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Original Article

Metformin alleviates the progression of oral submucous fibrosis through downregulation of metastasis associated lung adenocarcinoma transcript 1 (MALAT1)

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KEYWORDS
Oral submucous fibrosis;
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Abstract *Background: /purpose*: Emerging evidence demonstrates the anti-fibrotic properties of metformin, a first-line anti-diabetic drug; however, its inhibitory effects on oral fibrogenesis warrant further evaluation.
Materials and methods: The cytotoxic effects of metformin on normal and fibrotic buccal mucosal fibroblasts (fBMFs) derived from OSF tissues were evaluated using the MTT assay. Collagen gel contraction, wound healing, and transwell migration assays were carried out to assess myofibroblast features. In addition, the expression levels of alpha-1 type I collagen (COL1A1), alpha-smooth muscle actin (α-SMA) and Smad2 were measured. Moreover, RNA sequencing was performed to explore potential targets participating in the anti-fibrotic effects of metformin.
Results: A lower dose of metformin was sufficient to inhibit the proliferation of fBMFs without affecting normal BMFs, and to attenuate various myofibroblast characteristics, including collagen gel contraction, wound healing, transwell migration capacities, and the expression of fibrosis markers. Additionally, we showed that administration of metformin prevented the arecoline-induced myofibroblast activation. Most importantly, our results suggest that metformin may exert suppressive effects on myofibroblast activities by inhibiting ROS accumulation

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through the downregulation of MALAT1.
Conclusion: These findings indicate that metformin may serve as a preventive agent against OSF progression.

Introduction

Oral submucous fibrosis (OSF) has been recognized as an oral potentially malignant disorder with a variable risk of malignant transformation rate around 3—5 %.[1,2] This chronic, insidious scarring disease of the oral cavity is characterized by the progressive inability of mouth opening due to loss of elasticity and the development of fibrous bands in labial and buccal tissues. Several studies have indicated that the occurrence of OSF is attributed to areca nut chewing.[3,4] Stimulation of buccal mucosal fibroblasts (BMFs) with arecoline, the main alkaloid found in the areca nut, has been demonstrated to activate transforming growth factor (TGF)-β/Smads signaling and induce myofibroblast transdifferentiation.[5,6] Aside from the activation of the TGF-β/Smads pathway, the arecoline-induced reactive oxygen species (ROS) accumulation confers myofibroblast transdifferentiation and oral fibrogenesis as well.[7] Metformin, commonly prescribed as a first-line anti-diabetic agent, has demonstrated efficacy in reversing pulmonary fibrosis in various studies with its capacity to inhibit TGF-β1, collagen formation, ROS generation, and myofibroblast activation.[8—10] Nevertheless, its inhibitory effect on the development of OSF has yet to be determined.

Numerous studies have highlighted the critical roles of noncoding RNAs in various diseases, including OSF.[11,12] Among them, the long noncoding RNA MALAT1 (metastasis associated lung adenocarcinoma transcript 1) has been shown to contribute to the development and progression of oral cancer[13—15] and can serve as a salivary biomarker of oral cancer.[16] In fibrosis diseases, MALAT1 has been found to induce the TGF-β1/Smads-mediated fibrosis during the acute kidney injury to chronic kidney disease transition.[17] Besides, silencing of MALAT1 significantly reduced the expression levels of fibrosis-related proteins in renal interstitial fibrosis.[18] MALAT1 not only drives mouse fibroblast activation,[19] but also possess the capacity to regulate ROS production.[20,21] While MALAT1 is known to be overexpressed in oral cancer tissues compared to normal oral mucosa,[13,14] it remains unclear whether its aberrant upregulation also occurs in precancerous OSF lesions and contributes to disease progression. Notably, MALAT1 has been implicated in the therapeutic effects of metformin in breast and cervical cancers,[22,23] and whether metformin regulates MALAT1 to influence OSF development warrants further investigation.

As such, we aimed to investigate the effect of metformin on myofibroblast activities of fibrotic BMFs (fBMFs) derived from OSF tissues and myofibroblast transdifferentiation of BMFs stimulated with arecoline. Moreover, we examined the expression of MALAT1 to show if the metformin-mediated regulation of MALAT1 contributes to the treatment of oral fibrogenesis.

