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Original Article

Silencing miR-1291-LEFTY2 axis diminishes the myofibroblast activities and reactive oxygen species generation of fibrotic buccal mucosal fibroblasts

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KEYWORDS
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Abstract *Background/purpose:* Oral submucous fibrosis (OSF) is a potentially malignant disorder characterized by chronic inflammation and excessive collagen deposition, leading to fibrosis in the oral mucosa. This study aimed to explore the contribution of the miR-1291/LEFTY2 axis in the development of OSF progression.
Materials and methods: Expression of miR-1291 was evaluated in OSF tissues and primary myofibroblasts using RNA sequencing and qRT-PCR. The functional role of miR-1291 and LEFTY2 were investigated using miR-1291 inhibitor and lentiviral-mediated overexpression of LEFTY2, respectively. A luciferase reporter assay was conducted to examine the direct interaction between miR-1291 and LEFTY2. Myofibroblast activities were assessed by collagen gel contraction, wound healing, and transwell migration assays. Reactive oxygen species (ROS) production was measured by flow cytometry.

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Results: MiR-1291 was markedly upregulated in OSF tissues and myofibroblasts, and it was positively correlated with a couple of fibrosis markers, including α-SMA and TGF-β1. Inhibition of miR-1291 suppressed myofibroblast activities and ROS generation. Luciferase reporter assays confirmed that miR-1291 is directly bound to the three prime untranslated region (3′UTR) of LEFTY2, a negative regulator of TGF-β signaling. Overexpression of LEFTY2 attenuated phosphorylation of Smad, myofibroblast activities, and ROS production.
Conclusion: Our findings demonstrated that miR-1291 may promote fibrosis in OSF by suppressing LEFTY2 expression to increase myofibroblast activation via regulation of ROS accumulation and TGF-β/Smad signaling.

Introduction

Oral submucous fibrosis (OSF) is a precancerous disorder in the oral cavity with a malignant transformation rate of around 5 % in a retrospective cohort study between January 1973 and March 2024.[1] As the most prevalent oral potentially malignant disorders,[2] OSF has been known to be associated with the consumption of areca nut which leads to the accumulation of collagen deposition in the oral mucosa. Various cellular components coordinate fibrogenesis following injury, with myofibroblasts serving as the main extracellular matrix-secreting cells responsible for wound closure.[3] It has been proven that arecoline (a major areca nut alkaloid) induces the expression of a marker of myofibroblasts, α-smooth muscle actin (α-SMA), in buccal mucosal fibroblasts (BMFs).[4] Also, inflammatory niche and cytokines affect myofibroblast transdifferentiation.[5] Several studies have revealed that areca nut induces the activation of the transforming growth factor-β (TGF-β)/Smads pathway that contributes to the development of OSF.[6–8] Another study showed that arecoline induces latent TGF-β1 via mitochondrial reactive oxygen species (ROS) in BMFs,[9] and lower production of ROS has been shown to be accompanied by reduced myofibroblast activities.[10,11] Nevertheless, the detailed regulatory mechanism underlying the persistent activation of myofibroblasts in OSF still awaits elucidation.

In recent years, many studies have focused on the significance of non-coding RNAs in fibrotic diseases, as they can regulate myofibroblast transdifferentiation via various modes. The current consensus is that non-coding RNAs with modulatory roles can be divided into two categories by size: short non-coding RNAs (such as microRNAs; ∼21–23 nucleotides) and long non-coding RNAs (lncRNAs; more than 200 nucleotides). These non-coding RNAs are not translated into protein but possess the ability to post-transcriptionally regulate gene expression.[12] It has been revealed that microRNAs can bind to complementary sequences in mRNA molecules, thereby silencing these mRNA molecules through destabilizing, cleaving mRNAs or reducing their translation.[13] A large body of evidence suggests that numerous microRNAs involve in the regulation of ROS accumulation and myofibroblast transdifferentiation in OSF.[10,11,14,15] Since up to 1500 microRNAs have been identified in the human genome,[16] deciphering the potential role of unexplored microRNAs in OSF will contribute to a deeper understanding of microRNA-mediated pathogenesis in oral fibrogenesis.

