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Original Article

GAS5 decreased the arecoline-induced myofibroblasts activity in buccal mucosal fibroblasts via acting as a competing endogenous RNA for miR-21

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MicroRNA-21

Abstract *Background/purpose:* Oral submucous fibrosis (OSF) is recognized as a premalignant condition that increases the risk of oral cancer. Myofibroblasts are the primary cellular mediators of the pathological fibrosis characteristic of OSF. Consequently, understanding the molecular mechanisms that drive myofibroblast activation is crucial for the development of effective therapeutic interventions for this condition. Growth arrest-specific 5 (GAS5), a long non-coding RNA, represents a potential regulatory factor in OSF pathogenesis, although its specific role remains largely undefined.
Materials and methods: To validate the direct interaction between GAS5 and its target miR-21, a luciferase reporter assay was performed. qRT-PCR was employed to evaluate the expression levels of GAS5 in OSF tissues. Collagen gel contraction and transwell migration assays were utilized to assess myofibroblast functional activities.
Results: Our result validated overexpression of GAS5 inhibits TGF-β-induced myofibroblast activation, as evidenced by reduced collagen gel contraction, cell migration, and suppression

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of the TGF-β/Smad2 signaling pathway. GAS5 also attenuates arecoline-induced myofibroblast activation. Mechanistically, GAS5 directly interacts with and sponges miR-21, thereby modulating myofibroblast function.
Conclusion: Our findings suggest that GAS5 plays a crucial role in inhibiting myofibroblast activation in OSF by targeting the TGF-β/Smad2 signaling pathway and regulating the miR-21. GAS5 may represent a novel therapeutic target for the prevention and treatment of OSF.

Introduction

Oral submucous fibrosis (OSF) is a chronic inflammatory and potentially malignant disorder,[1] characterized by the progressive accumulation of dense fibrous connective tissue, which results in rigidity and restricted mouth opening. This can lead to difficulties in eating, which in turn affects the patient's overall quality of life. It has been indicated that OSF is associated with areca quid chewing habit.[2] The current therapies, such as anti-inflammatory/anti-oxidant injections,[3–5] physiotherapy[6] or surgery,[5] are symptomatic treatments, not acting on its etiology. As such, we sought to decipher the mechanism underlying OSF pathogenesis as well as identify the critical biomarkers to develop effective treatment modalities, leading to the reduction in cancer transformation from OSF.

Previous studies have shown that an imbalance in the synthesis and degradation of collagen, a component of the extracellular matrix (ECM), leads to the development of OSF.[7] And the activated myofibroblasts have been recognized as the key pathogenic cells that attributed to excessive deposition of ECM.[8] Increased activity of myofibroblast has been found in multiple tissue fibroses, such as heart,[9] liver,[10] lung,[11] and OSF.[12] It is well known that profibrogenic cytokines are critical mediators of fibrosis by differentiating fibroblasts to myofibroblast phenotype. The localized mucosal inflammation caused by areca quid leads to an increase in pro-fibrogenic cytokines, such as transforming growth factor (TGF)-β.[13–15] In fact, activation of TGF-β signaling has been postulated as one of the main causative events for induction of myofibroblast transdifferentiation in OSF.[13–15] Also, TGF-β induces cells to undergo epithelial to mesenchymal transition (EMT), which is crucial in a possible origin of activated myofibroblasts.[16] Previously, we have demonstrated that various EMT transcriptional factors have involved in dysregulation of myofibroblast in arecoline-induced OSF.[17,18] Where the current therapies only target the symptoms arise from OSF, but do not focus on the activation of myofibroblasts. Therefore, targeting molecules that regulate myofibroblast transdifferentiation may be a potential approach to understand pathogenesis of areca quid-associated OSF.

