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Vitamin D attenuates TGF-β1-induced lung fibroblast proliferation and migration through repression of *RasGRP3*

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Abstract: Background: Transforming growth factor-β1 (TGF-β1) is a pleiotropic cytokine that plays a central role in the pathogenesis of idiopathic pulmonary fibrosis (IPF). While previous studies have revealed a cross-talk between vitamin D and TGF-β1 signaling, it is still unclear how they interact with each other to regulate the progression of IPF. **Methods:** In this work, we searched for a novel mediator of TGF-β1 activity in lung fibroblasts and examined its regulation by vitamin D. In addition, we investigated the mechanism underlying the interaction between vitamin D and TGF-β1 signaling in lung fibroblast activation. Bioinformatic analysis was performed to identify TGF-β1 downstream target genes. Knockdown and overexpression expression experiments were conducted to determine gene function in the regulation of lung fibroblast proliferation and migration. **Results:** Analysis of publicly available datasets revealed that RAS guanyl releasing protein 3 (RasGRP3) was upregulated in TGF-β1-treated lung fibroblasts and lung tissues from IPF patients relative to healthy controls. Our data confirmed the upregulation of RasGRP3 by TGF-β1 in human MRC5 lung fibroblasts. Overexpression of RasGRP3 enhanced MRC5 cell proliferation and migration. Knockdown of RasGRP3 blocked TGF-β1-induced MRC5 proliferation and migration. Vitamin D abolished TGF-β1-induced RasGRP3 upregulation, which was reversed by inhibition of the vitamin D receptor (VDR). Mechanistically, vitamin D promoted VDR enrichment and prevented mothers against decapentaplegic homolog (SMAD) 2 and 3 occupancy at the promoter of *RasGRP3*. Additionally, overexpression of RasGRP3 reversed the suppressive effect of vitamin D on MRC5 cell proliferation and migration. **Conclusion:** In conclusion, vitamin D antagonizes TGF-β1-induced lung fibroblast activation by repressing RasGRP3 transcription.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive disease characterized by the excessive deposition of extracellular matrix (ECM) components in the lung, which can lead to lung dysfunction and death (Cui *et al.*, 2022; Staab-Weijnitz, 2022). IPF has a high mortality, with overall 3-year and 5-year cumulative survival rates of 61.8% and 45.6%, respectively (Zheng *et al.*, 2022). To date, no curative treatment options are available for IPF. Lung fibroblasts play a crucial role in the pathogenesis of IPF (Jeong *et al.*, 2022). Injured lung epithelial cells release multiple cytokines and growth factors to promote myofibroblast differentiation and expansion of fibroblasts, consequently causing a pathological remodeling of lung architecture (Wang *et al.*, 2022).

Transforming growth factor-β1 (TGF-β1) is an important regulator of tissue fibrosis (Meng *et al.*, 2016; Wang *et al.*, 2022). For example, TGF-β1 treatment stimulated fibroblast-myofibroblast transdifferentiation, as evidenced by increased expression of myofibroblast biomarkers α-smooth muscle actin (α-SMA) and collagen I (Jiang *et al.*, 2022). In another work, TGF-β1-treated lung fibroblasts acquired a profibrogenic phenotype and showed collagen I deposition (Chen *et al.*, 2022). TGF-β1 signaling has been reported to induce proliferation, migration, and myofibroblast transformation in lung fibroblasts (Wang *et al.*, 2021). In a study, TGF-β1 binding promoted the assembly between TGF-β type II receptor (TβR2) and TβR1, which triggered the activation of intracellular signaling pathways by phosphorylating receptor-associated SMADs including SMAD2 and SMAD3. Further, the phosphorylated SMAD2 and SMAD3 underwent nuclear translocation and promoted gene transcription (Zhong *et al.*, 2021). Inhibition of TGF-β1/SMAD signaling could elicit a strong

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anti-fibrotic effect on lung fibroblasts (Jiang *et al.*, 2021). Thus, these observations suggest that targeting TGF-β1/SMAD signaling may benefit fibrosis treatment.

Vitamin D deficiency has been shown to have an impact on the progression of IPF. Faverio *et al.* (2022) enrolled IPF patients and found that 56.3% of the patients (n = 90) have a low vitamin D level. Vitamin D deficiency was significantly correlated with all-cause mortality in patients with IPF (Tzilas *et al.*, 2019). In a high-fat-diet-induced obese mouse model, vitamin D supplementation attenuated obesity-associated lung fibrosis (Han *et al.*, 2021). Another study reported that vitamin D prevented bleomycin-induced lung fibrosis in a mouse model (Chang *et al.*, 2021). As a pleiotropic steroid hormone, vitamin D exerts its biological effects largely through interaction with the vitamin D receptor (VDR) (Rochel, 2022). VDR belongs to the nuclear receptor superfamily and can act as a heterodimer with retinoid X receptors (RXRs) to initiate gene transcription. VDR is expressed in a variety of cell types including lung fibroblasts (Stio *et al.*, 1997). Despite these findings, the mechanism for the protective effect of vitamin D on lung fibrosis is still unclear.

In the present study, we explored the cross-talk between vitamin D/VDR and TGF-β1 signaling in lung fibroblasts and investigated how vitamin D modulated TGF-β1-induced lung fibroblast proliferation and migration.

Materials and Methods

Cell culture and treatment

Human MRC5 pulmonary fibroblasts were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA).

