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Glycoprotein M6B Interacts with TβRI to Activate TGF-β-Smad2/3 Signaling and Promote Smooth Muscle Cell Differentiation

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Received March 2, 2018; accepted for publication October 8, 2018; first published online in STEM CELLS *EXPRESS* October 29, 2018.

http://dx.doi.org/10.1002/stem.2938

Key Words. Glycoprotein M6B • Stem cells • Smooth muscle cells • Cell differentiation • TGF-β pathway

ABSTRACT

Smooth muscle cells (SMCs), which form the walls of blood vessels, play an important role in vascular development and the pathogenic process of vascular remodeling. However, the molecular mechanisms governing SMC differentiation remain poorly understood. Glycoprotein M6B (GPM6B) is a four-transmembrane protein that belongs to the proteolipid protein family and is widely expressed in neurons, oligodendrocytes, and astrocytes. Previous studies have revealed that GPM6B plays a role in neuronal differentiation, myelination, and osteoblast differentiation. In the present study, we found that the GPM6B gene and protein expression levels were significantly upregulated during transforming growth factor-β1 (TGF-β1)-induced SMC differentiation. The knockdown of GPM6B resulted in the downregulation of SMC-specific marker expression and repressed the activation of Smad2/3 signaling. Moreover, GPM6B regulates SMC Differentiation by Controlling TGF-β-Smad2/3 Signaling. Furthermore, we demonstrated that similar to p-Smad2/3, GPM6B was profoundly expressed and coexpressed with SMC differentiation markers in embryonic SMCs. Moreover, GPM6B can regulate the tightness between TβRI, TβRII, or Smad2/3 by directly binding to TβRI to activate Smad2/3 signaling during SMC differentiation, and activation of TGF-β-Smad2/3 signaling also facilitate the expression of GPM6B. Taken together, these findings demonstrate that GPM6B plays a crucial role in SMC differentiation and regulates SMC differentiation through the activation of TGF-β-Smad2/3 signaling via direct interactions with TβRI. This finding indicates that GPM6B is a potential target for deriving SMCs from stem cells in cardiovascular regenerative medicine. STEM CELLS *2019;37:190–201*

SIGNIFICANCE STATEMENT

Understanding the molecular mechanisms of smooth muscle cell (SMC) differentiation is important to uncover the regulators of circulatory system development and angiogenesis in physiological and pathological processes. This study demonstrates that the glycoprotein M6B plays a crucial role in regulating SMC differentiation through the activation of TGF-β-Smad2/3 signaling via direct interactions with TβRI. The findings furthered the knowledge of the physiological function of glycoprotein M6B and uncovered a new regulator of SMC differentiation. Furthermore, GPM6B could be considered a potential target for influencing SMC differentiation and cardiovascular regenerative medicine.

INTRODUCTION

The differentiation of smooth muscle cells (SMCs) is an important component of vascular development. Through highly regulated contractile mechanisms, SMCs provide structural support to the vasculature and control blood pressure and blood flow. Alterations in the normal structure or function of differentiated VSMCs in adult animals contribute to a variety of cardiovascular pathologies, including hypertension, stroke, and atherosclerosis [1]. Therefore, characterizing the regulation of vascular SMC differentiation is essential for both the prevention and treatment of these diseases. Although recent studies have improved the current understanding of SMC differentiation and cardiovascular system development [2–12], the molecular mechanisms governing SMC differentiation from pluripotent stem cells remain largely unknown.

SMC differentiation is associated with the high expression of several SMC-specific

contractile and contractile-associated proteins, including smooth muscle α-actin (α-SMA), smooth muscle myosin heavy chain (MYH11), SM22, and calponin. Multiple environmental cues, such as growth factors, inflammatory mediators, and mechanical forces regulate the SMC phenotype [1, 13]. Among the factors, transforming growth factor-β1 (TGF-β1), a member of the TGF-β superfamily, induces SMC-specific gene expression in multiple pluripotent stem cells, including C3H10T1/2 (10T1/2) cells [14], neural crest Monc-1 cells [15], amniotic stem cells [16], and embryonic stem cells [17]. TGF-β1 is a multifunctional cytokine that regulates a large variety of cellular processes, including differentiation and proliferation [18]. TGF-β1 signals through TGF-β type I and type II receptors (TβRI and TβRII, respectively) to phosphorylate Smad2 and Smad3. Once activated by TGF-β receptors, phosphorylated Smad2 and Smad3 form a complex with Smad4 and translocate to the nucleus, where these proteins function as transcription factors alone or in association with other DNA binding factors to modulate target gene expression in a cell type-dependent manner [19, 20]. Many studies have revealed that the activation of Smad2 and Smad3 pathways plays a critical role in TGF-β1-induced SMC differentiation [21–23].

Many studies have shown that numerous regulators also mediate TGF-β signaling by various aspects during SMC differentiation. Sphingosylphosphorylcholine can induce human adipose-tissue-derived mesenchymal stem cells into smooth-muscle-like cell types by autocrine secretion of TGF-β [24]. miRNA-128 [25] and miRNA-18b [26] inhibit expression of SMC cellular marker proteins by translational inhibition of SMAD2 protein. Estrogen receptor-α (ERalpha) also reduces the SMC-specific marker expression by inhibition of Smad2 phosphorylation [27]. DeltaEF1, lncRNA growth arrest-specific 5 (GAS5), and brain cytoplasmic RNA 1 (BC1) can specifically bind to Smad3 protein and reduce Smad3 translocation to the nucleus [28] or bind to TGF-β-responsive SMC gene promoters [29]. In addition, miRNA-503 induces SMC differentiation through TGF-β signaling by inhibition of Smad7 transcription [30]. Although extensive studies have focused on TGF-β1-induced SMC differentiation, the molecular mechanisms controlling the TGF-β signaling during SMC differentiation are still not fully known.

Glycoprotein M6B (GPM6B) is a four-transmembrane protein that belongs to the proteolipid protein family, which mediates intercellular contact and regulates membrane growth, composition, and targeting [31–35]. GPM6B shows high protein sequence similarity with PLP1 and GPM6A and is widely expressed in neurons, oligodendrocytes, and a subset of activated astrocytes [36, 37]. Through two promoters and alternative exons, the GPM6B gene encodes at least eight GPM6B proteins and polypeptides with variable N- and C-terminal domains [38]. GPM6B plays a role in neuronal differentiation and myelination [33, 39]. Outside the nervous system, GPM6B regulates osteoblast differentiation by controlling the cytoskeleton and matrix vesicle release [40]. In addition, gene ontology analysis showed that GPM6B is highly related to smooth muscle contraction and transcription of smooth muscle contractile fibers. Meanwhile, including SMC-specific gene ACTA-2 and MYH-11, 67 gene probes were silenced in the GPM6B-KO osteoblastic hMSC [41], which implies that GPM6B is a regulator of SMC differentiation. However, to date, there have been no reports on the functional involvement of GPM6B in SMC differentiation and embryonic smooth muscle development. In the present study, we provide the first evidence that GPM6B plays an essential role in the TGF-β1-induced differentiation of 10T1/2 cells into SMCs. Furthermore, we found that GPM6B regulates SMC differentiation through the activation of TGF-β-Smad2/3 signaling by directly interacting with TβRI. These findings highlight the importance of GPM6B for SMC differentiation and the propagation of TGF-β signaling.