Among these regulatory molecules, non-coding RNAs (ncRNAs) have emerged as key players in gene expression control, influencing various physiological and pathological processes. It is known that ~70–80 % of the human genome is actively transcribed into RNA but only approximately 2 % is translated into protein.[19] Around 98 % of transcripts are noncoding RNAs (ncRNAs) and can be divided into small/short ncRNAs (such as microRNAs) and long non-coding RNAs (lncRNAs) according to their length of less or more than 200 nucleotides, respectively.[20] NcRNAs have been found to regulate gene expression that control diverse physiological and pathological processes. Several reports have revealed aberrant expression of ncRNA in various fibrotic diseases, such as liver,[21,22] renal,[23] pulmonary,[24,25] cardiac[26] fibroses and ncRNAs serve as modulators of these fibroses. Among the various ncRNAs implicated in fibrosis, lncRNA growth arrest-specific 5 (GAS5) has drawn attention due to its role in regulating fibrogenic pathways. GAS5 was first identified in 1988 during a search for novel tumor suppressors that are preferentially expressed during growth arrest. GAS5 is approximately 630 nt in length and localized at chromosome 1q25.1. GAS5 is a member of 5′-terminaloligopyrimidine (5′TOP) gene family and this motif is critical for translational control. Moreover, GAS5 represses liver fibrogenesis by competing with miR-222,[27] an indicative marker of fibrosis,[28] and acts as a negative regulator of pro-fibrogenic miR-21.[29,30]

Given that GAS5 functions as a negative regulator of profibrogenic microRNAs, its role in fibrosis may be particularly relevant in the context of TGF-β signaling. Notably, TGF-β activation has been shown to play a crucial role in mediating areca nut-induced myofibroblast characteristics by upregulating microRNA-21 (miR-21).[31] MiR-21 has been implicated in the pathogenesis of various fibrotic diseases and has been shown to be a potential biomarker for distinguishing oral tongue cancer from healthy individuals.[32] Building on these findings, our previous research confirmed that miR-21 is significantly overexpressed in OSF tissues and that arecoline exposure induces its upregulation in buccal mucosal fibroblasts (BMFs).[31] Given the interplay between GAS5 and miR-21 in fibrosis, we aimed to investigate whether GAS5 inhibited the myofibroblast differentiation in response to arecoline stimulation by suppressing miR21.

This study aimed to explore the relationship between GAS5 and miR-21 and elucidate the functional role of GAS5 in myofibroblast activation. Furthermore, we sought to determine if GAS5 modulated the myofibroblast transdifferentiation through the repression of miR-21. Our findings will provide insights into the contribution of the GAS5/miR-21 axis to the development of OSF.

Materials and methods

Tissues sample collections and primary cell culture

Primary human buccal mucosal fibroblasts (BMFs) and human fibrotic buccal mucosal fibroblasts (fBMFs) were

cultured. All tissue acquisition procedures followed the tenets of the Declaration of Helsinki and were reviewed by the Institutional Review Committee at Chung Shan Medical University. BMFs and fBMFs were cultivated as previously studied. Cell cultures between the third and eighth passages were used in this study.[33]

Collagen contraction assay

Cells were suspended in collagen gel solution (Sigma—Aldrich, St. Louis, MO, USA) and added into a 24-well-plate followed by incubation at 37 °C for 2 h. After polymerization, the gels were further incubated within 0.5 ml medium and 10 μg/ml arecoline for 48 h. The collagen gel size change (contraction index) was quantified using ImageJ software (NIH, Bethesda, MD, USA).[31]

Transwell migration assay

1×10^5 cells in a medium with low serum and 10 μg/ml arecoline were added into the upper chamber of a transwell (Corning, Acton, MA), and medium supplemented with higher serum was used as a chemoattractant in the lower chamber, followed by 24 h incubation. Cells on the lower surface of the insert membrane were stained with crystal violet. The number of migrated cells in a total of five randomly selected fields was measured.[31]

Quantitative real-time PCR (qRT-PCR)