For TGF-β1 treatment, MRC5 cells were exposed to different concentrations of TGF-β1 (0, 2, 5, 10, and 20 ng/mL; Peprotech, Hamburg, Germany) for 24 h. Unless stated otherwise, 10 ng/mL TGF-β1 was used. At the concentration, TGF-β1 was shown to effectively promote the proliferation of MRC5 fibroblasts (Ballester *et al.*, 2021). In some experiments, 1,25-dihydroxy vitamin D [1,25$(OH)_2$D], the active form of vitamin D (100 nM; Sigma-Aldrich) (Karkeni *et al.*, 2015) was added 24 h before exposure to TGF-β1 for another 24 h. 1,25$(OH)_2$D was dissolved in 99.8% ethanol (Sigma-Aldrich).

Quantitative real-time polymerase chain reaction analysis

After the above treatment, MRC5 cells were lysed in Trizol reagent (Invitrogen, Carlsbad, CA, USA), and total RNA was isolated. Reverse transcription from total RNA was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The levels of *IGF1*, *MYH11*, *SERPINE2*, *PLN*, *RasGRP3*, and *CCDC82* transcripts were measured via the quantitative real-time polymerase chain reaction (qRT-PCR) using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). *GAPDH* was used as an internal control. The primers are shown as follows: *IGF1* forward, 5′-GCTCTTCAGTTCGTGTGTGGA-3′ and reverse, 5′-GCCTCCTTAGATCACAGCTCC-3′; *MYH11* forward, 5′-GTCCAGGAGATGAGGCAGAAAC-3′ and reverse, 5′-GTCTGCGTTCTCTTTCTCCAGC-3′; *SERPINE2* forward, 5′-TTTGCAAAAATAACAACAGGGTCA-3′ and reverse, 5′-GCTGCTGAAGCTTTGGTTCC-3′; *PLN* forward, 5′-CAGGTCTTCACCAAGTATCA-3′ and reverse, 5′-TCCATACTTGATTCTCATCA-3′; *RasGRP3* forward, 5′-TCAGCCTCATCGACATATCCA-3′ and reverse, 5′-TCAGCCAATTCAATG GGCTCC-3′; *CCDC82* forward, 5′-GAGCTTGATAGTGAAGAATTT-3′ and reverse, 5′-CCGTTGCCAGAGTTAAT GAG-3′; *SMAD2* forward, 5′-GGAGCAGAATACCGAA GGCA-3′ and reverse, 5′-CTTGAGCAACGCACTGAAGG-3′; *SMAD3* forward, 5′-AGAAGACGGGGCAGCTGGAC-3′ and reverse, 5′-GACATCGGATTCGGGGATAG-3′; *VDR* forward, 5′-CGACCCCACCTACTCCGACTT-3′ and reverse, 5′-GGCTCCCTCCACCATCATTC-3′; *GAPDH* forward, 5′-GGAGCGAGATCCCTCCAAAAT-3′ and *GAPDH* reverse, 5′-GGCTGTTGTCATACTTCTCATGG-3′.

Western blot analysis

Cells were lysed with RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) supplemented with the Protease Inhibitor Cocktail (Cell Signaling Technology, Beverly, MA, USA). Protein concentration was determined using the BCA Protein Assay Kit (Cell Signaling Technology). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The membrane was then incubated at 4°C overnight with primary antibodies recognizing RasGRP3 (#3334; Cell Signaling Technology) or GAPDH (#5174; Cell Signaling Technology). After washing, the membrane was re-probed with secondary antibodies conjugated with horseradish peroxidase. Western blot bands were visualized using enhanced chemiluminescence reagents (Amersham GE Healthcare, Chicago, IL, USA) and subjected to densitometric analysis.

Plasmid details, small interfering RNAs, and cell transfection

Full-length human *RasGRP3* cDNA was cloned in pcDNA3.1(+) vector. Small interfering RNAs (siRNAs) specifically targeting human *RasGRP3*, *SMAD2*, *SMAD3*, and *VDR* and non-specific control siRNAs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

For transient transfection, MRC5 cells were seeded into 6-well plates (1×10^6 cells/well) 24 h before transfection. The RasGRP3-expressing plasmid and siRNAs (50 nM) were transfected to MRC5 cells using Lipofectamine 3000 (Invitrogen) in accordance with the manufacturer's protocol. After 24 h of transfection, the cells were subjected to gene expression analysis, proliferation, and migration assays.

Cell proliferation assay

For short term proliferation assays, 1×10^4 cells transfected with indicated constructs were seeded onto 12-well plates and cultured with or without TGF-β1 or vitamin D. After 3 or 5 days, the cells were harvested and counted using an automated cell counter.

Cell migration assay

For the assessment of cell migration, an *in vitro* wound-healing assay was performed. MRC5 cells were seeded onto

