miR-203 inhibits arecoline-induced epithelial-mesenchymal transition by regulating secreted frizzled-related protein 4 and transmembrane-4 L six family member 1 in oral submucous fibrosis

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Abstract. Oral submucous fibrosis (OSF) is a potentially malignant disease predominantly found in Asian people. The areca nut has been implicated in this disease. Arecoline, one of the areca alkaloids, induces epithelial-mesenchymal transition (EMT)-related factors in primary human buccal mucosal fibroblasts. Yet, the mechanisms of the underlying arecoline-induced EMT in OSF remain unknown. In the present study, we aimed to investigate the role of microRNAs (miRNAs) in arecoline-induced EMT in HaCaT cells. We found that miR-203 was significantly downregulated in OSF tissues compared to that in normal buccal mucosa tissues, and that miR-203 negatively regulated secreted frizzled-related protein 4 (SFRP4) and positively regulated transmembrane-4 L six family member 1 (TM4SF1). We observed that upregulation of miR-203 significantly decreased the cell proliferation of HaCaT cells, and significantly upregulated the expression of cytokeratin 19 (CK19) and E-cadherin proteins, whereas it significantly downregulated the expression of N-cadherin and vimentin compared to these levels in the vehicle control cells. Thus, we provide evidence to illustrate that miR-203 plays a role in the pathogenesis of OSF, which may be a target for OSF management.

Introduction

Oral submucous fibrosis (OSF) is a potentially malignant disease predominantly found in Asian people (1). OSF is characterized by submucosal fibrosis that affects most of the parts of the oral cavity and pharynx. OSF has been associated with oral squamous cell carcinoma, particularly in Taiwan and South-Central China where up to 80% of oral squamous cell carcinoma cases are associated with betel quid chewing (2). Possible etiological factors that have been implicated in this disease, include the areca nut, capsaicin in chillies, micronutrient deficiencies of iron, zinc and essential vitamins (3).

Arecoline, one of the areca alkaloids, is the main agent of the areca nut responsible for fibroblast proliferation (4). When influenced by slaked lime, arecoline is hydrolyzed to arecadine, which has pronounced effects on fibroblasts (5). It has been showed that arecoline exposure stimulates fibroblast growth (6). It was also reported that arecoline depleted cellular glutathione (GTH) levels, and subsequently induced various genotoxic and cytotoxic stimulation in oral mucosal fibroblasts (7). A recent study also demonstrated that arecoline induced epithelial-mesenchymal transition (EMT)-related factors in primary human buccal mucosal fibroblasts (6).

EMT is an indispensable mechanism during morphogenesis, and is also a crucial event in oral squamous cell carcinoma and OSF (8). Previous studies demonstrated that upregulation of several molecules involved in EMT, such as vimentin, were expressed in human buccal mucosal fibroblasts following arecoline treatment (9), suggesting that the EMT process may be directly involved in the pathogenesis of OSF. However, the mechanisms underlying the EMT induced by arecoline remain unknown.

MicroRNAs (miRNAs) are a class of short (~22 nt) non-coding RNAs, which have been implicated in multiple cellular processes, including survival, proliferation, apoptosis and EMT in various types of cells (10). Recently, a study found that arecoline induced expression changes in miRNAs in normal mucosal cells (11). However, few studies have investigated the functions and the mechanism of differentially expressed miRNAs in OSF.

In the present study, we aimed to investigate the role of miRNAs in arecoline-induced EMT in HaCaT cells. Furthermore, we also explored the potential regulated targets

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of the miRNAs. We found that miR-203 was significantly downregulated in OSF tissues compared to that in normal buccal mucosa tissues, and that miR-203 negatively regulated secreted frizzled-related protein 4 (SFRP4), which is correlated with EMT-related gene expression (12), and positively regulated transmembrane-4 L six family member 1 (TM4SF1), a small plasma membrane glycoprotein that regulates cell motility and proliferation (13). Subsequently, we observed that upregulation of miR-203 significantly decreased the capacity of cell proliferation of HaCaT cells, and significantly upregulated the expression of cytokeratin 19 (CK19) and E-cadherin proteins, whereas it significantly downregulated the expression of N-cadherin and vimentin compared to that of the vehicle control cells. Thus, we provide evidence to illustrate that miR-203 plays a role in the pathogenesis of OSF, which may be a target for OSF management.

Materials and methods

Sample collection. A total of 6 OSF tissue samples and 6 normal buccal mucosa tissues (Nor) were obtained from the Xiangya Hospital of Central South University according to the legislation and the Ethics Board of Xiangya Hospital. All subjects or their caregivers provided written informed consent. All samples were collected and identified by histopathological evaluation. All the samples were stored at -80˚C until being used.

Cell culture and treatment. HaCaT cells were purchased from ProCell Co. (Wuhan, China). All the cells were cultured in MEM supplemented with 15% fetal bovine serum in a 5% CO_2 humidified atmosphere at 37˚C. The cells were transfected with miR-203 mimics, miR-203 inhibitor, and a negative control (NC) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at a final concentration of 30 nM. To detect the effect of arecoline on EMT, the HaCaT cells were treated with arecoline (Selleck Chemicals, Houston, TX, USA) at the indicated concentrations for 72 h.