Materials and Methods

Cell Culture

C3H10T1/2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), penicillin (100 U/ml), and streptomycin (100 μg/ml), at 37°C and 5% CO_2. TGF-β1 was obtained from Novoprotein (Summit, New Jersey, USA). The culture medium was replaced with an identical medium containing only 1% fetal bovine serum (FBS). The C3H10T1/2 cells were incubated for 24 hours prior to incubation with 2 ng/ml TGF-β1 or TGF-β1 (2 ng/ml) + alantolactone (10 μmol/ml, MCE, NJ)/SB431542 (36 ng/ml, MCE, NJ).

Immunofluorescence Staining

Cultured C3H10T1/2 cells were fixed in 4% paraformaldehyde for 30 minutes. Subsequently, the cell membrane was permeabilized with 0.1% Triton X-100 for 30 minutes, followed by incubation with anti-Smad2/3 primary antibody (Abcam, Cambridge, U.K.; 1:100 dilution) overnight. After washing, the cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Abcam; 1:200 dilution) for 1 hour. The cells were then stained with 4′,6-diamidino-2-phenylindole for 5 minutes, mounted on glass slides with glycerol, and observed under a fluorescence microscope.

RT-PCR and qPCR Analysis

Total RNA was extracted from the differentiating cells at different stages by using TRIzol (Invitrogen, Carlsbad, CA). The cDNA was reverse-transcribed using Superscript II Reverse Transcriptase (TaKaRa). Real-time quantitative-polymerase chain reaction (PCR) was performed using TransStart SYBR Green qPCR Supermix (TaKaRa) to determine the expression levels of GPM6B (AGAGCGTGTCAGCCAATGAA; GATCCACCCCAGCAGTTTGA), SMA (GTACCACCATGTACCCAGGC; GTACCACCATGTACCCAGGC), Myh-11 (CGACACAGCCTACAGAAGCA; TCTTTCTTGCCCTTGTGGGA), and calponin (GCAGTGGACACACGCATTTT; AACAACTGGCCCCAA GACTC). β-Tubulin was used as an internal normalization reference for mRNA expression. The RT-PCR was performed with the following primers: gpm6b-1a (CAGACCTGCAAACTTGTGCC; AGCCTGCATTTTGCTTGCAT) and gpm6b-1b (TGAAGCCAGCCATGG AAACT; CACGCTCTGGCAAACACTTT). The RT-PCR conditions were predenaturation at 95°C (1 minute), denaturation at 95°C (30 seconds), annealing at 56°C (15 seconds), extension at 72°C (5 minutes), and a final extension at 72°C (3 minutes).

Western Blot Analysis

The cells were washed two times with phosphate-buffered saline (PBS), followed by protein extraction using RIPA buffer with cOmplete ULTRA Tablets and PhosSTOP (Roche). The protein concentration was measured using a BCA protein assay reagent. Equal amounts of proteins were resolved by SDS-PAGE

and transferred to nitrocellulose membranes. The membrane was blocked in 5% nonfat milk at room temperature for 1 hour prior to incubation with primary antibody. Detection was then performed by the enhanced chemiluminescent (ECL) system after addition of secondary antibodies diluted 1:5,000. The results were then quantified by using Quantity One software from Bio-Rad Laboratories (Hercules, CA).

Plasmid and Lentivirus Transfection

Approximately 4–6 × 10^5 293T cells were plated in 2 ml of growth medium without antibiotics 1 day prior to transfection to achieve 90%–95% confluence at the time of transfection. Then, 10 μl of Lipofectamine 2000 CD, 3 μg of psPAX2 plasmid, 1 μg of pMD2.G plasmid and 4 μg of pLKO.1 plasmid for each fragment of gpm6b1-5 or scrambled pLKO.1 (Sigma–Aldrich, St. Louis, MO) plasmid were added to 500 μl of DMEM and incubated for 30 minutes. The 293T cell growth medium was exchanged for DMEM without serum after washing the cells two times with PBS. The transfection mixtures were added to the medium, and the cells were incubated at 37°C in a CO_2 incubator. The medium was replaced after 6 hours, and the cells were further incubated for 48 hours. Approximately 70% of the C3H10T1/2 cell growth medium was replaced with filtered 293T cell supernatant, and the cells were incubated 96 hours. Western blotting was performed to examine the GPM6B expression level.

Immunoprecipitation

Immunoprecipitation (IP) was performed at 4°C with the addition of 1% phenylmethanesulfonyl fluoride (PMSF) to all protein samples and buffers. The lysates were precleared with 80 μl of 50% Control Agarose Resin. The samples were incubated with antibody overnight and precipitated with 40 μl of Pierce Protein A/G agarose resin for 2 hours, followed by washing with IP lysis/wash buffer. After an additional wash, the supernatant was removed, and the immunoprecipitates were analyzed by SDS-PAGE.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) analysis was performed according to the manufacturer's instructions using SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology, Danvers, MA). Cultured cells were lysed after fixing in 4% paraformaldehyde. The chromatin was then incubated with rabbit anti-SRF or myocardin antibody and protein G magnetic beads after shearing. The released DNA was subjected to PCR analysis. In addition, the rabbit IgG and 2% input was used as the negative and positive controls.

Statistical Analysis

Data are expressed as the mean values ± SD. Differences between groups were assessed using two-tailed Student's *t* test, and $p < .05$ was considered significant.

Results

GPM6B Is Upregulated During the TGF-β1-Induced Differentiation of 10T1/2 Cells into SMCs

A previous study showed that GPM6B regulates osteoblast differentiation and the actin cytoskeleton [40]. Therefore, we hypothesized that GPM6B is critical for SMC differentiation. To confirm this hypothesis, C3H10T1/2 cells were treated with TGF-β1 (2 ng/ml) for 1–3 days to induce SMC differentiation as previously described [42]. As shown in Figure 1, both the protein and mRNA levels of SMC differentiation-specific markers, smooth muscle α-actin (α-SMA), smooth muscle myosin heavy chain (MYH 11), and h1-calponin were significantly induced in the present differentiation model (Fig. 1A, 1C, 1E). Consistent with SMC-specific gene induction, the protein (Fig. 1A) and mRNA (Fig. 1B) levels of GPM6B were transiently elevated during SMC differentiation. The levels of GPM6B expression peaked at day 3 (Fig. 1A, 1B). Through two promoters and alternative exons, the GPM6B gene produces at least nine different M6B mRNAs encoding 6 isoforms with transmembrane domains (Supporting Information Fig. S1). Because exons 1A and 1B mark two alternative transcription start sites, we designed primers corresponding to exon 1A or exon 1B in combination with a primer specific for exon 10 to detect the levels of the nine isoforms of mRNA during SMC differentiation by RT-PCR. As shown in Figure 1F, the detected mRNA levels of the isoforms were upregulated during TGF-β1-induced SMC differentiation. These results suggest that GPM6B may play a role in SMC differentiation.

GPM6B Is Essential for the TGF-β1-Induced Differentiation of 10T1/2 Cells into SMCs

To determine the functional role of GPM6B in SMC differentiation, we generated 10T1/2 cell lines stably expressing shRNAs targeting GPM6B or expressing a nonspecific control hairpin construct. The 10T1/2 cells were then infected with lentiviruses expressing five independent hairpin shRNA sequences for GPM6B or control sh-scramble sequence, and stable cell lines with constitutive shRNA expression were established by selection with puromycin. The extent of the knockdown by the targeted shRNAs for GPM6B was confirmed by Western blotting and qPCR analysis. As shown in Figure 2A, 2B, each of the shRNA constructs, particularly sh-GPM6B1 and sh-GPM6B5, effectively knocked down the mRNA and protein expression of GPM6B. We then treated control (sh-scramble) and GPM6B-knockdown (sh-GPM6B1 or sh-GPM6B5) stable cell lines with TGF-β1 for 1–3 days. These data revealed that both mRNA and protein levels of α-SMA, MYH11, and calponin were markedly decreased at all time points examined in the GPM6B shRNA cell lines (Fig. 2C, 2F, Supporting Information Fig. S2), indicating that GPM6B knockdown inhibits the expression of SMC differentiation-specific markers. Taken together, these findings suggest that GPM6B gene expression is essential for the TGF-β1-induced differentiation of the 10T1/2 cells into SMCs.

