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Original Article

miR-1246 as a therapeutic target in oral submucosa fibrosis pathogenesis

Chia-Ming Liu [a,b], Yi-Wen Liao [a,b], Pei-Ling Hsieh [c], Chuan-Hang Yu [a,b], Pin Ju Chueh [d], Taichen Lin [a,b], Po-Yu Yang [a,b], Cheng-Chia Yu [a,b,e,**], Ming-Yung Chou [a,b,e,*]

[a] School of Dentistry, Chung Shan Medical University, Taichung, Taiwan
[b] Department of Dentistry, Chung Shan Medical University Hospital, Taichung, Taiwan
[c] Department of Anatomy, School of Medicine, China Medical University, Taichung, Taiwan
[d] Institute of Biomedical Sciences, National Chung Hsing University, Taichung, Taiwan
[e] Institute of Oral Sciences, Chung Shan Medical University, Taichung, Taiwan

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Background/purpose: Oral submucous fibrosis (OSF) is a precancerous condition of oral cancer with a complex etiology. Our previous work has demonstrated that non-coding RNA miR-1246 contributes to the cancer stemness of oral cancer. In the current study, we sought to investigate the effect of the inhibition of miR-1246 on the oral fibrogenesis.
Methods: The expression levels of miR-1246 in OSF tissues and fibrotic buccal mucosal fibroblasts (fBMFs) were examined by qRT-PCR. Collagen gel contraction and migration assays were conducted to evaluate the myofibroblast activities. The relationship between miR-1246 and type I collagen was assessed and the protein expression of type I collagen was determined by Western blot.
Results: MiR-1246 expression was upregulated in both OSF specimen and fBMFs compared to the normal counterparts. Inhibition of miR-1246 successfully suppressed the myofibroblast activities, including collagen gel contractility and migration capacity. Moreover, the expression of miR-1246 was positively correlated with type I collagen and the expression of type I collagen was abrogated by repression of miR-1246.
Conclusion: MiR-1246 is not only critical to the maintenance of oral stemness but also important to the activation of myofibroblasts. Our results showed that miR-1246 is positively associated with the type I collagen, which may be a downstream effector of miR-1246 and responsible for the fibrosis effect on fBMFs.

* Corresponding author. School of Dentistry, Institute of Oral Sciences, Chung Shan Medical University, No. 110, Sec. 1, Jianguo N. Rd., Taichung 40201, Taiwan. Fax: +886 4 24759065.
** Corresponding author. School of Dentistry, Institute of Oral Sciences, Chung Shan Medical University, No. 110, Sec. 1, Jianguo N. Rd., Taichung 40201, Taiwan. Fax: +886 4 24759065.
E-mail addresses: ccyu@csmu.edu.tw (C.-C. Yu), myc@csmu.edu.tw (M.-Y. Chou).

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Introduction

Oral submucous fibrosis is a premalignant disorder of the oral cavity, which is characterized by juxta-epithelial inflammation, atrophy and progressive fibrosis in the submucosal tissues. Although the precise etiology of OSF remains obscure, it has been indicated that areca nut chewing is strongly associated with OSF[1] and the abnormal collagen metabolism has been considered to be implicated in the susceptibility and pathogenesis of OSF.[2] It has been known that the key cellular mediator of fibrosis is myofibroblast, which serves as the primary collagen-producing cell when activated by areca nut stimulation.[3] Nevertheless, the detailed molecular mechanism of the aberrant expression of extracellular matrix (ECM) molecules such as collagen still needs further investigation.

MicroRNAs are important regulators of gene expression at the translational level that control both physiological and pathological processes, including fibrosis.[4] Our previous work also has shown that miR-200 family modulates the myofibroblastic transdifferentiation in buccal mucosal fibroblasts.[5,6] As a matter of fact, numerous miRs have been proven to be crucial in both fibrogenesis and carcinogenesis. For instance, miR-21[7,8] or miR-133a[9,10] been found to be implicated in various cancer and fibrosis diseases. One of our recent findings has shown that miR-1246 is critical to the oral cancer stemness,[11] but the understanding of its role in fibrosis has not been understood.

Hence, we sought to investigate the effect of miR-1246 on the myofibroblast activities and the expression of collagen to reveal its role in OSF. We examined the expression of miR-1246 in OSF tissues and assessed the myofibroblast activities after modulation of miR-1246. Last, we measured the expression of collagen in the myofibroblasts from OSF after miR-1246 inhibition.

Materials and methods

Tissues acquisition and cell culture

All procedures were conducted 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. Tissues from OSF patients were collected from Department of Dentistry, Chung Shan Medical University Hospital, Taichung, Taiwan. Specimens were excised from the histologically normal or fibrotic mucosa, and were minced and washed in PBS supplemented with antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml of amphotericin). Explants were placed into 60 mm Petri dishes and maintained in Dulbecco's-modified Eagle's medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco Laboratories) and antibiotics. Cell cultures between the third and eighth passages were used in this study.