MicroRNA-1291 (miR-1291) is localized within the SNORA34 (small nucleolar RNA H/ACA box 34) and has been reported to exert tumor-suppressive effects on multiple types of cancers, such as pancreatic cancer,[17] renal cancer,[18] and colorectal cancer.[19] In addition, it has been revealed that miR-1291 is involved in multidrug resistance as it regulates drug disposition and chemosensitivity via direct downregulation of ABCC1 expression.[20] Emerging evidence has shown that miR-1291 may also play a role in the development of fibrosis diseases. For instance, miR-1291 has been found to promote endometrial fibrosis, as evidenced by the amelioration of fibrosis through the use of miR-1291 antagomir in a murine model.[21] Another study showed that miR-1291 was upregulated in the TGF-β1-stimulated LX-2 cells (hepatic stellate cells; HSCs), as well as in tissues from mice and patients with hepatic fibrosis.[22] Besides, the expression levels of α-SMA and type I collagen were decreased in LX-2 cells overexpressing miR-1291.[22] Accordingly, we speculate that miR-1291 may also have the ability to regulate the development of OSF.

In the current study, we first examined the expression of miR-1291 in OSF tissues and fibrotic BMFs (fBMFs) derived from OSF specimens. Subsequently, we evaluated the functional role of miR-1291 in myofibroblast activities and investigate the potential mechanisms.

Materials and methods

Tissue collection and cell culture

Following approval by the Institutional Review Board of Chung Shan Medical University Hospital and informed consent from each participant, paired specimens of fibrotic buccal mucosa and adjacent normal mucosa were collected from OSF patients undergoing surgical resection. Primary fibroblasts were isolated from both OSF and normal tissues. Briefly, tissues were washed with phosphate-buffered saline (PBS), minced into 1 mm^2-sized pieces, and incubated with 0.05 % trypsin–EDTA (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) for 30–60 min. After centrifugation, the tissue pellets were resuspended in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin (Gibco). Fibroblasts, identified by their spindle-shaped morphology, were cultured at 37 °C in a humidified 5 % CO_2 atmosphere. Cells from passages 3–8 were used for all experiments.

Quantitative reverse transcription polymerase chain reaction

Tissue samples obtained from surgical resection were immediately snap-frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from the isolated RNA using the Superscript III First-Strand Synthesis System (Invitrogen Life Technologies). qRT-PCR was performed on an ABI StepOne™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) using the following primers: **α-SMA:** 5′-AGCACATGGAAAAGATCTGGCACC-3' (forward) and 5′-TTT TCTCCCGGTT GGCCTTG-3' (reverse);[14] **TGF-β1:** 5′-TCCGA-GAAGCGGTACCTGAA-3' (forward) and 5′-TGCTGTCACAG GAGCAGTGG-3' (reverse);[23] **GAPDH:** 5′-CTCATGAC CACAG TCCATGC-3' (forward) and 5′-TTCAGCTCTGGGATGACCTT-3' (reverse). Pearson's correlation analysis was used to assess the relationship between miR-1291 expression and the expression levels of fibrotic genes.

MiR-1291 overexpression or inhibition

MiR-1291 mimic, miR-1291 inhibitor, and a non-targeting miR-scramble control were obtained from ThermoFisher Scientific (Carlsbad, CA, USA). Cells were transfected with the miR-1291 mimic or inhibitor using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The miR-scramble served as a negative control for transfection. miR-1291 expression levels were determined by qRT-PCR using TaqMan miRNA assays with specific primer sets (Applied Biosystems).

