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Peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist inhibits collagen synthesis in human hypertrophic scar fibroblasts by targeting Smad3 *via* miR-145

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ABSTRACT

The transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ) functions to regulate cell differentiation and lipid metabolism. Recently, its agonist has been documented to regulate extracellular matrix production in human dermal fibroblasts. This study explored the underlying molecular mechanisms and gene interactions in hypertrophic scar fibroblasts (HSFBs) *in vitro*. HSFBs were cultured and treated with or without PPAR-γ agonist or antagonist for gene expression. Bioinformatical analysis predicted that miR-145 could target Smad3 expression. Luciferase assay was used to confirm such an interaction. The data showed that PPAR-γ agonist troglitazone suppressed expression of Smad3 and Col1 in HSFBs. PPAR-γ agonist induced miR-145 at the gene transcriptional level, which in turn inhibited Smad3 expression and Col1 level in HSFBs. Furthermore, ELISA data showed that Col1 level in HSFBs was controlled by a feedback regulation mechanism involved in PPAR-γ agonist and antagonist-regulated expression of miR-145 and Smad3 in HSFBs. These findings indicate that PPAR-γ-miR-145-Smad3 axis plays a role in regulation of collagen synthesis in HSFBs.

1. Introduction

Skin fibrosis results from progressive and uncontrolled deposition of extracellular matrix (ECM) proteins and leads to formation of abnormal scars in the skin. The abnormal scars could cause significant clinical problems, such as alteration of skin functions, hindering of joint movement, and cosmetic concerns [1]. Causes of skin fibrosis include multiple factors, such as physical trauma, injury, inflammation, and biological and chemical agents [2], but in some cases, the specific trigger remains unknown. Hypertrophic scars are a kind of skin fibrosis and characterized by abnormally high rates of collagen production and deposition and increase in levels of other ECM components (fibronectin and proteoglycans). Previous studies showed that there were differences between hypertrophic scar and normal skin fibroblasts in terms of potentials for collagen production and degradation, and expression and activity of collagenases [3,4]. Although fibrosis after skin injury is known to be a reactive and defensive action of the skin tissues and several different factors may modulate the relevant pathways, the underlying molecular and biochemical mechanisms are not fully understood [5].

Recent studies have identified that the nuclear receptor peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist was able to activate PPAR-γ to modulate expression of ECM proteins by targeting the TGF-β/Smad3 and MAPK signaling pathways [6–8]. Indeed, the canonial TGF-β/Smad signaling pathways play an important role in transcriptional regulation of type I collagen (Clo1) and other ECM protein expression [9]. In this signal pathway, Smad3 is a receptor-activated or pathway-restricted protein directly activated by TGF-β1 receptor ligands [10]. Targeting of Smad3 was able to suppress skin scar formation; for example, Smad3-null mice are resistant to irradiation-induced cutaneous

Abbreviations: ECM, extracellular matrix; HSFB, hypertrophic scar-derived fibroblast; RT-PCR, reverse transcriptase-PCR; UTR, untranslated region; PPAR-γ, peroxisome proliferator-activated receptor-γ; miRNA, microRNA; Col1, collagen type I.

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fibrosis, bleomycin-induced pulmonary fibrosis, carbon tetrachloride-induced hepatic fibrosis, or glomerular fibrosis *in vivo* [11,12]. Mechanistically, cell proliferation-inhibitory agents, inflammation modulators, or inhibition of Col1 production can all be suppressed by Smad3 [13–15]. Meanwhile, previous studies demonstrated that there is a potential PPRE (PPAR-γ-response element) (−1207 to−1194 bp) localized at miR-145 transcription starting site and Smad3 cDNA 3′-UTR contains a miR-145 binding site [16,18]. In addition, troglitazone, a PPAR-γ agonist reduces Col1 expression at both mRNA and protein levels (8). However, what is the role of miR-145 in the PPAR-γ mediated regulation of Smad3 expression especially overproduction of Col1 and other ECM proteins, thus modulating the process of cutaneous scar formation, remains unknown. Thus, in this study, we explored such underlying molecular mechanisms and gene interactions using hypertrophic scar fibroblasts (HSFBs) as an *in vitro* model. Our data demonstrated that PPAR-γ-regulated Col1 synthesis was through PPAR-γ-miR-145-Smad3 axis in HSFBs; therefore, it is plausible to develop PPAR-γ agonist as a therapeutic agent for the control of excessive skin scar formation in clinic.