Quantitative real-time polymerase chain reaction (qPCR). The RNeasy Plus Mini kit (Qiagen, Valencia, CA, USA) was used to extract total RNA according to the manufacturer's instructions. The miRNeasy Mini kit (Qiagen) was used for real-time PCR to detect the expression of miR-203, miR30c and miR-206. The specific primer sets for miRNA-203, miR30c, miR-206 and U6 were purchased from GeneCopoeia. miR-203, miR30c and miR-206 expression was normalized to that of U6. The FastLane Cell SYBR®-Green kit (Qiagen) was used for real-time PCR to detect the expression of TM4SF1 and SFRP4. The primers for TM4SF1, SFRP4 and GAPDH were: TM4SF1 sense, GCTGGAACAGGATGACTGCT and antisense, ACTCGGACCATGTGGAGGTA; SFRP4 sense, ATCTCGCCTGAAGCCATCG and antisense, GGGGCTTAG GCGTTTACAGT; GAPDH sense, CAATGACCCCTTCATTG ACC and antisense, GACAAGCTTCCCGTTCTCAG. TM4SF1 and SFRP4 expression was normalized to GAPDH. The $2^{-\Delta\Delta Ct}$ method was used to analyze the data.

Western blotting. RIPA lysis buffer (Cwbiotech, Wuhan, China) was used to extract the total protein from the tissues and cells. The BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the protein concentration. The total protein was separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were blocked with 8% non-fat milk for 1 h and incubated with the indicated primary antibody (anti-TM4SF1 and anti-SFRP4 from Epitomics; rabbit, 1:1,000; anti-N-cadherin and anti-vimentin from Abcam; rabbit, 1:500; anti-CK19, GAPDH and anti-E-cadherin from Santa Cruz; mouse, 1:500) overnight at 4˚C. The membranes were washed and incubated with the appropriate secondary antibody for 90 min at 37˚C. The signals on the membranes were detected by enhanced chemiluminescence (ECL) reagent. Data were analyzed by densitometry using Image-Pro Plus software 6.0 and normalized to internal control expression (GAPDH).

Dual luciferase reporter system. The wild-type 3'-UTR of TM4SF1 and SFRP4 was inserted into the dual luciferase reporter vector. For the luciferase assay, 10^5 cells were plated and cultured in 24-well plates to reach ~70% confluency. The cells were co-transfected with miR-203 mimics and the TM4SF1 or SFRP4 dual luciferase reporter vector, respectively. After a 48-h transfection, the Luciferase Reporter Gene Assay kit (Global Biotech, Shanghai, China) was used to determine the luciferase activitiy on a luminometer (Roche). *Renilla* luciferase activity was normalized to firefly luciferase activity.

CCK-8 cell proliferation assay. Cells (1,000) were seeded in each well of 96-well plates for 12 h. Then, following the indicated treatment, the cells were further incubated for 0, 12, 24, 48, 72 and 96 h, respectively. CCK-8 reagent (10 μl) (Dojindo, Tokyo, Japan) was added to the well at 1 h before the end of the incubation. The optical density (OD) value at 490 nm of each well was detected by an enzyme immunoassay analyzer.

Statistical analysis. Statistical analysis was performed by GraphPad Prism 5 and SPSS 16.0 softwares. The Student's t-test or one-way ANOVA was used depending on the experimental conditions. Data are expressed as mean ± SD. Compared to the controls, a P-value of <0.05 was considered to indicate a statistically significant result.

Results

miRNAs are differentially expressed in OSF tissues. We performed qPCR assay to detect the changes in miRNAs in 6 OSF tissues. Compared to the average expression in the normal oral mucosa tissues, the expression of miR-206 was significantly upregulated in 2 cases and slightly upregulated in 4 cases of OSF; the expression of miR-30c was significantly upregulated in 5 cases and slightly upregulated in 1 case of OSF; the expression of miR-203 was significantly upregulated in all of the 6 cases (Fig. 1A-C). We also detected the expression of potential target genes of these miRNAs by qPCR and western blotting in OSF tissues. We found that SFRP4 was increased in 5 of the 6 cases of OSF tissues at the mRNA level and upregulated in all the OSF tissues at the protein level compared with that in the normal tissues (Fig. 1D, F and G).