Collagen contraction assay

Myofibroblast contractility was assessed using a collagen contraction assay (Sigma—Aldrich, Merck, Darmstadt, Germany). Briefly, 2×10^5 cells were suspended in a 2 mg/mL collagen gel solution, and 0.5 mL of mixture was added to a 24-well plate. The plate was incubated at 37 °C for 2 h to allow for collagen polymerization. Polymerized gels were then gently detached from the well walls using a 200 μL pipette tip and incubated in the MEMα medium for 48 h. Images of the gels were captured at a fixed distance using a digital camera, and the change in gel size was quantified using ImageJ software (NIH, Bethesda, MD, USA).[6]

Wound healing assay

Cells were seeded in a 12-well plate and grown to confluence. A sterile 200 μL pipette tip was then used to create a linear scratch across the cell monolayer, generating a cell-free zone. The cells were washed to remove debris and incubated for 24 h to allow for migration into the wounded area. Images of the wound were captured at 0 and 24 h using a camera. Cell migration was quantified by measuring the closure of the wound area using image analysis software.[14]

Transwell migration assays

Cells were seeded in the upper chamber of a Transwell insert (Corning, Acton, MA, USA) in a serum-free medium. The lower chamber was filled with medium containing 10 % FBS to establish a gradient. After 24 h of incubation, non-migratory cells on the upper surface of the membrane were removed, and the cells that had migrated to the lower surface were fixed and stained with crystal violet. The number of migrated cells was quantified by counting five to ten randomly selected fields under a microscope.

Reactive oxygen species production

ROS production was measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), a fluorescent probe that is oxidized to 2′,7′-dichlorofluorescein (DCF) by intracellular ROS, particularly H_2O_2 and NO-based radicals.[1] Cells were incubated with 10 μM DCFH-DA for 60 min at 37 °C and then washed twice with PBS. DCF fluorescence was measured in 10,000 cells using a flow cytometer (BD, Becton Drive Franklin Lakes, NJ, USA) with excitation and emission wavelengths of 488 nm and 525 nm, respectively.

Western blot analysis

Whole-cell lysates were prepared using 10X RIPA buffer (Millipore, Merck, Darmstadt, Germany). Protein samples (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). Membranes were blocked with 5 % bovine serum albumin (Millipore) and incubated overnight at 4 °C with primary antibodies. After washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature.[1] Immunoreactive bands were visualized using enhanced chemiluminescence (ECL) substrate (Millipore) and a LAS-1000 plus analyzer (GE Healthcare Biosciences, Piscataway, NJ, USA). GAPDH served as a loading control for normalization. The antibodies used in this study include: mouse anti-GAPDH (MA5-15738), rabbit anti-smad (#51—1300), rabbit anti-p-smad (#44-244G) (Thermo Fisher Scientific, Waltham, MA, USA), mouse anti-α-SMA (sc-33251) (Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-LEFTY2 (ab229668) (Abcam, Cambridge, MA, USA).[24]

Luciferase reporter activity assay

The wild-type LEFTY2 three prime untranslated region (3′UTR) was cloned into a luciferase reporter plasmid. A mutant reporter construct was generated by site-directed mutagenesis, replacing the predicted miR-1291 binding site (CAGGGCC) with AUACCGG. Cells were co-transfected with the wild-type or mutant reporter plasmid along with either the miR-1291 mimic or a non-targeting miR-scramble control using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured and normalized to a co-transfected Renilla luciferase plasmid to control for transfection efficiency.

Lentiviral-mediated LEFTY2 overexpression

To overexpress LEFTY2, its cDNA was cloned into the pCDHI-MCS1-EF1-CopGFP lentiviral expression vector (System Biosciences, Mountain View, CA, USA). Lentiviral particles were produced by co-transfecting 293T cells with the LEFTY2-expressing plasmid and two helper plasmids (packaging and envelope plasmids) using Lipofectamine 2000 (Thermo Fisher Scientific). Following lentiviral transduction of target cells, GFP-positive cells were isolated by flow cytometry (BD Biosciences) to ensure successful transduction. Overexpression of LEFTY2 was confirmed by Western blot analysis. An empty pCDHI-MCS1-EF1-CopGFP vector served as a negative control.

Statistical analysis

Statistical analyses were performed using SPSS software (version 13.0; SPSS Inc., Chicago, IL, USA). Data are presented as the mean ± standard error of the mean (SEM) from at least three independent experiments. Student's t-test was used to compare differences between two groups. A *P*-value of less than 0.05 was considered statistically significant.

