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Original Article

Targeting microRNA-190a halts the persistent myofibroblast activation and oxidative stress accumulation through upregulation of Krüppel-like factor 15 in oral submucous fibrosis

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KEYWORDS
Oral submucous fibrosis;

Abstract *Background/purpose:* Oral submucous fibrosis (OSF) is a condition characterized by inflammation and excessive collagen deposition, which has been identified as a potentially malignant disorder. Recently, several microRNAs (miRNAs) have been shown to be implicated in various disorders associated with fibrosis. However, how these miRNAs modulate OSF

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development is poorly understood. Therefore, the study aimed to identify the specific miRNAs that contribute to the progression of OSF and to investigate their molecular mechanisms in promoting fibrosis.

Materials and methods: The expression and clinical significance of potential pro-fibrosis miRNA in the OSF cohort and primary buccal mucosal fibroblasts were confirmed through RNA sequencing and qRT—PCR. Luciferase reporter activity assay, miRNA mimic or inhibitor, and short-hairpin RNA silencing were used to elucidate the molecular mechanism of miRNA. Transwell migration, collagen contraction, and reactive oxygen species (ROS) generation detection were used to investigate the effects of this mechanism on the myofibroblast phenotype and cellular pro-fibrosis capacity.

Results: This study demonstrated that miR-190a was overexpressed in fibrotic buccal mucosal fibroblasts (fBMFs). Transfecting fBMFs with miR-190a inhibitor resulted in reduced cell migration, collagen gel contraction, ROS generation, and expression of fibrotic markers. Furthermore, miR-190a exerted this pro-fibrosis property by direct binding to its target, Krüppel-like factor 15 (KLF15). The results also indicated that the aberrant upregulation of miR-190a, in turn, downregulated the expression of KLF15, which resulted in the activation of myofibroblast.

Conclusion: Our findings demonstrated that miR-190a was involved in myofibroblast activation, suggesting that targeting the miR-190a/KLF15 axis may be a feasible approach in the therapy of OSF.

Introduction

Oral submucous fibrosis (OSF) is a chronic oral disorder that possesses the potential to malignantly transform into oral cancer. Persistent inflammation contributed to the formation of scar tissue in the oral mucosa, resulting in blanching, redness, and stiffness of the mucosal membrane.[1] Epidemiological studies have documented areca nut chewing as the primary etiological factors in contributing to OSF.[2] OSF is distinguished by an atypical accumulation of collagen and rigidity in the lips, tongue, and palate, which results in limited mouth opening (trismus), burning sensation, and difficulty in swallowing.[1] The high incidence of OSF is widely known in Southeast Asia. Yang et al. found that the prevalence of OSF doubled in Taiwan from 1996 to 2013,[3] and numerous therapeutic approaches have been employed to alleviate symptoms, such as the utilization of anti-inflammatory,[4] anti-oxidant agents,[5] physical therapy, and surgical intervention.[6] Nevertheless, these therapies have limitations in controlling OSF efficiently. Hence, it is imperative to elucidate the molecular mechanism behind OSF pathogenesis.

The expression of α-smooth muscle actin (α-SMA) has been recognized as a hallmark of mature myofibroblasts.[7] Due to their capacity to synthesize components of extracellular matrix (ECM; e.g. type I collagen alpha 1),[8] the contractile myofibroblasts have been found to play a pivotal role in the pathogenesis of fibrotic illnesses.[7]

Consistent myofibroblast activation also occurs during OSF progression, along with the accumulation of ECM as a result of an imbalance of collagen synthesis and degradation.[9—11] It is well known that activation of the transforming growth factor beta (TGF-β) pathway by areca nut constituents is a major event to induce myofibroblast transdifferentiation.[10] Moreover, arecoline-induced mitochondrial reactive oxygen species (ROS) contributed to the activation of TGF-β1 in buccal fibroblasts.[11]