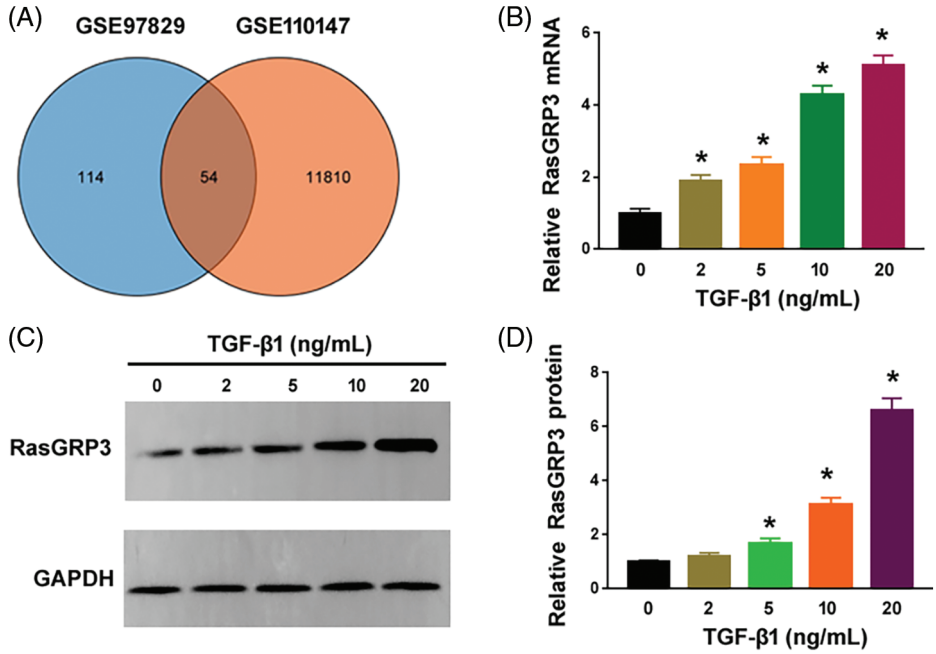

FIGURE 1. TGF-β1 stimulates the expression of RasGRP3 in human lung fibroblasts. (A) Venn diagram showing 54 differentially expressed genes commonly detected in the 2 publicly available datasets. (B) Expression of *RasGRP3* was measured by qRT-PCR in MRC5 cells treated with TGF-β1. *$p < 0.05$ using one-way analysis of variance with a Tukey's test ($n = 3$). (C) Western blot analysis of RasGRP3 protein levels in MRC5 cells treated with TGF-β1. Images were representative of three experiments. (D) Bar graph showing densitometry analysis of RasGRP3 protein in (C). Data are expressed as the fold change relative to 0 ng/mL TGF-β1. *$p < 0.05$ using one-way analysis of variance with a Tukey's test ($n = 3$). Key: TGF-β1-transforming growth factor beta-1; RasGRP3: RAS guanyl releasing protein 3; qRT-PCR: quantitative real-time polymerase chain reaction.

6-well plates and cultured to confluence. A sterile 10-μL pipette tip was used to make an artificial gap at the central part of the culture monolayer. The scratched cells were photographed at 0 and 24 h after scratching.

Transcription factor prediction

Putative transcription factors binding to the promoter region of the *RasGRP3* gene were predicted using the Animal Transcription Factor DataBase (AnimalTFDB) version 3.0 (http://bioinfo.life.hust.edu.cn/AnimalTFDB/#!/) (Hu *et al.*, 2019).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously (Liu *et al.*, 2019). Briefly, MRC5 cells with indicated treatments were cross-linked with 1% formaldehyde and sonicated on ice to yield 200–500 bp DNA fragments. The fragmented chromatin was incubated with anti-SMAD2/SMAD3 (#5678; Cell Signaling Technology), anti-VDR (ab3508; Abcam, Cambridge, MA, USA), or isotype control IgG (Cell Signaling Technology). The precipitated chromatin DNA was recovered and subjected to qRT-PCR analysis. The PCR primers are shown as follows: *RasGRP3* promoter, forward: 5′-CCATC CTGGCTAACATGGTGA-3′, and reverse: 5′-CGCTCATTA GCTAAACATGCT-3′.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). All statistical analyses were performed using the Student's *t*-test or one-way analysis of variance with a Tukey's test. $p < 0.05$ was considered to be significant.

Results

TGF-β1 stimulates the expression of RasGRP3 in human lung fibroblasts

To search for novel downstream genes mediating TGF-β1 profibrotic effects, we analyzed 2 publicly available datasets, i.e., GSE97829 and GSE110147. The GSE110147 dataset includes RNA profiling data from 22 IPF patients *vs.* 11 healthy controls. The GSE97829 dataset contains RNA sequencing data from human MRC5 lung fibroblasts treated with or without TGF-β1. The Venn diagram showed 54 differentially expressed genes (DEGs) commonly detected in both datasets (Fig. 1A). Among them, 6 genes (i.e., *IGF1*, *MYH11*, *SERPINE2*, *PLN*, *RasGRP3*, and *CCDC82*) were consistently upregulated in lung tissues from IPF patients and TGF-β1-treated fibroblasts. To validate the bioinformatic analysis, we performed qRT-PCR experiments using MRC5 cells after treatment with different concentrations of TGF-β1. While *IGF1*, *MYH11*, *PLN*, *RasGRP3*, and *CCDC82* were induced by 1.6–5.1 fold upon TGF-β1 treatment, *SERPINE2* levels remained unchanged (Fig. 1B and Suppl. Fig. S1). In this study, we focused on *RasGRP3* because it showed the highest fold change after TGF-β1 treatment. Western blot analysis also confirmed the upregulation of *RasGRP3* by TGF-β1 (Figs. 1C and 1D). These results suggest that *RasGRP3* may mediate the effect of TGF-β1 on lung fibroblasts.

