Journal of Dental Sciences 19 (2024) 79–85

Available online at www.sciencedirect.com

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journal homepage: www.e-jds.com

Original Article

Targeting histone deacetylase 9 represses fibrogenic phenotypes in buccal mucosal fibroblasts with arecoline stimulation

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Received 17 April 2023; Final revision received 22 May 2023
Available online 4 June 2023

KEYWORDS
Oral submucous fibrosis;
Histone deacetylase;
HDAC9

Abstract *Background/purpose:* Oral submucosal fibrosis (OSF) is a premalignant disorder positively associated with betel nut chewing. Recent studies supported the promising benefits of histone deacetylase (HDAC) inhibitors for fibrosis treatment. Here we aim to clarify the pro-fibrogenic role of HDAC9 in regulating OSF.
Materials and methods: Healthy and OSF specimens were collected to investigate the clinical significance of HDAC9. Chronic arecoline treatment process was used to induce arecoline-mediated myofibroblasts-related activation of primary buccal mucosa fibroblasts (BMFs). Functional analysis of collagen gel contraction, transwell migration, and wound-healing assays were performed to assess the change in pro-fibrogenic properties of BMFs and fibrotic BMFs (fBMFs).

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https://doi.org/10.1016/j.jds.2023.05.029
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Lentiviral-mediated HDAC9 knockdown was used to verify the role of HDAC9 in the pro-fibrogenic process.
Results: We found that arecoline significantly increased the mRNA and protein expression of HDAC9 of BMFs in a dose-dependent manner. Knockdown of HDAC9 in BMFs reversed the strengthened effects of arecoline on collagen gel contraction, cell migration, and wound-healing ability. We further demonstrated that knockdown of HDAC9 in fBMFs significantly attenuated its inherent pro-fibrogenic properties. Furthermore, we confirmed a significantly increased expression of HDAC9 mRNA in OSF compared to normal tissues, which suggested a positive correlation between the up-regulation of HDAC9 and OSF.
Conclusion: We demonstrated that silencing of HDAC9 inhibited arecoline-induced activation and inherent pro-fibrogenic properties, suggesting potential therapeutics by targeting HDAC9 in the OSF treatment.
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Introduction

Oral submucous fibrosis (OSF), recognized as a premalignant disease of the oral cavity, is a chronic progressive disorder prevalently in South Asia. It is characterized by chronic inflammation and submucosal fibrosis, resulting in reduced mouth opening, burning sensation, difficulty in swallowing, and restricted tongue movement. In histopathologically properties, OSF exhibits excessive collagen accumulation in the connective tissue, juxta-epithelial hyalinization, and epithelium atrophic. The habit of betel nut chewing has been identified as a major risk factor for the onset of OSF, which presents an increased risk of oral malignant transformation.[1] Among the elucidated pro-fibrogenic pathways, arecoline-induced activation of TGF-β signaling is the major contributor to OSF progression.[2] This dysregulated TGF-β signaling drives the overactive fibrogenic responses of fibroblasts and promotes the fibroblast-to-myofibroblast transformation.[3] The persistent existence of myofibroblasts causes the excessive production of profibrogenic factors and collagens, all of which ultimately lead to the development of OSF.[4–6] Despite many promising results reported, the available therapeutics for OSF are still lacking.

Histone deacetylases (HDAC) is an enzyme that regulates transcriptional activity by catalyzing the removal of acetyl groups from N-acetyl-L-lysine amino acids of histones. Recent studies indicated HDAC inhibitors (HDACi) as potent therapeutics for fibrosis due to HDACs expressed aberrantly in fibrotic tissues and regulated multiple pro-fibrogenic mechanisms, particularly the TGF-β signaling pathway, in a histone and non-histone targets manner.[7,8] For instance, the treatment with HDACi attenuated the TGF-β-induced activation of myofibroblasts.[9–11] However, understanding the various pathways by which HDAC9 regulates fibrosis is still insufficient. HDAC9 has been reported to involve some oral disease progression. For instance, overexpressed HDAC9 in oral squamous cell carcinoma cells (OSCCs) increased cell proliferation and prevented apoptosis by inhibiting nuclear receptor 4A1 (NR4A1) transcription.[12,13] In addition, the mutual inhibition between HDAC9 and miR-17 regulated inflammation-induced osteogenesis of human periodontal ligament stem cells.[14] Our previous study indicated the impact of HDAC8i on myofibroblast properties, highlighting that the HDAC-mediated pro-fibrogenic mechanisms are crucial for OSF development.[15] Therefore, this study aims to investigate the role of HDAC9 on arecoline-induced pro-fibrogenic properties of human buccal mucosa fibroblasts (BMFs) for the development of novel strategies to halt the process in OSF.

