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TGFβ1 induces myofibroblast transdifferentiation *via* increasing Smad-mediated RhoGDI-RhoGTPase signaling

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Abstract. This study serves to investigate the effects of the Smad pathway on TGFβ1-mediated RhoGDI expression and its binding to RhoGTPases in myofibroblast transdifferentiation. Myofibroblast transdifferentiation was induced by TGFβ1 *in vitro*. Cells were pretreated with different siRNAs or inhibitors. Myofibroblast transdifferentiation was detected by immunohistochemistry. Immunofluorescence was used to observe the nuclear translocation of Smad4, and PSR (Picrositius Red) staining was used to measure collagen concentration. TGFβ1 induced the phosphorylation of Smad2/3 and the nuclear translocation of Smad4 in human aortic adventitial fibroblasts (HAAFs). Furthermore, TGFβ1 increased the expression of RhoGDI and its binding to RhoGTPases. Nevertheless, inhibition of Smad2/3 phosphorylation decreased TGFβ1-induced RhoGDI1/2 expressions and RhoGDI2-RhoGTPases interactions. These data suggested that the inhibition of Smad phosphorylation attenuates myofibroblast transdifferentiation by inhibiting TGFβ1-induced RhoGDI1/2 expressions and RhoGDI-RhoGTPases signaling.

Key words: Smad — RhoGDI — RhoGTPase — Myofibroblast transdifferentiation — Transforming growth factor β1

Introduction

Myofibroblast transdifferentiation is an important feature of vascular remodeling. Recent studies have indicated that transforming growth factor β1 (TGFβ1) is a crucial profibrotic factor in vascular remodeling (Shang et al. 2017; Razdan et al. 2018). And TGFβ receptor participates in TGFβ1-induced myofibroblast transdifferentiation (Breton et al. 2018). Canonical TGFβ signaling concerns Smad2/3 phosphorylation, Co-Smad complexes formation, nuclear localization, and gene regulation (Zhang et al. 2017). Activated TGFβ receptor stimulates phosphorylation of Smad proteins. The Smad protein family is an intermediary molecule that transmits the signal generated by the binding of TGFβ and its receptor from the cytoplasm to the nucleus, thus playing an important role in signal transmission and regulating the transcription of downstream target genes (Luo 2017). Recent studies have demonstrated that Smad2/3-p38MAPK-ERK1/2 pathways participate in TGFβ1-induced myofibroblast transdifferentiation in human Tenon fibroblasts (Lin et al. 2018; Wang et al. 2019; Wen et al. 2019). Our previous study has shown that RhoGDI expression is involved in regulating TGFβ1-mediated myofibroblast transdifferentiation in human aortic adventitial fibroblasts (HAAFs) (Zhang et al. 2019). However, the molecular mechanism underlying TGFβ1-induced RhoGDI expression in HAAFs remains unclear.

The Rho-specific guanine nucleotide dissociation inhibitor (RhoGDI) is vital for the regulation of the Rho GTPase

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cycle (Boulter and Garcia-Mata 2010). Despite the wide diversity in the Rho GTPase family, there are only three genes encoding RhoGDI in mammals, including RhoGDI1, RhoGDI2, and RhoGDI3 (Dai et al. 2019). RhoGDI1 and RhoGDI2 interact with most Rho GTPases, such as Rac1, Cdc42, and RhoA, whereas RhoGDI3 only interacts with RhoB and RhoG (DerMardirossian and Bokoch 2005). It has been reported that that RhoGDIs are associated with invasion, migration, proliferation, and differentiation through the deregulation of RhoGTPase signaling (Harding and Theodorescu 2010; Xie et al. 2017). Meanwhile, RhoGDIs are differentially expressed in lots of human cancers and exert divergent roles in cancer malignancy (Cho et al. 2019). For instance, the expression of RhoGDI1 is upregulated in colorectal cancers and hepatocellular carcinoma (Zhao et al. 2008; Wang et al. 2014; Huang et al. 2017), while downregulated in lung and breast cancer (Forget et al. 2002; Luo and Bai 2014). RhoGDI2 expression is upregulated in ovarian and gastric cancers (Tapper et al. 2001; Cho et al. 2009, 2014), but downregulated in Hodgkin's lymphoma and bladder cancer (Gildea et al. 2002; Ma et al. 2007). Unfortunately, how RhoGDI expression is regulated is hardly known.

TGFβ/Smad signaling and RhoGDI have independently been implicated in vascular remodeling, but how they interact to regulate myofibroblast transdifferentiation is not adequately understood. We have recently confirmed that RhoGDI expression is involved in regulating myofibroblast phenotypic modulation and vascular remodeling as mediated by TGFβ1 and its receptor. In this research, we settled down to study the molecular mechanisms of how TGFβ1 regulates RhoGDI-RhoGTPases signaling in HAAFs.