RasGRP3 is required for TGF-β1-induced lung fibroblast proliferation and migration

Next, we investigated the role of *RasGRP3* in modulating the behavior of lung fibroblasts. Overexpression of *RasGRP3*

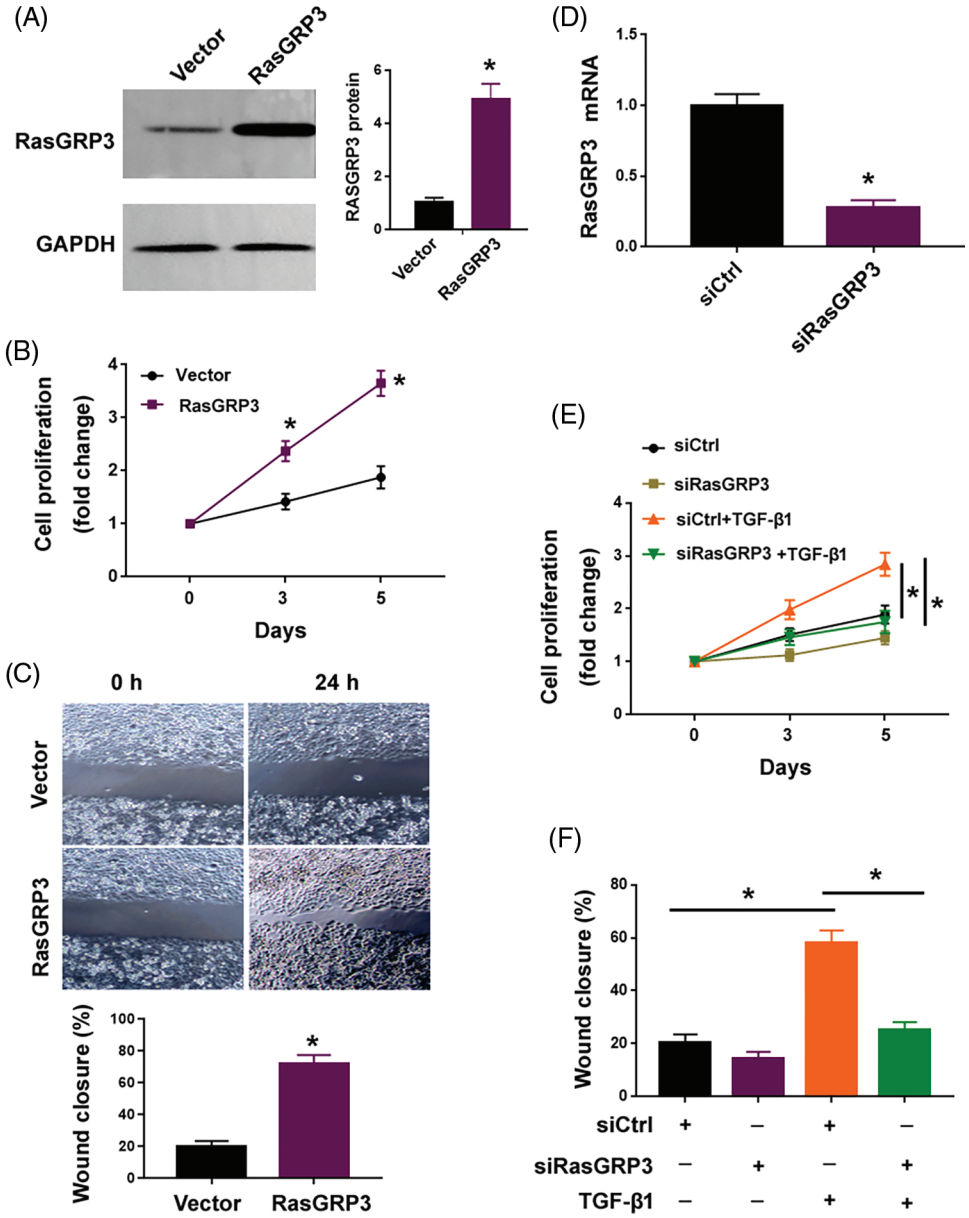

FIGURE 2. RasGRP3 is required for TGF-β1-induced lung fibroblast proliferation and migration. (A) RasGRP3 protein levels determined by Western blot analysis in MRC5 cells transfected with vector or RasGRP3-expressing plasmid. *Left*: images were representative of three experiments. *Right*: densitometry analysis of RasGRP3 protein. *$p < 0.05$ using the Student's *t*-test ($n = 3$). (B) Cell proliferation assay. *$p < 0.05$ using the Student's *t*-test ($n = 3$). (C) Wound-healing assay was performed to measure MRC5 cell migration. *$p < 0.05$ using the Student's *t*-test ($n = 3$). (D) qRT-PCR analysis of *RasGRP3* mRNA levels in MRC5 cells transfected with control siRNA (siCtrl) or RasGRP3-targeting siRNAs (siRasGRP3). *$p < 0.05$ using the Student's *t*-test ($n = 3$). (E) Cell proliferation assay. *$p < 0.05$ using one-way analysis of variance with a Tukey's test ($n = 3$). (F) Cell migration assay. *$p < 0.05$ using one-way analysis of variance with a Tukey's test ($n = 3$). Key: TGF-β1-transforming growth factor beta-1; RasGRP3: RAS guanyl releasing protein 3; qRT-PCR: quantitative real-time polymerase chain reaction; siRNA: small interfering RNA.

enhanced the proliferation and migration of MRC5 cells (Figs. 2A–2C). Previous studies (Jin *et al.*, 2019; Wang *et al.*, 2021) have shown that treatment with TGF-β1 led to a significant increase in MRC5 cell proliferation and migration. Of note, the knockdown of *RasGRP3* (Fig. 2D) reversed this TGF-β1-dependent proliferation and migration in MRC5 cells (Figs. 2E and 2F). These results indicate that *RasGRP3* is capable of inducing a profibrotic phenotype in terms of increased cell proliferation and migration in lung fibroblasts.