2. Materials and methods

2.1. Cell culture and gene transfection

Hypertrophic scar fibroblasts (HSFBs) were obtained from patients who were admitted to the Department of Burns and Cutaneous Surgery, Xijing Hospital between May 2009 and June 2014. Scar diagnosis was confirmed by pathological examination and cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), at 37 °C as described previously [16]. In this study, we performed our experiments using the third to the fifth passage of HSFBs unless otherwise indicated. For troglitazone analysis, HSFBs were exposed to DMEM alone (control group) or DMEM with 30 mmol troglitazone (Abcam, MA, USA). To determine whether the effects of troglitazone on HSFBs are PPAR-γ-dependent, HSFBs were co-incubated with 1 μmol PPAR-γ antagonist GW9662 (Abcam, MA, USA) for 48 h as described previously [7].

2.2. miRNA transfection

To assess the role of miR-145 in regulation of PPAR-γ-dependent effects on HSFBs, we chemically synthesized miR-145 mimic, miR-145 inhibitor, and control RNA; and purified them using the high-performance liquid chromatography as described previously [17]. We then transfected them into HSFBs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Forty-eight hours later, HSFBs were used for our experiments.

2.3. Quantitative real-time PCR

Total cellular RNA was isolated from HSFBs using the TRIzol reagent (Invitrogen) according to manufacturer's instructions. To detect miR-145 expression, these total RNA samples were reversely transcribed into cDNA using the miScript Reverse Transcription Kit (Qiagen, Hilden, Germany) with a miRNA specific primer and U6 RNA was used as an internal loading control. To assess level of Smad3, these RNA samples were reversely transcribed into cDNA using miScript Reverse Transcription Kit (Qiagen). GAPDH and U6 RNA were used as internal loading controls for mRNA and miRNA, respectively. The following primers were used for PCR amplification: a universal primer (UP) provided with the miScript Reverse Transcription Kit and 5′-GTCCAGTTTTCCCAGGAATCCCT-3′ or 5′-GTGCTCGCTTCGGCAGCACATAT-3′ for miR-145 or U6 RNA, respectively; 5′-TGGACGCAGGTTCTCCAAAC-3′ and 5′-CCGGCTCGCAGTAGGTAAC-3′ for Smad 3; 5′-GCCCAATACGACCAAATCC-3′ and 5′-AGCCACATCGCTCAGACAC-3′ for GAPDH. qPCR reactions were run in triplicate and relative expression levels of miRNA or mRNA were analyzed using the Bio-Rad C1000 Thermal Cycler (Bio-Rad, CA, USA).

2.4. Plasmid construction, gene transfection, and Luciferase reporter assay

The 3′UTR of *Smad3* containing the predicted target sites ofmiR-145 was amplified from HeLa cell cDNA using 5′-GAATTCTCAGGTAACCGTCTTCA-3′ and 5′-CTGCAGAAAGCCACTACACCCTC-3'as primers and then ligated to the pGL3 vector at *Eco*RI and *Pst*I sites to generate the pGL3-Smad3-UTR vector. The PCR products with the appropriate primers generated inserts with point substitutions of the miRNA complementary sites to generate the pGL3-Smad3-UTR-mut vector. Construction of the luciferase reporter containing the putative miR-145 (miR-145-wt) and miR-145 deletion mutant promoter (miR-145-mut) were performed as previously described [18]. All vectors were DNA sequence confirmed before being used in our *in vitro* experiments. After that, we performed luciferase assay using these vectors. The empty vector was used as control. Briefly, HSFBs were cultured in DMEM containing 10% FBS in 48-well plates and then co-transfected with 400 ng pGl3-Smad3-UTR DNA plasmid DNA (firefly luciferase reporter vector containing the survivin 3′-UTR) or pGl3-Smad3-UTR-mut DNA (firefly luciferase reporter vector containing the Smad3 3′-UTR mutant) and 20 ng of the control pRL-TK vector containing Renilla luciferase (Promega, Madison, WI, USA) in a final volume of 0.2 ml using Lipofectamine 2000 (Invitrogen). The Firefly and Renilla luciferase activities were measured 48 h after transfection. Luciferase reporter assay for evaluation of PPAR-γ transcription activity used the Cignal™ PPAR (Luc) reporter assay kit (QIAGEN) as previous report [18].