Materials and Methods

Materials

Recombinant human TGFβ1 was obtained from Novoprotein (#CA59-10; Shanghai, China), and the inhibitor of Smad2/3 phosphorylation SB505124 was from MCE (HY-13521; Shanghai, China). Fetal bovine serum (FBS; #F2442) and 4′,6-diamidino-2-phenylindole (DAPI; #28718-90-3) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). A total RNA Purification Kit was purchased from GeneMark (#TR01-150; Taichung, Taiwan). RevertAid First Strand cDNA Synthesis Kit (#K1622) was from Thermo Scientific (Shanghai, China). BeyoFastTM SYBR Green qPCR Mix (2×) was purchased from Beyotime Biotechnology (D7260, Shanghai, China). siRNAs (Table 1) and primers (Table 2) were purchased from Biomics Biotechnologies (Nantong, China). A Sirius Red staining solution kit was sourced from Solarbio (G1470; Beijing, China), while a Sirius Red collagen detection kit was purchased from Chondrex Inc. (#9062; Washington, USA). A DyLight 488-SABC SP kit was purchased from BosterBio (SA1094; Wuhan, China). RIPA lysis buffer (CW2333S) and a BCA protein assay kit (CW0014S) were both purchased from CWbio (Beijing, China), while an SDS-PAGE Gel Quick Preparation kit was sourced from Beyotime Biotechnology (P0012AC; Nantong, China). A dual-color, pre-stained protein marker was obtained from Epizyme Biological Technology (WJ101; Shanghai, China) and an anti-RhoGDI1 (A1214) antibody was obtained from ABclonal Technology (Wuhan, China). The antibody against α-SMA (ab124964), Smad3 (phospho S423+S425) (ab52904-40), and RhoGDI2 (ab181252) were purchased from Abcam Co. (Cambridge, UK). The primary antibody against GAPDH (#5174), anti-smad2 (#5339), anti-smad3 antibody (#9523), and anti-smad4 (#9515) were obtained from Cell Signaling Technology (Beverly, MA, USA). ZCL278 (Cdc42 inhibitor; #1177865-17-6) was from ChemCatch Co., Ltd. (Shanghai, China), and NSC23766 (Rac1 inhibitor; #587841-73-4) was from ApexBio Technology (Houston, TX, USA). HRP-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) was purchased from Proteintech (SA00001-2; Chicago, IL, USA) and Fluorescein (FITC)-conjugated AffiniPure Donkey Anti-Rabbit IgG (H+L) was purchased from BBI Life Sciences (D110051; Hong Kong, China). A SABC immunohistochemistry staining kit was sourced from BosterBio (SA1028; Wuhan, China). All other chemicals used in this study were from China and of analytical level.

Table 1. Sequences of siRNAs that successfully suppressed target gene expression

siRNAs	Sequence
siRhoGDI1	S: 5'- CUUUCCGGGUUAACCGAGAdTdT-3'
	A: 5'- UCUCGGUUAACCCGGAAAGdTdT-3'
siRhoGDI2	S: 5'-CACAAGAGAACAAGAAUAAdTdT-3'
	A: 5'-UUAUUCUUGUUCUCUUGUGdTdT-3'

S, sense; A, antisense.

Table 2. Sequences of primers used in Real-time PCR

Gene	Sequence
RhoGDI1	F: 5'-ATCCAGGAGGCTGGGTATTG-3'
	R: 5'-GCACGGACGGAGGCAATAAAT-3'
RhoGDI2	F: 5'-TTTATGGTTGGCAGCTATG-3'
	R: 5'-GAGGTAGGTCTTGCTTGTC-3'
GAPDH	F: 5'-ACAACTCTCTCAAGATTGTCAGCAA -3'
	R: 5'-ACTTTGTGAAGCTCATTTCCTGG-3'

F, forward primer; R, reversed primer.

Cell cultures and treatment

HAAFs were obtained from ScienCell Research Laboratories (Catalog#6120; CA, USA). The cells were grown in FM-2 medium supplemented with 10% FBS in a humidified atmosphere at 37°C under 5% CO_2 with replacement of culture medium every two-to-three days. Cells were used for experimentation in passages 3–7. HAAFs were first pretreated with 0.2 nM SB50514 (an inhibitor of Smad2/3 phosphorylation) for 24 h, with 50 μM NSC23766 or 50 μM ZCL278 for 30 min, and then exposed to 10 ng/ml TGFβ1 for another 72 h.

siRNA transfection

Cells were grown to 50% confluency and transfected using Lipofectamine 2000 (#11668019; Invitrogen). The synthetic siRNAs (20 μM in DEPC water) and Lipofectamine 2000 reagent were diluted using Opti-MEM and incubated at room temperature for 20 min. Afterward, the mixture was added to the cells and incubated for 48 h. Successful interference with the target gene was confirmed by Western blotting analysis. The transfected cells were then treated with 10 ng/ml TGFβ1 for 72 h.

Real-time PCR

Use a Total RNA Purification Kit to isolate total RNA according to the reagent specification. Use a First Strand cDNA Synthesis Kit to reverse transcribe one microgram of total RNA. The resulting cDNA was then mixed with gene-specific primers and Maxima SYBR Green qPCR Master Mix. The amplification conditions used for PCR cycling were as follows: 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s (GAPDH); 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s (RhoGDI1); 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s (RhoGDI2). Quantitative PCR was performed using a Corbett RG-6000 real-time PCR system (Corbett Life Sciences, Mortlake, Australia) following the manufacturer's guidelines. The relative expressions were shown after normalization to GAPDH.

Western blotting

Cells were harvested and lysed in 30–40 μl RIPA buffer on the ice. The lysates were centrifuged at 1500 × *g* for 15 min followed by incubation for 40 min. To isolate cytoplasmic and nuclear proteins, a Nuclear and Cytoplasmic Extraction kit was used following the manufacture's instructions. Then the protein concentrations were quantified using Bradford assays. Equal amounts of protein lysate were separated using SDS-PAGE gels and transferred to nitrocellulose membranes. Subsequently, the membranes were blocked with 5% nonfat milk for 2 h at room temperature and incubated overnight with different primary antibodies at 4°C. After incubation with HRP-conjugated secondary antibodies for 60 min, protein bands were detected by the ECL detection system (Amersham Biosciences). Histone H2 A, tubulin, and GAPDH were used as internal standards.

Co-immunoprecipitation (Co-IP)

HAAFs were lysed with a lysis buffer (150 mM of NaCl, 50 mM of Tris (pH 7.4), 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS) containing 25 mM NEM and 1 mM PMSF. The lysates were incubated on ice for 20 min and then were centrifuged for 15 min at 1500 × *g*. Subsequently, the protein concentrations were determined by a BCA Protein Assay Kit. After incubation with specific antibodies overnight at 4°C and Protein A/G PLUS-agarose at room temperature for another 2 h, the mixture was washed with normal washing buffer and high-salt washing buffer. Pulled down proteins were boiled in SDS loading buffer at 95°C for 5 min and analyzed by Western blotting.