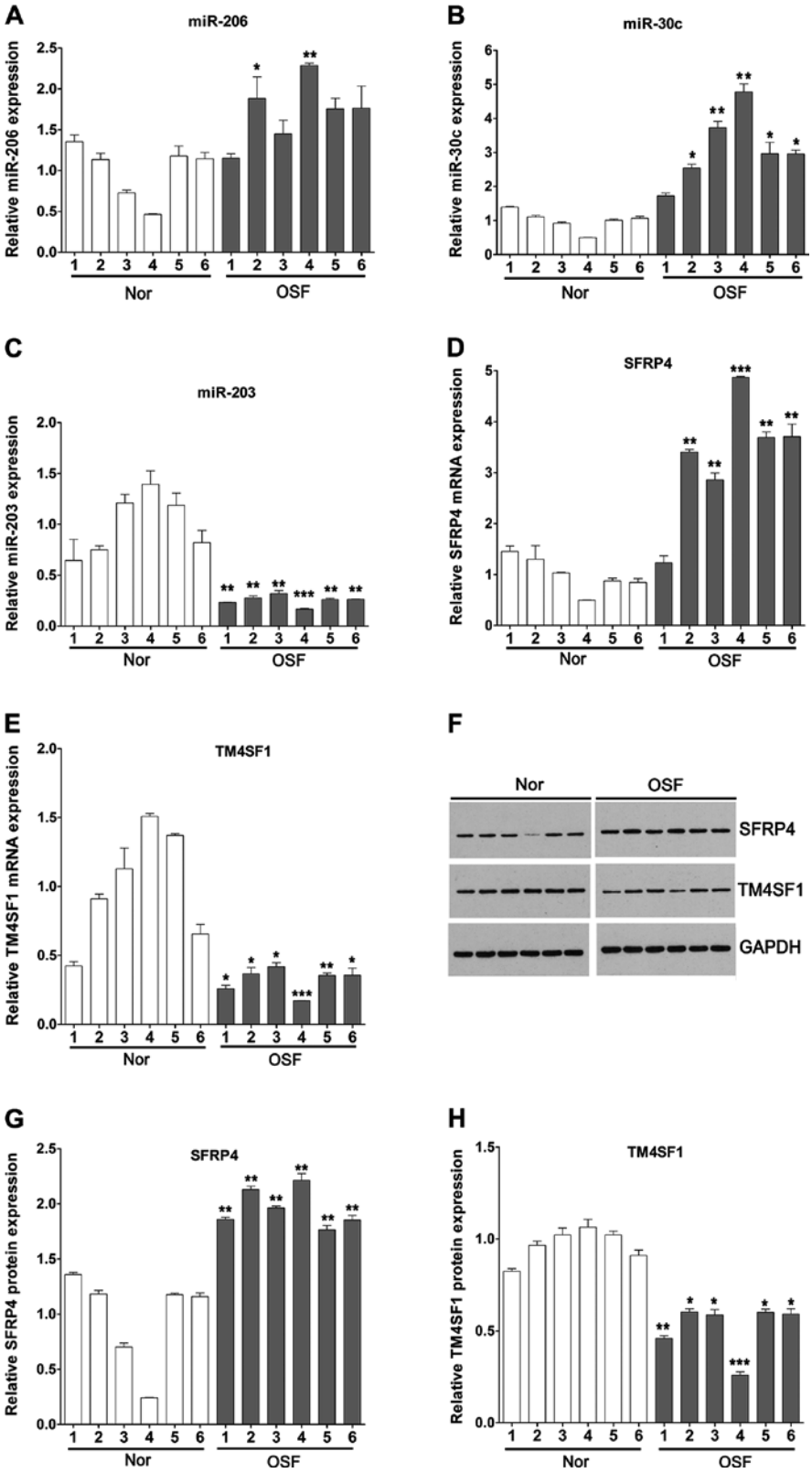

Figure 1. Expression of miR-206, miR-30c, miR-203, SFRP4 and TM4SF1 in OSF tissues and normal oral mucosa tissues. qPCR was utilized to detect the expression of (A) miR-206, (B) miR-30c, (C) miR-203, (D) SFRP4 and (E) TM4SF1 in OSF tissues and normal oral mucosa tissues. (F) Western blotting was used to analyze the expression of SFRP4 and TM4SF1 protein in OSF and normal oral mucosa tissues and quantification was carried out for (G) SFRP4 and (H) TM4SF1. Data are expressed as the means ± SD. *P<0.05, **P<0.01, ***P<0.001. SFRP4, secreted frizzled-related protein 4; TM4SF1, transmembrane-4 L six family member 1; OSF, oral submucous fibrosis.

In all of the 6 cases of OSF tissues and normal oral mucosa tissues, we found that TM4SF1 was significantly decreased at the mRNA and protein levels in the OSF tissues compared with levels in the normal tissues (Fig. 1E, F and H).