Results

The expression of miR-1291 is upregulated in OSF specimens and positively associated with fibrotic markers

As shown in Fig. 1A, the expression of miR-1291 was markedly higher in OSF tissues compared to normal counterparts using RNA sequencing. qRT-PCR analysis was used to validate that the expression of miR-1291 was increased in OSF specimens (Fig. 1B) and fBMFs (Fig. 1C) compared to normal tissues and BMFs, respectively. Moreover, we found that there was a positive correlation between the expression of miR-1291 and several fibrotic markers, including α-SMA (a hallmark of mature myofibroblasts) (Fig. 1D) and TGF-β1 (a major driver of fibrosis) (Fig. 1E). These results suggested that miR-1291 may be implicated in promoting fibrotic diseases.

Suppression of miR-1291 attenuates various myofibroblast activities

To investigate the functional role of miR-1291, we examined the effect of miR-1291 inhibitors on several myofibroblast activities and ROS generation. Wound closure is one of the features mediated by myofibroblasts as the wound matures and contractile forces elevate following myofibroblast activation. We showed that the collagen gel areas were increased in fBMFs transfected with miR-1291 inhibitors, indicating that the contractile forces of fBMFs decreased when miR-1291 was blocked (Fig. 2A). Given that activated myofibroblasts migrate into the lesion to aid in wound closure, we then conducted wound healing and transwell migration assays to examine cell mobility. Our results demonstrated that downregulation of miR-1291 diminished the wound healing capacity (Fig. 2B) and migration ability (Fig. 2C). In addition, we assessed ROS production using DCFH-DA, a cell-permeable and non-fluorescent precursor of DCF, as an intracellular probe for oxidative stress followed by flow cytometry. The proportion of cells with bright fluorescence was calculated and we showed that fBMFs with miR-1291 inhibitor exhibited substantially lower levels of ROS production (Fig. 2D). Collectively, our data demonstrated that

