulating PV-ADSCs differentiation. As MGP also occupied various biological effects in SMCs, we did not know whether MGP affected PV-ADSC characteristics through other mechanisms. Neointimal hyperplasia was a common pathological process in vascular remodeling. There have been a large number of studies on macrophages, ECs, SMCs, adventitial stem cells, and neointima formation[1], all of which are believed to have a contribution to neointima. A recent study verified that PV-ADSCs could differentiate toward SMCs *in vivo* and promoted neointima formation under vascular injury[8]. Our study demonstrated the contribution of MGP in PV-ADSCs to neointimal hyperplasia *in vivo*, which provided a supplement to the society.

The present study has few limitations. First, MGP is not uniquely expressed by PV-ADSCs. Another major source of

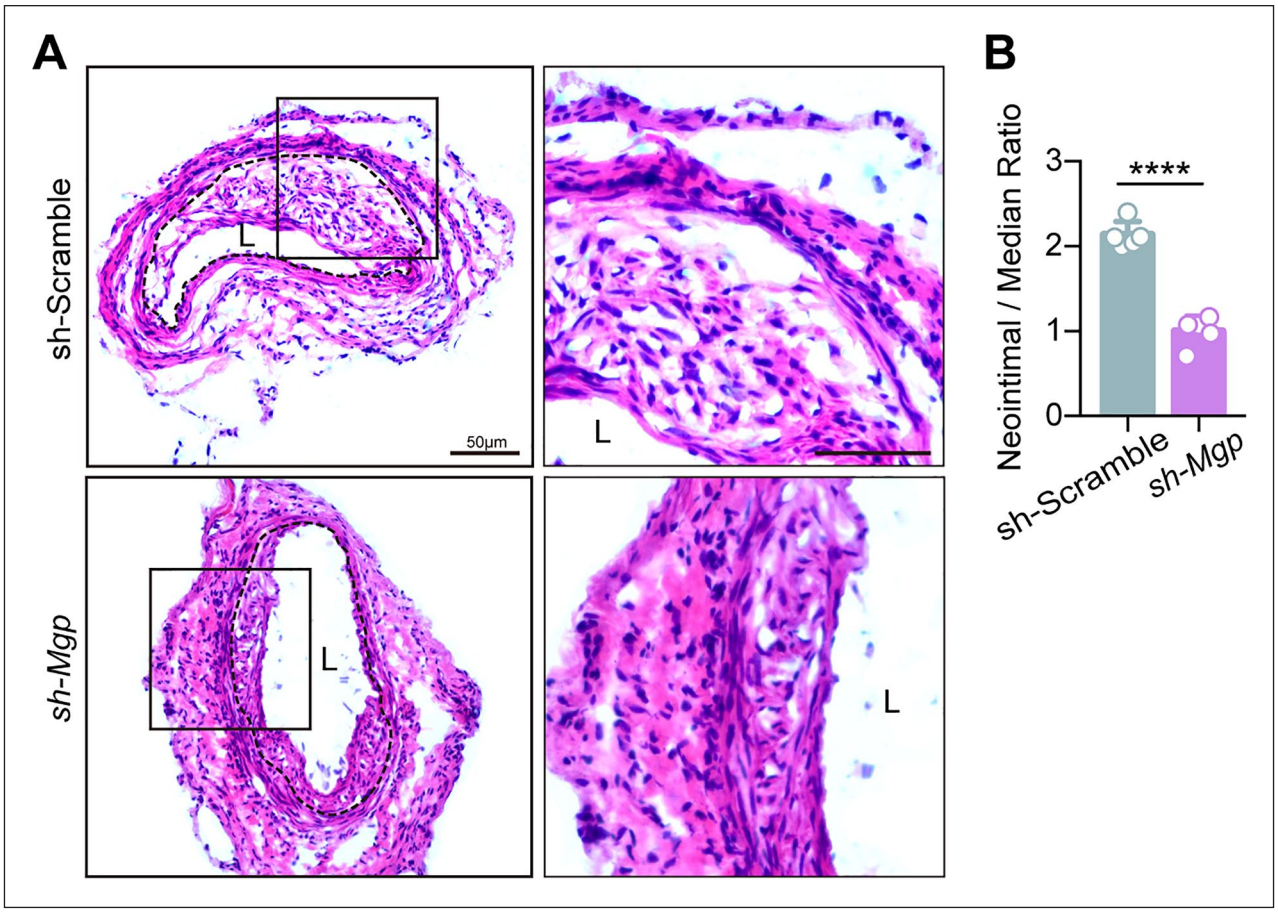

Figure 7. Knockdown of MGP in PV-ADSCs Alleviated Neointima Formation. (A) Hematoxylin–eosin staining of the injured femoral artery harvested 4 weeks after PV-ADSCs transplantation. Scale bar, 50 μm. (B) Quantification of neointima/media ratio ($n = 5$). MGP: matrix Gla protein; PV-ADSCs: perivascular adipose-derived stem cells. ****$P < 0.0001$.

MGP is SMCs. Here, we did not investigate the role of SMCs-derived MGP. We also did not know how much PV-ADSCs did contribute to neointimal formation. In addition, as we claimed that PV-ADSCs could secrete MGP, the effect of MGP on other stakeholders such as adventitial stem cells, macrophages, ECs, and so on was not evaluated. Third, PV-ADSCs lack unique markers, thus difficult to track *in vivo*. PDGFRA/PDGFRB may be potential markers for PV-ADSCs mentioned in the latest two publications[25,26], but it is still under debate. Therefore, there is no definite conclusion whether PV-ADSCs themselves would truly participate in neointimal formation *in vivo*. At last, the advantage of scRNA-sequencing is not well taken. Cellular subpopulation regarding MGP expression is not addressed. Deeper analysis could be applied if further studies with respect to MGP effects on other PV-ADSCs' biological function are designed.

Author Contributions

Hui Ni contributed to all *in vitro* experiments, data analysis, and manuscript writing. Chang Liu and Yuwen Chen contributed to figure layout and manuscript revision. Yunrui Lu contributed to scRNA dataset analysis. Yongli Ji contributed to manuscript revision. Meixiang Xiang and Yao Xie contributed to scientific hypothesis, study design, *in vivo* experiments, critical manuscript revision, and language polishing.

Ethical Approval

This study was approved by the Animal Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine (Hangzhou, China).

Statement of Human and Animal Rights

All procedures involving animals were approved by the Animal Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine (Hangzhou, China).

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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