MicroRNAs (miRNAs) are composed of 20—22 nucleotides which are central regulators for post-transcriptional modifications.[12] MiRNAs exert regulatory control on messenger RNA (mRNA) expression by binding to their 3 prime-untranslated region (3′-UTR) and causing degradation or inhibition of the process of translation. Given that miRNAs regulate diverse biological mechanisms including cellular proliferation, differentiation, and apoptosis,[13] it is widely acknowledged that aberrant expression of miRNAs contributes to the pathogenesis of various diseases, including OSF.[14,15] In the present study, we aimed to investigate the expression of miR-190a in myofibroblasts and OSF samples as miR-190a has been proven to promote the migration capacity of hypertrophic scar-derived fibroblasts,[16] but its significance in OSF development has not been evaluated. In addition, we examined the potential impact of miR-190a suppression on myofibroblast activity and oxidative stress. Last, we discovered a putative target of miR-190a and further ascertained its expression and impact on the activation of myofibroblasts.

Materials and methods

Tissue collection, cell culture, and reagents

Specimens of fibrotic buccal mucosa (OSF; n = 25) and adjacent normal mucosa (N; n = 25) were collected from patients undergoing resection at the Department of Dentistry, Chung Shan Medical University Hospital according to a procedure approved by the Institutional Review Board

in Chung Shan Medical University Hospital, Taichung, Taiwan, and obtained the informed written consent from each individual. Normal buccal mucosal fibroblasts (BMFs) and fibrotic BMFs (fBMFs) were respectively obtained from the normal and fibrotic buccal mucosa specimens of the same patients. After being washed with phosphate-buffered saline (PBS), tissues were minced into 1 mm^2-sized pieces and incubated with Trypsin-EDTA (0.05%) for 30—60 min. Removed the supernatants after the centrifugation at 1200 rpm for 5 min; the tissue pellets were suspended in culture medium containing 90% [v/v] Dulbecco's Modified Eagle's Medium (DMEM), 10% [v/v] fetal bovine serum (FBS), and 1 % [v/v] penicillin-streptomycin (Gibco, Grand Island, NY, USA). Cells with a spindle-shaped morphology that migrated from the tissue were identified as fibroblasts. All cells were routinely maintained in a culture medium at 37 °C/5% CO_2, and cells at the third to eighth passages were used in the following experiments.

Quantitative reverse transcription polymerase chain reaction

For the preparation of clinical specimens, tissues excised immediately from the surgery were placed in liquid nitrogen and stored frozen at −80 °C. Total RNA is prepared from tissues and cells using Trizol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Quantitative reverse transcription polymerase chain reaction (qRT—PCR) of mRNAs were reverse-transcribed using the Superscript III first-strand synthesis system for RT—PCR (Invitrogen). QRT—PCR reactions on resulting complementary DNAs (cDNAs) were performed on an ABI StepOne™ Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). The primer sequences are listed below: α-SMA, 5′-AGCACATGGAAAAGATCTGGCACC-3' (forward) and 5′-TTTTCTCCCGGTTGGCCTTG-3' (reverse); COL1A1, 5′-GGGTGACCGTGGTGAGA-3' (forward) and 5′-CCAGGAGAGCCAGAGGTCC-3' (reverse); GAPDH, 5′-CTCATGACCACAGTCCATGC-3' (forward) and 5′-TTCAGCTCTGGGATGACCTT-3' (reverse). Pearson's correlations analysis between miR-190a expression and fibrotic gene levels were determined by qRT—PCR analysis.

Micro RNA-190a overexpression or inhibition

Synthesized oligonucleotides of miR-190a mimic, miR-190a inhibitor, and miR-scramble were purchased from ThermoFisher Scientific (Waltham, MA, USA). To overexpress and inhibit the endogenous miR-190a, miR-190a mimic and miR-190a inhibitor were transfected into cells, respectively, using Lipofectamine 2000 in accordance with the manufacturer's instructions (LF2000, Invitrogen), while the miR-scramble was used as a negative control. For miR-190a levels detection, qRT—PCR was performed using TaqMan miRNA assays with specific primer sets (Applied Biosystems).