Immunofluorescence

HAAFs grown in multiwell plastic chamber slides were washed with PBS and then fixed with 4% para-formaldehyde for 20 min on ice. Afterward, the cells were permeabilized in 0.5% Triton X-100 for 10 min and incubated with primary antibodies overnight at 4°C. After a brief rinse, cells were stained with FITC-conjugated anti-rabbit IgG for 2 h and 0.5 μg/ml DAPI for 20 min in the dark. Cells were washed with PBS and viewed by a fluorescence microscope (Nikon, Japan).

Immunohistochemistry

Immunohistochemical staining against RhoGDI1/2 and α-SMA was performed using a streptavidin-biotin complex (SABC) immunohistochemistry staining kit following the manufacturer's guidelines. HAAFs grown on glass coverslips were washed with cold PBS and fixed in 4% para-formaldehyde for 60 min. The cells were then incubated in a mixture of 30% H_2O_2 and methanol for 15 min and blocked in serum blocking solution for 30 min. The samples were incubated with primary antibodies at 4°C overnight followed by biotinylated anti-rabbit IgG for 2 h and SABC for 1 h. After visualization with a diaminobenzidine (DAB) staining kit, cells were counter-stained with hematoxylin to stain target proteins brown. The images were captured by an Olympus digital camera (Olympus, Tokyo, Japan).

Picrosirius Red collagen detection

Total collagen (type I to V) content was detected by a Sirius Red collagen detection kit following the manufacture's in-

structions. In short, prepare standards and samples using a 1× acetic acid solution. A 100 μl volume of blanks, standards, and samples were transferred to 1.5 ml centrifuge tubes and mixed with 500 μl of Sirius Red solution for 20 min. All assays were conducted in duplicate. Upon removing supernatants, the tubes were centrifuged at 1042 × *g* for 3 min. Pellets were dissolved in 250 μl of extraction buffer, followed by three washes with 500 μl of washing solution. The solutions were added to 96-well plates. Then the absorbance was measured at 530 nm using an ELISA plate reader (Bio Tek Instruments, Vermont, USA).

Statistical analysis

All data were expressed as means ± SD. Statistical analysis was evaluated by one-way analyses of variance followed by Tukey *post hoc* tests in the Graphpad Prism software. A value of $p < 0.05$ was considered to be statistically significant.

Results

TGFβ1 induced RhoGDI expression via Smad signaling

RhoGDI has previously been reported to participate in myofibroblast transdifferentiation (Wei et al. 2019). Our earlier study has demonstrated that TGFβ1 stimulates RhoGDI expression through TGFβ1 receptor activation, thereby inducing myofibroblast transdifferentiation (Zhang et al. 2019). To clarify the specific molecular mechanisms by which TGFβ1 regulates RhoGDI expression, cells were treated with SB505124 (an inhibitor of Smad2/3 phosphorylation). Smad expression and phosphorylation were investigated by Western blotting, Real-time PCR, and immunofluorescence. We found that SB505124 exactly notably inhibited TGFβ1-induced Smad2/3 phosphorylation compared with the control cells, which is consonant with the results as originally reported (Fig. 1A and B). Apart from Smad2/3 phosphorylation, we continually investigated whether TGFβ1 affects the nuclear translocation of the Smad complex. Smad4 nuclear expression was examined by Western blotting and immunofluorescence. TGFβ1 increased the nuclear expression of Smad4 but reduced its cytoplasmic expression (Fig. 1C). Similarly, Smad4 immunoreactivity was found in the cytoplasm in control cells, while little was found in the nucleus. Strong Smad4 staining in the nuclear showed that the majority of intracellular Smad4 translocated from the cytoplasm to the nucleus in response to TGFβ1 (Fig. 1D). Therefore, TGFβ1 phosphorylates Smad2 and/or Smad3, which in turn bind to Smad4 to induce translocation into the nucleus in HAAFs.

Due to the regulation of TGFβ1 on Smad signaling, we surmised that Smad signaling participates in TGFβ1-induced RhoGDI expression. Consequently, the transcription levels and expressions of both RhoGDI1 and RhoGDI2 were detected. The outcome reveals that TGFβ1 exerted remarkable effects on RhoGDI1/2 transcription levels and expressions, but the effects were reversed by SB505124 pretreatment (Fig. 1E–G), which confirmed our hypothesis. Accordingly, TGFβ1 activated downstream RhoGDI signaling by stimulating Smad2/3 phosphorylation and Smad4 nuclear expression.

Smad signaling participates in TGFβ1-induced myofibroblast transdifferentiation

Our former research has demonstrated that RhoGDI expression is involved in the altering of the fibroblast phenotype by stimulating transdifferentiation into myofibroblast as mediated by TGFβ1. Nevertheless, the role of Smad signaling in myofibroblast phenotypic modulation is still unknown. Myofibroblasts are the major source of extracellular matrix (ECM), which are identified by the expression of α-SMA (Hu et al. 2019). In this research, we further assessed α-SMA expression and collagen secretion in HAAFs using Western blotting, immunohistochemistry, and collagen detection assays. As illustrated in Figures 2A and B, TGFβ1 dramatically increased the expression of α-SMA, while SB505124 effectively inhibited TGFβ1-induced α-SMA expression. Likewise, Figure 2C shows that the increased collagen secretion induced by TGFβ1 was inhibited by SB505124. So Smad signaling is associated with RhoGDI-mediated myofibroblast phenotypic modulation.