Knockdown of GPM6B Represses the Activation of Smad2/3 Signaling During SMC Differentiation

Previous studies using microarrays assays have demonstrated that GPM6B controls cytoskeleton organization and the expression of extracellular matrix (ECM) genes [40]. TGF-β-Smad2/3 signaling, which plays an important role in SMC differentiation, also participates in ECM protein production and cytoskeletal organization [43, 44]. Therefore, we tested whether GPM6B is involved in Smad2/3 activation. Control (sh-scramble) and GPM6B-knockdown (sh-GPM6B1) stable cell lines were starved for 24 hours in serum-free medium and subsequently treated with TGF-β1 for 15–60 minutes prior to collection for Western blotting to test for Smad phosphorylation. As shown in

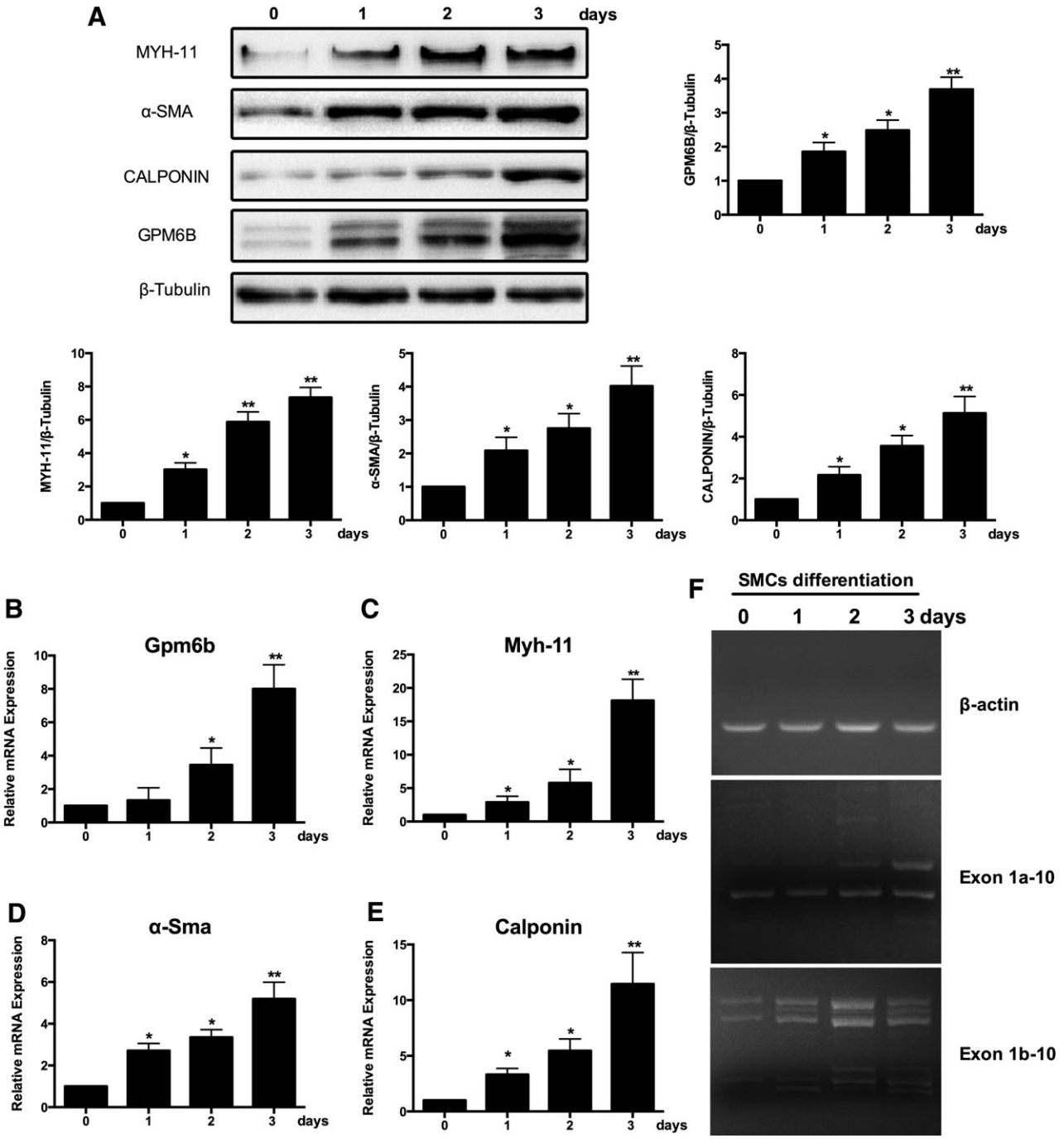

Figure 1. GPM6B expression is increased during transforming growth factor-β1 (TGF-β1)-induced smooth muscle cell (SMC) differentiation. The 10T1/2 cells were seeded onto 60-mm plates with 10% fetal bovine serum (FBS) medium. Samples were harvested at 1 day, 2 days, or 3 days after incubation with 2 ng/ml TGF-β1. Undifferentiated cells starved with 1% FBS medium for 24 hours were used as day 0 controls. Western blotting revealed the GPM6B as well as the SMC differentiation markers SM-MHC, α-SMA, and calponin **(A)** during differentiation. β-Tubulin was used as an internal control. Quantification of the Western blot images (A) and the Q-PCR analysis of the mRNA levels of the former genes **(B–E)** revealed that the expression of GPM6B as well as SM-MHC, α-SMA, and calponin was elevated at the transcriptional level. RT-PCR **(F)** using primers for actin, GPM6B-1a, and GPM6B-1b showed the various GPM6B mRNA splice forms were expressed during SMC differentiation. The data are presented as the mean ± SEM of three independent experiments. *, $p < .05$; **, $p < .01$ (vs. control).

Figure 3A, GPM6B knockdown significantly reduced the phosphorylation of Smad2/3 at 15 minutes, 30 minutes, or 1 hour after TGF-β1 induction. We also examined the expression of the TGF-β signaling markers, Pai-I and fibronectin, before or after incubation with TGF-β1 for 3 days by Q-PCR analysis. GPM6B knockdown significantly reduced the expression of Pai-I and fibronectin after treatment with TGF-β1 for 3 days (Fig. 3C, 3D). To further determine the role of GPM6B in Smad activation, we examined Smad2/3 responses to TGF-β1 by immunofluorescence staining in control or GPM6B-knockdown cells. As expected,