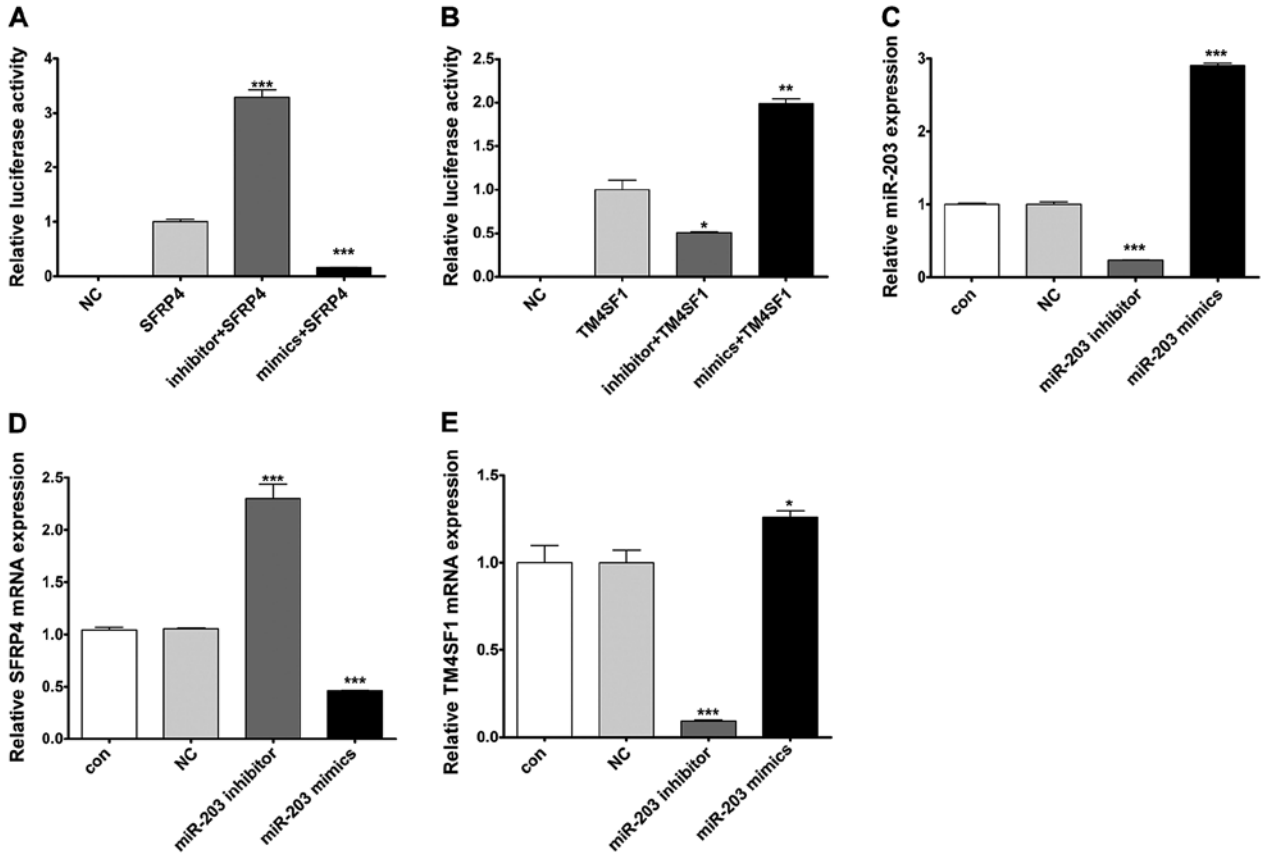

Figure 2. miR-203 negatively regulates SFRP4 and positively regulates TM4SF1. (A) The luciferase activity in cells transfected with SFRP4 3'UTR was decreased by the miR-203 mimics, whereas increased by miR-203 inhibitor. (B) The luciferase activity in cells transfected by TM4SF1 3'UTR was increased by miR-203 mimics, whereas decreased by miR-203 inhibitor. qPCR was used to detect the expression of (C) miR-203, (D) SFRP4 and (E) TM4SF1 mRNA after transfection with miR-203 mimic, miR-203 inhibitor or NC. Data shown are means ± SD, *P<0.05, **P<0.01, ***P<0.001. SFRP4, secreted frizzled-related protein 4; TM4SF1, transmembrane-4 L six family member 1.

miR-203 negatively regulates SFRP4 and positively regulates TM4SF1. To investigate whether miR-203 regulates the expression of SFRP4 and TM4SF1, we cloned the 3'UTR of SFRP4 and TM4SF1 downstream to the luciferase reporter gene. The constructed vectors were co-transfected with miR-203 mimics or inhibitor into the HaCaT cells. The luciferase activity of cells transfected with the miR-203 mimics and SFRP4 was significantly decreased compared with the cells that were transfected with SFRP4 alone, whereas the luciferase activity of cells transfected with miR-203 inhibitor and SFRP4 was significantly increased compared with the cells that were transfected with SFRP4 alone (Fig. 2A). In contrast to SFRP4, however, the luciferase activity of cells transfected with TM4SF1 was induced by co-transfection with the miR-203 mimics, while it was reduced by co-transfected with the miR-203 inhibitor (Fig. 2B). In order to further illustrate the regulatory relationship between miR-203 and SFRP4 and TM4SF1, we transfected the miR-203 mimics or inhibitor into HaCaT cells to upregulate or downregulate miR-203 expression. The efficiency of transfection was satisfied for further analysis (Fig. 2C). qPCR showed that enhanced or repressed miR-203 significantly decreased or increased SFRP4 mRNA levels, respectively, compared to the cells transfected with NC in the HaCaT cells (Fig. 2D). In addition, upregulation or downregulation of miR-203 significantly induced or reduced TM4SF1 mRNA levels, respectively, compared to the cells transfected with NC in the HaCaT cells (Fig. 2E). These results revealed that miR-203 negatively regulated SFRP4 and positively regulated TM4SF1 at the transcriptional levels.