Materials and methods

Reagents

Arecoline and collagen solution from bovine skin were purchased from Sigma—Aldrich (St. Louis, MO, USA).

Primary BMFs and fBMFs culture

All procedures for tissue acquisition adhered to the Declaration of Helsinki and were reviewed and approved by the Institutional Review Committee of Chung Shan Medical University. The isolation and culture of normal buccal mucosal fibroblasts (BMFs) and fibrotic BMFs (fBMFs) from OSF tissues were conducted as previously described. Cells between the third and eighth passages were used for all experiments to ensure phenotypic consistency.[11]

Cell proliferation and survival assay

Cells were seeded into 96-well plates at a density of 1×10^4 cells per well and incubated overnight to allow adhesion. Cells were then treated with metformin at concentrations of 10, 20, 40, 80, and 160 μM for 48 h. After treatment, the proliferation rate and IC50 value were assessed using the MTT assay, following the manufacturer's instructions (Sigma—Aldrich). Absorbance at 570 nm was measured with a microplate reader (Molecular Devices, San Jose, CA, USA).

Collagen gel contraction assay

Cells were suspended in collagen gel solution (Sigma—Aldrich) and seeded into a 24-well plate, followed by incubation at 37 °C for 2 h to allow gel polymerization. Subsequently, 0.5 mL of medium was added, and gels were cultured for 48 h. The gel surface area was analyzed using ImageJ software (NIH, Bethesda, MD, USA), and contraction was quantified relative to initial area.[24]

Transwell migration assays

A total of 1×10^5 cells in medium with low serum were placed in the upper chamber of transwell inserts with 8 μm pores (Corning, Acton, MA, USA). The lower chamber was

filled with medium containing higher serum. After 24 h, cells that migrated to the underside of the membrane were stained with crystal violet and counted in five randomly selected fields.[25]

Wound healing assay

Cells were grown to approximately 80 % confluence in 12-well plates. A linear scratch was created using a sterile 200 μL pipette tip, and cells were cultured for another 48 h in serum-containing medium. Crystal violet staining was used to visualize the migration of cells into the wound area at 0 and 24 h under a microscope.[26]

Western blot analysis

Total protein extraction, SDS-PAGE, and immunoblotting were performed using standard protocols. Primary antibodies included α-SMA, COL1A1, phosphorylated Smad2 (p-Smad2), and total Smad2. GAPDH was used as a loading control.[11]

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. First-strand cDNA was synthesized using the SuperScript III first-strand synthesis system (Invitrogen Life Technologies), and quantitative PCR was performed on an ABI StepOne™ Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) using the following primers: MALAT1, GCCTGGAAGCTGAAAAACGG (forward) and TGGAAAACGCCTCAATCCCA (reverse); GAPDH (internal control), CTCATGACCACAGTCCATGC (forward), TTCAGCTC TGGGATGACCTT (reverse). Relative gene expression levels were calculated using the $2^{-\Delta Ct}$ method.

Silencing MALAT1

Lentiviral vectors encoding short hairpin RNAs (shRNAs) targeting MALAT1 were constructed using the pLV-RNAi plasmid (Biosettia, San Diego, CA, USA), following the manufacturer's protocol. Synthetic oligonucleotides were cloned into the vector to generate lentiviral particles for cell transduction.

Overexpression of MALAT1

MALAT1 cDNA was cloned into the lentiviral vector pLV-EF1a-MCS-IRES-Puro (BioSettia, Cat. No: cDNA-pLV01). Lentiviral particles were produced by co-transfecting the expression vector along with helper plasmids (VSVG and Gag-Pol) into 293T cells (American Type Culture Collection, Manassas, VA, USA) using Lipofectamine 2000 (Invitrogen).

Intracellular reactive oxygen species (ROS) level assessment

The intracellular levels of reactive oxygen species (ROS) were measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) assay (Sigma—Aldrich). BMFs and fBMFs were seeded and treated with or without metformin at the indicated concentrations for 48 h. In some experiments, fBMFs were transduced with lentiviral constructs expressing either MALAT1 shRNA or MALAT1 cDNA prior to metformin treatment to examine the role of MALAT1 in ROS regulation. After treatment, 1×10^5 cells were collected, resuspended in serum-free medium containing 10 μM DCFH-DA, and incubated at 37 °C for 60 min in the dark. Following two washes with phosphate-buffered saline (PBS), the fluorescence intensity of 10,000 cells was recorded using a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA, USA) and analyzed using FlowJo software.[27]

Statistical analysis

All data were expressed as mean ± SD. Student's t-test or one-way analysis of variance (ANOVA) was used to compare differences between groups. A *P*-value less than 0.05 was considered statistically significant.