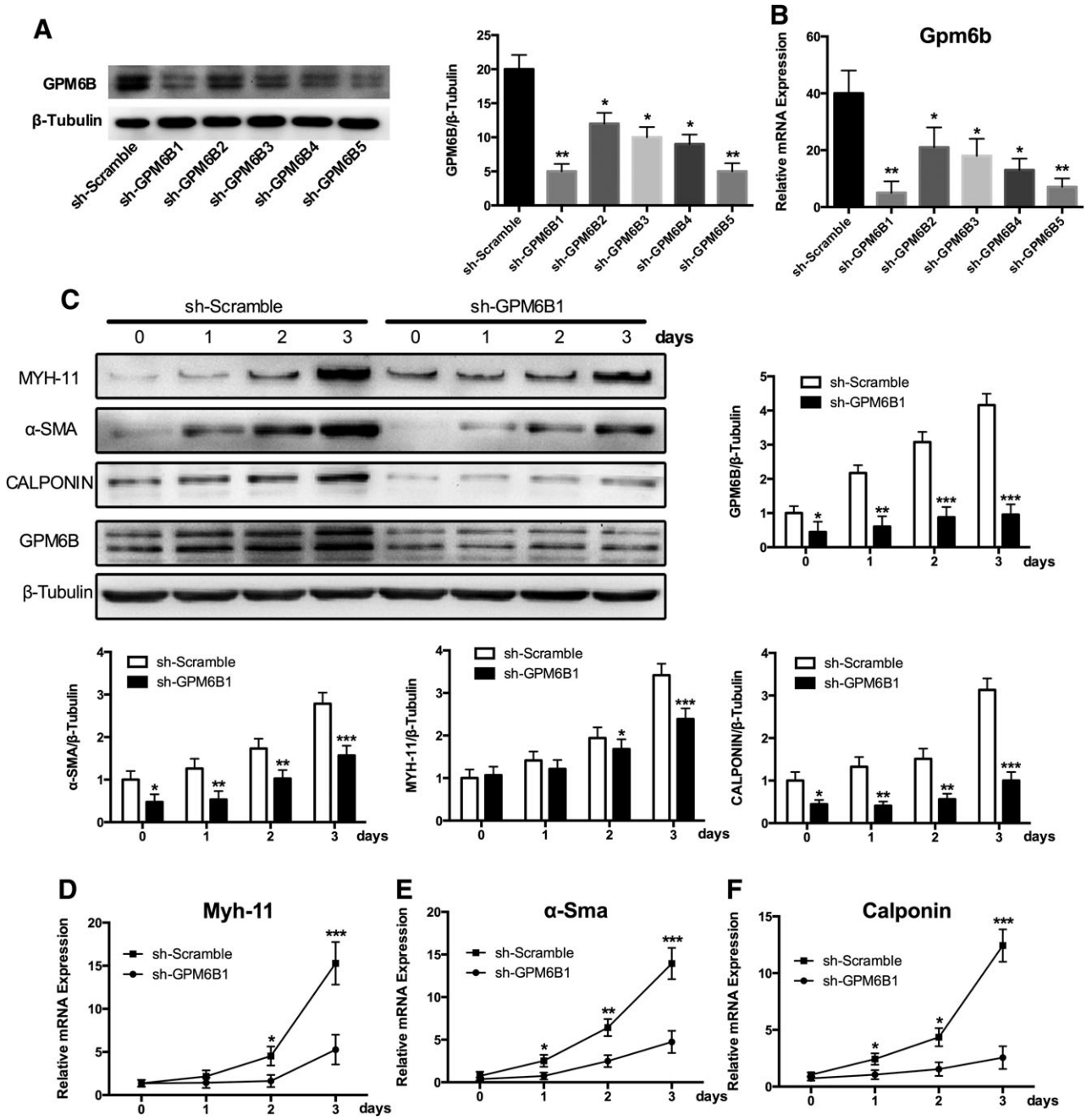

Figure 2. Knockdown of GPM6B blocks the smooth muscle cell (SMC)-specific marker expression. For the knockdown of GPM6B experiment, 10T1/2 cells were infected with lentiviruses expressing five independent hairpin shRNA sequences for GPM6B (sh-GPM6B1-5) or a control sh-scramble sequence. Western blot analysis examined the GPM6B expression level of the sh-scramble cells and sh-GPM6B1-5 cells **(A)** after incubation with 2 ng/ml transforming growth factor-β1 (TGF-β1) for 3 days. Quantification of Western blot images (A) and Q-PCR analyzed mRNA levels of GPM6B **(B)** show that the sh-GPM6B1 and sh-GPM6B5 were the most downregulated cell lines. Differentiated samples of the sh-scramble group and sh-GPM6B1 group were harvested at 1 day, 2 days, or 3 days after incubation with 2 ng/ml TGF-β1 and were subjected to Western blot **(C)** and Q-PCR **(D–F)** analysis for SMC specific markers and GPM6B. Undifferentiated cells starved with 1% fetal bovine serum medium for 24 hours were used as a day 0 control. Quantification of Western blot images (C; mean ± SE; $n = 3$) showed a significant downregulation of the SMC-specific markers as well as GPM6B at the protein level in the sh-GPM6B1 cells compared with the sh-scramble cells. The data are presented as the mean ± SEM of three independent experiments. *, $p < .05$; **, $p < .01$ (vs. control).

Smad2/3 was translocated into the nuclei within 30 minutes after TGF-β1 treatment in control cells (Fig. 3D). However, Smad2/3 nuclear translocation was significantly inhibited in most GPM6B-knockdown cells, suggesting a role for GPM6B in regulating Smad location or function (Fig. 3D).

GPM6B Regulates SMC Differentiation by Controlling TGF-β-Smad2/3 Signaling

To determine whether the attenuation of TGF-β-Smad2/3 signaling underlies the defect in SMC differentiation in the GPM6B-knockdown in 10T1/2 cells, we used the TGF-β signaling agonist