Collagen contraction assay

Cells were suspended in 0.5 ml of 2 mg/ml collagen solution (Sigma-Aldrich, St. Louis, MO, USA) and added into one well of a 24-well plate. The plate was incubated at 37 °C for 2 h, which caused the polymerization of collagen cell gels. After detaching the gels from the wells, the gels were further incubated in 0.5 ml medium for 48 h. The contraction of the gels was photographed and measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA) to calculate their areas.

Wound healing assay

Once the cells in a 12-well culture plate had reached around 80% confluence, the monolayer was scraped across the center of the well using a sterile 200 μL pipette tip to create a denuded region. After allowing the cells to develop for an additional 24 h, the cell migration toward the incision site was captured on camera at 0 and 24 h.

Transwell migration assays

Cells were exposed to serum-free media in the upper chamber of a transwell (Corning, NY, USA). A 10% FBS medium was added to the lower chamber, followed by a 24-h incubation period for the cells. Crystal violet was used to stain the cells that were connected to the other side of the membrane. A random selection of five to ten fields was counted.

Reactive oxygen species generation analysis

The reactive oxygen species (ROS) generation was assessed by flow cytometry as the fluorescence of 2′,7′-dichlorofluorescein (DCF) which are the oxidation products of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) with a sensitivity for H_2O_2/NO-based radicals (Invitrogen). Cells were incubated with 10 μM DCFH-DA for 60 min at 37 °C then washed twice with PBS. DCF fluorescence of 10,000 cells were analyzed by flow cytometry (Becton—Dickinson and Company, Franklin Lakes, NJ, USA) at excitation and emission wavelengths of 488 and 525 nm, respectively.

Western blotting

Whole-cell lysates were obtained by using NP-40 lysis buffer (ThermoFisher Scientific). Twenty-five μg of total protein of whole-cell lysates were separated by 10% SDS-PAGE electrophoresis and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Burlington, MA, USA) The membranes sequentially underwent blocking with 5% bovine serum albumin (BSA) in TBST (Tris-buffered saline with 0.1% Tween-20) and incubation with the KLF15 antibody (Abcam, Cambridge, UK) and the corresponding secondary anti-goat horseradish peroxidase-conjugated-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, USA). The ECL-plus chemiluminescence substrate (Millipore) was used for developing the signals of immunoreactive bands, which were captured using a LAS-1000plus Luminescent Image Analyzer (GE Healthcare Biosciences, Piscataway, NJ, USA) and were quantified using ImageJ software (National Institutes of Health). All antibodies were purchased from ThermoFisher Scientific.

Luciferase reporter activity assay

The wild-type KLF15-3′UTR was cloned into the β-gal control plasmid according to the manufacturer's protocol. The mutant reporter was generated by replacing the original sequence ACAUAUCA in the wild-type reporter with UGUAUAGU. The β-galactosidase activity of vector alone plasmid, the wild-type reporter, and the mutant reporter were normalized using the luciferase activity of a cotransfected plasmid expressing luciferase in order to represent background reporter activity. The reporter plasmid and miR-190a mimic or miR-Scramble were co-transfected into cells using a Lipofectamine 2000 reagent (Invitrogen). Firefly luciferase activity after normalizing to transfection efficiency represented reporter activity.

Lentiviral-mediated RNA interference for silencing KLF15

The pLV-RNAi vector was purchased from Biosettia Inc. (San Diego, CA, USA). The method of cloning the double-stranded short hairpin (sh) RNA sequence followed the manufacturer's protocol. Oligonucleotide sequence of lentiviral vectors expressing shRNA that targets human *KLF15* was synthesized and cloned into pLVRNAi to generate a lentiviral expression vector. The target sequences for *KLF15* are listed as follows: Sh-KLF15-1: 5′-AAAAGCCGCAGAACTCATCAAAATTGGATCCAATTTTGATGAGTTCTGCGGC -3′,Sh-KLF15—2:5′-AAAAGGGTGTGAGAGAACTAGATTTGGATCCAAATCTAGTTCTCTCACACCC -3'. Lentivirus production was performed by co-transfection of plasmid DNA mixture with lentivector plus helper plasmids (VSVG and Gag-Pol) into 293T cells (American Type Culture Collection, Manassas, VA, USA) using Lipofectamine 2000 (Invitrogen).