Arecoline affects the expression of EMT-related genes in a dose-dependent manner. To determine the effects of arecoline on EMT in HaCaT cells, the HaCaT cells were treated with a series of increased doses of arecoline for 72 h. Western blotting was used to evaluate the changes in expression of the EMT-related genes, including CK19, E-cadherin, N-cadherin and vimentin. The results showed that CK19 expression was significantly decreased with increased concentrations of arecoline; E-cadherin expression was markedly reduced at 0.08 mM; while the expression of N-cadherin and vimentin was significantly upregulated with increasing concentrations of arecoline (Fig. 3). Downregulated expression of CK19 and E-cadherin, and upregulated expression of N-cadherin and vimentin are important promotive factors of EMT. Thus, the concentration of arecoline at 0.08 mM was chosen for further analysis.

Effects of arecoline on miR-203, SFRP4 and TM4SF1 in the HaCaT cells. To further analyze the effects of arecoline on

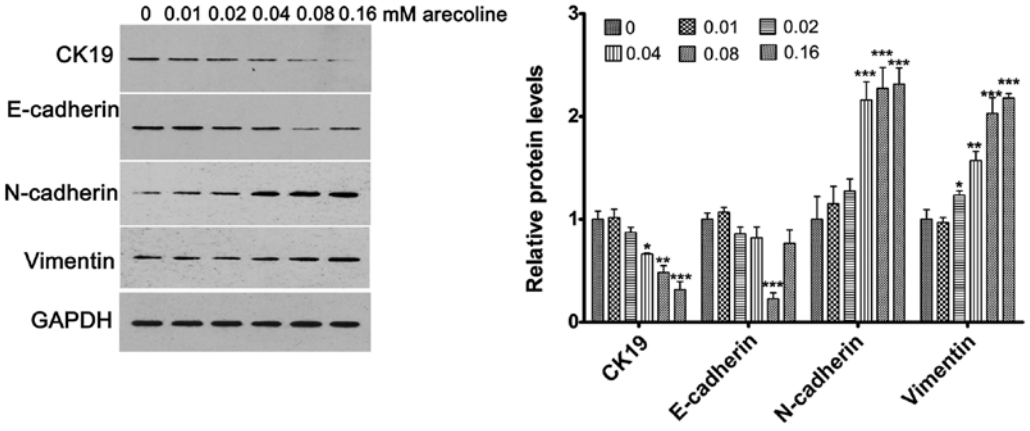

Figure 3. Arecoline affects expression of epithelial-mesenchymal transition-related genes in a dose-dependent manner. Expression of epithelial-mesenchymal transition-related genes (CK19, E-cadherin, N-cadherin and vimentin) in HaCaT cells was determined by western blotting and the results were quantified. Data shown are means ± SD, *P<0.05, **P<0.01, ***P<0.001. CK19, cytokeratin 19.