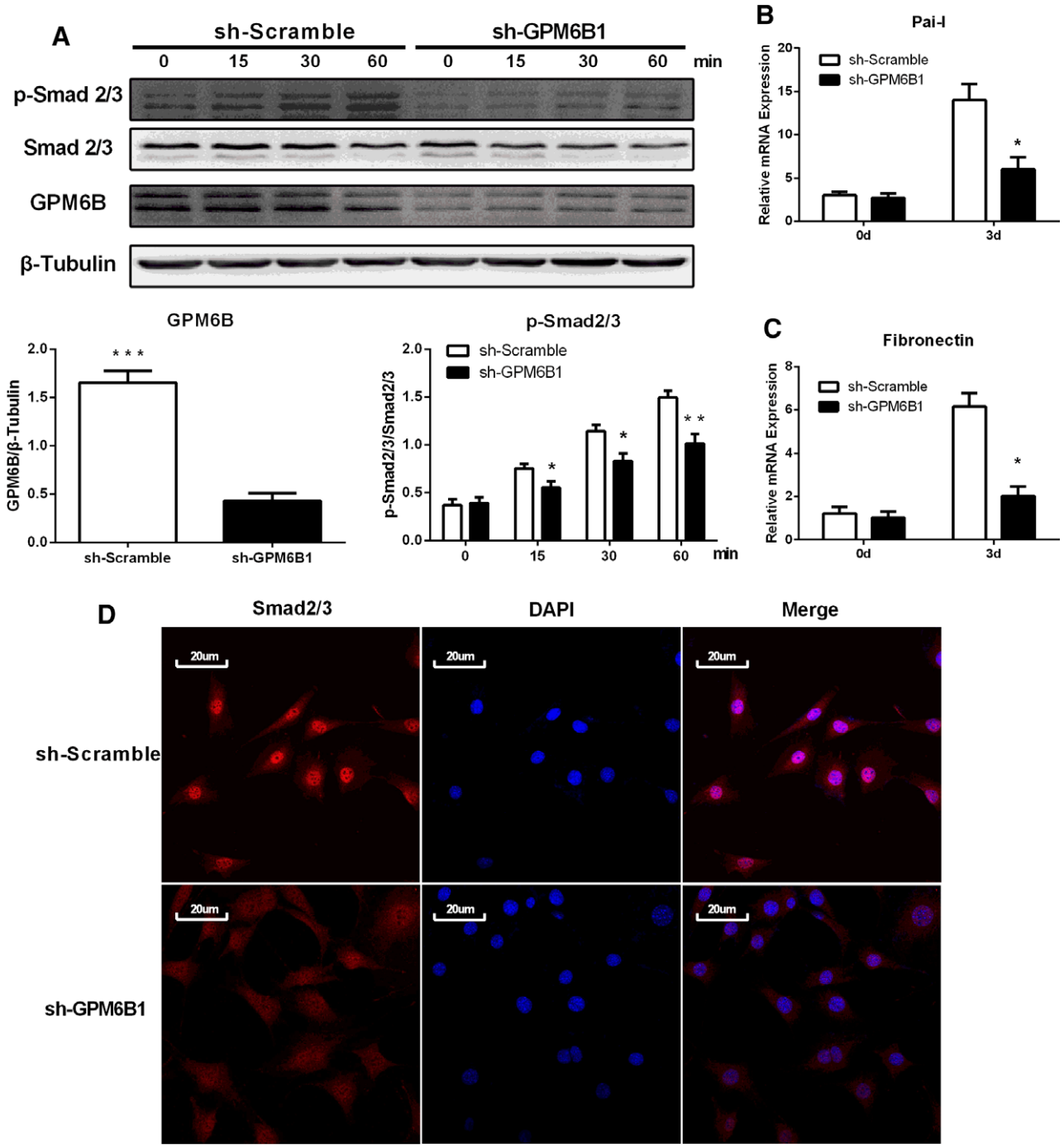

Figure 3. Knockdown of GPM6B represses the activation of Smad2/3 during TGF-β1-induced smooth muscle cells (SMCs) differentiation. The sh-scramble cells and sh-GPM6B cells were incubated with 5 ng/ml transforming growth factor-β1 (TGF-β1) after starving with 1% fetal bovine serum (FBS) medium for 24 hours, and the samples were harvested at 15, 30, and 60 minutes for Western blot analyses **(A)**. Untreated cells were starved with 1% FBS medium for 24 hours and used as day 0 controls. Quantification of the Western blot images (A; mean ± SE; $n = 3$; *, $p < .05$; **, $p < .01$) showed a significant repression of Smad2/3 phosphorylation. The sh-scramble cells and sh-GPM6B cells were incubated with 2 ng/ml TGF-β1 after starving with 1% FBS medium for 24 hours, and the samples were harvested at 3 days for Q-PCR analyses of the TGF-β signaling specific protein Pai-I **(B)** and fibronectin **(C)**. Immunofluorescence staining **(D)** revealed more Smad2/3 was translocated into the nuclei in the sh-scramble cells compared with the sh-GPM6B cells.

alantolactone and inhibitor SB431542. Control (sh-scramble), GPM6B-knockdown (sh-GPM6B1), and GPM6B-overexpression (Gpm6b-sgRNA) stable cell lines were starved for 24 hours in serum-free medium and treated with TGF-β1, alone or in combination with alantolactone/SB431542 for 30 minutes prior to collection for Western blotting. The immunoblots showed that the phosphorylation of Smad2/3 and the protein expression of α-SMA, SM-MHC, and calponin was inhibited in the GPM6B-knockdown cells and strengthened in the GPM6B-overexpression cells. Furthermore, stimulation with alantolactone rescued the

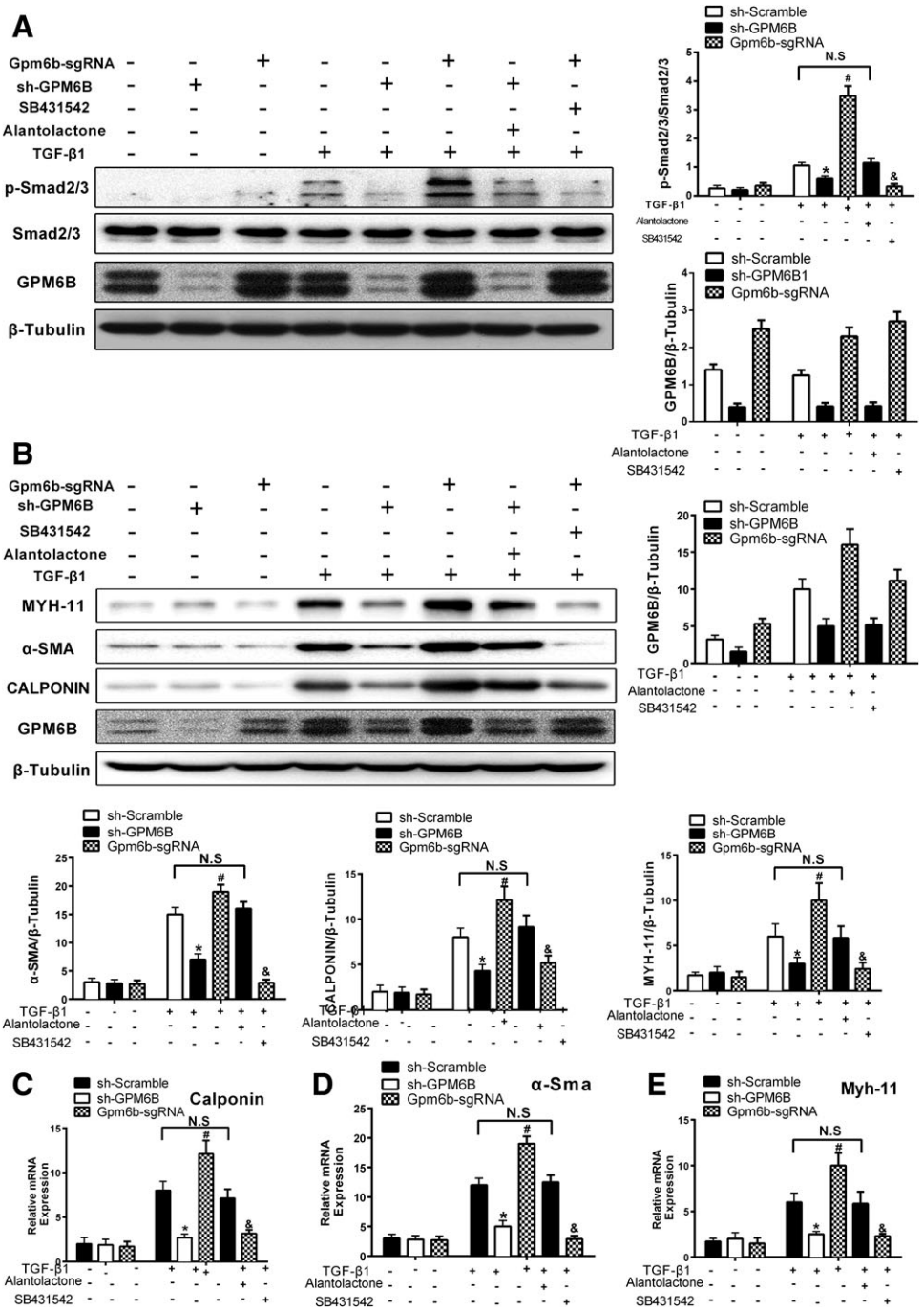

Figure 4. GPM6B regulates smooth muscle cell (SMC) differentiation by controlling transforming growth factor-β (TGF-β)-Smad2/3 signaling. The sh-scramble cells, sh-GPM6B cells, and GPM6B-sgRNA cells were treated with TGF-β1 (5 ng/ml) or TGF-β1 (5 ng/ml) + alantolactone (10 μmol/ml)/SB431542 (36 ng/ml) for 60 minutes after starving with 1% fetal bovine serum (FBS) medium for 24 hours. Untreated cells starved with 1% FBS medium for 24 hours were used as day 0 controls. Quantification of Western blot images **(A)** showed Smad2/3 phosphorylation was lower than baseline in the sh-GPM6B cells and higher than baseline in the GPM6B-sgRNA cells. The phosphorylation of Smad2/3 in the sh-GPM6B TGF-β1 group was elevated by alantolactone and the phosphorylation was reduced by treatment with SB431542. Western blot **(B)** and Q-PCR **(C–E)** analyses showed the repression of SMC specific markers in the sh-GPM6B TGF-β1 group were abolished by alantolactone in the sh-GPM6B and the enrichment of SMC specific markers in the GPM6B-sgRNA cells was canceled by treatment with SB431542. Untreated cells starved with 1% FBS medium for 24 hours were used as day 0 controls. The data are presented as the mean ± SEM of three independent experiments. *, $p < .05$; **, $p < .01$ (vs. control).