TGF-β1-SMAD2/3 signaling drives the transcription of RasGRP3

Since TGF-β1 treatment augmented the expression of *RasGRP3* transcript (Fig. 1B), we hypothesized whether TGF-β1 signaling can directly initiate the transcription of *RasGRP3*. Using the AnimalTFDB 3.0 software, we predicted that the promoter region of the *RasGRP3* gene harbored binding sites for 72 transcription factors. Intriguingly, there was one binding site for SMAD2/

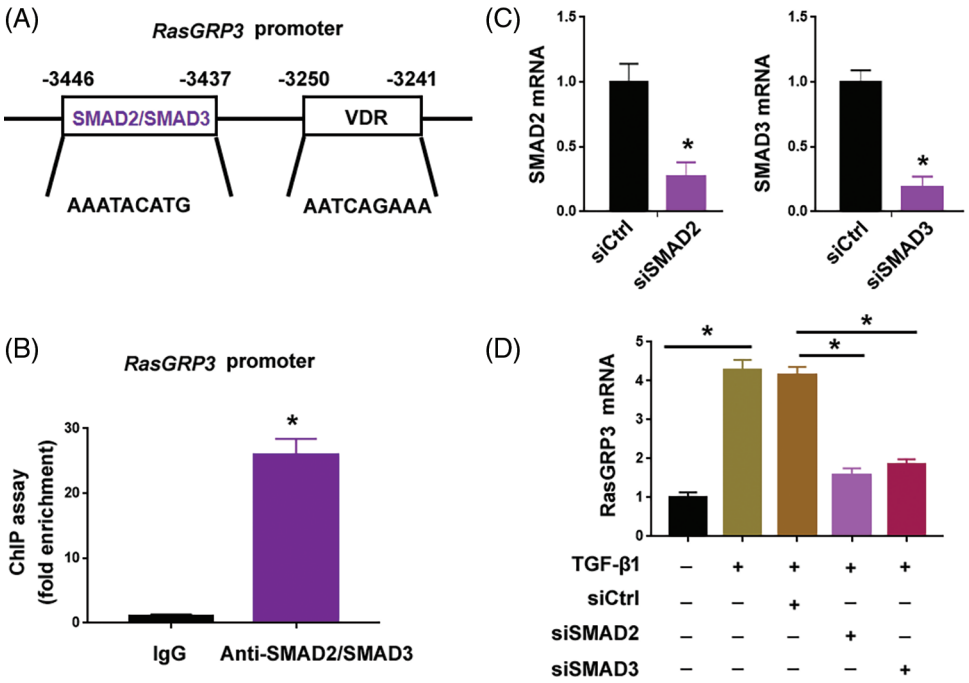

FIGURE 3. TGF-β1-SMAD2/3 signaling drives the transcription of *RasGRP3*. (A) Using the AnimalTFDB 3.0 software, the promoter region of the *RasGRP3* gene was predicted to have 1 binding site for SMAD2/SMAD3. (B) ChIP assays of SMAD2/SMAD3 on the promoter of *RasGRP3* in MRC5 cells. *$p < 0.05$ using the Student's *t*-test ($n = 3$). (C) Knockdown of SMAD2 and SMAD3 using specific siRNAs. *$p < 0.05$ using the Student's *t*-test ($n = 3$). (D) qRT-PCR analysis of *RasGRP3* mRNA in MCR5 cells with indicated treatments. *$p < 0.05$ using one-way analysis of variance with a Tukey's test ($n = 3$). Key: TGF-β1-transforming growth factor beta-1; RasGRP3: RAS guanyl releasing protein 3; qRT-PCR: quantitative real-time polymerase chain reaction; ChIP: chromatin immunoprecipitation; siRNA: small interfering RNA.

SMAD3: AAATACATG (-3446/-3437 bp) (Fig. 3A). ChIP assay confirmed the enrichment of SMAD2/SMAD3 at the promoter region of *RasGRP3* (Fig. 3B). Knockdown of SMAD2 or SMAD3 (Fig. 3C) significantly impaired the TGF-β1-induced upregulation of RasGRP3 (Fig. 3D). These data indicate that TGF-β1 promotes RasGRP3 expression through SMAD2/SMAD3-dependent transcription.

Vitamin D blocks TGF-β1-mediated RasGRP3 upregulation through the VDR

Vitamin D has been shown to modulate TGF-β1/SMAD signaling in different biological settings (Subramaniam *et al.*, 2001; Tao *et al.*, 2015). Thus, we checked whether vitamin D could regulate the expression of *RasGRP3*. Interestingly, we found that vitamin D significantly inhibited the upregulation of *RasGRP3* in TGF-β1-treated MRC5 cells (Fig. 4A). ChIP assay demonstrated that the enrichment of SMAD2/SMAD3 at the promoter region of *RasGRP3* was impaired when treat MRC5 cells were treated with vitamin D (Fig. 4B). One putative VDR binding site, AATCAGAAA (-3250/-3241 bp) localized nearby the predicted SMAD2/SMAD3 binding site at the *RasGRP3* promoter (Fig. 3A). Notably, vitamin D treatment promoted the enrichment of the VDR at the *RasGRP3* promoter (Fig. 4C). In addition, the depletion of the VDR using specific siRNAs (Fig. 4D) abolished the suppression of RasGRP3 by vitamin D (Fig. 4E). Taken together, vitamin D prevents TGF-β1-induced RasGRP3 upregulation likely by interfering with the binding of SMAD2/SMAD3 to the *RasGRP3* promoter.