Statistical analysis

Statistical Package of Social Sciences software (version 13.0, SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data from at least triplicate analysis was shown as mean ± standard error of the mean (SEM). Student's *t* test was used to determine statistical significance of the differences between experimental groups; *P*-value less than 0.05 was considered statistically significance.

Results

First, we discovered a higher expression of miR-190a in fibrotic buccal mucosal fibroblasts (fBMF) obtained from OSF patients using qRT—PCR (Fig. 1A). To assess the effect of miR-190 on myofibroblast activities, miR-190a inhibitor was administered to decrease the expression of miR-190a in fBMF (Fig. 1B) and the abilities of migration and collagen gel contraction with miR-190a inhibitor were all decreased (Fig. 1C—D). Moreover, suppression of miR-190a reduced the generation of reactive oxygen species (ROS) in fBMFs (Fig. 1E). Additionally, suppression of miR-190a markedly decreased the ability of wound healing (Fig. 2A). It is well-accepted that α-SMA is a marker of myofibroblasts, and we found that the expression of α-SMA was downregulated in fBMFs with miR-190a inhibitor (Fig. 2B). Furthermore, results from RNA sequencing and qRT—PCR showed that the expression of miR-190a was indeed aberrantly upregulated in OSF tissues (Fig. 2C—D). Besides, Pearson correlation analysis showed that there was a positive correlation between miR-190a and various fibrotic markers, including alpha smooth muscle actin (α-SMA), collagen type I alpha 1 chain (COL1A1), and transforming growth factor beta 1 (TGF-β1) (Fig. 2E—G). These findings indicated that an increase in the expression of miR-190a in fBMFs might potentially result in the transformation of myofibroblast and reduced ROS generation.

Next, we used the bioinformatic TargetScan database to predict biological targets of miR-190a and found a putative binding site in the 3′-UTR of Kruppel-like factor 15 (KLF15). To determine whether there was a specific binding, KLF15 mRNA 3′-UTR-driven luciferase reporter (wild type) and KLF15 mRNA 3′-UTR mutant luciferase reporter (mutant) constructs were generated (Fig. 3A) and transiently transfected into cells along with miR-190a. As shown in Fig. 3B, the luciferase activity was markedly declined in fBMFs transfected with wild-type KLF15, while the mutated form was not affected. Also, the expression of KLF15 in fBMFs was increased when miR-190a was repressed (Fig. 3C), suggesting that KLF15 was a direct target of miR-190a.

In order to explore the function role of KLF15, fBMFs were transfected with KLF15 plasmids to increase its expression (Fig. 4A). We showed that various myofibroblast phenotypes, such as collagen gel contractility (Fig. 4B) and transwell migration (Fig. 4C), were all attenuated in fBMFs with KLF15 overexpression. In addition, we found that overexpression of KLF15 diminished ROS production in fBMFs using the DCFH-DA assay (Fig. 4D). Taken together, these results indicated that miR-190a may regulate myofibroblast activation through direct binding of KLF15.

To substantiate our assumption, we conducted rescue experiments to examine whether the effect of miR-190a on myofibroblast was mediated by KLF15. As shown in Fig. 5A, collagen gel contractility was downregulated in fBMFs transfected with miR-190a inhibitor compared to miR-Scr., whereas silencing of KLF15 abrogated this activity. Likewise, the transwell migration ability was suppressed when miR-190a was inhibited, while the knockdown of KLF15 blocked it (Fig. 5B). Aside from myofibroblast activities, silencing of KLF15 also impeded the repressive property of miR-190a inhibitor on the expression of fibrosis markers, including α-SMA, COL1A1, connective tissue growth factor (CTGF, Fig. 5C).

