科技部補助專題研究計畫成果報告
期末報告

微小RNA及其標的基因於口腔黏膜下纖維化症之致病機轉研究（第3年）

計 畫 類 別 ： 個別型計畫
計 畫 編 號 ： MOST 103-2314-B-040-016-MY3
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執 行 單 位 ： 中山醫學大學牙醫學系（所）

計 畫 主 持 人 ： 張育超
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中 華 民 國 106 年 10 月 22 日

中 文 摘 要 ： 口腔黏膜下纖維化症(oral submucous fibrosis) 為一種口腔癌發生之癌前病變(precancerous condition)，流行病學指出口腔黏膜下纖維化症主要與檳榔嚼食習慣相關，檳榔中的主要成分檳榔素(arecoline)對於口腔黏膜下纖維化症所造成的病理病灶已經被確立，然而口腔黏膜纖維化症之分子致病機轉至今仍尚未清楚有待進一步研究探討。微小RNA (microRNAs)是一類長度約19-24個核苷酸之非編碼RNA，可利用鹼基配對的方式與標靶mRNA的3’ UTR (untranslation region)結合而抑制mRNA的轉譯，許多證據亦顯示微小RNA在組織纖維化中扮演重要角色，但微小RNA於口腔黏膜下纖維化症之角色仍未知。本計畫成果發現正常頰黏膜纖維母細胞在處理不同檳榔鹼處理下會降低miR-200b的表現且初代培養之口腔黏膜纖維化細胞之miR-200b表現量較正常頰黏膜纖維母細胞初代培養低。過度表現miR-200b可反轉檳榔鹼在正常頰黏膜纖維母細胞誘發之膠體收縮能力及細胞移動性，同樣地，過度表現miR-200b可抑制口腔黏膜纖維化初代培養細胞膠體收縮能力及細胞移動性。過度表現miR-200b可降低肌纖維纖維母細胞標記α-SMA表現量且可直接標靶上皮間質轉化轉錄因子ZEB2。降低miR-200b可增強膠體收縮能力及細胞移動性但同時降低ZEB2可反轉這些現象。miR-200b表現低下可透過標靶ZEB2來促進口腔黏膜下纖維化症。

中 文 關 鍵 詞 ： 口腔黏膜纖維化症; 微小RNA; miR-200b; ZEB2

英 文 摘 要 ： Oral submucous fibrosis (OSF) is a progressive scarring disease. MicroRNA-200b (miR-200b) has been reported as a tumor suppressor, but its role in the precancerous OSF remains unknown. In this study, we investigated the impact of miR-200b on myofibroblastic differentiation activity. Arecoline is a major areca nut alkaloid and has been employed to induce the elevated myofibroblast activity in human buccal mucosal fibroblasts (BMFs). Treatment of arecoline in BMFs dose-dependently reduced gene expression of miR-200b, which corresponded with the decreased expression of miR-200b in fBMFs. The arecoline-induced myofibroblast activities were abolished by overexpression of miR-200b in BMFs, and the same results were observed in fBMFs. In addition, α-SMA was inhibited by an increase in miR-200b. We further demonstrated that miR-200b-mediated decrease in ZEB2 led to down-regulation of α-SMA, vimentin. Loss of miR-200b resulted in enhanced collagen contraction and migration capabilities, and knockdown of ZEB2 reversed these phenomena. Lastly, we showed the expression of miR-200b was significantly less and ZEB2 was markedly higher in OSF tissues. These results suggested that down-regulation of miR-200b may contribute to the pathogenesis of areca quid-associated OSF through regulation of ZEB2 and myofibroblast hallmarks.