Overexpression of RasGRP3 antagonizes the anti-fibrotic effect of vitamin D on lung fibroblasts

Supplementation of vitamin D significantly attenuated the proliferation and migration of MRC5 cells treated with TGF-β1 (Figs. 5A and 5B). The exogenous expression of RasGRP3 restored MRC5 cell proliferation and migration in the presence of vitamin D (Figs. 5A–5C). These results suggest that the anti-fibrotic effect of vitamin D involves the modulation of *RasGRP3*.

Discussion

TGF-β1 is a potent cytokine that shows the ability to promote lung fibrosis (Mackinnon *et al.*, 2012; Boutanquoi *et al.*, 2020). Treatment with TGF-β1 is able to elicit genome-wide expression changes in a variety of cell types including lung fibroblasts (Lu *et al.*, 2020). In this respect, it is important to screen for DEGs upon TGF-β1 treatment. In the current study, we show that RasGRP3 is stimulated by TGF-β1 in lung fibroblasts. Analysis of publically available data from 22 IPF patients *vs.* 11 healthy controls suggests that RasGRP3 is upregulated in lung tissues from IPF patients relative to healthy controls. This upregulation of RasGRP3 may contribute to IPF progression. RasGRP3 belongs to the RASGRP family of guanine nucleotide exchange factors (GEFs). Many studies have reported the role of RasGRP3 in malignant diseases (Yang *et al.*, 2010; Lee *et al.*, 2015). For example, RasGRP3 was highly expressed in glioblastoma and accelerated cell migration and invasion (Lee *et al.*,