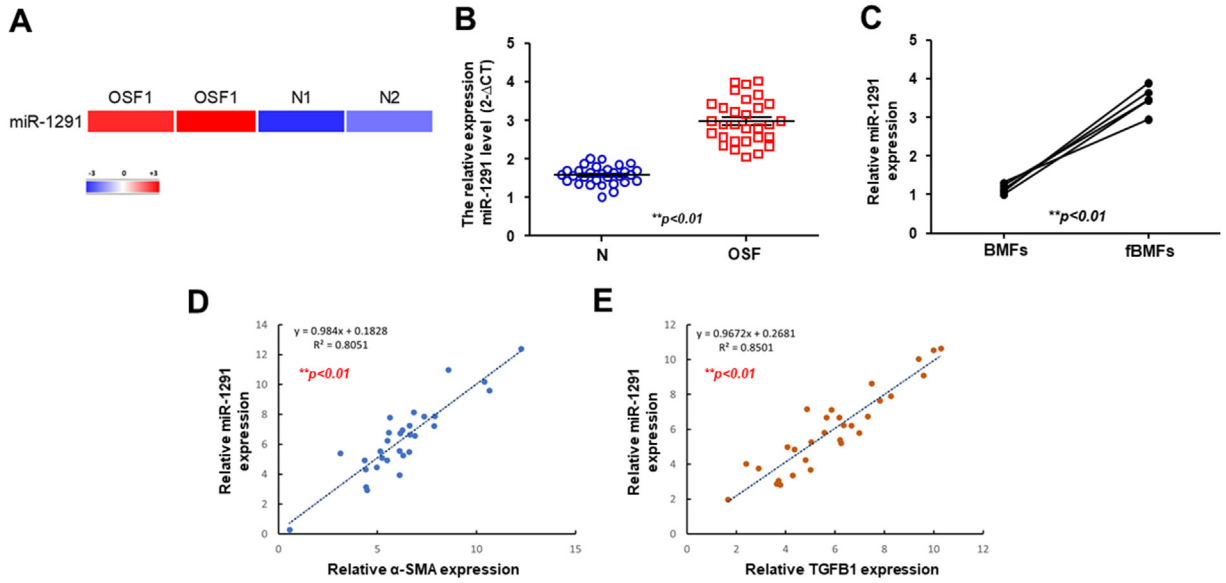

Figure 1 MiR-1291 is upregulated in OSF and correlates with fibrosis markers. (A) A heatmap showing differentially expressed miR-1291 in OSF tissues (OSF1, OSF2) and normal tissues (N1, N2) by RNA sequencing. (B) qRT-PCR analysis of miR-1291 expression in OSF tissues (n = 30) and normal tissues (n = 30). (C) qRT-PCR analysis of miR-1291 expression in fibrotic buccal mucosal fibroblasts (fBMFs) isolated from OSF tissues and buccal mucosal fibroblasts (BMFs) from normal tissues. The relationship between miR-1291 expression and the expression of α-SMA (D) and TGF-β1 (E) in OSF tissues was assessed by Pearson correlation measures.

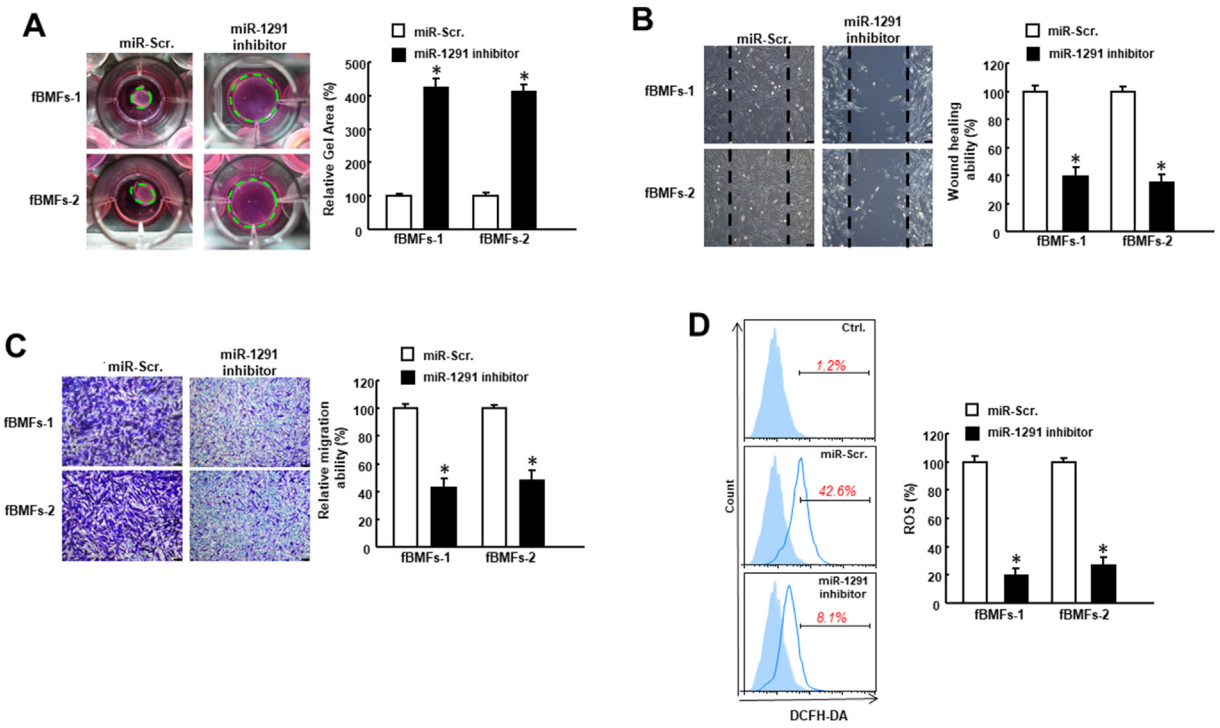

Figure 2 Inhibition of miR-1291 suppresses myofibroblast activities and ROS generation in fBMFs. (A) Collagen gel contraction assay showing the effect of miR-1291 inhibition on fBMF contractility. (B) Wound healing and (C) transwell migration assays were conducted to evaluate the effect of miR-1291 inhibition on fBMF migration and proliferation. (D) fBMFs transfected with miR-1291 inhibitor or scrambled control were stained with the fluorescent DCFH-DA probe, followed by flow cytometry analysis to detect ROS production. *$P < 0.05$ compared with miR-Scramble (miR-Scr.).

suppression of miR-1291 weakened the myofibroblast activities of fBMFs.