Materials and methods

Tissue collection and cell culture

Extraction of fBMFs and BMFs were from the normal buccal mucosa in OSF patients recruited in Chung Shan Medical University Hospital. All operations were under the adherence to the precepts of the Helsinki Declaration and evaluation by Chung Shan Medical University's Institutional Review Committee. Fibrotic BMFs and BMFs were cultured as previously reported, having cell cultures used between the third and eighth passages.[16] Patients with an areca-using history who were first diagnosed with OSF at our hospital were included in this study; those with systemic diseases, severe periodontal diseases, oral malignant tumors, or smoking history were excluded. The healthy oral mucosa (N) samples removed undergoing the surgical operation were obtained from healthy individuals without areca-using habits. After the final diagnosis by two experienced pathologists, a total of 30 cases of normal and OSF tissues were collected.

Quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted using Trizol Reagent from cells and tissues, and the RNAs were reverse-transcribed to cDNAs using Superscript III first-strand synthesis system (Invitrogen Life Technologies). The resultant cDNAs were then amplified by qRT-PCR in ABI StepOne™ Real-Time PCR Systems (Applied Biosystems, Waltham, MA, USA). PCR was performed under the following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Relative mRNA expression was presented by the $2^{-\Delta\Delta CT}$ method using GAPDH.[16] The primer sequences used in this

study were listed below: HDAC9: forward primer sequence: 5′-CCTCCTCGAAAGATGGCTGT-3′ and reverse primer sequence: 5′- TTCCCAGGGCTTTCCTCAAG-3′; GAPDH: forward primer sequence: 5′-AGACCACAGTCCATGCCATC-3′ and reverse primer sequence: 5′-CAGGGCCCTTTTTCTGAGCC-3′.

Knockdown of HDAC9

The pLV-RNAi vector was purchased from Biosettia Inc. (Biosettia, San Diego, CA, USA). The construction of pLV-RNAi vector expressing scramble sequence and short-hairpin RNA (shRNA) that targets human HDAC9, virus particle production, and cell infection was described in our previous study.[17] The target sequences for HDAC9 are listed as follows: Sh-HDAC9-1: 5′- AAAAGCTTCTGATAGCAGAGTTTCTTGGATCC AAGAAACTCTGCTATCAGAAGC-3′and Sh-HDAC9-2: 5′-AAAA GGCACGTGTTGTTTACATTTTGGATCCAAAATGTAAACAACACG TGCC-3′.

Western blot analysis

Western blotting was conducted according to the previous description previously.[17] In brief, 20 μg protein of cell lysates were run on 10% SDS-PAGE and transferred onto a PVDF membrane (Millipore, Billerca, MA, USA). The membranes were then blocked with 5% bovine serum albumin (BSA) followed by successive incubation with primary (HDAC9 and GAPDH: Thermo Scientific, Waltham, MA, USA) and secondary antibodies. The immunoreactive bands were developed using Pierce ECL Western Blot Substrate (Thermo Scientific, Waltham, MA, USA) and captured using LAS-1000plus Luminescent Image Analyzer (GE Healthcare Biosciences, Piscataway, NJ, USA). All antibodies were purchased from ThermoFisher Scientific.