Total RNA was prepared from cells using Trizol reagent according to the manufacturer's protocol (Invitrogen Life Technologies, Carlsbad, CA, USA). qRTPCRs of mRNAs were reverse-transcribed using the Superscript III first-strand synthesis system for RT-PCR (Invitrogen Life Technologies). qRT-PCR reactions on resulting cDNAs were performed on an ABI StepOne™ Real-Time PCR System (Applied Biosystems, Waltham, MA, USA).[31] The primer sequences used in this study were listed as follows: GAS5 F: 5′- CAG AGCGGTTGGCATTCATC -3'; R: 5′- CTTTGCGAATGTTGCGGG TT -3'.GAPDH F: 5′- CTCATGACCACAGTCCATGC-3'; R: 5′- TT CAGCTCTGGGATGACCTT -3'.

Western blot analysis

All procedures in this assay followed the previously described protocols. The primary antibodies against Smad2, p-Smad2, and GAPDH were applied.[34]

Overexpression of GAS5

GAS5 cDNA was cloned into pLV-EF1a-MCS-IRES-Puro (Bio-Settia, San Diego, CA, USA). 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 Life Technologies).

TGF-β secretion ELISA assay

BMF cells were plated in 24-well plates (5 × 10−4 cells/well) and grown with DMEM containing 10 % FBS. After adhesion, cells underwent serum deprivation for 48 h (DMEM with 1 % FBS). Cell culture supernatant for ELISA was collected. A human TGF-β ELISA kit (R&D Systems, Minneapolis, MN, USA) was used for ELISA with optical density measurements performed at 450 nm in ELISA reader to determine TGF-β levels.

Lentiviral-mediated RNAi for silencing GAS5

The pLV-RNAi vector was purchased from Biosettia Inc. The method of cloning the double-stranded shRNA sequence is described in the manufacturer's protocol. The oligonucleotide sequence of lentiviral vectors expressing shRNA that targets human GAS5 was synthesized and cloned into pLVRNAi to generate a lentiviral expression vector. The target sequence for GAS5 was 5′-AAAAGGAAGGATGAGAA-TAGCTATTGGATCCAATAGCTATTCTCATCCTTCC-3'.

Luciferase activity assay

The pmirGLO-GAS5-Wt reporter was generated by cloning the wild-type putative target region of GAS5 into pmirGLO plasmids (Promega, Madison, WI, USA) following the manufacturer's instructions. The pmirGLO-GAS5-mut reporter was generated by mutagenesis. Cells co-transfected with the pmirGLO-GAS5-Wt reporter, the pmirGLO-GAS5-mut reporter, or miR-Scr using Lipofectamine 2000 reagent were analyzed for luciferase activity.

Statistical analysis

Data were presented as mean ± SD. A Student's T test or analysis of variance (ANOVA) test will be used to compare the continuous variables among groups. $P < 0.05$ will be considered statistically significant.

Results

Accumulating evidence has suggested that lncRNAs are essential regulatory factors in fundamental physiology processes and dysregulation of lncRNAs may contribute to pathological outcomes, including fibrosis.[35,36] In order to identify the association between lncRNAs and OSF pathogenesis, we used RNA sequencing to demonstrate reduction in the expression level of the lncRNA GAS-5 in OSF tissues compared to normal buccal mucosa (Fig. 1A). Afterwards, qRT-PCR analysis revealed significantly lower expression of GAS in OSF tissues compared to N tissues (Fig. 1B). In the same way, the relative expression level of GAS5 was lower in fBMFs relative to BMFs (Fig. 1C). This finding suggests a potential role for GAS-5 in the pathogenesis of OSF.