英 文 關 鍵 詞 ： Oral submucous fibrosis; microRNAs; miR-200b; ZEB2

科技部補助專題研究計畫成果報告

（□期中進度報告/■期末報告）

微小 RNA 及其標的基因於口腔黏膜下纖維化症之致病機轉研究

計畫類別：■個別型計畫　□整合型計畫

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計畫主持人：張育超 教授

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本計畫除繳交成果報告外，另含下列出國報告，共 ___ 份：

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□出席國際學術會議心得報告

□出國參訪及考察心得報告

中　華　民　國 106 年 10 月 22 日

中文摘要

口腔黏膜下纖維化症(oral submucous fibrosis) 為一種口腔癌發生之癌前病變(precancerous condition)，流行病學指出口腔黏膜下纖維化症主要與檳榔嚼食習慣相關，檳榔中的主要成分檳榔素(arecoline)對於口腔黏膜下纖維化症所造成的病理病灶已經被確立，然而口腔黏膜纖維化症之分子致病機轉至今仍尚未清楚有待進一步研究探討。微小RNA (microRNAs)是一類長度約19-24個核苷酸之非編碼RNA，可利用鹼基配對的方式與標靶mRNA的3'UTR (untranslation region)結合而抑制mRNA的轉譯，許多證據亦顯示微小RNA在組織纖維化中扮演重要角色，但微小RNA於口腔黏膜下纖維化症之角色仍未知。本計畫成果發現正常頰黏膜纖維母細胞在處理不同檳榔鹼處理下會降低miR-200b的表現且初代培養之口腔黏膜纖維化細胞之miR-200b表現量較正常頰黏膜纖維母細胞初代培養低。過度表現miR-200b可反轉檳榔鹼在正常頰黏膜纖維母細胞誘發之膠體收縮能力及細胞移動性，同樣地，過度表現miR-200b可抑制口腔黏膜纖維化初代培養細胞膠體收縮能力及細胞移動性。過度表現miR-200b可降低肌纖維纖維母細胞標記α-SMA表現量且可直接標靶上皮間質轉化轉錄因子ZEB2。降低miR-200b可增強膠體收縮能力及細胞移動性但同時降低ZEB2可反轉這些現象。miR-200b表現低下可透過標靶ZEB2來促進口腔黏膜下纖維化症。

關鍵字：口腔黏膜纖維化症; 微小 RNA; miR-200b; ZEB2

Abstract

Oral submucous fibrosis (OSF) is a progressive scarring disease. MicroRNA-200b (miR-200b) has been reported as a tumor suppressor, but its role in the precancerous OSF remains unknown. In this study, we investigated the impact of miR-200b on myofibroblastic differentiation activity. Arecoline is a major areca nut alkaloid and has been employed to induce the elevated myofibroblast activity in human buccal mucosal fibroblasts (BMFs). Treatment of arecoline in BMFs dose-dependently reduced gene expression of miR-200b, which corresponded with the decreased expression of miR-200b in fBMFs. The arecoline-induced myofibroblast activities were abolished by overexpression of miR-200b in BMFs, and the same results were observed in fBMFs. In addition, α-SMA was inhibited by an increase in miR-200b. We further demonstrated that miR-200b-mediated decrease in ZEB2 led to down-regulation of α-SMA, vimentin. Loss of miR-200b resulted in enhanced collagen contraction and migration capabilities, and knockdown of ZEB2 reversed these phenomena. Lastly, we showed the expression of miR-200b was significantly less and ZEB2 was markedly higher in OSF tissues. These results suggested that down-regulation of miR-200b may contribute to the pathogenesis of areca quid-associated OSF through regulation of ZEB2 and myofibroblast hallmarks.

Key words: Oral submucous fibrosis; microRNAs; miR-200b; ZEB2

Introduction

Oral submucous fibrosis (OSF) is a chronic progressive scarring disease and has been recognized as one of the oral potentially malignant disorders. This pre-cancerous condition of the oral mucosa is associated with inflammatory cell infiltration followed by the accumulation of connective tissue in lamina propria and epithelial atrophy [1,2], leading to difficulty of mouth opening. Epidemiological evidence has attributed areca quid chewing habit as the main aetiological factor for OSF [3,4]. However, the precise pathogenesis of OSF still remains elusive and effective treatment is lacking.