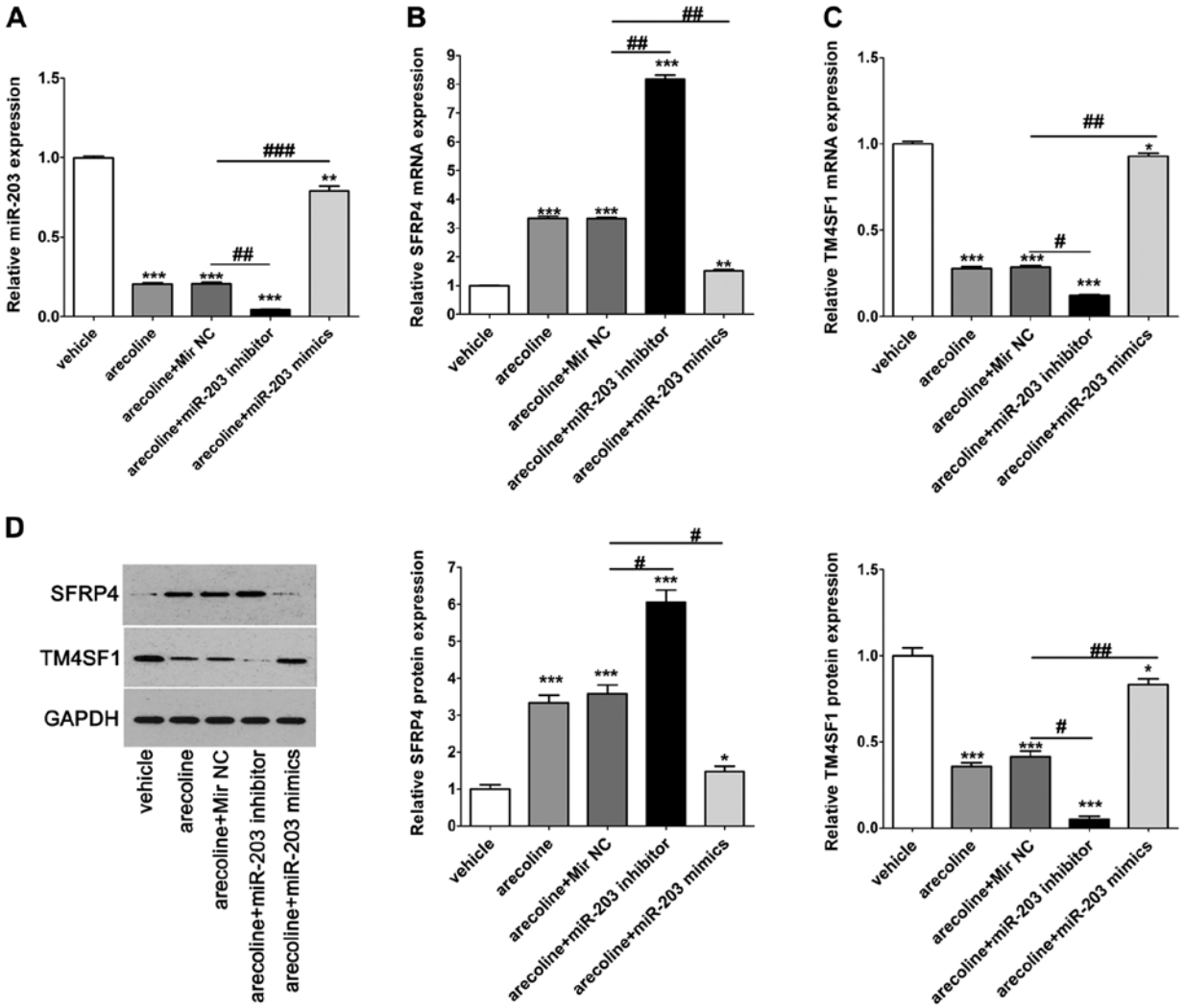

Figure 4. Effects of arecoline and miR-203 on SFRP4 and TM4SF1 in the HaCaT cells. qPCR was used to detect the expression of (A) miR-203, (B) SFRP4 mRNA and (C) TM4SF1 mRNA. (D) Western blotting was used to detect the expression of SFRP4 and TM4SF1 and the results were quantified. Data shown are means ± SD, *P<0.05, **P<0.01, ***P<0.001; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001. SFRP4, secreted frizzled-related protein 4; TM4SF1, transmembrane-4 L six family member 1.

miR-203, SFRP4 and TM4SF1 in the HaCaT cells, cells were treated with arecoline alone or co-treated with miR-203 mimics/inhibitor. We found that arecoline significantly decreased the expression of miR-203 compared with the vehicle control,