Given GAS5's reduced expression in OSF tissues and its potential role in fibrosis, we further investigated its relationship with TGF-β, a key regulator of fibrotic processes. To investigate whether GAS5 modulates TGF-β-induced

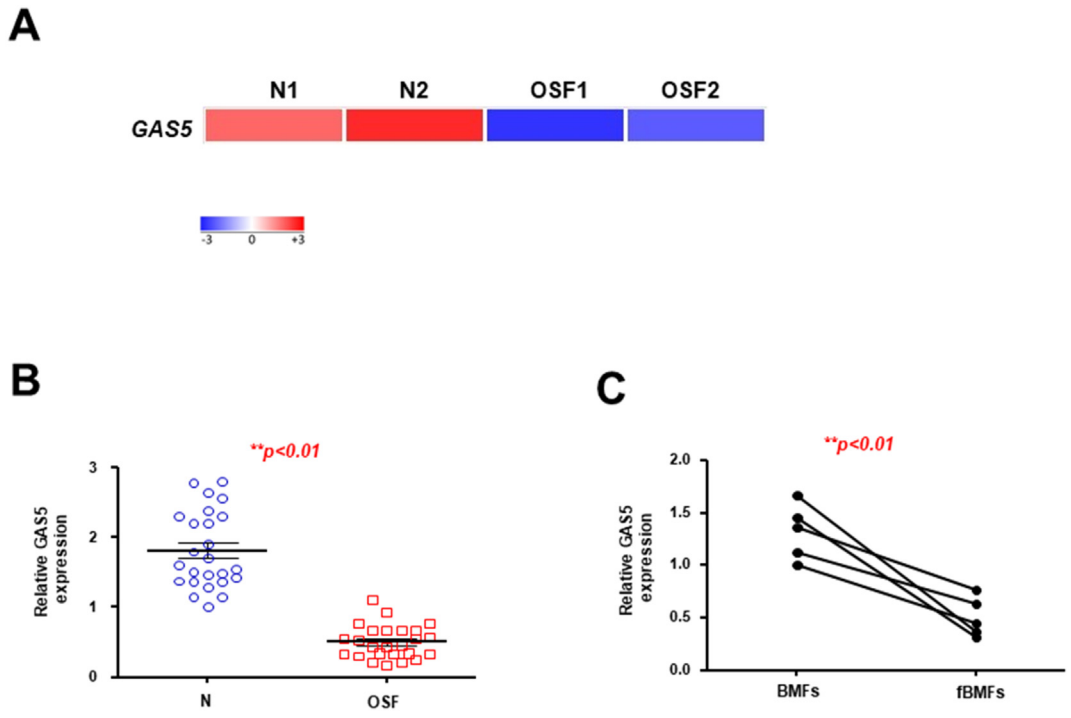

Figure 1 Downregulation of GAS5 in OSF tissues and fBMFs. (A) RNA sequencing analysis demonstrated reduction in the expression level of the lncRNA growth arrest-specific 5 (GAS5) in oral submucous fibrosis (OSF) tissues compared to normal buccal mucosa (N). (B) A significant decrease in GAS5 expression was found in OSF compared to N tissues by qRT-PCR analysis. (C) The relative expression level of GAS5 was lower in human fibrotic buccal mucosal fibroblasts (fBMFs) relative to human buccal mucosal fibroblasts (BMFs). Student's T test statistical analysis $^{**}P < 0.01$ compared to control group.