Quantitative RT-PCR

Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) was used to isolate total RNA of cells according to the manufacturer's instruction. Superscript III first-strand synthesis system (Invitrogen Life Technologies, Carlsbad, CA, USA) was utilized to reversely transcribe the total RNAs. qRT—PCR was performed using TaqMan miRNA assays with specific primer sets (Applied Biosystems, Carlsbad, Calif). qRT-PCR reactions on resulting cDNAs were performed on an ABI StepOne™ Real-Time PCR Systems (Applied Biosystems).

Collagen contraction assay

Cells (2×10^5 cells) were suspended in 0.5 ml of 2 mg/ml collagen solution (Sigma—Aldrich, St. Louis, MO, USA) and added into one well of 24-well-plate followed by incubation at 37 °C for 2 h for the polymerization of collagen cell gels. After detaching gels from wells, the gels were further incubated for 48 h. Contraction of the gels was photographed and measured using ImageJ software (NIH, Bethesda, MD, USA) to calculate their areas.

Transwell migration and invasion assays

The 24-well Transwell system with a polycarbonate filter membrane of 8-μm pore size (Corning, United Kingdom) was employed. For the invasion capacity analysis, we coated the membrane of upper chamber with Matrigel. Cells will be placed in the upper chamber of transwell (Corning, Acton, MA) with serum free medium and medium containing 10% FBS was added to the lower chamber. After 24 h of incubation, cells attached to the reverse side of the membrane were stained with crystal violet subsequent to the filter membrane fixation. These cells were counted from five different visual areas of 100-fold magnification under a microscope.

Western blot

Western blot analysis was carried out as previously described.[12] The sample was separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Amersham, Arlington Heights, IL, USA). The primary antibody against ColA1 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Following the primary antibody incubation, the corresponding secondary antibody was added to the membrane. GAPDH was used as protein

loading control. 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).

Statistical analysis

Three replicates of each experiment were performed. Data were expressed as the mean ± SD and analyzed by Student's t-test. $p < 0.05$ was considered statistically significant.

Results

In order to determine the expression of miR-1246, we analyzed the relative expression levels of miR-1246 in OSF tissues (n = 20) and buccal mucosal fibroblasts (BMFs) derived from OSF specimen. qRT-PCR revealed that miR-1246 was aberrantly upregulated in fibrosis tissues (Fig. 1A) and fBMFs (Fig. 1B) compared to their normal counterparts. Subsequently, we utilized the inhibitor of miR-1246 to suppress its expression (Fig. 2A) and found that down-regulation of miR-1246 significantly inhibited collagen contractility, a characteristic of activated myofibroblasts, using collagen gel contraction assay in two individual fBMFs (Fig. 2B). Likewise, we observed another feature of activated myofibroblasts, the migration (Fig. 3A) and invasion (Fig. 3B) capacity, was impeded by the administration of a miR-1246 inhibitor, suggesting that regulation of miR-1246 may be an effective approach to modulate the activation of myofibroblasts.

It has been known that the disturbance in homeostatic equilibrium between synthesis and degradation of extracellular matrix, wherein collagen represents a major component, is implicated in OSF. According to one of the previous studies, the excessive production and reduced degradation of type I collagen by myofibroblasts derived from OSF may contribute to the collagen deposition.[13] As such, we first analyzed the data of oral cancer from The Cancer Genome Atlas (TCGA) and see whether there was any connection between miR-1246 and type I collagen. As expected, our results showed that miR-1246 was positively correlated with collagen type I α1 (COL1A1), 2 (COL1A2), and 3 (COL1A3) chain (Fig. 4A—C). Next, we assessed the expression of type I collagen in fBMFs with or without miR-1246 inhibitor to evaluate the effect of miR-1246. Our results demonstrated that suppression of miR-1246 significantly blocked the expression of type I collagen in two fBMFs (Fig. 4D). Taken together, these findings indicated that miR-1246 may affect the myofibroblast activities via regulation of type I collagen.

Discussion

Various studies have shown that miR-1246 functions as an oncogenic factor and could serve as a biomarker for tracking disease progression.[14—16] It has been demonstrated to drive Wnt/β-catenin activation in liver cancer stem cells[17] and numerous experimental results have revealed that miR-1246 exerts its ability through exosomes. Exosomal miR-1246 increased breast cancer proliferation, invasion, drug resistance via CCNG2,[18] and it promoted cell motility by directly targeting DENN/MADD Domain Containing 2D in oral cancer cells.[19] One of the recent studies revealed that TP53 mutant colon cancer cells shed miR-1246-enriched exosomes for the neighboring macrophages, triggering the miR-1246-dependent reprogramming into a cancer-promoting state.[20] Although these findings all support the oncogenic role of miR-1246, its effect on the precancerous condition has not been investigated yet. In the present study, we showed the expression of miR-1246 was upregulated in OSF tissues and fBMFs, and down-regulation of the aberrantly elevated miR-1246 successfully reduced the myofibroblast activities with lower expression of type I collagen in fBMFs. Since myofibroblasts share similar expression profiles of cancer-associated fibroblasts and we observed high expression of miR-1246 in both oral cancer cells and myofibroblasts, it is very likely that miR-1246 participates in tumorigenesis and progression, and it is worthy to investigate whether miR-1246 is also crucial to the refractory to conventional therapy.