Myofibroblasts, the α-smooth muscle actin (SMA)-expressing contractile fibroblasts [5], play a critical role in wound healing and tissue remodeling [6]. However, persistent activation of myofibroblasts often leads to pathological fibrosis as it has been implicated in the dysregulation of extracellular matrix (ECM) synthesis and degradation[6,7]. Myofibroblast accumulation indeed has been found in multiple tissue fibroses, including OSF [8]. Among multiple sources of myofibroblast precursors, it has been indicated that epithelial–mesenchymal transition (EMT) contributes to tissue fibroses [6,7,9]. EMT is a process in which epithelial cells transdifferentiate into motile mesenchymal cells, and it is mediated by various key transcription factors, including Snail (SNAI1), Slug (SNAI2), zinc-finger E-box-binding (ZEB) and other basic helix-loop-helix transcription factors [10,11]. Also, intermediate filaments, such as vimentin, play a critical role in the induction of mesenchymal cell shape and motile behavior [12]. Our previous findings have shown that arecoline-induced myofibroblast transdifferentiation is mediated by ZEB1 [13] and inhibition of EMT transcription factor suppressed the pathogenesis of areca quid-induced OSF through downregulation of myofibroblast activity [14]. Hence, approaches to decrease the EMT regulating factors may be a promising strategy to inhibit the activation of myofibroblasts after arecoline stimulation, therefore preventing OSF pathogenesis.

MicroRNAs (miRNAs) are small, endogenous non-coding RNAs of 21–25 nucleotides in length that mediate the post-transcriptional regulation of gene expression through partial complementary binding to the 3’untranslated region (UTR) in target transcripts [15,16]. Aberrant expression of miRNAs in fibrotic diseases have been reported in various studies (see Review[17]), and numerous miRNAs have been suggested to modulate EMT process, thereby contributing or inhibiting tissue fibroses [18-20]. For instance, the EMT-inducing transcriptional factors ZEB1 has been found to be a target of miRNA-192 and inhibition of miR-192 resulted in decreased collagen expression as well as reduced renal fibrosis [21]. In addition, accumulating studies have demonstrated that miR-200 family reduce fibrosis by inhibiting EMT and preventing the deposition of ECM [22-24]. Also, it has been revealed that miR-200b is associated with cancer metastasis [25], chemoresistance [26] and prognosis [27]. Hence, the impact of miR-200b on precancerous OSF needs to be clarified.

In this study, we explored the functional role of miR-200b in the pathogenesis of areca quid-associated OSF and the associated mechanism. We tested the expression of miR-200b in fibrotic cells and tissues, and investigated its contribution in arecoline-induced and fibrotic myofibroblast activity, including higher collagen gel contractility and migration ability. Moreover, we assessed the expression of EMT-associated factors in accordance with overexpression of miR-200b, and examined the relationship between miR-200b and ZEB2 in BMFs. Our data revealed the anti-fibrotic potential of miR-200b in preventing the progression of OSF.

Materials and Methods

Chemicals and cell culture

Arecoline and collagen solution from bovine skin were purchased from Sigma-Aldrich (St. Louis, MO, USA). All methods applied in this study were carried out in accordance with the approved guidelines from the Institutional Review Board of Chung Shan Medical University Hospital and informed written consent was obtained from each individual prior to commencing the study. Tissue specimens from areca quid chewers were collected from Department of Dentistry, Chung Shan Medical University Hospital, Taichung, Taiwan. Biopsy specimens were taken from the histologically normal oral mucosa and fibrotic mucosa at the time of surgical third molar extraction. Fibroblast cultures were grown and maintained by using the explant method as described previously [28]. Cell cultures between the third and eighth passages were used in this study.

Collagen contraction assay

2×10^5 BMFs were suspended in 0.5 ml of 2 mg/ml collagen solution (Sigma-Aldrich) and then added into 24-well-plate followed by incubation at 37°C for 2 hours for the polymerization of collagen cell gels. After detaching gels from wells, the gels were further incubated in 0.5 ml MEMα medium with or without arecoline for 48 hours. Contraction of the gels was photographed and measured using ImageJ software (NIH) to calculate their areas [29].

Migration assay

Cell migration assay was conducted using 24-well plate Transwell® system with a polycarbonate filter membrane of 8-μm pore size (Corning, Acton, MA, USA)) assay kit as previously described [30]. The migrated cancer cells were then visualized and counted from 5 randomly selected fields under 100-fold magnification using an inverted microscope.

Wound healing assay

Cells were seeded into 6-well culture dishes. Wounds were introduced to the confluent monolayer of cells with a sterile 200 μL plastic pipette tip to create a denuded area. Cell movement toward the center of the wound area was photographed at 0 and 48hr under a microscope [14].