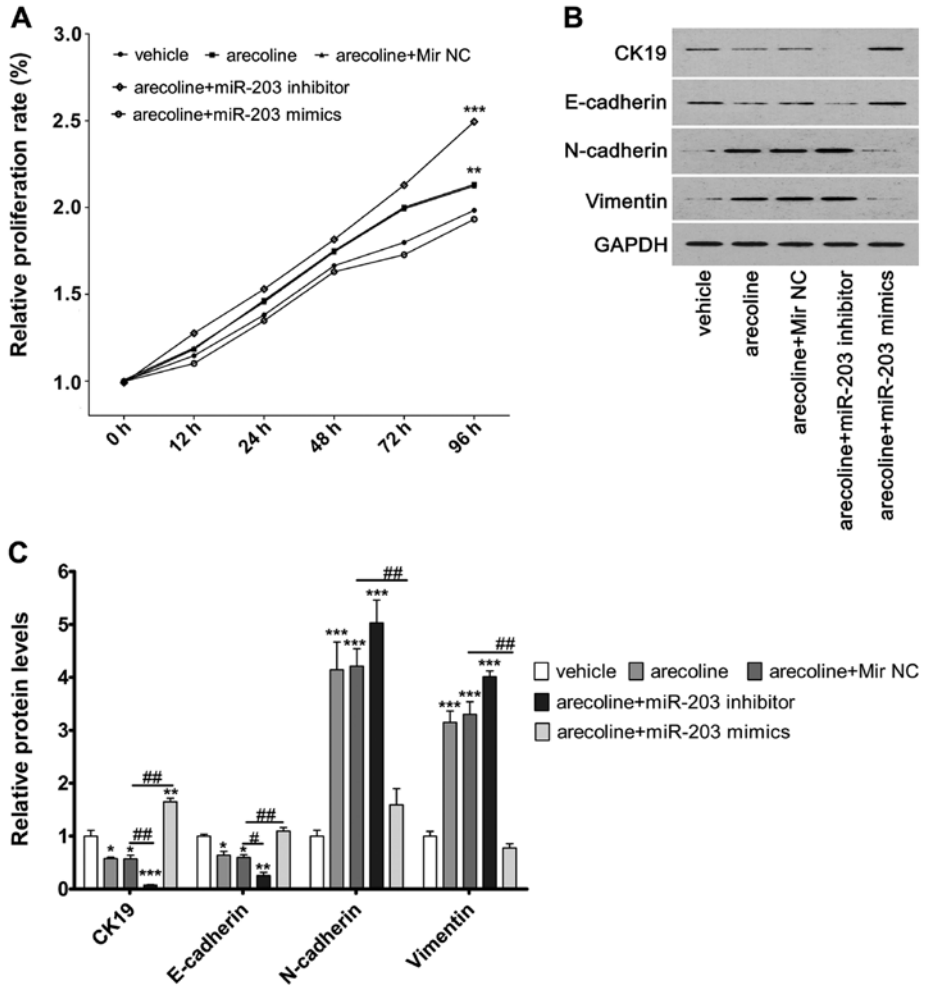

Figure 5. Effects of arecoline and miR-203 on HaCaT cells. (A) CCK-8 cell proliferation assay was used to evaluate cell proliferation. Data are expressed as 450 nm optical density in the different groups. (B and C) Expression of epithelial-mesenchymal transition-related genes (CK19, E-cadherin, N-cadherin and vimentin) in the HaCaT cells was determined by western blotting and quantified. Data shown are means ± SD, *P<0.05, **P<0.01, ***P<0.001; $^{\#}$P<0.05, $^{\#\#}$P<0.01. CK19, cytokeratin 19.

and had a synergistic effect with the miR-203 inhibitor while co-treatment with miR-203 mimics rescued the expression of miR-203 that was decreased by arecoline (Fig. 4A). qPCR and western blotting were performed to detect the expression of SFRP4 and TM4SF1 in the HaCaT cells after the indicated treatments. We found that arecoline significantly increased the expression of SFRP4 compared with that in the vehicle control, and had a synergistic effect with the miR-203 inhibitor; while co-treatment with miR-203 mimics reversed the upregulated expression of SFRP4 that was induced by arecoline (Fig. 4B). Inversely, arecoline significantly reduced the expression of TM4SF1 compared with the vehicle control, and had a synergistic effect with the miR-203 inhibitor; while co-treatment with miR-203 mimics reversed the downregulated expression of TM4SF1 that was decreased by arecoline (Fig. 4C). The results of the western blotting further validated the phenomenon mentioned above at the translational level (Fig. 4D). These results indicated that arecoline affected the expression of miR-203, and subsequently regulated the expression of SFRP4 and TM4SF1.

miR-203 regulates cell proliferation, and regulates the expression of CK19, E-cadherin, N-cadherin and vimentin in the HaCaT cells. We observed that downregulation of miR-203 mediated by arecoline or co-treatment with the miR-203 inhibitor significantly increased cell proliferation ability in the HaCaT cells compared to the vehicle control cells. Furthermore, we observed that co-treatment of miR-203 mimics and arecoline significantly decreased the upregulation of cell proliferation induced by arecoline (Fig. 5A). This indicated that miR-203 regulated the proliferation of HaCaT cells.

To explore the potential molecular mechanisms underlying miR-203-induced cell proliferation and EMT, we assessed the expression of CK19, E-cadherin, N-cadherin and vimentin in HaCaT cells. Downregulation of miR-203 induced by arecoline or miR-203 inhibitor significantly downregulated the expression of CK19 and E-cadherin proteins compared to these levels in the vehicle control cells, whereas it significantly upregulated the expression of N-cadherin and vimentin. Reversal of the downregulation of miR-203 in the arecoline-treated cells by miR-203 mimic transfection significantly upregulated the expression of CK19 and E-cadherin proteins compared to these levels in the vehicle control cells, whereas it significantly downregulated the expression of N-cadherin and vimentin (Fig. 5B and C).

Discussion

miRNAs have been implicated in inflammation, fibrosis, EMT and various types of cancers, such as oral cancer. Some miRNA families have gained attention for their clear function in tissue fibrosis. miR-30c has been reported to be a tumor suppressor in endometrial cancer (14) and to act as an independent predictor for the clinical benefit of tamoxifen therapy in patients with advanced breast cancer (15). A previous study showed that miR-206 overexpression inhibited estrogen receptor-α-dependent proliferation, impaired invasiveness and induced cell cycle arrest of estrogen receptor-α-positive endometrial endometrioid adenocarcinoma cell lines (16). miR-203 has previously been shown to play an important role in epithelial cell biology (17). miR-203 was differentially regulated in gingival epithelial cells in the presence of *P. gingivalis*. Silencing of miR-203 diminished the activation of signal transducer and activator of transcription 3 (Stat3), suggesting that alterations in miRNAs modulate important host signaling responses in pathological processes of severe periodontal disease (18). During master transcription factor-induced EMT in MCF7 breast cancer cells, miR-203 was repressed in a time-dependent manner. Dynamic simulations revealed stable epithelial and mesenchymal states, and underscored the crucial role of miR203 in state transitions underlying epithelial plasticity (19). Thus, according to these findings, we hypothesized that miR-203 plays a critical role in oral submucous fibrosis by regulating EMT-related genes via downstream target molecules.