Collagen gel contraction analysis

Cells were mixed with 2 mg/ml collagen solution (Sigma-Aldrich, St. Louis, MO, USA). The 0.5 ml cell/collagen mixture was then added into a 24-well-plate followed by incubation at 37 °C for 2 h. After polymerization, the gels were incubated with 0.5 ml serum-free medium for another 48 h. The extent of gel contraction was quantified using ImageJ software (NIH, Bethesda, MD, USA).[18]

Transwell migration assay

Cell migration ability was evaluated using Transwell migration assays with a polycarbonate filter membrane of 8-μm pore size (Corning Incorporated, Kennebunk, ME, USA). Cells suspended in serum-free medium were seeded into the upper insert of the chamber, and the growth medium with 10% fetal bovine serum (FBS) were then added into the lower chamber for serving as a chemoattractant. After 48 h of incubation, the migrated cells on the lower surface of the membranes were stained with 0.1% crystal violet and quantified using ImageJ software (NIH, Bethesda, MD, United States).[19]

Wound-healing assay

Once cells reached 90% confluence in 12-well plates, a wound gap was introduced by scratching the length of the well with a 200-μl pipette tip. The growth medium was then replaced with a 2% FBS medium and incubated cells for another 48 h. The cell movement towards the wound gap was imaged under a microscope, and the area not covered by cells was quantified using ImageJ (NIH, Bethesda, MD, USA).[19] The change in wound-healing ability was assessed by calculating the ratio of the cell-uncovered area in each group to that of the control group.

Statistical analysis

All data were obtained from at least three independent experiments. Statistical analysis was performed using either a Student's t test or one-way ANOVA with Tukey's multiple comparison. Data were represented as mean ± SD, and $P < 0.05$ was considered statistically significant.

Results

The chronic-stimulated experimental model with arecoline has been widely utilized to induce the transformation of fibroblast-to-myofibroblast.[2,6,20] Results showed that the mRNA (Fig. 1A) and protein (Fig. 1B) expression levels of HDAC9 in the patient-derived BMFs were significantly increased after treatment with arecoline for 24 h in a dose-dependent manner.

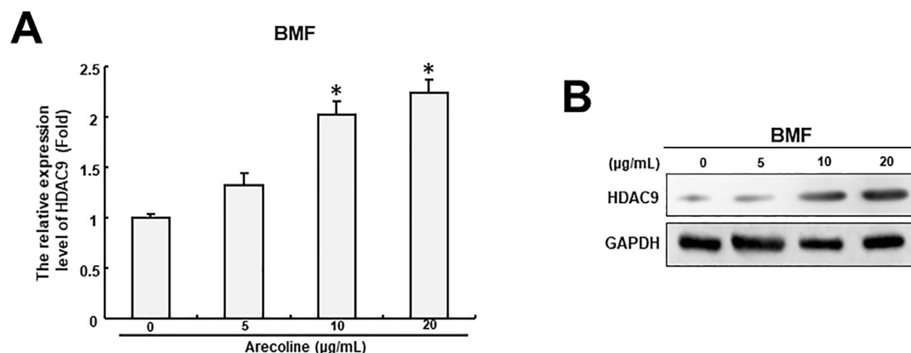

Figure 1 HDAC9 expression is increased by arecoline treatment of BMFs. The relative expression of HDAC9 (**A**) mRNA level and (**B**) protein level of BMFs after treatment with arecoline for 24 h *$P < 0.05$ compared with control without arecoline treatment.