Western blot analysis

Western blot analysis was conducted as previously described [30]. The primary antibodies against α-SMA, ZEB2, vimentin, and Slug were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Following primary antibodies, the membranes were incubated with corresponding secondary antibodies. The immunoreactive bands were developed using an ECL-plus chemiluminescence substrate (Perkin-Elmer, Waltham, MA, USA) and captured by LAS-1000 plus Luminescent Image Analyzer (GE Healthcare, Piscataway, NJ, USA)

Apoptotic Assay

Apoptotic cells were detected with an Annexin V-APC kit (Calbiochem, Darmstadt, Germany) according to manufacturer's guidelines. After staining, the cells incubated with 20 μg/ml propidum iodide (PI) were analyzed by FACS Calibur apparatus (Becton Dickinson, San Diego, CA, USA).

Statistical analysis

Quantitative data were presented as mean ± SD and analyzed with Student's *t*-test using SPSS Statistics version 13.0. *p* value less than 0.05 was considered as statistically significant.

Results

Mir-200b is significantly down-regulated in arecoline-stimulated BMFs and fBMFs

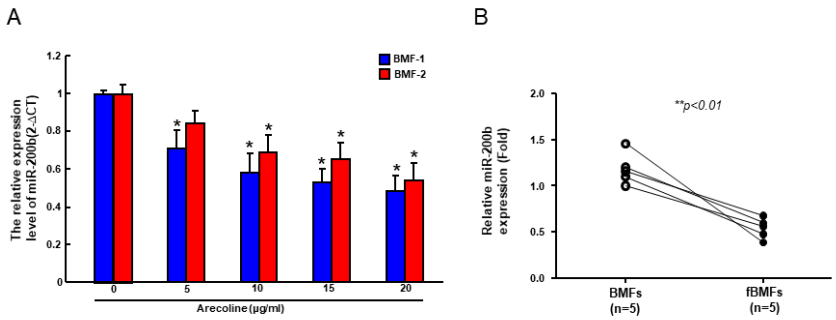

Figure 1. miR-200b is down-regulated in arecoline-stimulated BMFs and fBMFs.
The expression of miR-200b was assessed in BMFs in response to various concentration of arecoline exposure (A); the expression of miR-200b was compared between normal buccal mucosal fibroblasts (BMFs) and fibrotic BMFs using qRT-PCR. * $p < 0.05$ compared with no treatment control; ** $p < 0.01$ compared with BMFs.

Overexpression of miR-200b successfully hinders the arecoline-induced myofibroblast activities

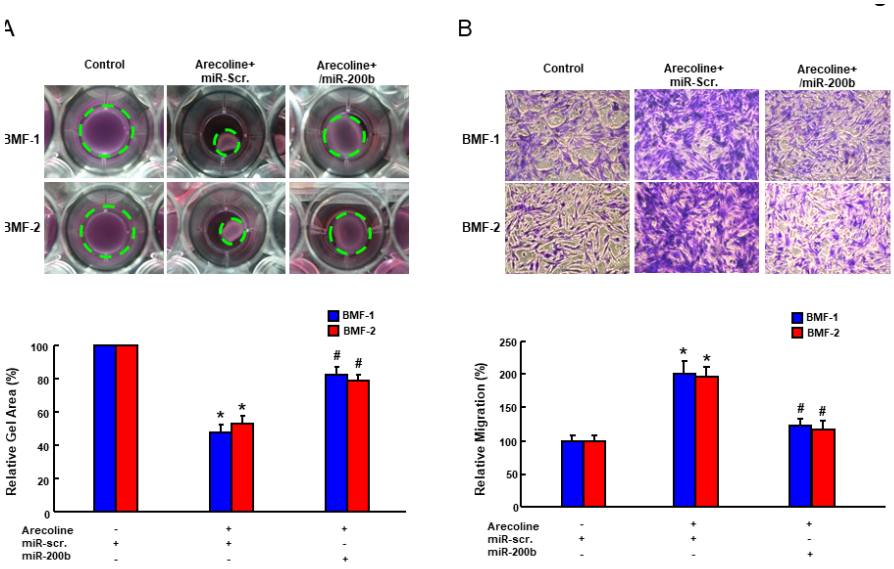

Figure 2. Arecoline-induced myofibroblast activities are ameliorated by overexpression of miR-200b
BMFs were treated with arecoline coordinately overexpression of miR-200b followed by collagen gel contraction (A) and transwell migration (B) assays. * $p < 0.05$ compared with no treatment control; # $p < 0.05$ compared with arecoline + miR-scr. group