Discussion

MiR-190a is located in the intronic region of the talin2 gene (TLN2) on chromosome 15q22.2[17] and was first reported in 2003 as one of the newly identified miRNAs using the human osteoblast sarcoma cell line.[18] The aberrant expression of miR-190a was discovered in various types of cancer, such as meningioma,[19] breast cancer,[20] and prostate cancer.[21] MiR-190a also mediates several diabetic complications, including diabetic vascular dysfunction[22] and diabetic neuropathic pain.[23] It has been shown that miR-190a was one of the miRNAs that were greatly associated with the

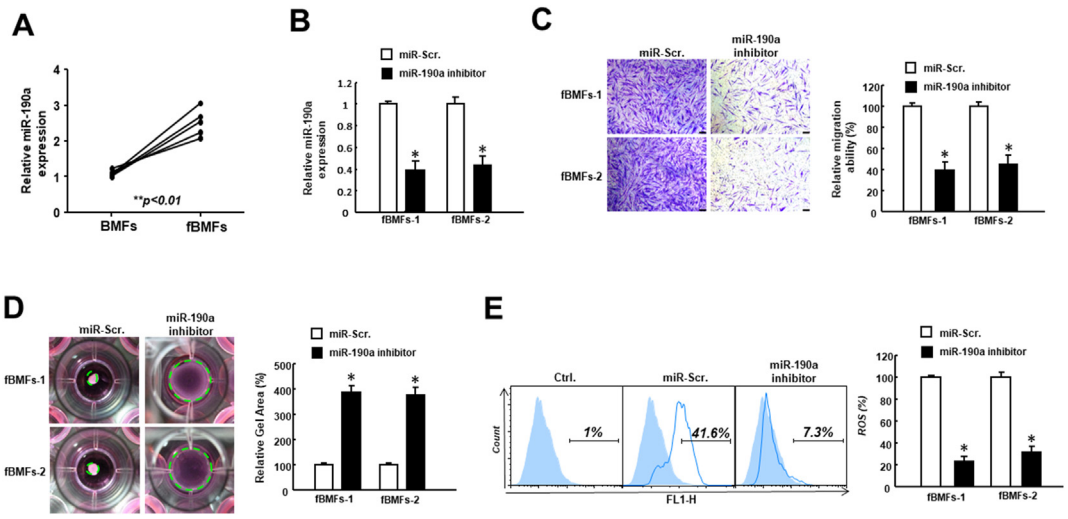

Figure 1 Inhibition of miR-190a possesses a suppressive effect on myofibroblast activation and reactive oxygen species generation. (A) The relative expression level of miR-190a was evaluated by qRT—PCR in buccal mucosal fibroblasts (BMFs) and fibrotic BMFs (fBMFs). (B) Downregulation of miR-190a in fBMFs with inhibitor. (C—E) The migration ability (C), collagen gel contraction (D), and reactive oxygen species (ROS) generation (E) were examined in fBMFs transfected with scramble control (miR-Scr.) and miR-190a inhibitor. *$P < 0.05$ when compared to the miR-Scr. The green dashed circles indicate the area of the gel. Ctrl.: Non-transfected control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