TGF-β1-induced phosphorylation of Smad2/3 in the GPM6B-knockdown cells and SB431542 inhibits the Smad2/3 phosphorylation in the GPM6B-overexpression cells (Fig. 4A). As expected from this restoration of Smad2/3 activation, alantolactone also ameliorated the abolishment of the TGF-β1-induced protein expression of α-SMA, SM-MHC, and calponin in the GPM6B-knockdown cells and SB431542 inhibited protein expression in the GPM6B-overexpression cells (Fig. 4B, 4E). In summary, these

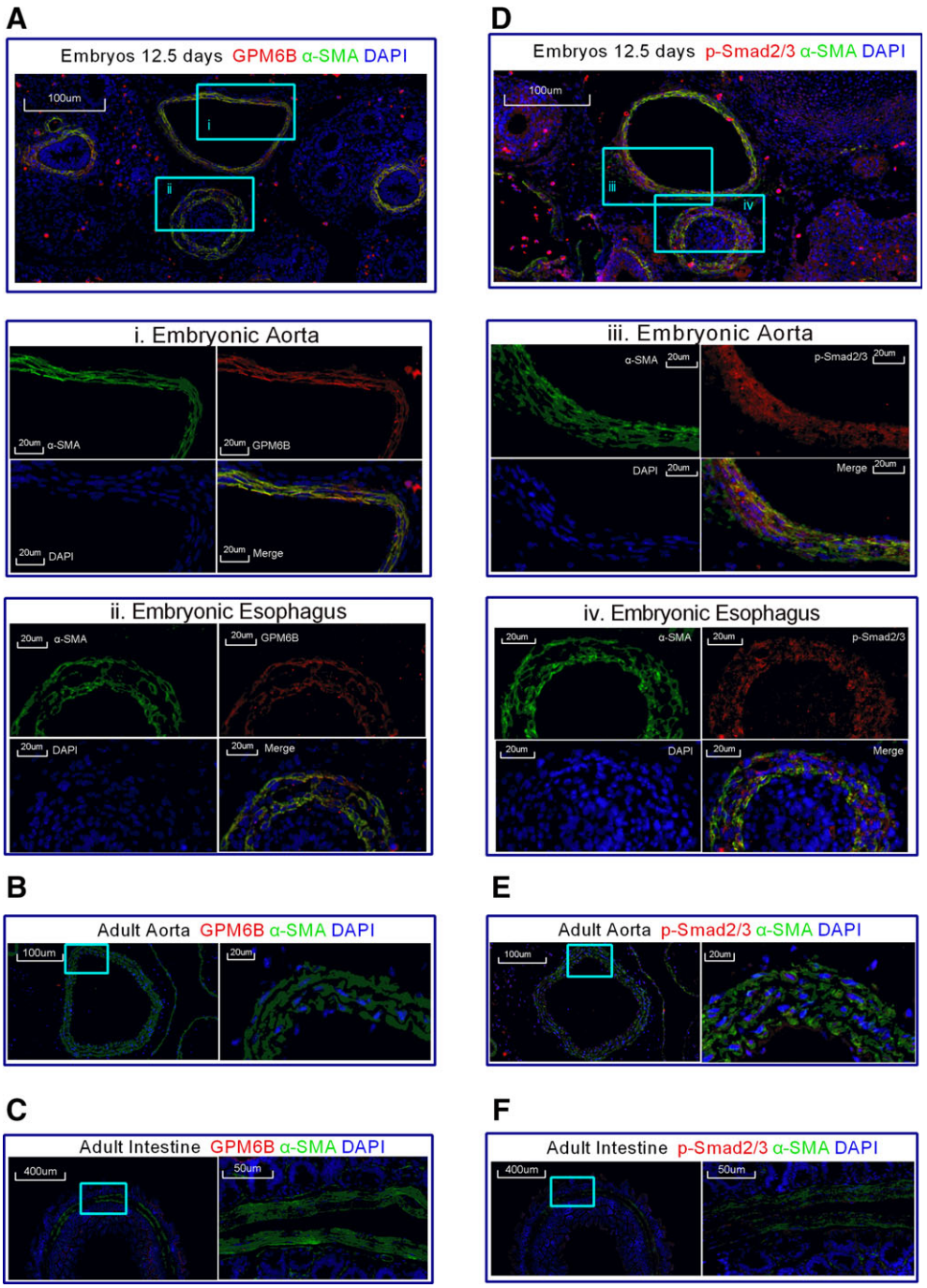

Figure 5. GPM6B and p-Smad2/3 are expressed in embryos and colocalize with smooth muscle cell (SMC) specific markers, but the colocalization was reduced in the adult SMCs. GPM6B is expressed with SMC-specific markers in embryonic (12.5 days) aorta and esophagus **(A)** but little was expressed in the adult (8 weeks) aorta **(B)** and small intestine **(C)**. Phosphorylation of Smad2/3 is elevated with the SMC specific marker in the embryonic (12.5 days) aorta and esophagus **(D)** but little in the adult (8 weeks) aorta **(E)** and small intestine **(F)**.

findings indicate that GPM6B regulates SMC differentiation through the activation of TGF-β-Smad2/3 signaling.

GPM6B Expression Pattern in Embryonic and Adult SMCs

To investigate the involvement of GPM6B during smooth muscle development, double immunofluorescence staining for GPM6B or p-Smad2/3 with α-SMA was performed on cross-sections of mouse embryonic (day 12.5) aorta, esophagus, adult aorta, and small intestine. Similar to the p-Smad2/3 expression pattern in embryonic mice (Fig. 5D), GPM6B was profoundly expressed in cells transformed/differentiated into α-SMA-positive cells (cells positive for both GPM6B and α-SMA) on day 12.5 in the mouse embryo aorta (Fig. 5Ai) and esophagus (Fig. 5Aii). Notably, GPM6B was localized to the cytomembrane as well as the cytoplasm in embryonic SMCs, consistent with its transmembrane protein character. In addition, similar to p-Smad2/3, GPM6B was not or weakly, if any,