MiR-200b reduces the characteristics of myofibroblasts

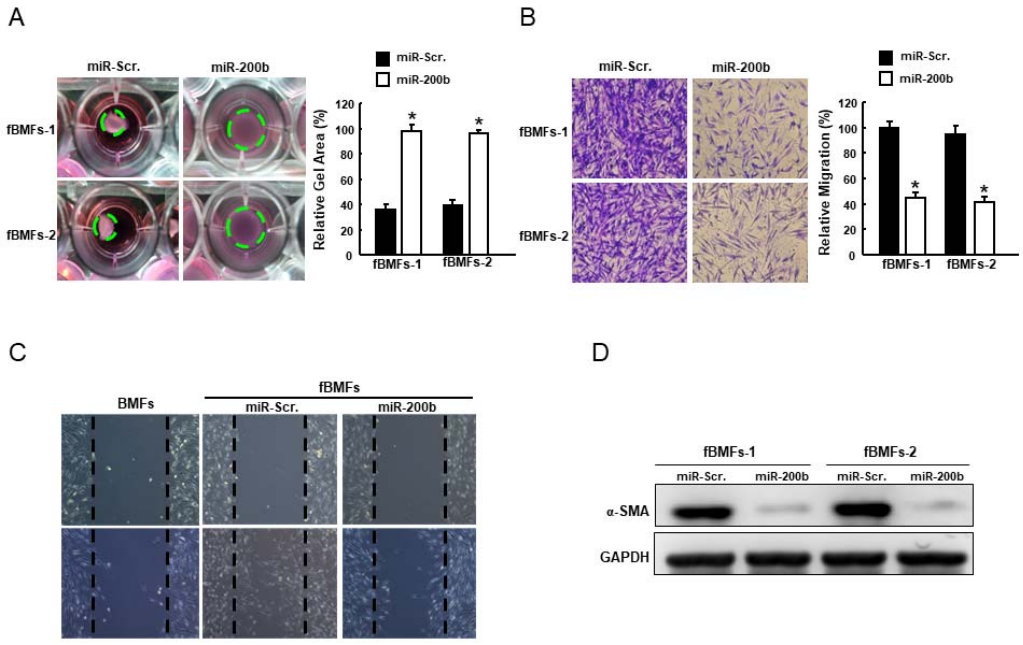

Figure 3. Overexpression of miR-200b diminishes the elevated myofibroblast activities

Collagen gel contraction (A), transwell migration (B) and wound healing (C) assays were applied to examine the myofibroblast activities in two lines of patient-derived fBMFs with or without overexpression of miR-200b. The expression of α-SMA was evaluated by Western blot. * $p < 0.05$ compared with miR-scr control.

MiR-200b directly targets ZEB2 and mediates ZEB2-regulated expression of α-SMA and vimentin in fBMFs