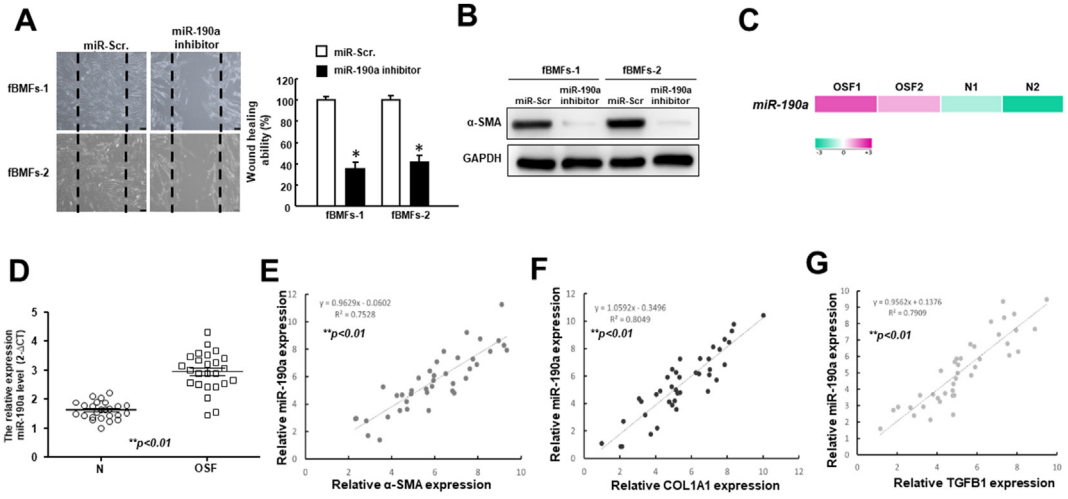

Figure 2 MiR-190a is aberrantly elevated in oral submucous fibrosis tissues and positively correlated with markers associated with myofibroblasts and fibrosis. (A) Wound healing ability and (B) the protein expression of alpha smooth muscle actin (α-SMA) were examined in fBMFs transfected with miR-190a inhibitor. (C) RNA sequencing was applied to detect differential expression of miR-190a in OSF and normal samples. (D) QRT—PCR analysis revealed a higher expression level of miR-190a in OSF compared to adjacent normal mucosa (N, n = 25). (E—G) Pearson correlation analysis revealed a positive correlation between the expression of miR-190a and markers of myofibroblasts, including α-SMA (E), collagen type I alpha 1 chain (COL1A1, F), and transforming growth factor beta 1 (TGF-β1, G). *$P < 0.05$ compared to the miR-Scr.

overall survival of patients with squamous cell carcinoma of the tongue.[24] Another study showed that miR-190 suppressed the TGF-β-induced epithelial-mesenchymal transition (EMT) of breast cancer and prevented metastasis *in vitro* and *in vivo* by targeting SMAD2.[25] Given that miR-190a was related to the progression of oral cancer and TGF-β-elicited EMT, a potential source of myofibroblasts,[26] we postulated that miR-190a may also participate in the development of precancerous OSF as well. To date, only a few studies have investigated the effect of miR-190a on fibrosis diseases. For instance, it has been demonstrated that injection of miR-190a-5p agomir plasmid into the mice to suppress it resulted in attenuation of liver fibrosis *in vivo*.[27] Another study revealed that overexpression of miR-190a-3p promoted the migratory activity and fibronectin 1 secretion in hypertrophic scar-derived fibroblasts

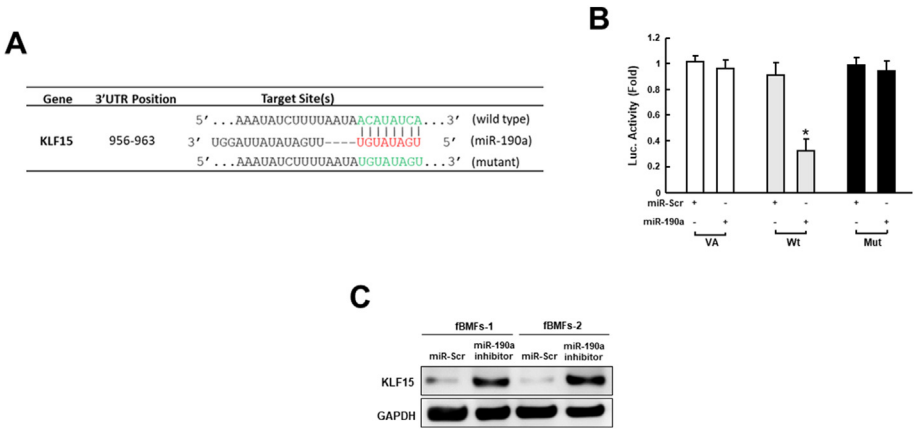

Figure 3 KLF15 is a direct target of miR-190a. (A) The illustration of the complementarity between the 3′UTR portions of both full-length (wild type) and mutant KLF15 and the miR-190a seed sequence, as predicted by the TargetScan in silico browser. (B) The luciferase (Luc.) activity of KLF15 performed with vector alone (VA), wild-type (Wt), and mutated (Mut) constructs was assessed. (C) The expression of KLF15 in fBMFs transfected with miR-Scr or miR-190a inhibitor. *$P < 0.05$ when compared to the miR-Scr.