TGFβ1 promotes RhoGDI-Cdc42 and RhoGDI-Rac1 interactions

When Rho proteins are bound to GTP, they interact with downstream effector proteins, thus facilitating a series of cellular processes (Jaffe and Hall 2005). The classical RhoGTPases contain Rho, Rac, Cdc42, and so on (Heasman and Ridley 2008). To clarify the effects of TGFβ1 on the interaction between RhoGDI and Cdc42 or Rac1 in HAAFs, we tested the interaction of RhoGDI1/2 with Cdc42 or Rac1 by Co-IP analysis after TGFβ1 treatment. RhoGDI1 and RhoGDI2 expressions were suppressed by siRNA. As illustrated in Figure 3, there was an increase in the interaction of RhoGDI1/2 with both Cdc42 and Rac1in response to TGFβ1 treatment; however, the effect was significantly reduced by RhoGDI1/2 suppression.

Smad signaling is involved in TGFβ1-mediated RhoGDI2-RhoGTPases interactions

To demonstrate whether Smad signaling participated in the regulation of RhoGDI-RhoGTPases interactions mediated by TGFβ1, we next measured the interaction of RhoGDI1/2 with Cdc42 or Rac1 by Co-IP analysis after SB505124

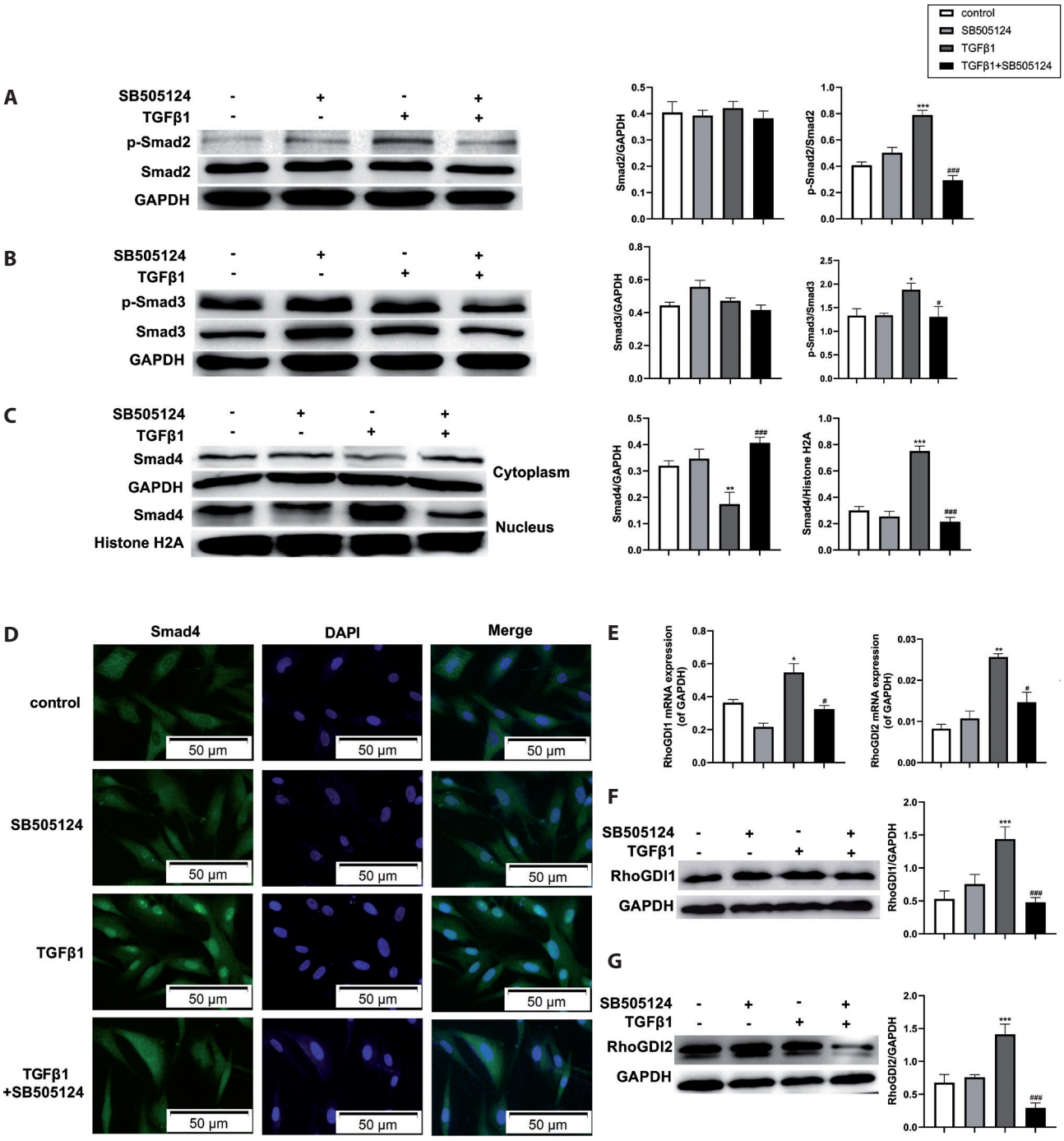

Figure 1. The effects of Smad signaling on RhoGDI. **A, B**. Western blot of the expression and phosphorylation of Smad proteins. Cells were pretreated with 0.2 nM SB5050124 for 24 h and then exposed to 10 ng/ml TGFβ1 for an additional 72 h. Untreated cells were used as the control group. Histograms show the ratios of phospho-Smad2 to Smad2 and phospho-Smad3 to Smad3. * $p < 0.05$, *** $p < 0.001$ *vs.* control group; $^{\#}$ $p < 0.05$, $^{\#\#\#}$ $p < 0.001$ *vs.* TGFβ1-treated group ($n = 3$). **C.** Western blot of Smad4 expression in the cytoplasm and nucleus. Histograms show the ratios of Smad4 to GAPDH or Histone H2A. Untreated cells were used as controls. ** $p < 0.01$, *** $p < 0.001$ *vs.* control group; $^{\#\#\#}$ $p < 0.001$ *vs.* TGFβ1-treated group ($n = 3$). **D.** Immunofluorescence assay of Smad4 (green) using fluorescence microscope. Nuclei were stained with DAPI (blue). **E.** Real-time PCR analysis of RhoGDI1 and RhoGDI2 mRNA levels. HAAFs were treated with TGFβ1 for 72 h. Histograms show the ratios of RhoGDI1 or RhoGDI2 mRNA levels to GAPDH mRNA levels ($n = 3$). * $p < 0.05$, ** $p < 0.01$ *vs.* control group; $^{\#}$ $p < 0.05$ *vs.* TGFβ1-treated group ($n = 3$). Western blot of RhoGDI1 (**F**) and RhoGDI2 (**G**) expressions. Untreated cells were used as controls. *** $p < 0.001$ *vs.* control group; $^{\#\#\#}$ $p < 0.001$ *vs.* TGFβ1-treated group ($n = 3$).