Results

To explore the potential role of metformin in suppressing myofibroblast proliferation, we assessed the cytotoxic effect of metformin on normal BMFs and fBMFs derived from OSF tissues with various concentrations of metformin using an MTT assay. As shown in Fig. 1, metformin exhibited a dose-dependent inhibition on cell survival in both BMFs and fBMFs, and the IC_{50} values for metformin in BMFs and fBMFs were 51.3 ± 8.6 and 22.6 ± 2.2 μM, respectively. A lower concentration of metformin was sufficient to reduce the viability of fBMFs, with minimal effects on normal BMFs. Hence, the lower concentrations of metformin (0—20 μM) were used in the following experiments to determine the anti-fibrosis effect of metformin.

Under conditions of tissue injury or in response to certain signals, fibroblasts acquire reparative features to stabilize and close wounds by producing a collagen-rich extracellular matrix (ECM) during a phenotypic switch known as fibroblast-to-myofibroblast activation. Apart from the expression of α-smooth muscle actin (α-SMA), the increased collagen contraction and migration capacities are also the hallmarks of mature myofibroblasts.[28,29] In Fig. 2, we showed that treatment of metformin markedly mitigated the collagen gel contractility (Fig. 2A), wound healing capacity (Fig. 2B), and transwell migration ability (Fig. 2C) in a dose-dependent manner. Besides, the expression levels of fibrosis-associated markers, such as alpha-1 type I collagen (COL1A1), alpha-smooth muscle actin (α-SMA), and phosphorylated Smad2 were all downregulated in response to metformin. Furthermore, we demonstrated that metformin administration attenuated the arecoline-induced myofibroblast activation in BMFs (Fig. 3A and B). These results indicate that metformin treatment may ameliorate the areca nut-associated oral fibrogenesis.

To elucidate the mechanism underlying the anti-fibrotic effects of metformin, RNA sequencing was performed to identify targets involved in metformin-mediated inhibition of myofibroblast activation. As shown in Fig. 4A, MALAT1 is differentially expressed long noncoding RNAs in the metformin-treated fBMFs. To validate the RNA-sequencing results, qRT-PCR was conducted and we found that the expression of MALAT1 was downregulated in fBMFs treated with metformin in a dose-dependent fashion (Fig. 4B).

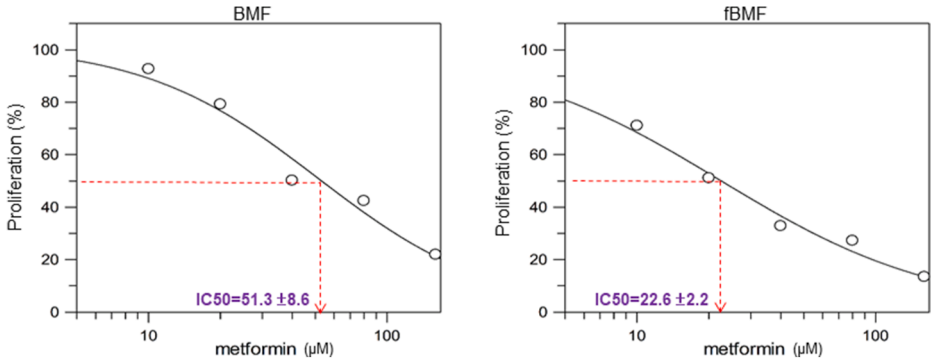

Figure 1 Cytotoxic effect of metformin on the cell viability of buccal mucosal fibroblasts (BMFs) and fibrotic BMFs (fBMFs) An MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was utilized to evaluate cell survival/proliferation in response to various concentrations of metformin. The half-maximal inhibitory concentration (IC_{50}) values were calculated by GraFit software.