2.5. Chromatin immunoprecipitation assay (ChIP)

HSFBs were grown and treated with 30 μM troglitazone for 48 h and then washed twice with phosphate buffered saline (PBS) and cross-linked using 1% formaldehyde (BIO-RAD, Hercules, CA, USA) for 15 min at 37 °C. Next, the cells were washed again twice with PBS, lysed, digested with micrococcal nuclease, immunoprecipited, and eluted. After that, the DNA was recovered. ChIP assay was performed using the Pierce™ Agarose ChIP Kit (Thermo Scientific, MA, USA) according to the manufacturer's instructions. An anti-PPAR-γ antibody (Cell Signaling Technology, MA, USA) or Rabbit IgG (as the negative control) were used to pull down protein-bound DNA. ChIp recovered DNA was then amplified by qPCR using primers covered a segment containing −1194 to −1207 bp upstream of the transcription start site of the miR-145 promoter (5′-GCTTCCTTCCTCCTACC-3′ and 5′-ACTGCCCTCTGCTGTCT-3′). ChIP-recovered protein was subjected to Western blotting analysis of Smad3 protein expression as described previously [14] using a rabbit monoclonal anti-Smad3 antibody (Abcam, MA, USA) or mouse monoclonal anti-β-actin antibody (Sigma–Aldrich, WI, USA).

2.6. ELISA detection of collagen type 1 (Col1) levels

The cell growth-conditioned medium from the above experiments was collected and then analyzed for levels of collagen type I triple helix using Human Collagen Type I enzyme-linked

immunosorbent assay (ELISA) kits (ACEL, Kanagawa, Japan). The data were summarized as mean ± SEM.

2.7. Statistical analysis

All statistical analyses were performed using the PRISM Software, Version 4 (GraphPad Software, CA, USA). All data were expressed as the mean ± SEM of at least three separate experiments. The difference between groups was analyzed using two-sided Student's *t*-test and a $p < 0.05$ value was considered statistically significant.

3. Results

3.1. PPAR-γ agonist suppresses expression of Smad3 and Col1 in HSFBs

In this study, we first assessed the effect of PPAR-γ agonist on regulation of Col1 and Smad3 expression in HSFBs. After culture and treatment of HSFBs with 30 μmol of a PPAR-γ agonist troglitazone for 72 h, Col1 expression was significantly reduced at both mRNA and protein levels (Fig. 1A). Interestingly, we also found significant decrease in Smad3 levels, which was parallel to downregulation of Col1 mRNA and protein level (Fig. 1B).

3.2. PPAR-γ-induced miR-145 targets Smad3 expression in HSFBs

We found that PPAR-γ agonist was able to induce miR-145 expression in HSFBs and that miR-145 expression in PPAR-γ agonist-treated HSFBs was significantly upregulated after troglitazone treatment compared to the control cells (Fig. 2A). However, PPAR-γ antagonist GW9662 was able to abolish the effects of troglitazone on miR-145 expression (Fig. 2A). As shown in Fig. 2B, troglitazone-reduced expression of Smad3 mRNA was rescued by transfection of miR-145 inhibitor into HSFBs. Furthermore, GW9662 increased the basal level of Smad3 expression in HSFBs, but transfection of miR-145 mimics was able to downregulate Smad3 mRNA level in these GW9662-treated cells (Fig. 2C).

We performed a luciferase assay using a vector carrying Smad3 cDNA 3′-UTR (Fig. 2D) and found that after inhibition of miR-145 expression using miR-145 inhibitor, the relative luciferase activity was increased by 80% (Fig. 2E). In contrast, the mutated Smad3 cDNA 3′-UTR construct completely abolished this increase (Fig. 2E), suggesting that miR-145 directly binds to Smad3 cDNA 3′-UTR. Furthermore, Western blot data showed that transfection of miR-145 inhibitor not only abolished the effects of PPAR-γ agonist, but also induced Smad3 expression further (Fig. 2F), while miR-145 mimics had the opposite effects (Fig. 2F).