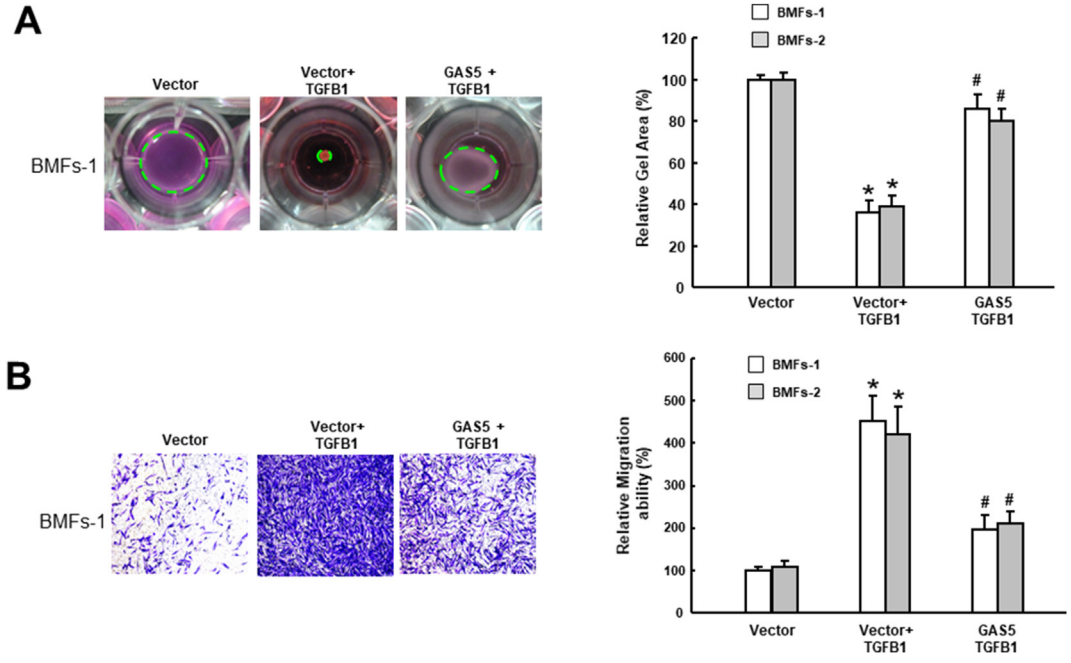

Figure 2 GAS5 repressed TGF-β1-induced collagen contractility and migration in BMFs. Human buccal mucosal fibroblasts (BMFs) treated with or without transforming growth factor (TGF)-β were transduced with lentiviral vectors overexpressing growth arrest-specific 5 (GAS5). (A) TGF-β1 significantly increased the contractile activity in BMFs and this was restored when GAS5 overexpression. (B) Transwell migration assay revealed that GAS5 overexpression reversed the increased migration in the TGF-β1-stimulated BMFs. ANOVA test statistical analysis $^{*}P < 0.05$ compared to Vector group; $\#P < 0.05$ compared to Vector + TGFB1 group.

fibrosis, we treated BMFs with TGF-β and restored GAS5 expression to observe changes in cell phenotype. The results show that overexpression of GAS5 inhibited TGF-β-induced collagen gel contractility and migration ability in BMFs (Fig. 2B). On the other hand, it is well known that TGF-β/Smad2 signaling plays a central role in various fibroses.[37] Therefore, to further elucidate the mechanism by which GAS5 regulates fibrosis, we examined the impact of GAS5 on the TGF-β/Smad2 pathway and TGF-β secretion through Western blotting (WB) and ELISA. The results showed that the overexpression of GAS5 attenuated the TGF-β-induced phosphorylation of Smad2 (Fig. 3A). The secretion of TGF-β was significantly reduced in the GAS5 overexpression group compared to the control group (Fig. 3B). According to the findings above, the TGF-β secretion and Smad2 phosphorylation were significantly inhibited by GAS5 overexpression, suggesting that GAS5 may regulate fibrosis processes by suppressing the TGF-β/Smad2 signaling pathway.

To simulate the process of OSF caused by betel nut chewing, we will treat BMF with arecoline, a major component of betel nut, and overexpress GAS. The changes in contractile and migratory abilities will be observed. The results show the overexpression of GAS5 inhibited arecoline-induced collagen gel contractility and migration ability in BMFs (Fig. 4). These results suggest that GAS5 may play a protective role against arecoline-induced fibrosis, potentially contributing to the understanding and treatment of OSF.