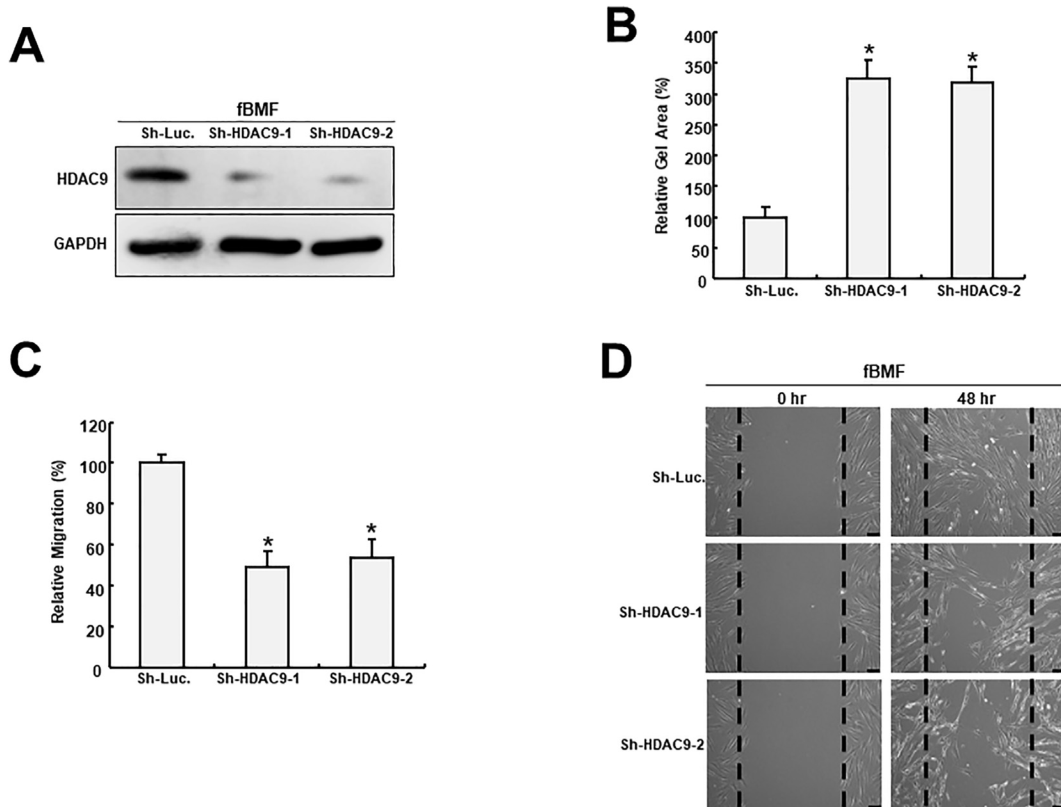

Figure 2 **Knockdown of HDAC9 in fBMFs attenuates the pro-fibrogenic properties**. (**A**) Verification of HDAC9 protein expression of sh-HDAC9 expressing fBMFs (Sh-HDAC9-1/-2). The relative ability of (**B**) collagen gel contraction, (**C**) cell migration, and (**D**) wound-healing of fBMFs (Sh-Luc.) and sh-HDAC9 expressing fBMFs (Sh-HDAC9-1/-2). *P $<$ 0.05 compared with Sh-Luc.

To confirm the potential of targeting HDAC9 for the OSF treatment, we collected patient-derived fibrotic BMFs (fBMFs) and established the shHDAC9-expressing fBMFs (Fig. 2A). As reported in our previous studies, these primary fBMFs showed the increased expression of myofibroblasts markers, including α-SMA and collagen type I, as well as several pro-fibrogenic properties, including excessive secretion of TGF-β and the ability of collagen gel contraction. Results showed that the knockdown of HDAC9 significantly inhibited the collagen gel contraction (Fig. 2B), cell migration (Fig. 2C), and wound-healing abilities (Fig. 2D) of fBMFs.

To determine the key role of HDAC9 in the arecoline-induced fibrogenesis in BMFs, the lentiviral vectors expressing shHDAC9 was performed to knockdown the expression of HDAC9 in BMFs. As expected, the arecoline was