3.3. PPAR-γ transcriptional regulation of miR-145 expression in HSFBs

We assessed whether PPRE consensus sequence is functional to induce miR-145 expression by vector construction (Fig. 3A) and luciferase assay. Our data showed that the transcriptional activity of the plasmid carrying the wild type miR-145 promoter increased upon troglitazone treatment, while there was no change in luciferase activity observed with the mutant plasmid, indicating that the predicted PPRE in miR-145 promoter is responsible for PPAR-γ transactivation of miR-145 expression (Fig. 3B). We then performed a chromatin immunoprecipitation (ChIP) and qRT-PCR assays. The data showed that troglitazone treatment induced PPAR-γ recruitment to miR-145 promoter PPRE to induce miR-145 upregulation compared to controls in HSFBs (Fig. 3C).

3.4. Effects of miR-145 on regulation of Col1 synthesis in HSFBs

After that, we further assessed whether miR-145 expression could regulate Col1 expression in HSFBs. Our data showed that the miR-145 inhibitor suppresses the effects of PPAR-γ agonist on inhibition of Col1 (Fig. 4A), while PPAR-γ antagonist-induced Col1 expression was suppressed by miR-145 mimics (Fig. 4B). Taken together, these ELASA data indicate that potential miR-145-driven pathways may be affected by PPAR-γ agonist.

4. Discussion

In the past decades, emerging evidence has demonstrated that ECM proteins and tissue fibrosis are associated with carcinogenesis and fibrosis diseases [19,20]. PPAR-γ was reported to modulate expression of ECM proteins and therefore, suppress fibrosis [6–8]. In this study, we explored the underlying molecular mechanism. We found that PPAR-γ agonist suppressed expression of Col1 and Smad3 and induced miR-145 targets Smad3 expression in HSFBs. Further study showed that PPAR-γ transcriptionally regulated miR-145 expression and that miR-145 regulated Col1 synthesis through targeting Smad3 expression in HSFBs.

Indeed, previous studies demonstrated that PPAR-γ-regulated ECM protein expression by targeting of the TGF-β/Smad3 and MAPK signaling pathways [6–9]. In the TGF-β/Smad3 pathway, TGF-β1 receptor ligands downregulate Smad3 expression [10] and in turn to suppress skin scar formation [21]. It is necessary to point out that the strategy in our study for collagen synthesis was not in agreement with previous work that the TGF-β1-initialed pathway affected collagen synthesis [22,23]. It is true that TGF-β1 is able to impact several pathways and modulate miRNA expression [20,24]. miR-145 was directly regulated by TGF-β1 treatment [25]. In the current study, we found an additional signaling by which PPAR-γ

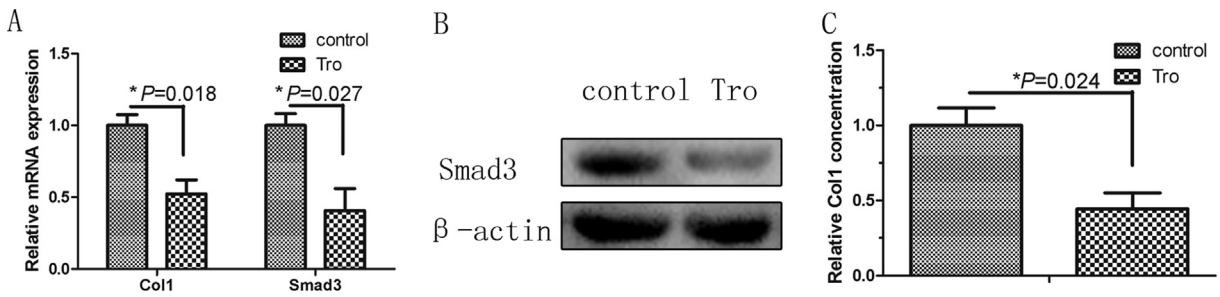

Fig. 1. Effects of troglitazone treatment on regulation of Col1 and Smad3 expression in HSFBs. (A) qRT-PCR. HSFBs were grown and treated with 30 μM of troglitazone for 72 h and then subjected to qRT-PCR analysis of Col1 and Smad3 levels. The experiments were performed in triplicate and repeated three times. Data are presented as means ± SEM. (B) Western blot. The duplicate cells were subjected to Western blot analysis. The graph is quantitative data of Western blots.