MiR-1291 may regulate the expression of LEFTY2/p-Smad2 signaling

We predicted that left-right determination factor 2 (LEFTY2) is one of the target genes of miR-1291 using TargetScan analysis, and the binding site of miR-1291 was identified within the 3′UTR of LEFTY2 (Fig. 3A). A luciferase reporter assay was performed using a wild-type LEFTY2 3′UTR reporter construct and a mutant construct with a disrupted miR-1291 binding site. Co-transfection of the miR-1291 mimic with the wild-type reporter significantly reduced luciferase activity, whereas the mutant reporter was unaffected (Fig. 3B). Aside from demonstrating the possible interaction between miR-1291 and LEFTY2, we also showed that inhibition of miR-1291 in fBMFs downregulated the expression of α-SMA and phosphorylated Smad 2 (p-Smad 2) while upregulating LEFTY2 (Fig. 3C). Based on these findings, we postulated that miR-1291 may regulate myofibroblast activation through interrupting the TGF-β1/Smads pathway via direct binding to LEFTY2.

Overexpression of LEFTY2 mitigates myofibroblast activation and ROS production

In an effort to verify our hypothesis, we assessed the expression of p-Smad2 and LEFTY2 in fBMFs with lentiviral-mediated overexpression of LEFTY2. We showed that the expression of p-Smad2 was abrogated in fBMFs when LEFTY2 was overexpressed (Fig. 4A). Besides, the collagen gel contraction capacity was alleviated in fBMFs with ectopic expression of LEFTY2 (Fig. 4B). Similarly, the transwell migration ability of fBMFs was reduced following LEFTY2 upregulation (Fig. 4C). Furthermore, we demonstrated that ROS generation was markedly diminished in fBMFs overexpressing LEFTY2 (Fig. 4D). Taken together, our results suggested that the aberrantly upregulated miR-1291 in OSF may confer persistent myofibroblast activation via negatively regulating LEFTY2 and subsequently modulating the TGF-β1/Smads signaling.

Discussion

To date, there is still a lack of investigation on miR-1291 in precancerous OSF or head and neck cancers. In the present study, we showed that miR-1291 was aberrantly overexpressed in OSF tissues and fBMFs. We not only found that miR-1291 was positively correlated with fibrosis markers (α-SMA and TGF-β1), but also demonstrated that miR-1291 may induce myofibroblast activation through direct regulation of LEFTY2, affecting the TGF-β1/Smads pathway and modulating ROS production. Our results were consistent with previous studies showing that miR-1291 was upregulated in tissues of endometrial fibrosis[21] and hepatic fibrosis.[22]

We showed that suppression of miR-1291 attenuated the expression α-SMA and phosphorylated Smad, myofibroblast