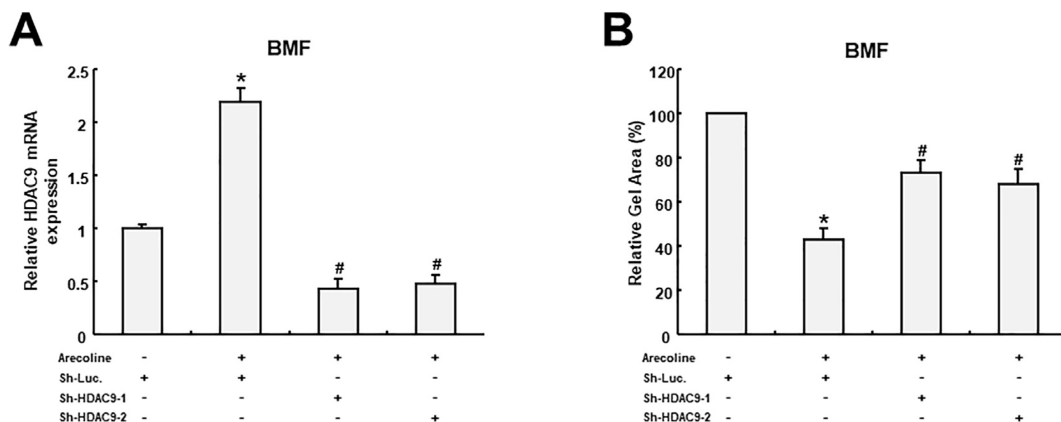

Figure 3 **Knockdown of HDAC9 in BMFs reverses the arecoline-stimulated increase in HDAC9 expression and gel contraction.** The relative (**A**) mRNA level of HDAC9 and (**B**) collagen gel contraction ability of BMFs (Sh-Luc.) and sh-HDAC9 expressing BMFs (Sh-HDAC9-1/-2) after treatment with arecoline for 24 h *$P < 0.05$ compared with Sh-Luc. without arecoline treatment. #$P < 0.05$ compared with Sh-Luc. with arecoline treatment.

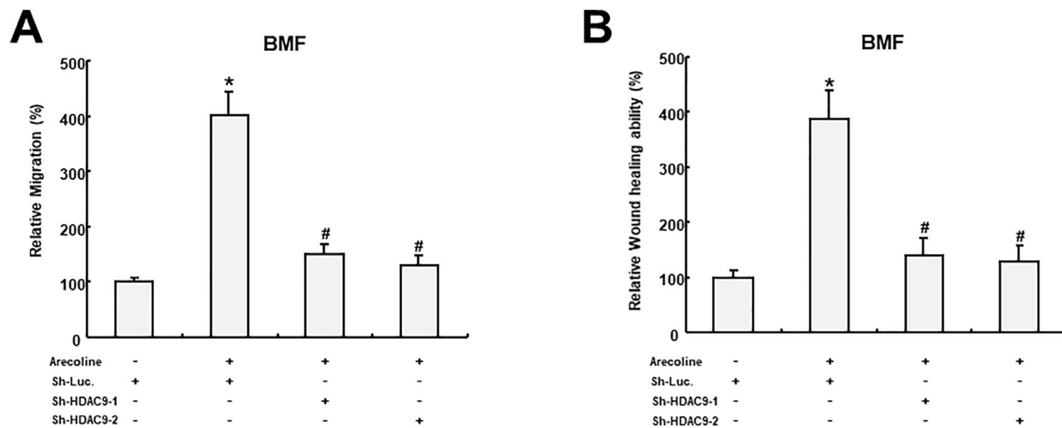

Figure 4 Knockdown of HDAC9 in BMFs reverses the arecoline-stimulated increase in cell migration and wound-healing ability. The relative ability of (**A**) cell migration and (**B**) wound-healing of BMFs (Sh-Luc.) and sh-HDAC9 expressing BMFs (Sh-HDAC9-1/-2) after treatment with arecoline for 24 h *$P < 0.05$ compared with Sh-Luc. without arecoline treatment. #$P < 0.05$ compared with Sh-Luc. with arecoline treatment.

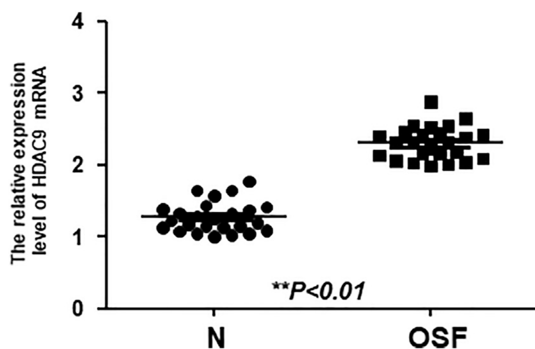

Figure 5 Significant increased expression of HDAC9 in clinical OSF tissues. The relative expression level of HDAC9 mRNA of normal (N) and fibrotic (OSF) buccal tissues obtain from healthy individuals (n = 30) and OSF patients (n = 30) by qRT-PCR analysis.

failed to induce the up-regulation of HDAC9 in shHDAC9-expressing BMFs (Fig. 3A). Collagen gel contraction, transwell migration, and wound healing assays are the typical methods for quantifying myofibroblasts properties and phenotypes in vitro. Results showed that the arecoline-induced collagen gel contraction (Fig. 3B), cell migration (Fig. 4A), and wound-healing abilities (Fig. 4B) of BMFs were reversed in shHDAC9-expressing BMFs (Fig. 3B and 4). These results suggested that the increased expression of HDAC9 conferred BMFs with myofibroblast properties that may contribute to pro-fibrogenic processes triggered by arecoline.