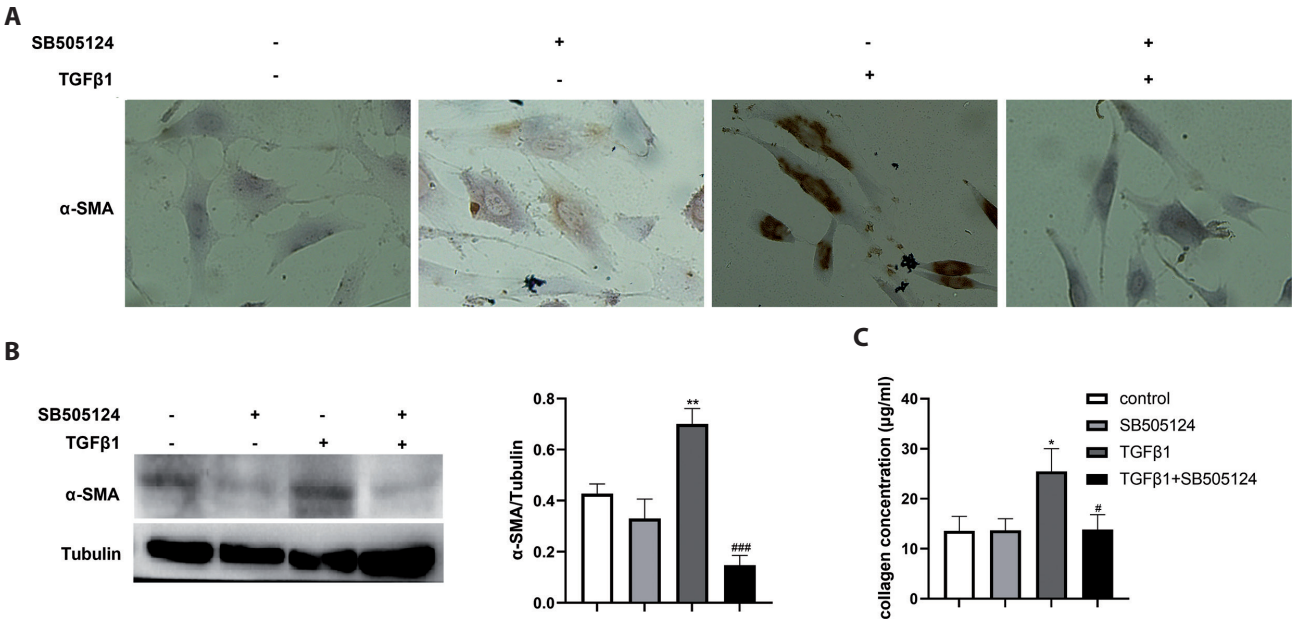

Figure 2. The effect of Smad signaling on myofibroblast transdifferentiation. **A.** Immunohistochemistry staining of α-SMA in HAAFs. Cells were pretreated with 0.2 nM SB505124 for 24 h and then exposed to 10 ng/ml TGFβ1 for an additional 72 h. Untreated cells were used as controls. α-SMA-positive cells are indicated by brown coloration. **B.** Western blot showing the expression of α-SMA. Histogram shows the ratio of α-SMA to tubulin. ** $p < 0.01$ *vs*. control group; ### $p < 0.001$ *vs*. TGFβ1-treated group ($n = 3$). **C.** Detection of collagen secretion into media. Histogram shows collagen concentrations in each group based on absorbance at 530 nm. *$p < 0.05$ *vs*. control group; # $p < 0.05$ *vs*. TGFβ1-treated group ($n = 3$).

pretreatment. Similar to the findings in Figure 3, Figure 4 implies that there was an evident improvement in the interaction of RhoGDI1/2-RhoGTPases in the TGFβ1-treated cells. Nevertheless, SB505124 pretreatment attenuated RhoGDI2-RhoGTPases interactions in response to TGFβ1 stimulation without affecting RhoGDI1-RhoGTPases interactions. These data suggested that TGFβ1 promoted the interactions of RhoGDI2 with Cdc42 or Rac1 *via* Smad signaling.

RhoGTPases participate in TGFβ1-induced myofibroblast transdifferentiation

To investigate whether Cdc42 or Rac1 activation is involved in TGFβ1-induced myofibroblast transdifferentiation, we inhibited the activities of Cdc42 or Rac1 using ZCL278 and NSC23766. Figure 5A and B show that Cdc42 or Rac1 inhibition (ZCL278 or NSC23766 pretreatment) significantly reduced the expression of α-SMA in TGFβ1-treated HAAFs, regardless of performing immunohistochemistry or Western blotting assay. Meanwhile, collagen secretion induced by TGFβ1 was greatly downregulated in HAAFs after ZCL278 or NSC23766 pretreatment. These results conveyed that RhoGTPases are responsible for TGFβ1-induced myofibroblast transdifferentiation.