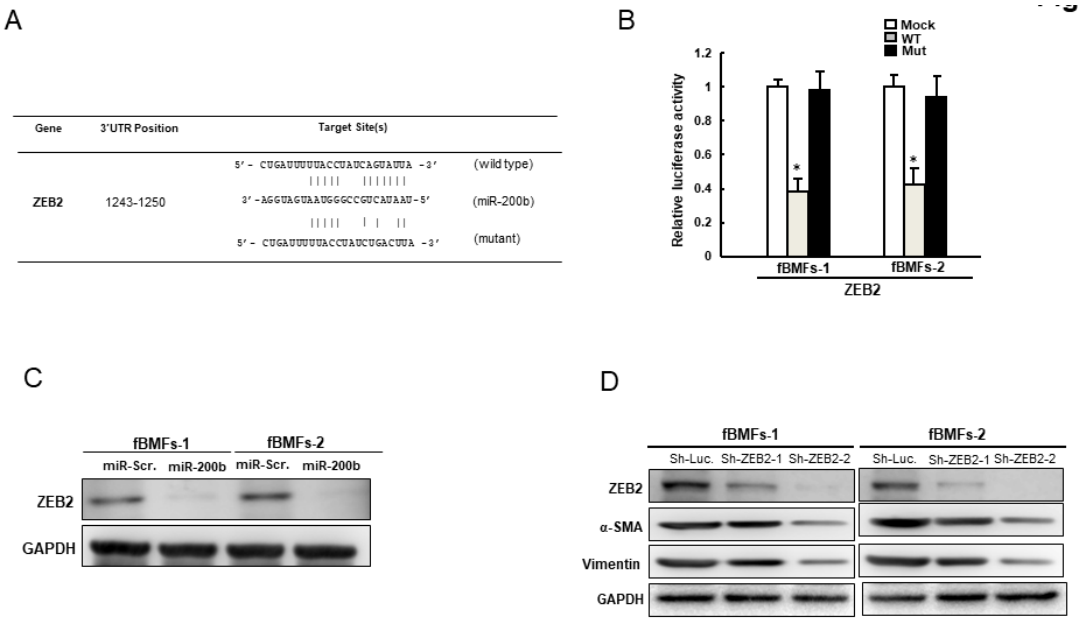

Figure 4. ZEB2 is a direct target of miR-200b and knockdown of ZEB2 reduces the fibrosis markers

The illustration of 3′UTR regions of full length, mutated ZEB2 and miR-200b (A); The luciferase activity of ZEB2 using mock, wild-type and mutated construct was assessed (B); Protein expression of ZEB2 with miR-200b overexpression in fBMFs was detected by Western blot (C); Protein expression of ZEB2, α-SMA,

and vimentin was assessed following knockdown of ZEB2 (D). * $p < 0.05$ compared with mock control.

Loss of miR-200b stimulates the activation of BMFs and increases the expression of fibrosis markers