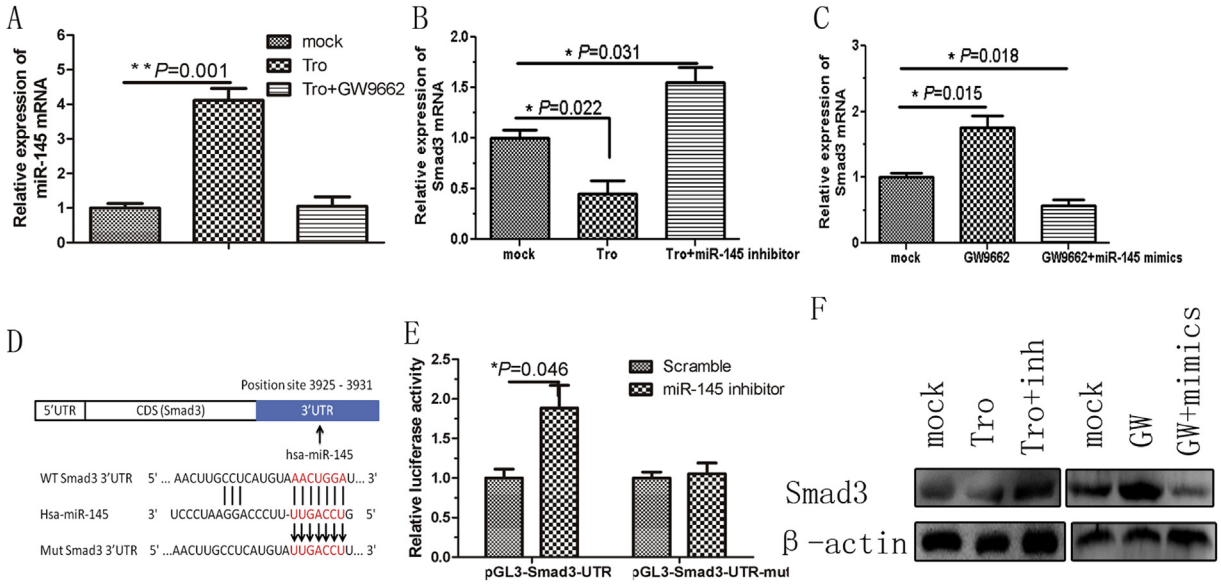

Fig. 2. miR-145 directly targets and inhibits Smad3 expression in HSFBs after treated with troglitazone. (A) qRT-PCR. HSFBs were grown and treated with or without 30 μM of troglitazone and 1 μmolGW9662 for 48 h and then subjected to qRT-PCR. (B) qRT-PCR. HSFBs were grown and treated with 30 μM of troglitazone and then transfected with miR-145 inhibitor for 48 h and subjected to qRT-PCR. (C) qRT-PCR. HSFBs were grown and treated with 30 μM of troglitazone and then transfected with miR-145 inhibitor for 48 h and subjected to qRT-PCR. (D) Alignment of Smad3 3′-UTR with miR-145 sequences. Bioinformatical analysis was performed to identify the interaction of miR-145 with Smad3. (E) Luciferase assay. HSFBs were grown and transiently transfected with wide-type or mutated Smad3 3′-UTR luciferase reporter construct and then with miR-145 inhibitor for 48 h and then subjected to Luciferase assay. (F) Western blot. HSFBs were grown and transiently transfected with miR-145 inhibitor or mimics and treated with or without 30 μM of troglitazone and 1 μmol GW9662 for 48 h and then subjected toWestern blot.