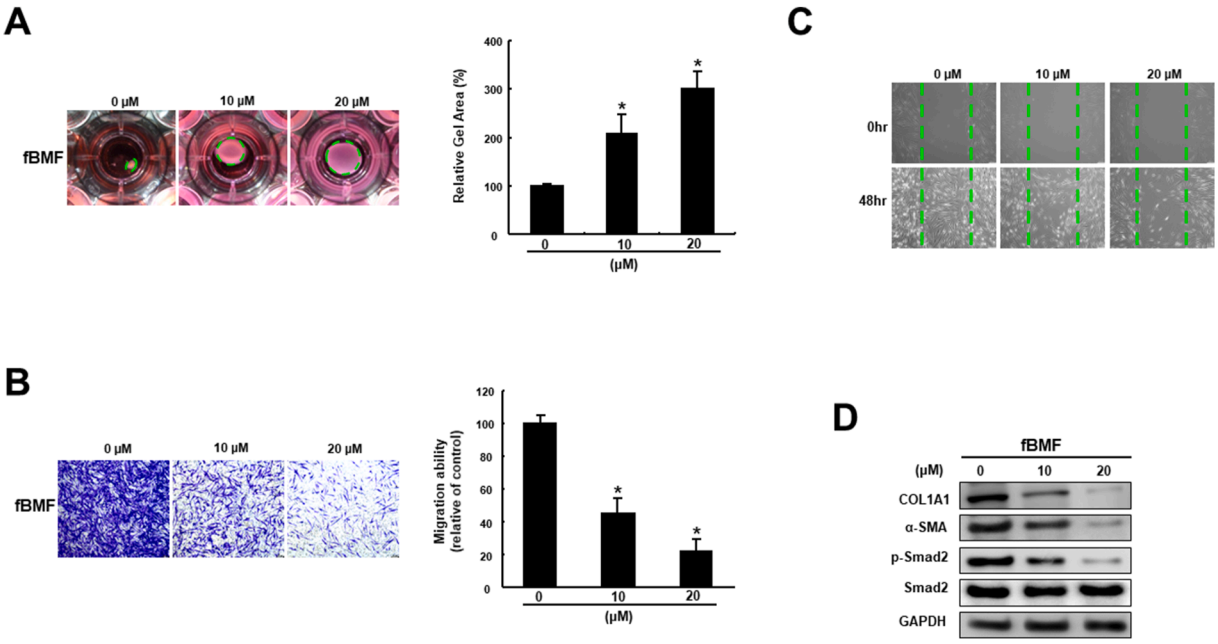

Figure 2 Effects of metformin on myofibroblast characteristics in fBMFs. The fBMFs were subjected to collagen gel contraction (A), wound healing (B), and transwell migration (C) assays. Collagen gel contraction and migration were assessed 48 h after metformin treatment. The expression levels of alpha-1 type I collagen (COL1A1), alpha-smooth muscle actin (α-SMA), and both phosphorylated and total Smad2 were analyzed. All experiments were independently repeated three times, and representative results are shown. Data are presented as mean ± SD. *$P < 0.05$ compared to the untreated control group.

Furthermore, we showed that the suppressive effect of metformin on ROS production was mediated by diminishing MALAT1 (Fig. 5). Collectively, our findings suggest that metformin may diminish myofibroblast activation by reducing ROS accumulation through the downregulation of MALAT1.

Discussion

Metformin, a prototypical biguanide compound, has long been employed in the management of diabetes mellitus. Besides glucose control, metformin also offers multifaceted benefits that can mitigate inflammation and oxidative stress. Beyond its conventional use, metformin has garnered increasing attention for its therapeutic potential in lowering cancer risk and improving clinical outcomes in cancer patients. Several studies have demonstrated the anti-cancer effects of metformin against head and neck/oral squamous carcinoma cells.[30–33] It may also serve as an adjuvant drug for oral cancer due to its synergistic effects with cisplatin and its radiosensitizing properties.[30,33] Moreover, there is growing enthusiasm for the use of metformin in mitigating pathological fibrosis, including pulmonary,[8–10] ovarian,[34] and liver[35] fibrosis. Extensive studies underpin this paradigm shift by demonstrating the ability of metformin to inhibit key fibrotic and stress-related pathways, including AMPK activation, TGF-β1