To validate the direct interaction between GAS5 and miR-21, we first employed bioinformatics databases, miRBase and starBase, to predict potential target genes of GAS5. Subsequently, a mutant GAS5 sequence was designed to enable its binding to miR-33a (Fig. 5A). Following the

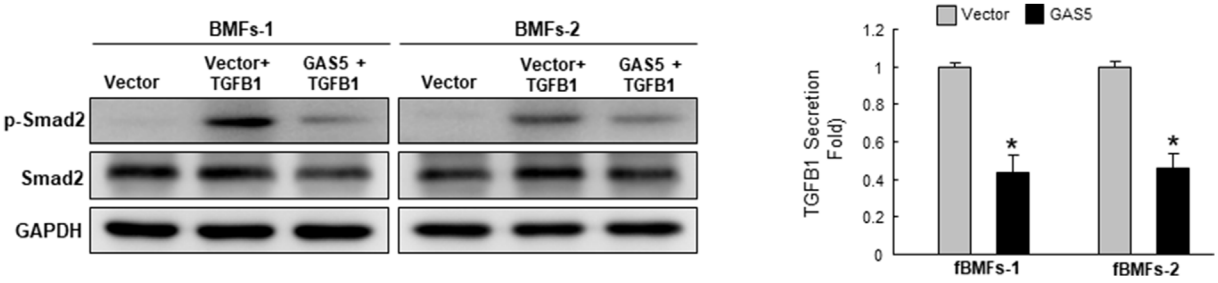

Figure 3 GAS5 repressed TGF-β1/Smad 2 signaling. **(A)**The protein expression levels of p-Smad2 and Smad2 in GAS5-transfected TGFB1 stimulated BMFs were analyzed by western blotting. (B) The secretion of TGFB1 in control and GAS5-overexpressing human fibrotic buccal mucosal fibroblasts (fBMFs) by ELISA analysis. Student's T test statistical analysis $^{*}P < 0.05$ compared to Vector group.

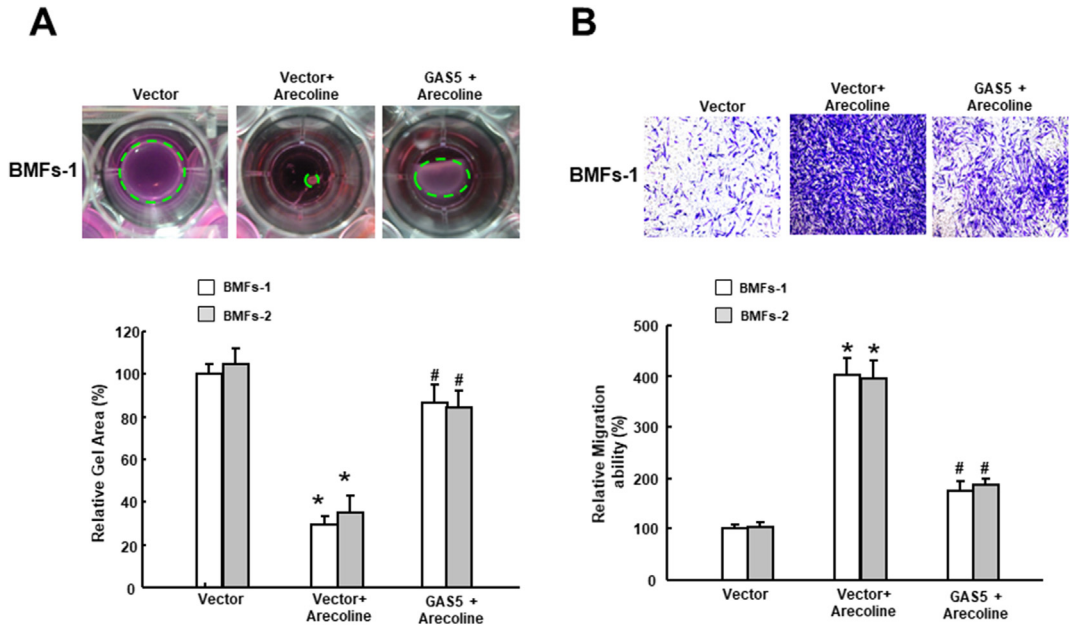

Figure 4 GAS5 repressed arecoline-induced collagen contractility and migration in BMFs. Human buccal mucosal fibroblasts (BMFs) treated with or without arecoline were transduced with lentiviral vectors overexpressing growth arrest-specific 5 (GAS5). (A) Arecoline significantly increased the contractile activity in BMFs and this was restored when GAS5 overexpression. (B) Transwell migration assay revealed that GAS5 overexpression revers the increased migration in the arecoline-stimulated BMFs. ANOVA test statistical analysis $^{*}P < 0.05$ compared to Vector group; $\#P < 0.05$ compared to Vector + Arecoline group.