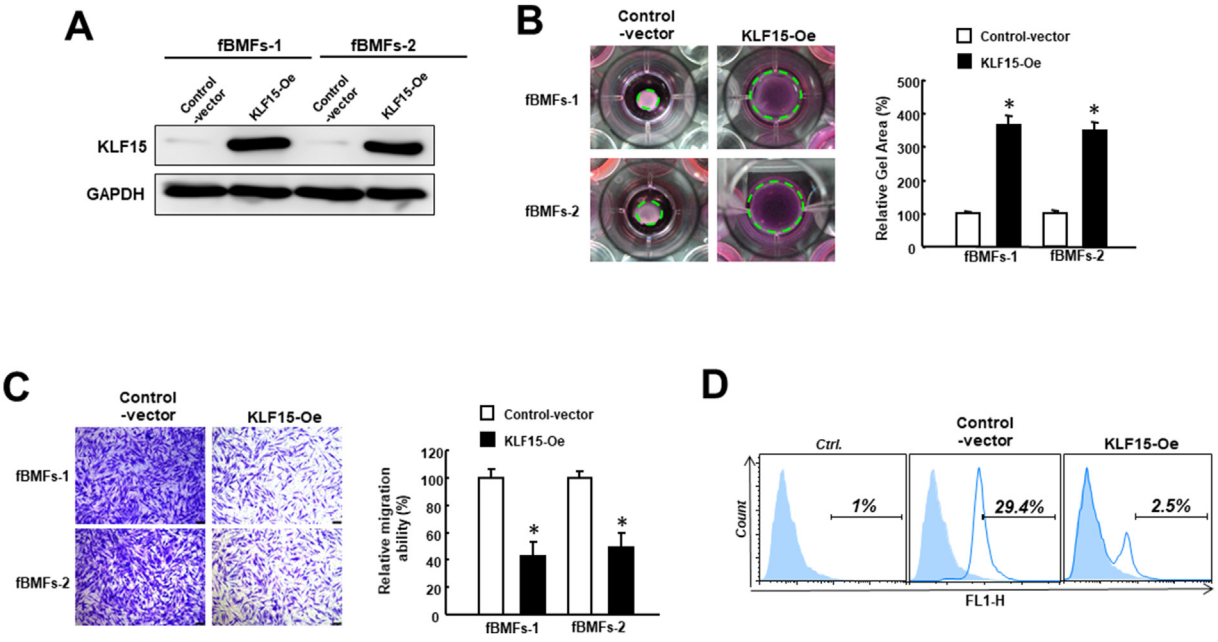

Figure 4 KLF15 overexpression eliminates myofibroblast characteristics in fibrotic buccal mucosal fibroblasts. (A) Western blotting analysis was used to confirm the overexpression impact of KLF15 in fBMFs. (B—D) In fBMFs, overexpression of KLF15 leads to suppression of collagen gel contraction (B), transwell migration (C), and ROS generation (D). The experiments were replicated thrice, and the representative outcomes were displayed. *$P < 0.05$ when compared to the Control-vector. KLF15-Oe: Overexpression of KLF15.

via direct binding to the 3′-UTR of the CUB and Sushi multiple domains 1 (CSMD1).[16] In agreement with these findings, we showed that suppression of miR-190a reduced myofibroblast activities and ROS production of fBMFs. Moreover, we verified that the regulatory effect of miR-190a on myofibroblast features was mediated by KLF15.