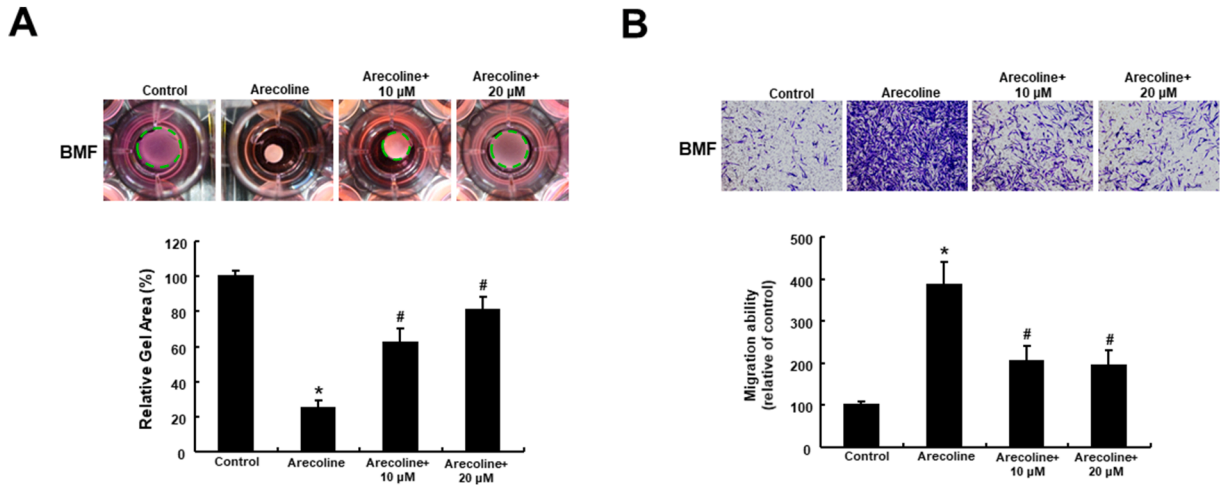

Figure 3 Suppressive effect of metformin on arecoline-induced myofibroblast activation (A) Collagen gel contraction and (B) transwell migration assays were performed following arecoline stimulation to assess myofibroblast transdifferentiation in the absence or presence of metformin. All experiments were independently repeated three times, and representative results are shown. Data are presented as mean ± SD. *$P < 0.05$ compared to the untreated control group. #$P < 0.05$ compared to the arecoline-only group.

Figure 4 Metformin treatment downregulates the expression of MALAT1. (A) A heatmap showing that MALAT1 is differentially expressed genes in fBMFs treated with or without metformin. (B) The expression level of MALAT1 in two fBMF lines was dose-dependently diminished in response to metformin treatment. Data are presented as mean ± SD. *$P < 0.05$ compared to the untreated control group.

signaling, collagen synthesis, ROS generation, and myofibroblast activation.[8—10,36] One of the previous studies has shown that metformin prevents the development of oral cancer from carcinogen (4NQO)-induced premalignant lesions.[37] Another study demonstrated that both the clinical and histological findings provide encouraging evidence supporting the potential chemopreventive role of metformin in individuals with oral premalignant lesions.[38] In line with these findings, we also demonstrated that metformin may ameliorate the progression of premalignant OSF, as evidenced by the suppression of myofibroblast features in fBMFs and the inhibition of arecoline-induced myofibroblast activation in BMFs, possibly through the downregulation of MALAT1.

MALAT1, also known as LINC00047 or NEAT2, is a long noncoding RNA of more than 8000 nucleotides, expressed from chromosome 11q13.[39] MALAT-1 was first identified in 2003 and found to be associated with metastasis in non-small cell lung cancer, from which its name is derived.[39] Multiple studies have demonstrated that MALAT1 is overexpressed in oral cancer tissues[40,41] and is implicated in its development through various mechanisms, including the maintenance of epithelial—mesenchymal transition,[13,14] inhibition of apoptosis,[14] and functioning as a competing endogenous RNA (ceRNA).[15,41] A number of studies have shown that the regulation of MALAT1 is critical for the therapeutic effects of metformin in the treatment of cervical and breast cancers.[22,23] In agreement with these findings, we demonstrated that metformin downregulated the expression of MALAT1 in fBMFs. It has been found that MALAT1 regulated the TGF-β1/Smad-mediated fibrosis in various diseases, such as chronic kidney disease[17] or diabetic cardiomyopathy.[42] MALAT1 has also been shown to drive myofibroblast activation by acting as a ceRNA for miR-335—3p,[19] regulating CXCL5 expression,[43] or promoting the TGF-β signaling pathway.[44] In our results, metformin treatment reduced the expression of phosphorylated Smad2, collagen, and the myofibroblast marker α-SMA in fBMFs. This effect may be linked to MALAT1 suppression. Furthermore, we demonstrated that a reduction of MALAT1