Discussion

As the most abundant cell type of vascular adventitia, fibroblasts are the primary "sensing cells" of injury stimulation (Heasman and Ridley 2008). Fibroblasts can be activated by multiple pathways. Activated fibroblasts exhibit cancerous phenotypes, which include excessive proliferation, anti-apoptosis, and pro-inflammatory and metabolic abnormalities (Boulter and Garcia-Mata 2010). They can not only differentiate into myofibroblasts, which migrate to the middle membrane or even the intima, hence promoting the thickening of the vascular wall, but also participate in vascular inflammatory response by secreting chemokines, cytokines, and growth factors (Song et al. 2017; Anderluh et al. 2019). Among them, TGFβ1 induces fibroblasts to differentiate into myofibroblasts by stimulating the expression of α-SMA and the production of collagen (Avouac et al. 2017; Sun and Chan 2018). There are reports that RhoGDI is of vital importance in a series of tumor cellular functions, which consist of proliferation, differentiation, and tumor biological behaviors (Boulter et al. 2010). Our previous results manifested that RhoGDI is involved in myofibroblast transdifferentiation that is mediated by TGFβ1 (Zhang et al. 2019). However, the mechanisms behind the effects of RhoGDI on myofibroblast phenotypic modulation are worth further research.

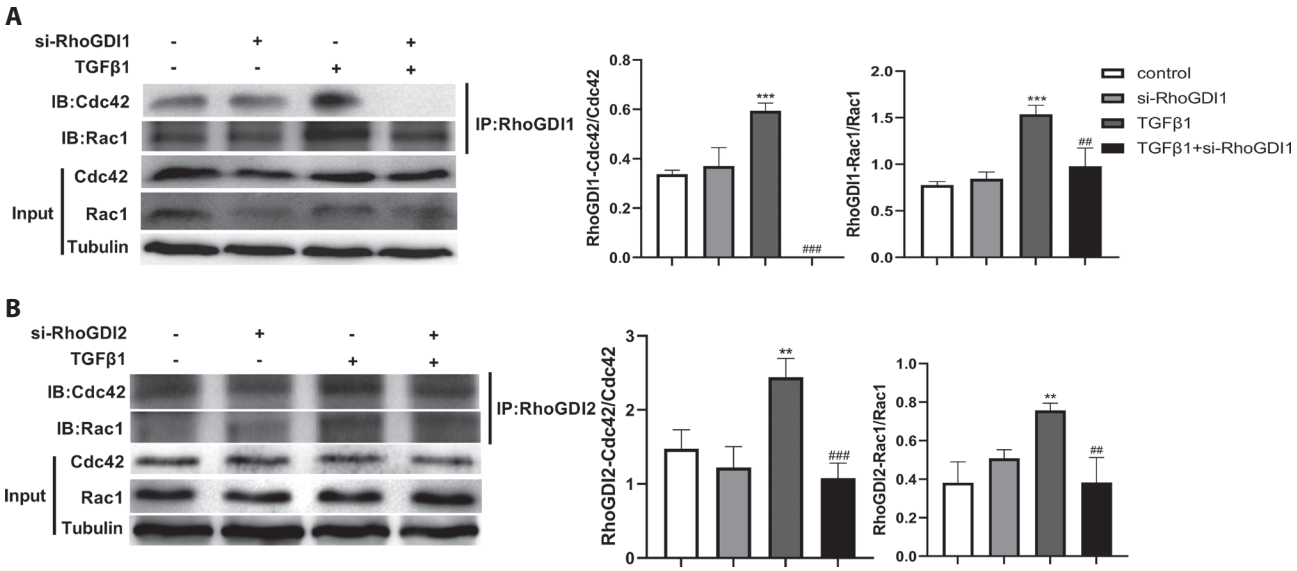

Figure 3. Both RhoGDI1 (**A**) and RhoGDI2 (**B**) suppression inhibit their binding to Cdc42 or Racl. HAAFs were tranfected with si-RhoGDI1 or si-RhoGDI2 for 48 h, followed by exposure to 10 ng/ml TGFβ1 for another 72 h. Untreated cells were used as control group. Analysis of the interaction of RhoGDI1 and RhoGDI2 with Cdc42 or Rac1 using Co-IP analysis. Immunoprecipitate RhoGDI1 or RhoGDI2 from the cell lysates with specific antibodies, and then analyze the immunoprecipitated proteins using Western blot. Both RhoGDI1 and RhoGDI2 suppression inhibited their binding with Cdc42 or Rac1 induced by TGFβ1. Histograms show the ratios of RhoGDI-binding Cdc42 or Rac1 to total Cdc42 or Rac1. ** $p < 0.01$, *** $p < 0.001$ *vs*. control group; $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ *vs*. TGFβ1-treated group ($n = 3$).

On the basis that certain inflammatory factors such as TGFβ1, which is important for the regulation of cell growth and differentiation (Skeen et al. 2012), may stimulate fibroblasts to differentiate into myofibroblasts, we constructed a TGFβ1-induced myofibroblast transdifferentiation model *in vitro*. Our findings suggest that TGFβ1 enhances RhoGDI1