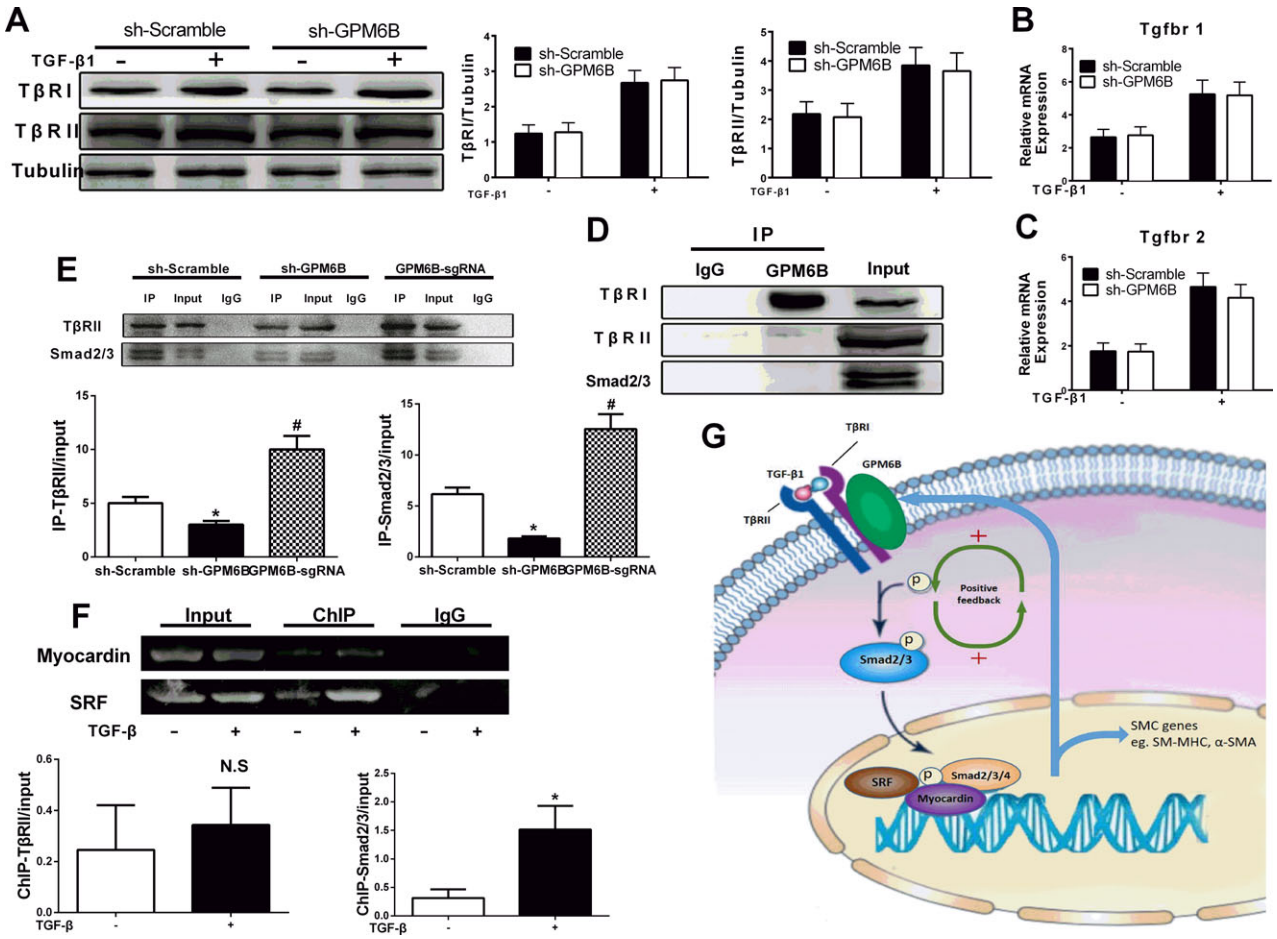

Figure 6. GPM6B activates transforming growth factor-β (TGF-β)-Smad2/3 signaling through interactions with TβRI. The sh-scramble cells and sh-GPM6B cells treated with TGF-β1 (2 ng/ml) were harvested at 3 days. Untreated cells, after starving with 1% fetal bovine serum (FBS) medium for 24 hours, were used as controls. Western blot analyses **(A)** and Q-PCR **(B, C)** analyses showed that the expression level of TβRI and TβRII was elevated after incubation with TGF-β1, but the knockdown of GPM6B did not downregulate the expression levels of TβRI and TβRII. Coimmunoprecipitation assay **(D)** showed that the GPM6B protein interacts with TβRI but does not interact with TβRII or Smad2/3. Coimmunoprecipitation assay **(E)** using TβRI antibody showed that the interaction between TβRI and TβRII or Smad2/3 was suppressed in the sh-GPM6B cells and enhanced in the GPM6B-sgRNA cells. ChIP assay **(F)** showed that the interaction between SRF and the GPM6B promotor was enhanced during smooth muscle cell (SMC) differentiation and myocardin was loosely bound to the GPM6B promotor. **(G)**: Proposed model of GPM6B-mediated SMC differentiation.

expressed in the aortic SMCs (Fig. 5B, 5E) and small intestine muscle tissues (Fig. 5C, 5F). Taken together, these results suggest that similar to p-Smad2/3, GPM6B was profoundly expressed and coexpressed with SMC differentiation markers in embryonic SMCs, indicating that GPM6B might play a role in smooth muscle development.

GPM6B Promotes the Interactions between TβRI and Smad2/3, and TGF-β-Smad2/3 Signaling Regulates the Expression As Well

As GPM6B activates TGF-β-Smad2/3 signaling, we examined whether GPM6B upregulates TβRI and TβRII expression. Control (sh-scramble) and GPM6B-knockdown (sh-GPM6B1) stable cell lines were starved for 24 hours in serum-free medium and treated with TGF-β1 for 3 days prior to collection for Western blotting and q-PCR analyses. As expected, both the mRNA and protein levels of TβRI and TβRII were elevated in the control and GPM6B-knockdown stable cell lines incubated with TGF-β1 for 3 days (Fig. 6A, 6C). However, there was no significant difference in TβRI and TβRII expression between the control and GPM6B-knockdown cell lines before or after incubation with TGF-β1 (Fig. 6A, 6C), indicating that GPM6B does not activate TGF-β signaling through the upregulation of TβRI and TβRII expression. Because GPM6B belongs to the PLP family, which interacts with other transmembrane proteins, including cell adhesion molecules, growth factors, and integrins [45, 46], we considered that GPM6B might interact with TβRI or TβRII to activate Smad2/3 signaling. To confirm this hypothesis, we performed coimmunoprecipitation with the GPM6B antibody in 10T1/2 cells incubated with TGF-β1 for 3 days. As shown in Figure 6D, GPM6B directly binds to TβRI, whereas there was no association with TβRII or Smad2/3. Meanwhile, we performed coimmunoprecipitation with the TβRI antibody in the control 10T1/2 cells, sh-GPM6B, or LV-Gpm6b-sgRNA cells induced with TGF-β1 for 30 minutes. The direct interaction between TβRI and Smad2/3 as well as TβRI and TβRII was elevated in the LV-Gpm6b-sgRNA cells and inhibited in the sh-GPM6B cells compared with the control 10T1/2 cells (Fig. 6E).

The former results revealed that GPM6B expression was significantly increased after incubation with TGF-β1 for 3 days, which implies that the expression of GPM6B may be initiated by TGF-β signaling. To test this hypothesis, we conducted a ChIP experiment in TGF-β1 treated 10T1/2 cells to explore the relationship between the GPM6B promotor and TGF-β signaling-specific translation factors (SRF and myocardin) in SMC differentiation. The ChIP results demonstrate that SRF can directly bind with the GPM6B promotor after treatment with TGF-β1 for 3 days, whereas there was no direct interaction with myocardin (Fig. 6F).