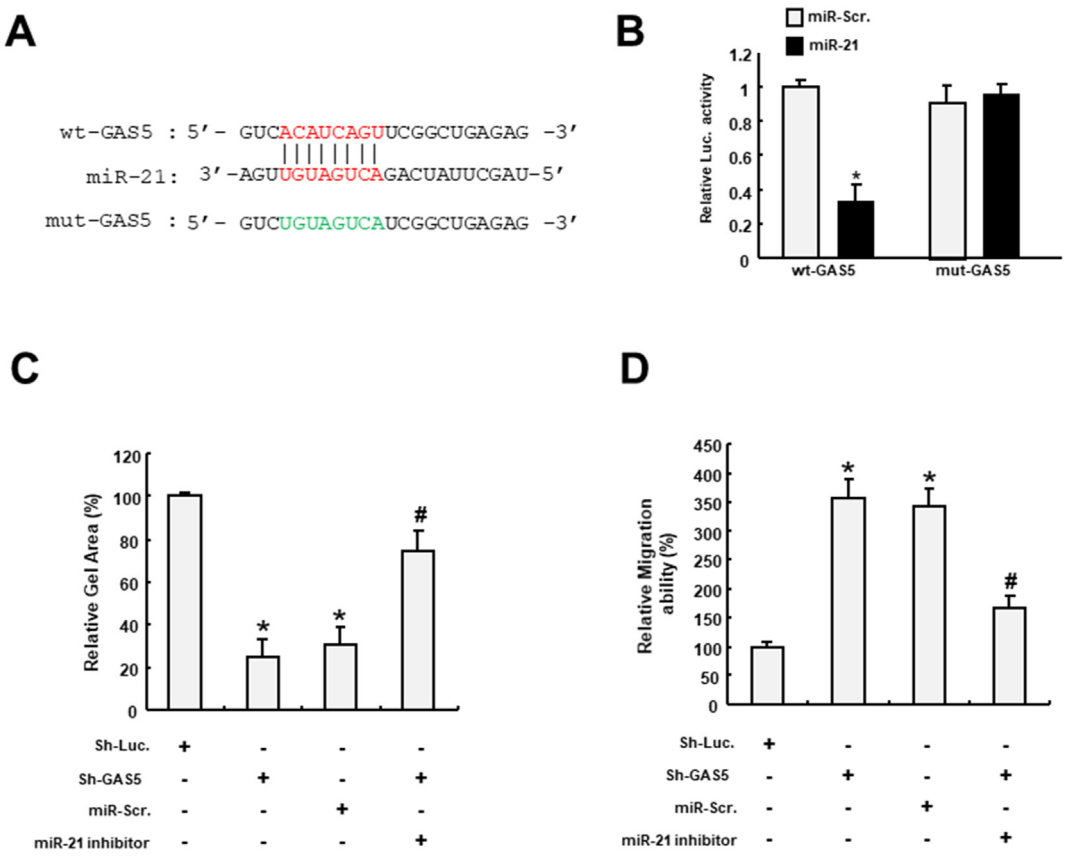

Figure 5 Silencing of GAS5 in BMFs enhanced cell contraction and migration, an effect reversed by miR-21 inhibition. (A) Binding sequences between growth arrest-specific 5 (GAS5) and miR-21 were analyzed using bioinformatics databases (miRBase and starBase), and a luciferase reporter gene sequence was designed accordingly. (B) The luciferase reporter assay confirmed that the designed sequence could not bind to miR-21. *$P < 0.05$ compared to wt-GAS + miR-Scr. group (C) Collagen contraction assays were performed to analyze the effects of miR-21 inhibitor and Sh-GAS5 on collagen contraction. (D) Transwell migration assays were used to analyze the effects of miR-21 inhibitor and Sh-GAS5 on cell migration. ANOVA test statistical analysis *$P < 0.05$ compared to Sh-Luc. group; #$P < 0.05$ compared to miR-Scr. group.