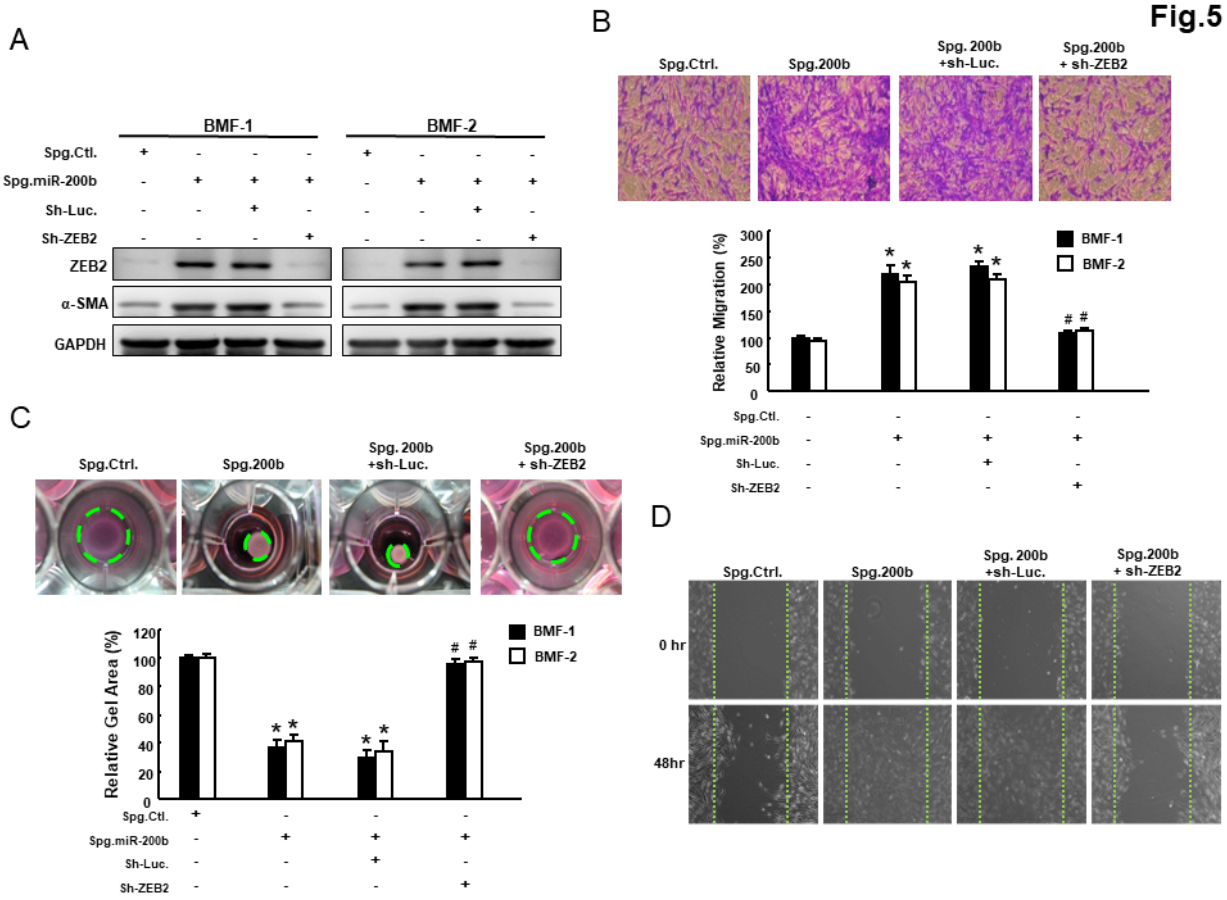

Figure 5. Loss of miR-200b promotes myofibroblast differentiation through ZEB2

BMFs were transduced with miR-200 sponge vector and/ or shZEB2 lentivirus and examined for the expression of Slug and α-SMA (A), transwell migration (B), collagen gel contraction (C) and wound healing (D) capacities. Contraction of the gels and migration ability were quantified using ImageJ software. * $p < 0.05$ compared with no treatment control; # $p < 0.05$ compared with Spg-miR-200b.

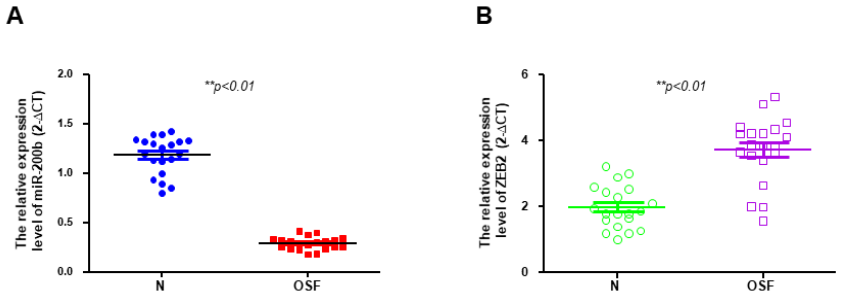

Figure 6. The expression of miR-200b is down-regulated and ZEB2 is upregulated in OSF tissues

The relative expression levels of miR-200b (A) and ZEB2 (B) were detected and compared between normal (N) and OSF tissues. ** $p < 0.01$ compared with normal mucosal tissues.

Discussion

Despite emerging evidence has suggested that miR-200 family members may involve in regulating multiple pathophysiological processes, the role of miR-200b in the precancerous OSF has not been well characterized. In the current study, we demonstrated that miR-200b is a critical factor in modulation of myofibroblast activity. Our results showed that overexpression of miR-200b can prevent the elevated

myofibroblast activity in BMFs after arecoline stimulation and we observed the similar effect in fBMFs with reduced expression of myofibroblast marker, α-SMA. Additionally, we proved that miR-200b repressed Slug, vimentin, and α-SMA via directly binding to ZEB2, leading to diminished myofibroblast activity.

Over the past few years, the mechanism for the anti-fibrotic effects of miR-200b has been examined and discussed in several fibrotic diseases. It has been shown that miR-200b ameliorated TGF-β1-induced fibrosis in colorectal epithelial cells [38]. In hypertrophic scars, miR-200b has been proved to decrease the expression of fibrosis markers, such as collagen type 1 and α-SMA, as well as ZEB1 in fibroblasts [39]. Another study showed that miR-200b affected hypertrophic scarring through regulating the fibroblasts proliferation and apoptosis by affecting the collagen synthesis, fibronectin expression and TGF-β1/α-SMA signaling [40]. As for pulmonary fibrosis, it has been found that the expression of miR-200 was reduced in fibrotic lungs, and down-regulation of miR-200 may contribute to EMT and enhance pulmonary fibroblast accumulation [41]. In contrast, overexpression of miR-200b markedly attenuated TGF-β1-induced expression of fibronectin and α-SMA in lung fibroblasts [41]. Furthermore, overexpressing miR-200b in intestinal epithelial cells led to inhibition of EMT characterized by downregulation of vimentin and upregulation of E-cadherin through targeting ZEB1 [42]. In consistent with these studies, we revealed the reduced expression of miR-200b in OSF tissues and demonstrated that overexpression of miR-200b reduced the hallmarks of myofibroblasts in arecoline-stimulated BMFs and fBMFs.