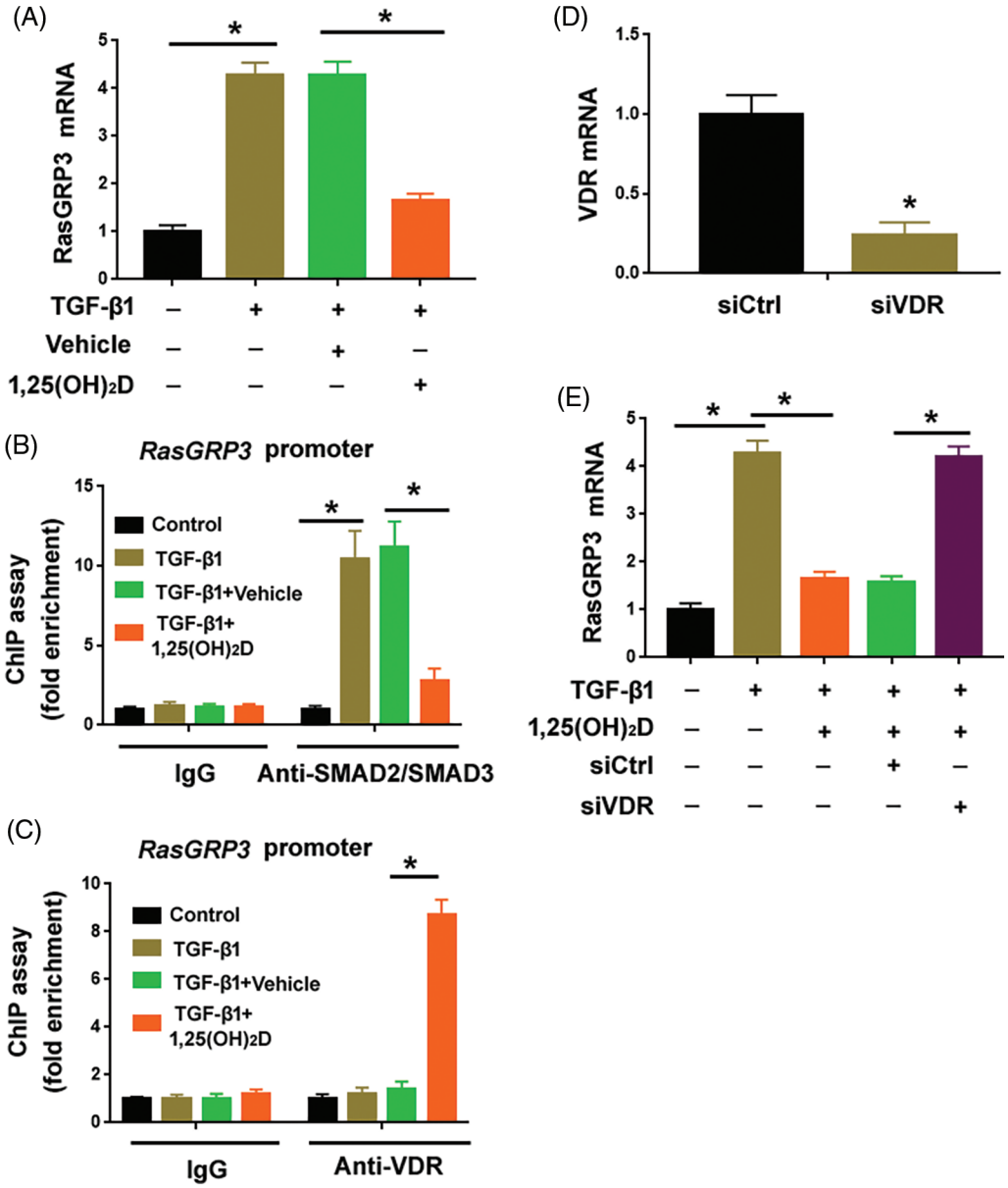

FIGURE 4. Vitamin D blocks TGF-β1-mediated RasGRP3 upregulation through VDR. (A) MCR5 cells were treated with 100 nM $1,25(OH)_2D$ or 99.8% ethanol (vehicle) 24 h before exposure to 10 ng/mL TGF-β1 for another 24 h. *RasGRP3* mRNA levels were determined by qRT-PCR analysis. *$p < 0.05$ using one-way analysis of variance with a Tukey's test ($n = 3$). (B) ChIP assays of SMAD2/SMAD3 on the promoter of *RasGRP3* in MRC5 cells treated as in (A). *$p < 0.05$ using one-way analysis of variance with a Tukey's test ($n = 3$). (C) ChIP assays of VDR on the promoter of *RasGRP3* in MRC5 cells treated as in (A). *$p < 0.05$ using one-way analysis of variance with a Tukey's test ($n = 3$). (D) Knockdown of VDR using specific siRNAs. *$p < 0.05$ using the Student's *t*-test ($n = 3$). (E) qRT-PCR analysis of *RasGRP3* mRNA in MCR5 cells with indicated treatments. *$p < 0.05$ using one-way analysis of variance with a Tukey's test ($n = 3$). Key: TGF-β1-transforming growth factor beta-1; RasGRP3: RAS guanyl releasing protein 3; qRT-PCR: quantitative real-time polymerase chain reaction; ChIP: chromatin immunoprecipitation; siRNA: small interfering RNA; VDR: vitamin D receptor.

2015). Yang *et al.* (2010) reported that the knockdown of RasGRP3 inhibited prostate cancer cell proliferation and migration. RasGRP3 also regulates the behaviors of nonmalignant cells such as endothelial cells (Randhawa *et al.*, 2011) and macrophages (Tang *et al.*, 2014). Randhawa et al. (2011) reported that activation of RasGRP3 impairs endothelial cell migration. Using lung fibroblasts, we demonstrate in this work that RasGRP3 mediates the TGF-β1-induced profibrotic phenotype. Knockdown of RasGRP3 attenuated the proliferation and migration of lung fibroblasts stimulated by TGF-β1. Therefore, our work highlights the importance of RasGRP3 in regulating the phenotype of lung fibroblasts. However, it should be noted that the cell line MRC5 used in this study is a fetal fibroblast cell line. Given that IPF is a condition affecting older adults, the present results seen in MRC5 cells should be validated in adult human lung fibroblasts.

Bioinformatic analysis reveals that the promoter of the *RasGRP3* gene contains a putative binding site for SMAD2/SMAD3. When SMAD2 or SMAD3 is depleted, TGF-β1-induced upregulation of RasGRP3 is reversed. These data suggest that TGF-β1 stimulates RasGRP3 upregulation likely

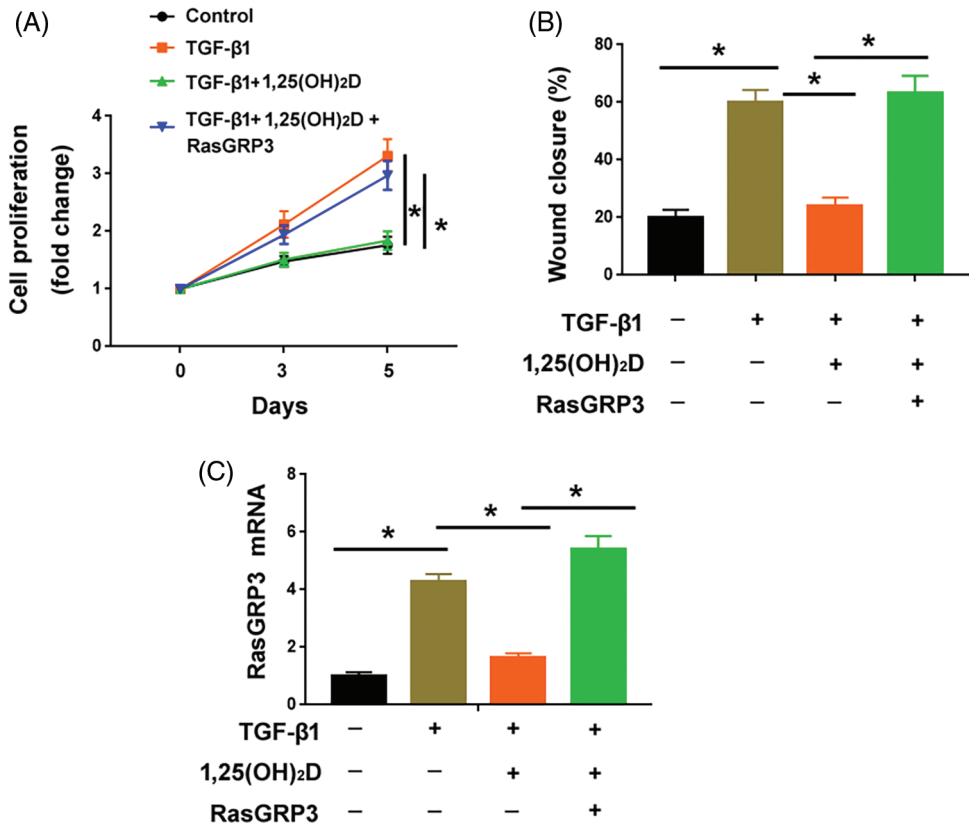

FIGURE 5. Overexpression of RasGRP3 antagonizes the anti-fibrotic effect of vitamin D on lung fibroblasts. (A) Assessment of the proliferation of MRC5 cells with indicated treatments. *$p < 0.05$ using one-way analysis of variance with a Tukey's test ($n = 3$). (B) Wound-healing assays were performed to determine the migration of MRC5 cells with indicated treatments. *$p < 0.05$ using one-way analysis of variance with a Tukey's test ($n = 3$). (C) qRT-PCR analysis of *RasGRP3* mRNA in MCR5 cells with indicated treatments. *$p < 0.05$ using one-way analysis of variance with a Tukey's test ($n = 3$). Key: TGF-β1-transforming growth factor beta-1; RasGRP3: RAS guanyl releasing protein 3.