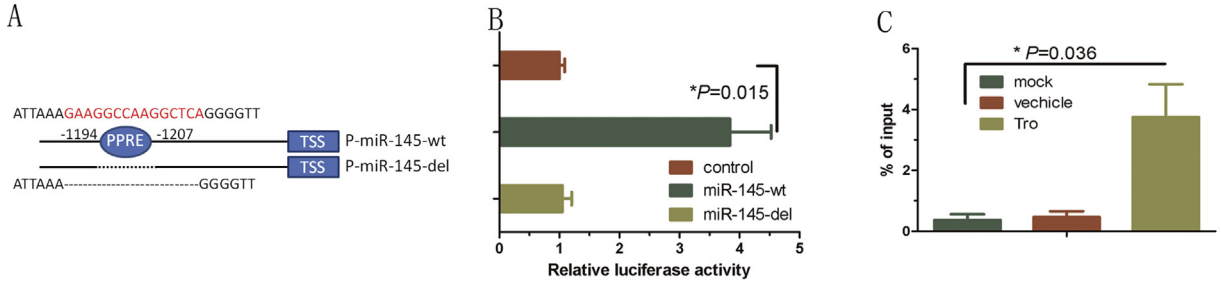

Fig. 3. PPAR-γ transcriptional regulation of miR-145 expression in HSFBs. (A) Bioinformatical analysis of miR-145 promoter containing a putative PPAR response element (PPRE) binding site (between −1194 to−1207 bp). The promoter named P-miR-145, and the other promote P-miR-145-del contains a deleted sequences listed in the bottom. (B) Luciferase assays. HSFBs were grown and transiently transfected with miR-145-wt and miR-145-del reporter construct and then subjected to Luciferase assay. The raw luciferase activity was first normalized to the internal transfection control Renilla. (C) ChIP assay. HSFBs were grown and treated with or without troglitazone subjected to ChIP and qPCR analysis of the PPAR-γ enrichment at the miR-145 promoter (PPRE). The relative enrichment data is normalized to IgG as control. The assay in B and C were performed in triplicate.

inhibition of Smad3 expression was able to upregulate miR-145 expression.

miRNA is a group of endogenous small non-coding RNA molecules that function to silence or knockdown protein translation by binding to complementary sequences in 3′-UTR of the targeting mRNAs to degrade them and/or repress their translation [26]. miR-145 is reported to target multiple genes that inhibited the synthesis of ECM proteins and cell proliferation and migration [27–29]. These

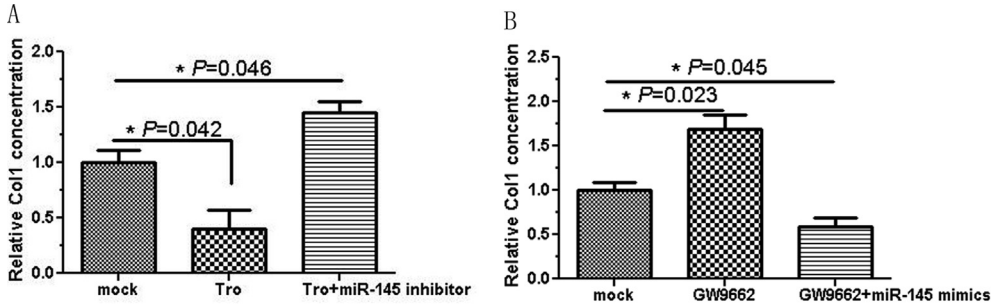

Fig. 4. Effect of miR-145 on Col1 expression in HSFBs. (A) ELISA. The amount of Col1 released from cultured HSFBs was detected at 72 h after treated with troglitazone or transiently transfected miR-145 inhibitors. (B) ELISA. The amount of Col1 released from cultured HSFBs was detected at 72 h after treatment with GW9662 or transiently transfected miR-145 mimics.

findings pretty well fit our hypothesis and support our current data, suggesting that PPAR-γ-regulated Col1 synthesis was by regulation of the PPAR-γ-miR-145-Smad3 axis in HSFBs. However, opposite data do occur; for example, a previous study showed that overexpression of miR-145 was recently described as a fibrosis promoter in lung fibrosis [30]. In that study, Yang et al. found miR-145 overexpression in lung fibroblasts increased SMA-α expression, enhanced contractility, and promoted formation of focal and fibrillar adhesions, while miR-145 inhibition diminished TGF-β1-induced SMA-α expression. MiR-145 expression was not affecting TGF-β1 activity, but promoting activation of the latent TGF-β1 [30]. This study used a lung fibroblast as a model to study miR-145-TGF-β1 signaling in lung fibrosis; the remaining question or concern is that this study didn't include PPAR-γ and Smad3 in the picture. Thus, it is essential for us to fully assess the biological functions and molecular mechanism of miR-145 action.

Conflict of interest

The authors declare that there is no conflict of interest in this work.

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