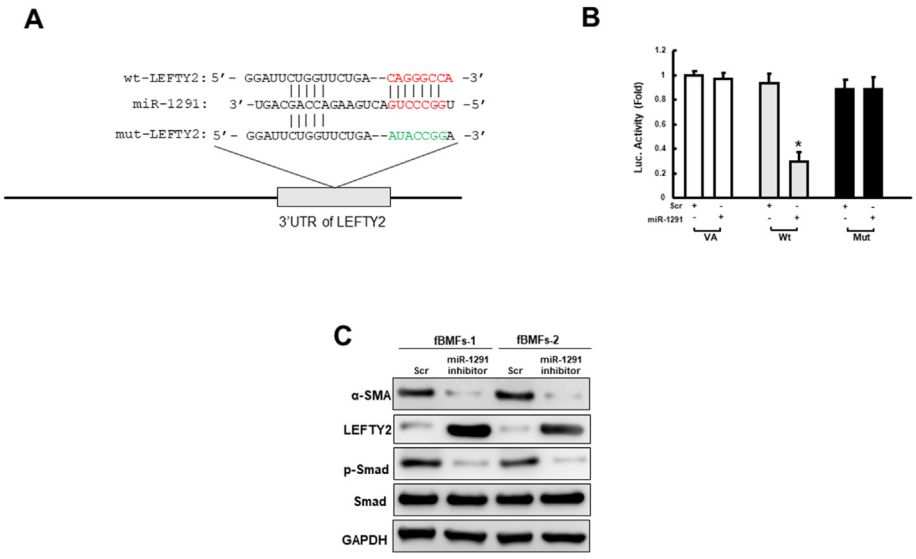

Figure 3 MiR-1291 directly targets LEFTY2 and affects the phosphrylation of Smad2. (A) Schematic showing the predicted miR-1291 binding site within the 3′UTR of LEFTY2 (TargetScan). The mutated sequence used in the luciferase reporter assay is also shown. (B) A luciferase reporter assay showing the effect of miR-1291 on the activity of wild-type (Wt) and mutant (Mut) LEFTY2 3′UTR reporter constructs. VA, vector alone. (C) Western blot analysis of α-SMA, LEFTY2, phosphorylated Smad2 (p-Smad), and total Smad2 in fBMFs transfected with miR-1291 inhibitor or scrambled control. *$P < 0.05$ compared with miR-Scr.

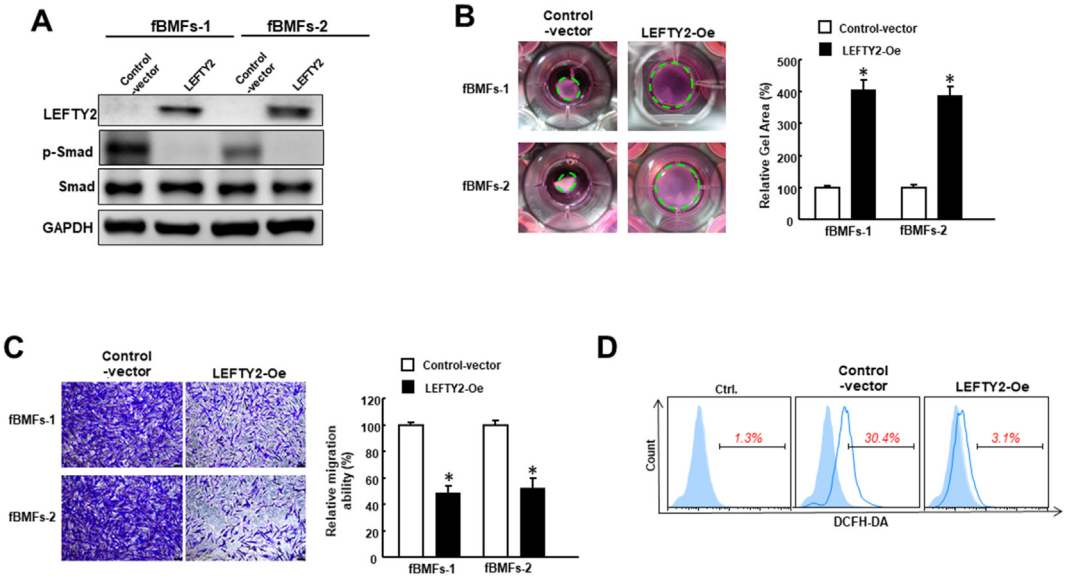

Figure 4 Overexpression of LEFTY2 attenuates myofibroblast activities and ROS production. (A) Western blot analysis of LEFTY2, phosphorylated Smad2 (p-Smad), and total Smad2 in fBMFs with lentiviral-mediated overexpression of LEFTY2 (LEFTY2-Oe) or control vector. (B) Collagen gel contraction and (C) transwell migration assays were carried out to assess the effect of LEFTY2 overexpression on fBMF migration and proliferation. (D) Flow cytometry analysis of ROS production in fBMFs with LEFTY2 overexpression or control vector. *$P < 0.05$ compared with control vector.