Collectively, these results demonstrate that GPM6B directly interacts with TβRI to facilitate the activation of Smad2/3 signaling and thus promotes SMC differentiation (Fig. 6D). Knockdown or overexpression of GPM6B can regulate TGF-β-Smad2/3 signaling and SMC differentiation by regulating the tightness between TβRI and TβRII or Smad2/3. While the TGF-β-Smad2/3 signaling was regulated by GPM6B, the expression of GPM6B also regulated TGF-β-Smad2/3 signaling via the direct interaction of SRF and the GPM6B promotor. Therefore, TGF-β-Smad2/3 signaling and GPM6B constitute a positive loop, by which the TGF-β signaling was amplified during SMC differentiation.

Discussion

SMC differentiation is a complicated process involving various signaling pathways and molecules, such as the Myocd-SRF complex, ECM-integrin signaling, retinoid signaling, Nox4-ROS signaling, TGF-β signaling, Notch signaling, PDGF signaling, microRNAs, and HDACs [47]. Furthermore, over the last decade, enormous efforts have been made in this field, but the current understanding of the molecular mechanisms involved in SMC differentiation is still far from complete. In the present study, we successfully identified GPM6B as an important SMC differentiation regulator. GPM6B was upregulated during SMC differentiation, and its knockdown inhibits SMC differentiation. Moreover, GPM6B regulates SMC differentiation through the activation of TGF-β-Smad2/3 signaling. Furthermore, similar to p-Smad2/3, GPM6B was highly expressed and coexpressed with SMC differentiation markers in embryonic SMCs, indicating that GPM6B might play a role in smooth muscle development. Importantly, GPM6B can regulate the tightness between TβRI and TβRII or Smad2/3 by directly binding to TβRI to activate Smad2/3 signaling during SMC differentiation. Taken together, the present findings provide the first evidence that GPM6B promotes SMC differentiation through the activation of TGF-β-Smad2/3 signaling by directly interacting with TβRI to increase the binding between TβRI and TβRII or Smad2/3.

GPM6B, a member of the proteolipid protein family, is predominantly expressed in embryonic, neonatal and adult central nervous system (CNS) regions [38]. Although the precise molecular function of GPM6B remains elusive, this protein has been implicated in CNS myelination and neuronal differentiation [33, 39]. In addition to abundant expression in the brain, GPM6B mRNA expression has also been detected in many tissues, including lung, liver, muscle, spleen, and heart of adult mice [38]. However, aside from its role in osteoblast differentiation [40], there are no studies on the function of GPM6B outside the nervous system so far. In the present study, we provided the first evidence that GPM6B plays an essential role in SMC differentiation. In addition, GPM6B was profoundly expressed and coexpressed with SMC differentiation markers in embryonic SMCs, indicating that GPM6B might play a role in smooth muscle development. Previous studies have demonstrated that GPM6B is a good vascular marker in breast carcinoma and ovarian carcinoma and have shown that the expression of this protein correlates with the intensity of neovascularization during tumor growth [48–50], suggesting its role in tumor angiopoiesis. As SMC differentiation is a critical step in the formation of the vascular system, the essential role of GPM6B in SMC differentiation may implicate this protein as a tumor vascular marker.

GPM6B is a four-transmembrane domain protein that belongs to the proteolipid protein family. Other members of this protein family include PLP/DM20 and GPM6A. One of the distinct functional features of proteolipids and tetraspanins is their ability to interact with multiple proteins and form lateral associations with each other as well as with other transmembrane proteins [45, 46]. GPM6A interacts with the μ-opioid receptor and facilitates receptor endocytosis and recycling [51]. GPM6B interacts with the serotonin transporter (SERT) to regulate serotonin uptake [32]. Here, we report that GPM6B is a novel binding partner of TβRI. The physical interaction between GPM6B and TβRI was verified by coimmunoprecipitation experiments. Recent studies have shown that TβRI interacts with a number of different proteins. Most of these proteins enhance TβRI degradation [52–57]. In the present study, we found that GPM6B knockdown repressed the activation of Smad2/3 signaling. Moreover, the knockdown of GPM6B did not change either the protein or the mRNA levels of TβRI. These results indicated that GPM6B interacts with TβRI to phosphorylate Smad2/3 but not to change its protein level. Furthermore, we explored the mechanism by which GPM6B promotes the interaction between TβRI, TβRII, and Smad2/3 to stimulate the activation of TGF-β signaling. In addition, activating TGF-β signaling can facilitate GPM6B expression, which forms a positive feedback loop to help SMCs differentiate efficiently.

TGF-β signaling plays a pivotal role during vascular development and SMC differentiation and proliferation [52–57]. TGF-β1, the most abundant isoform of TGF-β, stimulates SMC differentiation through the canonical Smad signaling pathway [14, 15, 17]. Upon TGF-β1 stimulation, TβRII is rapidly autophosphorylated, resulting in the recruitment of and heterodimerization with TβRI. After recruitment, TβRI directly phosphorylates and activates Smad2 and Smad3, which form heteromeric complexes with Smad4. The resulting Smad2/3/4 complex translocates to the nucleus and initiates the activation of SMC-specific marker genes. Previous studies have indicated that other signaling pathways, including Notch, PI3K, RhoA and some microRNAs, may participate in the regulation of SMC differentiation by interacting with TGF-β signaling molecules or downstream targets [47, 58]. In the present study, we found that GPM6B is a novel positive regulator of TGF-β signaling during SMC differentiation. GPM6B activated TGF-β signaling by directly interacting with TβRI to increase the binding between TβRI and TβRII or Smad2/3. Although no studies have reported that GPM6B regulates TGF-β signaling, a deficiency in another binding partner, SERT, causes the sudden death of

newborn mice through the activation of TGF-β1 signaling [59]. Whether SERT regulates TGF-β1 signaling through interactions with GPM6B will require additional studies.

Conclusion

In summary, the present study revealed that GPM6B plays a crucial role in SMC differentiation. We also demonstrated that GPM6B activated TGF-β signaling by directly interacting with TβRI to increase the binding between TβRI and TβRII or Smad2/3.These findings provide new insights into the biological function of GPM6B, SMC differentiation and the propagation of TGF-β signaling. Therefore, GPM6B could be considered a potential target for influencing SMC differentiation and cardiovascular regenerative medicine.

Acknowledgments

This work was financially supported by the Program for National Science Funds of China (Grant Nos. 81600683, 81730011, 81470478, and 81400201), Program for Changjiang Scholars and Innovative Research Teams in the University (Grant No. PCSIRT-14R08), the Fourth Military Medical University's Young Talent Project (the first level), National Science Fund for Distinguished Young Scholars of China (Grant No. 81225001), National Key Basic Research Program of China (973 Program, Grant No. 2013CB531204), Key Science and Technology Innovation Team in the Shaanxi Province (Grant No. 2014KCT-19), Major Science and Technology Project of China "Significant New Drug Development" (Grant No. 2012ZX09J12108-06B), Key Problems of Social Development Science and Technology of the Shaanxi Province (2018SF-129), and Shaanxi Key Laboratory of Ischemic Cardiovascular Disease (2016ZDKF09).

Author Contributions

X.Z., H.X., P.C.: collection and assembly of the data, data analysis and interpretation; H.Z., Y.X.: collection and assembly of the data, manuscript writing; L.Z.: provision of study materials; X.G., C.H., F.Y.: collection and assembly of the data, data analysis and interpretation, final approval of the manuscript; L.H., C.L., Y.Y., Z.X., X.W., G.L.: collection and assembly of the data, manuscript writing; S.W.: conception and design, administrative support, manuscript writing, final approval of the manuscript; L.T.: administrative and financial support, final approval of the manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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