To further defined the clinical significance of HDAC9 in OSF, qRT-PCR analysis was conducted to detect the HDAC9 mRNA level in normal and OSF tissues obtained from healthy individuals and OSF patients, respectively. Results showed that HDAC9 expression was significantly increased in OSF tissues (Fig. 5). Collective, our data suggested that HDAC9 plays a crucial contributor to OSF and that targeting HDAC9 may be novel therapeutics for OSF treatment.

Discussion

This study sheds light on the pro-fibrogenic role of HDAC9 in OSF for the first times. Our results showed that HDAC9 expression was up-regulated in patients-derived fBMFs and OSF tissue, while inhibition of HDAC9 might reverse the fibroblast-to-myofibroblasts transformation to combat OSF. Previous studies attempt to explore the pathogenic mechanisms of fibrosis from various aspects. Among them, TGF-β is considered the clearest pro-fibrogenic factor, which drives fibroblast-to-myofibroblast transformation and exacerbates ECM deposition via canonical SMAD and non-canonical signaling pathways.[3] Although TGF-β inhibitors have been applied in clinical studies, it not always shown efficacy and might cause unacceptable adverse effects.[3] Recently, HDACi have been recognized as alternative methods to inhibit fibrosis due to the involvement of HDACs in regulating TGF-β pathway activity through various processes.[7,8] Numerous studies revealed that administration with HDACi directly impacted TGF-β-induced myofibroblast transformation. For instance, Jones et al. established a dataset of epigenetic small-molecule modulators for identifying the potent molecules that could reverse TGF-β-mediated fibroblast activation.[10] Their results showed that pan-HDACi treatment or knockdown of HDAC7 of lung fibroblasts suppressed the TGF-β-mediated epigenetic inhibition of PGC1α and caused fibroblasts quiescence.

HDAC family is divided into Zn^{+}-dependent HDACs (classes I, IIa, IIb, and IV) and NAD^{+}-dependent class III HDACs.[7] Our previous works shown that HDAC8, classified as a class I HDAC, was overexpressed in fBMFs and OSF tissue.[15] Inhibition of HDAC8 significantly inhibits the pro-fibrogenic properties of fBMFs, including TGF-β secretion,

TGF-β1/Smad signaling, collagen gel contraction, and cell migration. In this study, we focused on the pro-fibrogenic effects of HDAC9, which belonged to class IIa HDAC, in arecoline-induced activated BMFs and OSF tissue-derived fBMFs. Altogether, results from our both studies suggested that HDACs mediated pro-fibrogenic mechanisms are crucial for OSF development, and that also explains the significant therapeutic effects of pan-HDACi on various types of fibrosis.[8,10]

The evidence of how HDAC9 regulating fibrosis progression is still unclear. Yang et al. reported that the activation of hepatic stellate cells (HSCs) was accompanied by increased expression of HDAC9 and several myofibroblast markers, including TGF-β, α-SMA, and COL1A1.[21] Knockdown of HDAC9 significantly suppressed the expression of pro-fibrogenic genes induced by TGF-β1 and hepatic stellate cells the activation. Moreover, overexpression of HDAC9 was detected in several human liver disease tissues, indicating that HDAC9 may be an early important event in the pathological progression toward fibrosis. Notably, this study also showed that inhibition of HDACs significantly reduced the expression of myocyte enhancer factors 2 (MEF2), which were upregulated during HSCs activation. The mutual regulation of HDAC9 and MEF2 regulates various biological processes, including muscle differentiation,[22] cancer progression,[23,24] and fibrosis.[24] In OSCCs, the HDAC9 interacted with MEF2D to form a transcriptional complex that inhibited the expression of the MEF2D-targeted pro-apoptotic gene, NR4A1. Therefore, knockdown of HDAC9 significantly impeded cell proliferation and induced cell apoptosis. This evidence implied that the HDAC9/MEF2s axis might be important for the evasion of apoptosis of myofibroblasts in OSF tissue and indicating the potential for malignant transformation.