activities and ROS production in fBMFs, which was in line with the results of lower α-SMA and type I collagen expression in HSCs (the main source of myofibroblasts in liver fibrosis) overexpressing miR-1291.[22] The research on the role of miR-1291 in oxidative stress is still limited, and our findings indicated that the regulatory effects of miR-1291 on ROS generation may be mediated by LEFTY2 as we showed that overexpression of LEFTY2 resulted in downregulation of ROS.

The human LEFTY locus consists of a potential pseudogene and two functional genes, LEFTY1 and LEFTY2.[25] Although LEFTY proteins are members of the TGF-β family, they have a unique structure compared to other TGFβ molecules as they lack a long α-helix and a critical cysteine

residue that is essential for stabilizing TGF-β homodimers and heterodimers.[26] One of the previous studies has shown that LEFTY1 inhibits the TGF-β1-induced fibroblast-myofibroblast transdifferentiation of normal rat kidney interstitial fibroblast cells.[27] In agreement with this finding, we demonstrated that overexpression of LEFTY2 in fBMFs downregulated myofibroblast activities, phosphorylation of Smad and ROS generation. LEFTY proteins have been found to inhibit a subset of TGF-β signaling by antagonizing EGF-CFC coreceptors.[28] Additionally, LEFTY proteins are known to act as negative modulators of Nodal signaling, which involves phosphorylation and activation of the effectors Smad2/3.[29—31] A previous study has demonstrated that LEFTY2 blocked the activation of HSCs and liver fibrosis by regulating the TGF-β1/Smad3 pathway.[32] Our finding was in accordance with these studies and showed that ectopic expression of LEFTY2 in fBMFs abrogated phosphorylation of Smad2. The expression of LEFTY2 was downregulated in fibrosis liver tissues,[22,32] which was consistent with our results that almost no expression of LEFTY2 was detected in two fBMFs. However, whether its expression was dysregulated in OSF tissues needs further assessment. Likewise, LEFTY2 has been reported as one of the oxygen-sensitive transcriptional programs in human embryonic stem cells,[33] but its implication in ROS generation or oxidative stress requires further investigation. Despite the significant role of LEFTY2 in regulating TGF-β, the involvement of the miR-1291/LEFTY2 axis in lung or kidney fibrosis remains underexplored; however, our OSF findings suggest a conserved mechanism potentially applicable to other tissues. This highlights the miR-1291/LEFTY2 axis as a promising target for cross-tissue antifibrotic therapies, warranting further investigation into its role in diverse fibrotic conditions.

While our study demonstrates the effects of miR-1291 inhibition on myofibroblast activation and ROS production at a single time point, the effect over prolonged periods remains unexplored due to the scope of the current work. Previous studies suggest that modulating miRNAs to suppress TGF-β signaling has been shown to produce sustained antifibrotic outcomes in the short-to-intermediate term (miR-29b reducing fibrosis *in vivo* for 28 days).[34] The direct targeting of LEFTY2 by miR-1291 in our study supports the potential for stable suppression of TGF-β/Smad signaling. However, investigations assessing the long-term dynamics of miR-1291 inhibition could further elucidate its therapeutic durability in OSF.

Considering that areca nut-induced ROS accumulation in BMFs[9] and activation of the TGF-β/Smads pathway contributes to the development of OSF,[6—8] we then infer that the aberrantly overexpressed miR-1291 in OSF may contribute to persistent myofibroblast activation via direct targeting LEFTY2 to affect ROS accumulation and TGF-β/Smads signaling. In the future, research could focus on unraveling how miR-1291/LEFTY2 influences ROS production and whether targeting this axis can delay the progression of OSF.

Declaration of competing interest

All authors have no conflicts of interest relevant to this article.

Acknowledgments

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