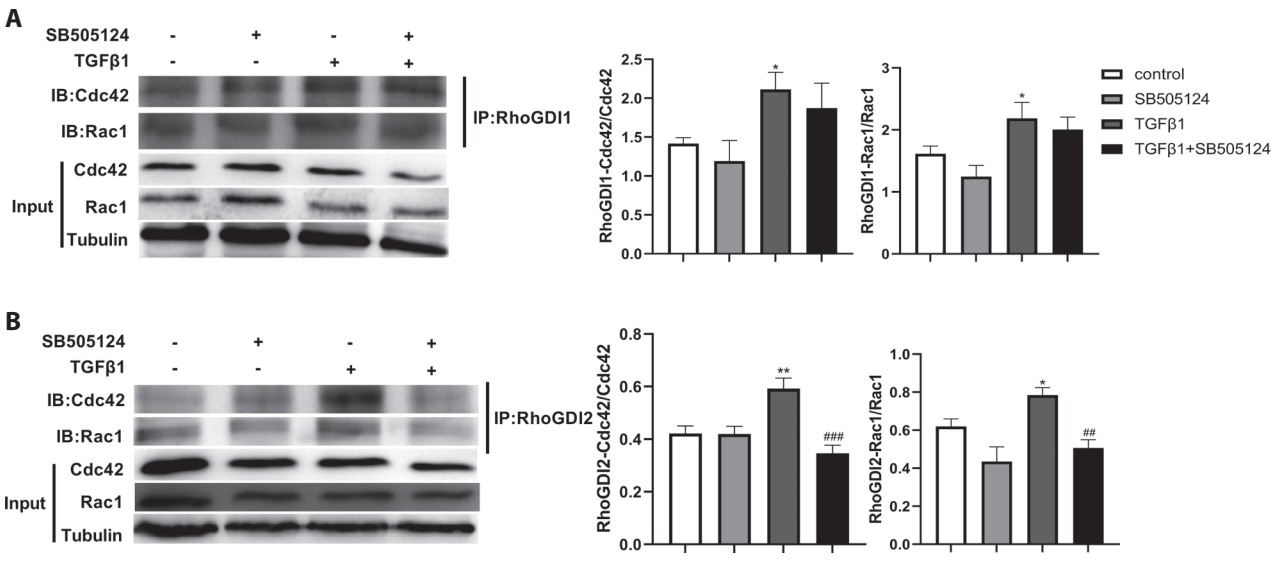

Figure 4. SB505124 pretreatment inhibit the binding of RhoGDI2 with Cdc42 or Rac1. Cells were pretreated with 0.2 nM SB505124 for 24 h and then exposed to 10 ng/ml TGFβ1 for an additional 72 h. Untreated cells were used as controls. Analysis of the interaction of RhoGDI1 (**A**) and RhoGDI2 (**B**) with Cdc42 or Rac1 using Co-IP analysis. SB505124 pretreatment inhibited the binding of RhoGDI2, rather than RhoGDI1, with Cdc42 or Rac1 induced by TGFβ1. Histograms show the ratios of RhoGDI-binding Cdc42 or Rac1 to total Cdc42 or Rac1. * $p < 0.05$, ** $p < 0.01$ *vs*. control group; $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ *vs*. TGFβ1-treated group ($n = 3$).

and RhoGDI2 expression by activating Smad signaling in TGFβ1-induced HAAF cells. Moreover, the inhibition of Smad2/3 phosphorylation remarkably decreased TGFβ1-induced α-SMA expression and collagen secretion in HAAFs. These results indicated that activated Smad signaling affects the phenotypic modulation of RhoGDI-mediated myofibroblast. SB505124 is an inhibitor of Smad2/3 phosphorylation and has been widely used in various experiments (DaCosta et al. 2004; Yao et al. 2015; Kim et al. 2016). SB505124 treatment contributed to decreasing in the expression of RhoGDI and collagen production in TGFβ1-induced HAAFs. These results illustrate that inhibition of Smad2/3 phosphorylation can reduce the expression of downstream RhoGDI, thereby inhibiting myofibroblast transdifferentiation. We have confirmed that TGFβ1 promotes RhoGDI expression through TGFβ receptor activation (Zhang et al. 2019). In this study,