EMT is regarded as a key process in fibrosis progression. Many studies have attempted to modulate OSF through EMT.[5,25] Notably, increased expression of HDAC9 contributed to endothelial-to-MT phenotype and promoted the vascular pathology of atherosclerosis.[26] Endothelial-specific knockout of *hdac9* and treatment with class IIa HDAC inhibitor suppressed End-MT and improved aorta plaque stability in vivo. It suggested that HDAC9 confers cells to acquire mesenchymal phenotype, explaining the multiple origins of myofibroblasts. The involvement of Wnt signaling in regulating EMT process has been demonstrated in various types of cells.[27,28] Recent study indicated that inhibition of HDAC9 impeded EMT of $CD133^{+}$ prostate cancer cells through the suppression of β-catenin activation and its translocation from the cytoplasm to nucleus. However, the conclusions regarding how HDAC9 regulates Wnt signaling are still inconsistent. It seems to depend on tissue subtype or environmental conditions. For instance, HDAC9-mediated epigenetic suppression of autophagy-related gene restricted the autophagosome formation under hypoxic condition, resulting in the dephosphorylation of GSK3β and Wnt signaling inactivation. Another study revealed that HDAC9-mediated β-catenin deacetylation restrained its nuclear translocation and decreased EMT in non-serous ovarian cancer. In contrast, HDAC9-mediated FOXO1 nuclear translocation in serous ovarian cancer promotes the transcription of TGFβ and its signaling activation, thereby increasing EMT and metastasis.[29]

Our previous study found a significant role of early growth response protein 1 (Egr-1) in promoting OSF progression.[30] The Egr-1 overexpression was controlled by non-coding mediated epigenetics regulation, promoting pro-fibrogenic properties and resulting in the fibroblast-to-myofibroblasts transformation. Consistent with our works, Li et al. reported that the chronic treatment with arecoline induced cell proliferation of BMFs via the Egr-1-dependent Wnt5a activation.[31] Furthermore, their transcriptome analysis of BMFs also indicated that the ECM-related and Wnt signaling were the distinct core signaling pathways that responded to the chronic treatment with arecoline.[32] However, other studies have shown that Wnt5a-mediated Wnt signaling activity is negatively associated with HDACs activity.[33,34] Notably, the available ChIP-Seq data from 8 cell lines revealed that the proximal promoter of HDAC9 contains transcription factor binding sites of not only MEF2A and MEF2C but also Egr-1 and NF-κB.[35] Based on this data, we suggested that the increased HDAC9 could be partially driven by arecoline-induced Egr-1, thereby increasing EMT properties and pro-fibrogenic properties through non-Wnt signaling pathways.

There have some limitations in this study. We were considering that with an increasing passage number could result in senescence of primary oral fibroblasts, which may lead to the overinterpretation of data of the arecoline-induced phenotypes.[36] Therefore, in this study, we only stimulated BMFs with arecoline for 24 h, which has been reported in our previous studies to be sufficient for inducing the pro-fibrogenic phenotypes, such as increased α-SMA expression and collagen gel contraction ability.[30,37] Moreover, the arecoline-induced transcriptome profile of BMFs reported in a recent study supports our findings, indicating that low-dose arecoline-induced differentially expressed genes in BMFs were involved in ECM remodeling.[32] However, this is a relatively short duration compared to the OSF pathogenesis progression that may not reflect the potential long-term consequences of arecoline. Therefore, it is necessary to investigate the long-term treatment with arecoline on BMFs, such as conducting a stimulation period of at least a month in vitro, as well as arecoline-induced OSF animal models.

Collectively, we concluded that targeted inhibition of HDAC9 successfully reversed the arecoline-induced activation of BMFs and suppressed the pro-fibrogenic properties of fBMFs. It supports the significant role of HDAC9 in the initiation and progression of oral fibrosis and provides new insights into the development of therapeutic targets for OSF.

Declaration of competing interest

The authors have no conflicts of interest to this article.

Acknowledgment

This work was funded by grants from the Chung Shan Medical University Hospital, Taichung, Taiwan (CSH-2021-C-015); Kaohsiung Armed Forces General Hospital, Kaohsiung, Taiwan (KAFGH-A-110012); Wan Fang Hospital, Taipei, Taiwan (112-wf-eva-22) in Taiwan.

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