through SMAD2/SMAD3-dependent transcription. Vitamin D has been reported to antagonize TGF-β signaling in multiple cell types such as human hepatic stellate cells (Beilfuss *et al.*, 2015), fibroblasts, and epithelial cells (Ramirez *et al.*, 2010). Xu *et al.* (2020) provided evidence for the physical interaction between VDR and SMAD3. Our data demonstrate that vitamin D treatment suppresses TGF-β1-induced upregulation of RasGRP3. Moreover, vitamin D prevents SMAD2/SMAD3 binding to the promoter of *RasGRP3*. Instead, the enrichment of the VDR at the promoter of *RasGRP3* is potentiated by vitamin D. Ablation of the VDR reverses the suppression of RasGRP3 by vitamin D. These results suggest that activated VDR may interfere with SMAD2/SMAD3 binding to the promoter of *RasGRP3*, thus inhibiting RasGRP3 transcription.

A number of molecular mechanisms have been suggested to be involved in the anti-fibrotic effects of vitamin D (Liu *et al.*, 2014; Chang *et al.*, 2021; Zhu *et al.*, 2021). Zhu *et al.* (2021) reported that VDR binding to the promoter of *PSAT1* leads to the downregulation of phosphoserine amino transferase 1 (PSAT1), consequently inhibiting the mitogen-activated protein kinase (MAPK) pathway and preventing lung fibrosis. Chang *et al.* (2021) demonstrated that vitamin D suppresses lung fibrosis through the regulation of the local renin-angiotensin system in the lung. In this study, our data show a novel mechanism by which vitamin D regulates lung fibroblast activation. We demonstrated that vitamin D suppresses the transcription of RasGRP3 to block lung fibroblast proliferation and migration. Ectopic expression of RasGRP3 reverses the anti-fibrotic effect of vitamin D on lung fibroblasts. Vitamin D-mediated suppression of RasGRP3 is dependent on VDR. Ding *et al.* (2013) reported a receptor-dependent mechanism for the suppression of liver fibrosis by vitamin D. They found that in the presence of vitamin D, VDR binding interferes with SMAD3 occupancy at the promoter of profibrotic genes, thus inhibiting liver fibrosis. Our results reveal a similar mechanism in the context of lung fibroblast activation. Taken together, vitamin D counteracts TGF-β1-induced lung fibroblast proliferation and migration through VDR-mediated repression of RasGRP3 transcription.

Despite these *in vitro* findings, there is no direct evidence for the role of RasGRP3 in animal models of IPF yet. It is still unclear whether RasGRP3 could be used as a biomarker for IPF diagnosis and prognosis. In addition, it remains to be explored in future work how RasGRP3 regulates lung fibrosis.

In summary, our data suggest RasGRP3 as a novel mediator of TGF-β1 profibrotic activity in lung fibroblasts. Vitamin D can reduce TGF-β1-induced lung fibroblast activation, which is at least partially mediated through inhibition of SMAD2/SMAD3 occupancy and VDR enrichment at the promoter of *RasGRP3*. The significance of RasGRP3 in IPF deserves further investigation.

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Author Contributions: GQH, RSH, and XXC designed the study and analyzed the data. GQH, RSH, LLL, and QSP conducted experiments and collected data. GQH was responsible for manuscript drafting. All authors read and approved the final draft of the manuscript.

Availability of Data and Materials: All data generated or analyzed during this study are included in this published article.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Supplementary Materials

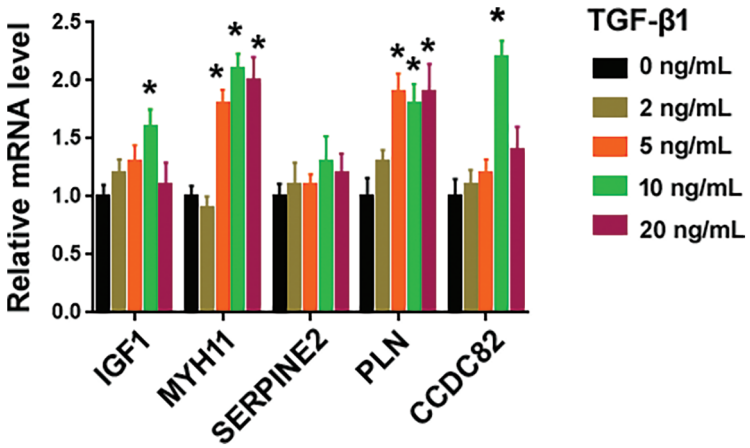

FIGURE S1. Effect of TGF-β1 on the expression of indicated genes. Quantification of indicated transcripts in MRC5 cells treated with different concentrations of TGF-β1 using qRT-PCR assays. *$p < 0.05$ using one-way analysis of variance with a Tukey's test ($n = 3$).