Experimental data have identified various genes as the targets of miRNA-200b. It has been indicated that miRNA-200b regulated EMT via inhibition of ZEB1 and ZEB2 [23,34,43,44]. ZEB2 is known as Smad-interacting protein 1 (SIP1), and it has been shown that miRNA-200b directly targets and represses Smad 2 in intestinal epithelial cells[42] as well. One of the recent studies has discovered that level of miR-200b was upregulated by miR-192 in the mesangial cells, and miR-192-miR-200 cascade induced TGF-β1 expression [45]. They showed that suppression of miR-200b reversed the inhibitory effect of TGF-β1 on ZEB1 protein expression and upregulated collagen via E-box enhancer [45]. Interestingly, another study reported that inhibition of miR-192 caused elevated expression of ZEB1/2 and decreased collagen in diabetic renal fibrosis [21]. It is worthy to examine the expression of miR-192 in OSF tissues and investigate whether it affects the aberrant expression of miR-200b in the future. In summary, this study demonstrated that miR-200b regulated arecoline-induced myofibroblast transdifferentiation by directly binding to ZEB2. We showed the anti-fibrotic potential of miR-200b in BMFs. Our results also provided evidence supporting that aberrant expression miR-200b contributed to the pathogenesis of OSF and miR-200b may function as a screening factor and therapeutic target for OSF.

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autoregulation in renal glomerular mesangial cells. *Kidney Int*. 2011; 80: 358-68.

103年度專題研究計畫成果彙整表

計畫主持人：張育超	**計畫編號：**103-2314-B-040-016-MY3
計畫名稱：微小RNA及其標的基因於口腔黏膜下纖維化症之致病機轉研究	

成果項目					量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
	技術移轉	件數			0	件	
		收入			0	千元	
國外	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			1	篇	submitted to JCMM
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		

	技術移轉	件數	0	件	
		收入	0	千元	
參與計畫人力	本國籍	大專生	0	人次	
		碩士生	0		
		博士生	0		
		博士後研究員	1		實驗規劃與執行、實驗技術指導、實驗數據分析、收集文獻、撰寫論文報告及實驗室管理。
		專任助理	1		實驗數據分析、收集文獻、及實驗室管理。
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）					

科技部補助專題研究計畫成果自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
   　□實驗失敗
   　□因故實驗中斷
   　□其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   論文：□已發表　□未發表之文稿　■撰寫中　□無
   專利：□已獲得　□申請中　■無
   技轉：□已技轉　□洽談中　■無
   其他：（以200字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性，以500字為限）
   本計畫成果發現正常頰黏膜纖維母細胞在處理不同檳榔鹼處理下會降低miR-200b的表現且初代培養之口腔黏膜纖維化細胞之miR-200b表現量較正常頰黏膜纖維母細胞初代培養低。過度表現miR-200b可反轉檳榔鹼在正常頰黏膜纖維母細胞誘發之膠體收縮能力及細胞移動性，同樣地，過度表現miR-200b可抑制口腔黏膜纖維化初代培養細胞膠體收縮能力及細胞移動性。過度表現miR-200b可降低肌纖維纖維母細胞標記α-SMA表現量且可直接標靶上皮間質轉化轉錄因子ZEB2。降低miR-200b可增強膠體收縮能力及細胞移動性但同時降低ZEB2可反轉這些現象。miR-200b表現低下可透過標靶ZEB2來促進口腔黏膜下纖維化症。

4. 主要發現
   本研究具有政策應用參考價值：■否　□是，建議提供機關
   （勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
   本研究具影響公共利益之重大發現：□否　□是
   說明：（以150字為限）