In the present study, we found that the expression of miR-203 was significantly upregulated in all 6 OSF tissues, whereas upregulated expression of miR-206 and miR-30c was observed in 2 and 5 cases, respectively. Collectively, we therefore focused on the role of miR-203 in EMT of HaCaT cells for further analysis. We also found that SFRP4 was increased, while TM4SF1 was significantly decreased in OSF tissues at the mRNA and protein levels compared with that in the normal tissues. By dual luciferase report assay, we demonstrated that miR-203 negatively regulated SFRP4 and positively regulated TM4SF1 at the transcriptional levels. Secreted frizzled related protein (SFRP) proteins are characterized by a frizzled-like cysteine-rich domain and form a family of soluble proteins (SFRP1-5) (20). Upregulation of SFRP4 has been observed both in the skin of systemic sclerosis patients (21) and kidney fibrosis (22). Matsushima *et al* demonstrated that intramuscular administration of recombinant SFRP4 reduced fibrosis scar size and ameliorated cardiac function after ischemic injury (23). By oligonucleotide microarray that containing 15 cases of OSF tissues and 14 normal buccal mucosa tissues, our previously study found that expression of EMT-related gene SFRP4 was significantly upregulated in the OSF tissues compared with that in the normal mucosa tissues, which was validated by RT-PCR (24). This suggested that SFRP4 abnormalities in EMT may play an important role in the pathogenesis and malignant transformation of OSF. Transmembrane-4 L six family 1 (TM4SF1), the founding member of the L6 family, was originally identified as a protein abundantly expressed in a variety of epithelial cancer cells, and was found to share certain tetraspanin functions including roles in cell growth, motility and metastasis (25,26). Previous findings indicated that TM4SF1 serves as a surface protein marker which identified mesenchymal stem cells from diverse cell sources, in particular, fibroblast-rich connective tissues (27). Thus, these data suggest that TM4SF1 is involved in the cell proliferation of epithelial cells during the processes of EMT. miRNAs regulate gene expression by inhibition of gene translation or facilitation of mRNA degradation. Our recent results showed that miR-203 directly targets the 3'UTR of SFRP4, and subsequently downregulated its expression. However, we also found that miR-203 upregulated TM4SF1 expression. We infer that miR-203 regulates the expression of TM4SF1 in an indirect manner, and further research should validate this.

CK19 is expressed exclusively by epithelial cells and derived cancers (28). Previous research indicated that CK19 expression may be implicated in the retention of proliferative potential or undifferentiated character in oral non-keratinized mucosa (29). We previously reported that the expression of CK19 at the mRNA or protein levels was shown to be significantly downregulated in the OSF basal cell layer, which is the proliferative invasive layer, than levels in normal buccal non-keratinized mucosa, indicating that the self-renewal capacity of the basal cell layer of OSF through stem cells was obviously inhibited (30). Similarly, stimulation by the areca nut also depressed the constant regeneration of OSF mucosa and promoted the atrophy of the oral epithelium. E-cadherin, a calcium-dependent cell-surface glycoprotein, is critical for maintaining epithelial cell-cell adhesion, cellular polarity differentiation, growth, cell migration and seems to be the most common target for various EMT signaling pathways (31). Downregulation of E-cadherin has been considered as a hallmark of EMT (32). During EMT, the epithelium expresses mesenchymal markers and becomes motile, and causes expression of N-cadherin which is present in mesenchymal cells (33). Enhanced expression of N-cadherin in epithelial cells has been shown to decrease the endogenous levels of E-cadherin (34). Vimentin, one of the four types of intermediate filaments, is considered to play an important role in structural maintenance and adhesion in many cells originating from the mesenchymal (35). A previous immunohistochemical assay revealed that vimentin expression is significantly increased in OSF specimens than that in normal buccal mucosa. The upregulation of vimentin following arecoline exposure was consistent with that noted for fibroblasts cultured from OSF patients (9). By evaluating the changes in EMT hallmarks, CK19 and E-cadherin, and mesenchymal markers, N-cadherin and vimentin, using western blotting, we validated that arecoline induced EMT in the HaCaT cells. We found that arecoline significantly decreased the expression of miR-203 and SFRP4, and increased the expression of TM4SF1. Furthermore, arecoline treatment markedly enhanced the cell growth of HaCaT cells. Importantly, we found that restoration of downregulated miR-203 induced by arecoline increased the expression of SFRP4, and decreased the expression of TM4SF1, and attenuated the cell proliferation of HaCaT cells. Moreover, the restoration of miR-203 expression in the arecoline-treated cells significantly upregulated the expression of CK19 and E-cadherin proteins, whereas it significantly downregulated the expression of N-cadherin and vimentin. Our results suggest that miR-203 inhibits EMT by regulating the expression of SFRP4 and TM4SF1 in HaCaT cells.

In conclusion, the present study provides evidence that miR-203 plays a critical role in arecoline-induced OSF by regulating the process of EMT, at least partially, via targeting SFRP4 and TM4SF1. Thus, miR-203 may be a target for the prevention and therapy of OSF.

Acknowledgements

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