above design, we performed a luciferase reporter assay to validate the target site. The results demonstrated a significant decrease in luciferase activity in the wt-GAS5 group transfected with miR-21 compared to the miR-Scr control group. However, in the mut-GAS5 group, where the miR-21 binding site was mutated, the inhibitory effect of miR-21 was significantly attenuated. There was no significant difference in luciferase activity between the mut-GAS5 group transfected with miR-21 and the control group (Fig. 5B). Collagen contraction assays showed that silencing GAS5 significantly enhanced the contractile ability of BMFs. However, co-treatment with miR-21 inhibitor suppressed the increased contractile ability caused by GAS5 silencing (Fig. 5C). Similar results were observed in cell migration assays (Fig. 5D). Collectively, these findings demonstrate that GAS5 directly interacts with miR-21 to regulate myofibroblast function, supporting its role as a negative regulator of fibrosis in OSF.

Discussion

Oral submucous fibrosis (OSF) is a chronic, potentially malignant condition characterized by progressive fibrosis of the oral cavity.[1] While the exact etiology remains unclear, various factors, including betel nut chewing, are implicated in its pathogenesis.[2] Among the various molecular regulators implicated in fibrosis, long non-coding RNAs (lncRNAs) have emerged as key players. In this study, we identified GAS5 as a significantly downregulated lncRNA in OSF tissues and investigated its role in fibrosis.

Our findings revealed that GAS5 overexpression significantly inhibited TGF-β-induced collagen gel contraction and cell migration in BMFs. Furthermore, GAS5 attenuated TGF-β-induced phosphorylation of Smad2 and reduced TGF-β secretion, suggesting that GAS5 exerts its anti-fibrotic effects by inhibiting the TGF-β/Smad2 signaling pathway. These findings are consistent with previous studies that have implicated TGF-β as a key mediator of fibrosis in various tissues.[13–15]

To further explore the mechanisms underlying the anti-fibrotic effects of GAS5, we investigated its interaction with miRNAs. Bioinformatics analysis and luciferase reporter assays confirmed that GAS5 directly interacts with miR-21. Given that our previous studies demonstrated that arecoline upregulates miR-21 expression,[31] which in turn promotes OSF by targeting PDCD4,[38] we explored whether GAS5 interacts

with miR-21 to modulate fibrosis. Functional assays further revealed that GAS5 silencing significantly enhanced BMF contractility and migration, whereas co-treatment with a miR-21 inhibitor effectively reversed these effects. These results suggest that GAS5 functions as a molecular sponge for miR-21, and the miR-21/GAS5 axis plays a crucial role in modulating cellular behaviors in BMFs.

By identifying GAS5 as a key regulator of fibrosis, our study provides new insights into OSF pathogenesis. Given the interplay between GAS5 and miR-21, targeting this axis may offer a novel therapeutic strategy for OSF. However, several limitations of this study should be acknowledged. Firstly, the study primarily focused on in vitro experiments. Further in vivo studies are necessary to validate these findings in animal models of OSF. While GAS5 appears to regulate fibrosis through TGF-β/Smad2 signaling, future studies should assess its role in alternative pathways such as PI3K/Akt or MAPK, which have also been implicated in OSF progression.

In conclusion, our study demonstrates that GAS5 plays a crucial role in regulating fibrosis in BMFs by inhibiting the TGF-β/Smad2 signaling pathway. The GAS5/miR-21 axis represents a novel therapeutic target for the prevention and treatment of OSF. Future studies should validate these findings in vivo models and explore targeted therapies that enhance GAS5 expression, paving the way for clinical applications in OSF management.

Declaration of competing interest

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

Acknowledgments

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