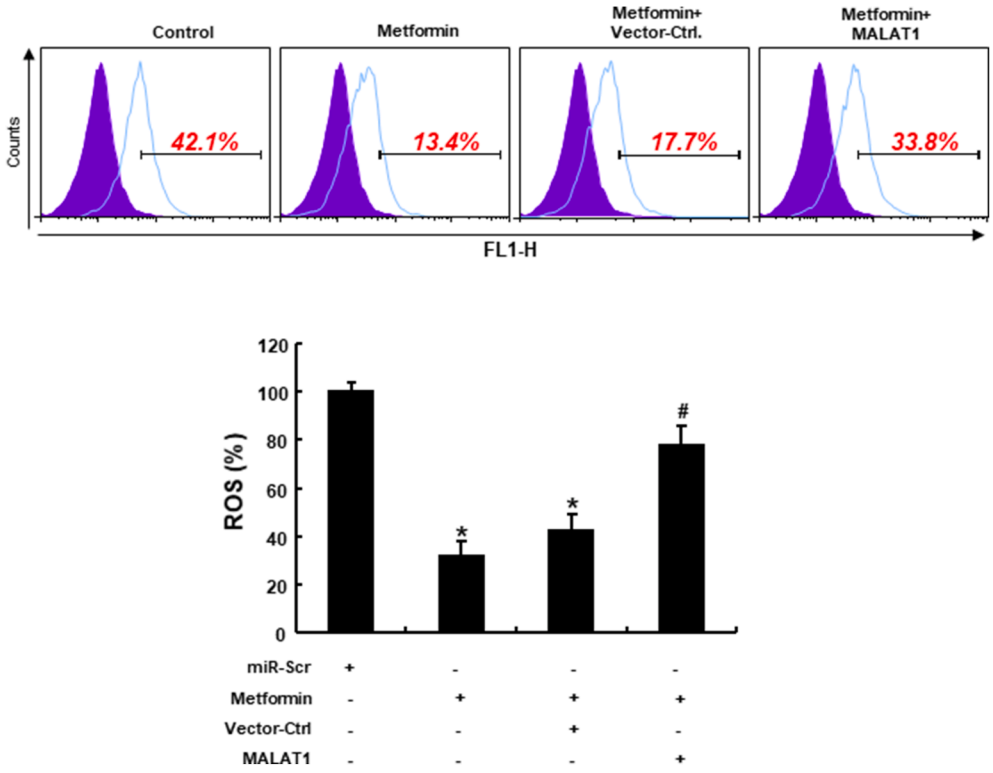

Figure 5 Overexpression of MALAT1 counteracts the metformin-induced reduction in ROS production in fBMFs. Intracellular reactive oxygen species (ROS) levels were measured using the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) assay to investigate whether MALAT1 suppression is required for metformin-induced downregulation of ROS generation. All experiments were independently repeated three times, and representative results are shown. Data are presented as mean ± SD. *$P < 0.05$ compared to the untreated control group. #$P < 0.05$ compared to the metformin-only group.

was required for the inhibition of ROS accumulation following metformin treatment. It has been known that arecoline-elicited ROS may contribute to the TGF-β activation in BMFs.[45] Therefore, it is reasonable to infer that the metformin-induced reduction in ROS levels is one of the contributing factors to the decreased expression of myofibroblast markers and reduced activation of the TGF-β signaling pathway. Further investigations are needed to explore whether MALAT1 may also influence myofibroblast activation or the TGF-β pathway through mechanisms beyond ROS regulation.

Taken together, this study demonstrates that metformin holds the potential to prevent the exacerbation of OSF by regulating MALAT1, thereby reducing ROS production and the subsequent persistent activation of myofibroblasts.

Declaration of competing interest

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

Acknowledgments

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