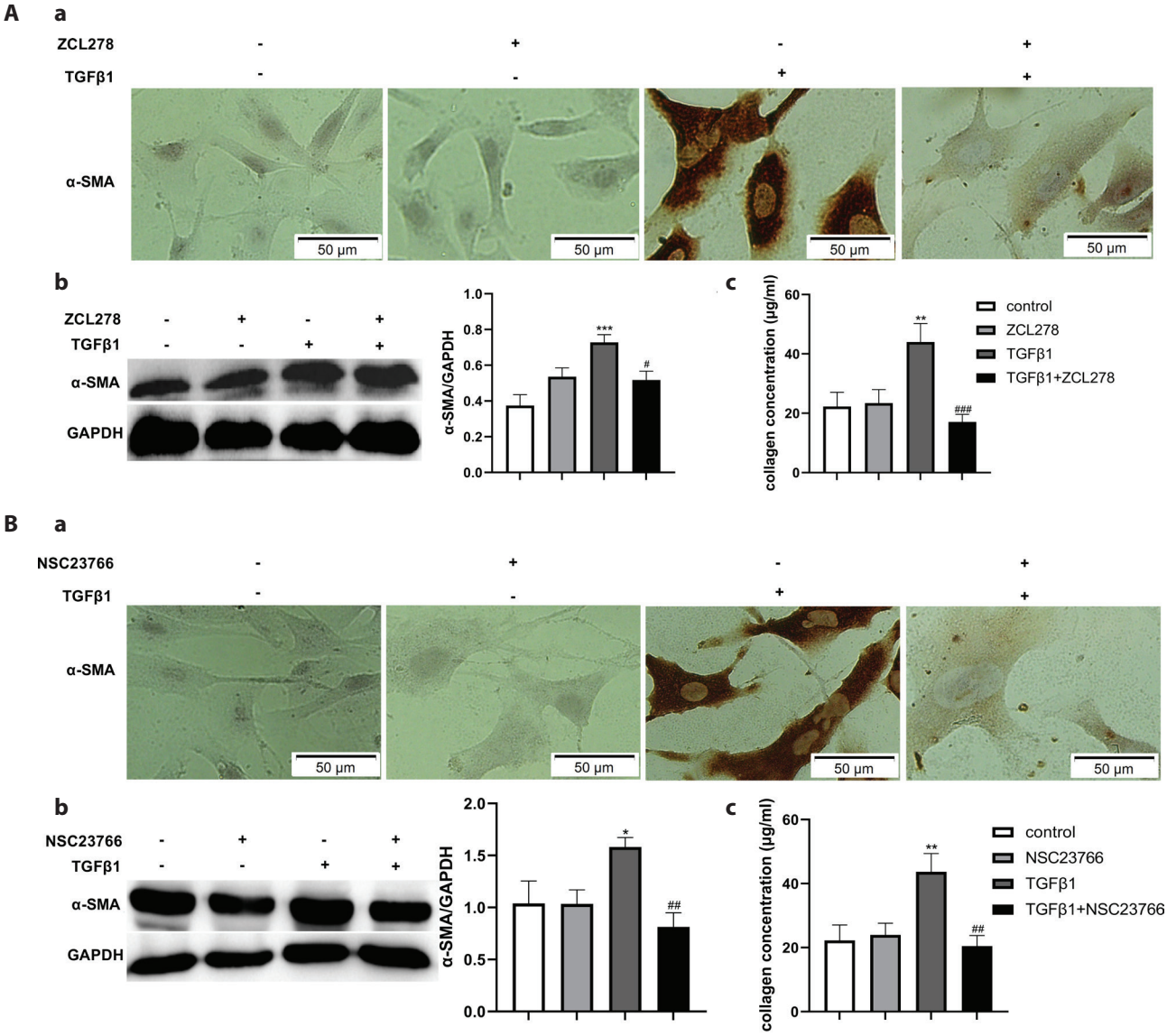

Figure 5. The effects of RhoGTPases on myofibroblast transdifferentiation using inhibitor of Cdc42 (ZCL278) (**A**) and inhibitor of Rac1 (NSC23766) (**B**). Human aortic adventitial fibroblasts (HAAFs) were pretreated with 50 μM ZCL278 or 50 μM NSC23766 for 30 min followed by treatment with 10 ng/ml TGFβ1 for 72 h. Immunohistochemistry staining of α-SMA in HAAFs (**Aa, Ba**). α-SMA-positive cells are indicated by brown coloration. Western blot showing the expression of α-SMA (**Ab, Bb**). Histogram shows the ratio of α-SMA to GAPDH. * $p < 0.05$, *** $p < 0.001$ *vs.* control group; # $p < 0.05$, ## $p < 0.01$ *vs.* TGFβ1-treated group ($n = 3$). Detection of collagen secretion into media (**Ac, Bc**). Histogram shows collagen concentrations in each group based on absorbance at 530 nm. ** $p < 0.01$ *vs.* control group; ## $p < 0.01$, ### $p < 0.001$ TGFβ1-treated group ($n = 3$).

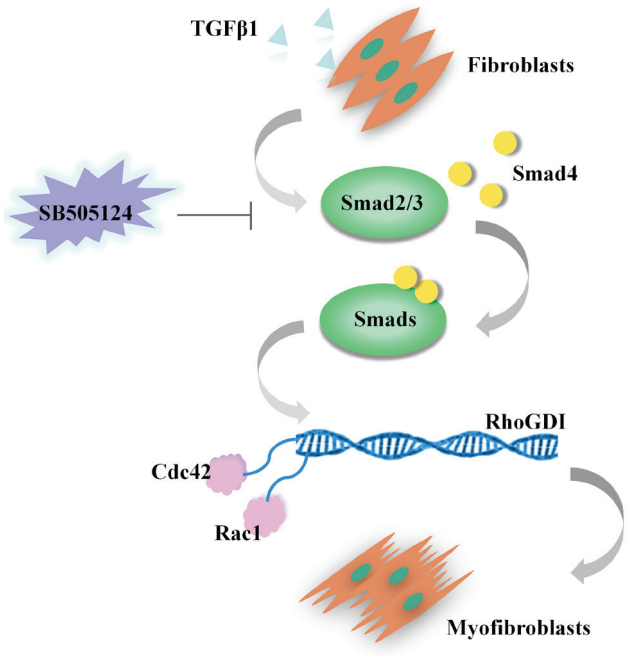

Figure 6. Diagram of the effects of inhibition of Smad2/3 phosphorylation on TGFβ1-mediated RhoGDI-RhoGTPases interactions and myofibroblast transdifferentiation.

TGFβ1 increased the mRNA expressions of both RhoGDI1 and RhoGDI2 in HAAFs, which illustrates that TGFβ1 regulates RhoGDI expression at the transcription level.

RhoGDIs has been proven to regulate the crosstalk between RhoGTPases, as well as Rho-family GTPase activation (Stultiens et al. 2012). The small Rho GTPase family, which comprises three major G-proteins Cdc42, Rac1, and RhoA, plays pivotal parts in regulating a variety of common cellular functions (Boureux et al. 2007). Our previous research has claimed that TGFβ1 promotes myofibroblast transdifferentiation *via* increasing the expression of RhoGDI1/2 (Zhang et al. 2019). Moreover, degradation of RhoGDI1inhibites Cdc42 activation in HA-VSMAs according to our other report (Qi et al. 2021). But so far, the regulatory effects of RhoGDI on RhoGTPases in HAAFs remain to be elucidated. For the first time, we confirmed that TGFβ1 facilitates the interaction of RhoGDI2 with Cdc42 or Rac1 *via* Smad signaling in HAAFs for the first time. TGFβ1 accelerates the interaction of RhoGDI1/2 with Cdc42 or Rac1. Interestingly, Smad signaling merely affects the interaction between RhoGTPases with RhoGDI2, but not RhoGDI1. These suggest that RhoGDI2, rather than RhoGDI1 participates in TGFβ1 induced myofibroblast transdifferentiation.

On the whole, the major findings of this research are: (1) the inhibition of Smad phosphorylation attenuates TGFβ1-induced RhoGDI1/2 expressions and transcription levels, thereby inhibiting myofibroblast phenotypic modulation; (2) RhoGDI2-RhoGTPases interaction, which is regulated by Smad signaling, participates in myofibroblast transdifferentiation (Fig. 6).

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Conflict of interest. The authors have no conflicts of interest to declare about this article.

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