KLF15 belongs to the KLF family, a subclass of Cys2/His2 zinc-finger DNA-binding proteins, and is involved in various cellular events, such as metabolism[28,29] and differentiation.[30] Numerous studies have revealed that KLF15 acts as a repressor of pathological fibrosis. In cardiac fibrosis, KLF15 inhibited the recruitment of the co-activator P/CAF to the CTGF promoter following TGF-β stimulation in neonatal rat ventricular fibroblasts.[31] Overexpression of KLF15 has been found to attenuate the ISO-induced cardiac fibrosis via the Akt/mTOR signaling,[32] and interaction of KLF15 with Smad2/3 suppressed CCL2 expression and fibroblast activation in angiotensin II-treated adventitial fibroblasts.[33] A similar finding was reported in renal fibrosis that KLF15 directly bound to the co-activator P/CAF and repressed its recruitment to the CTGF promoter in angiotensin II-stimulated NRK-49F cells.[34] Upregulation of KLF15 reduced the TGF-β1-induced fibronectin, type III collagen, and CTGF in NRK-49F cells via the ERK/MAPK and JNK/MAPK

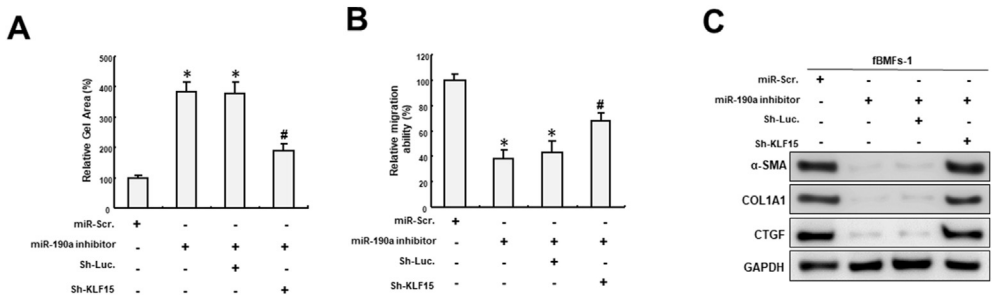

Figure 5 KLF15 mediates the suppressive effect of miR-190a inhibitor on myofibroblast features. (A–C) Collagen gel contraction (A), transwell migration (B), and expression of fibrosis-associated factors (C) were decreased in fBMFs transfected with the miR-190a inhibitor, while silencing of KLF15 reversed these effects. $^{*}P < 0.05$ when compared to the miR-190 inhibitor; $^{\#}P < 0.05$ when compared to the miR-190 inhibitor with Sh-Luc. The green dashed circles indicate the area of the gel.

pathways.[35] Induction of podocyte-specific KLF15 also reduced kidney fibrosis by upregulating wilms tumor 1, a transcription factor critical for podocyte differentiation.[36] Another study showed that knockdown of KLF15 in mouse embryonic fibroblasts activated the canonical Wnt/β-catenin signaling and increased various profibrotic transcripts.[37] Suppression of proximal tubule-specific Klf15 exacerbated kidney fibrosis through loss of fatty acid β-oxidation gene transcription.[38] Our findings were in line with these results and showed that KLF15 served as an anti-fibrosis factor in OSF. Moreover, we showed suppression of miR-190a may inhibit the production of ROS, possibly through upregulation of KLF15. It has been shown that the Elabela-mediated alleviation of oxidative stress in the doxorubicin-stimulated aortic adventitial fibroblasts was mediated by the KLF15-dependent Nrf2/SLC7A11/GPX4 signaling.[39] KLF15 also has been shown to be involved in cardiac ROS clearance through the modulation of NAD^{+} levels.[40] Therefore, it may be beneficial to investigate whether the preservation of KLF15 in fBMFs can reduce oxidative stress through the abovementioned mechanisms and suppress persistent myofibroblast activation in the future.

In summary, our results demonstrated that the down-regulation of miR-190a may halt the persistent activation of myofibroblast and accumulation of oxidative stress through upregulation of KLF15. These findings provide insight into the pathogenesis of OSF and possible treatment direction.

Declaration of competing interest

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

Acknowledgments

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