There is no doubt that collagen-related genes play an important role in the homeostasis of collagen and fibrogenesis. Evidence has been accumulated to suggest that collagen-related genes are altered due to ingredients in the areca nut[21] and collagen biosynthesis plays a part in the accumulation of collagen in OSF lesions.[13] It has been demonstrated that OSF cells produced about 85% type I collagen and 15% type III collagen, and the ratio of α1(I) to

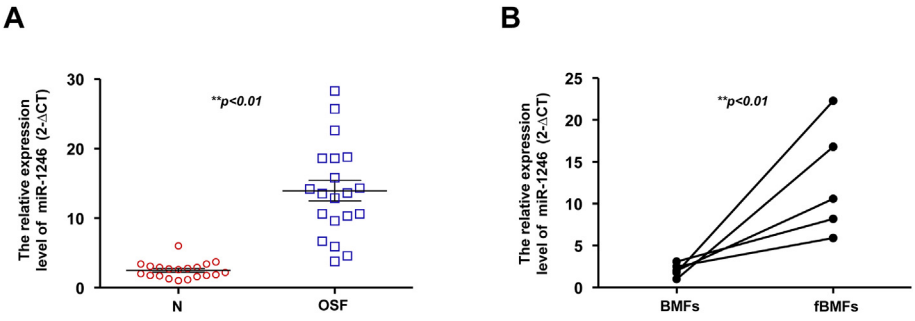

Figure 1 miR-1246 is up-regulated in OSF tissues and fibrotic buccal fibroblasts (fBMFs). The relative expression levels of miR-1246 in (A) OSF specimen and its normal counterparts, or (B) fBMFs and normal BMFs were determined by qRT-PCR analysis. **represents significant difference from control values with $p < 0.01$.

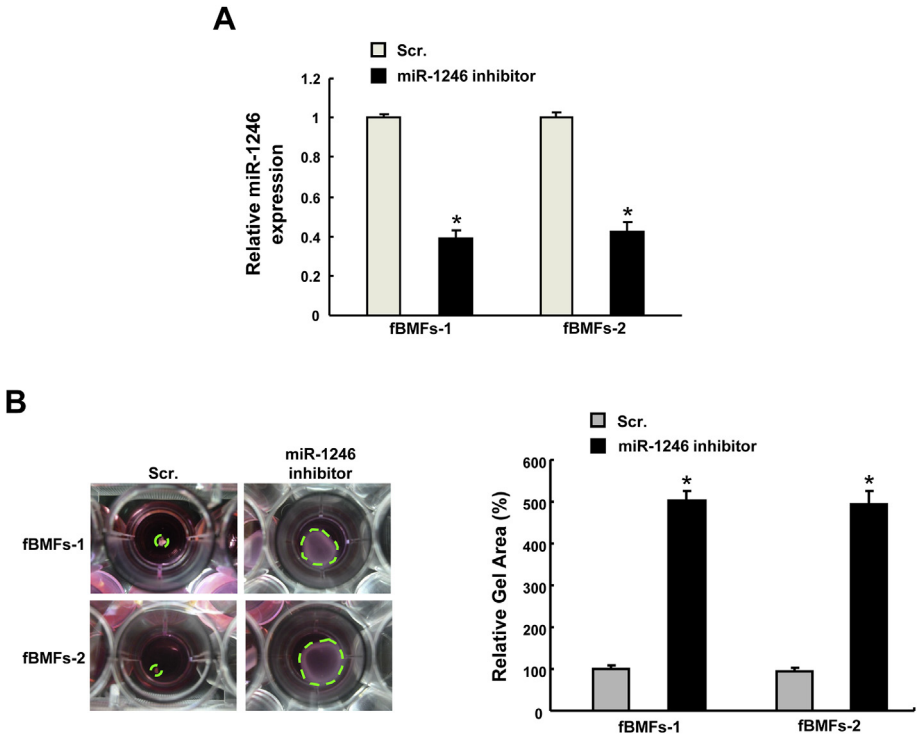

Figure 2 Inhibition of miR-1246 relieves the collagen gel contractility. (A) The knockdown efficiency of miR-1246 in fBMFs was determined by qRT-PCR analysis. (B) The collagen gel contraction assay was used to evaluate their contractility in two fBMFs with or without a miR-1246 inhibitor. *$p < 0.05$ compared with scramble control.

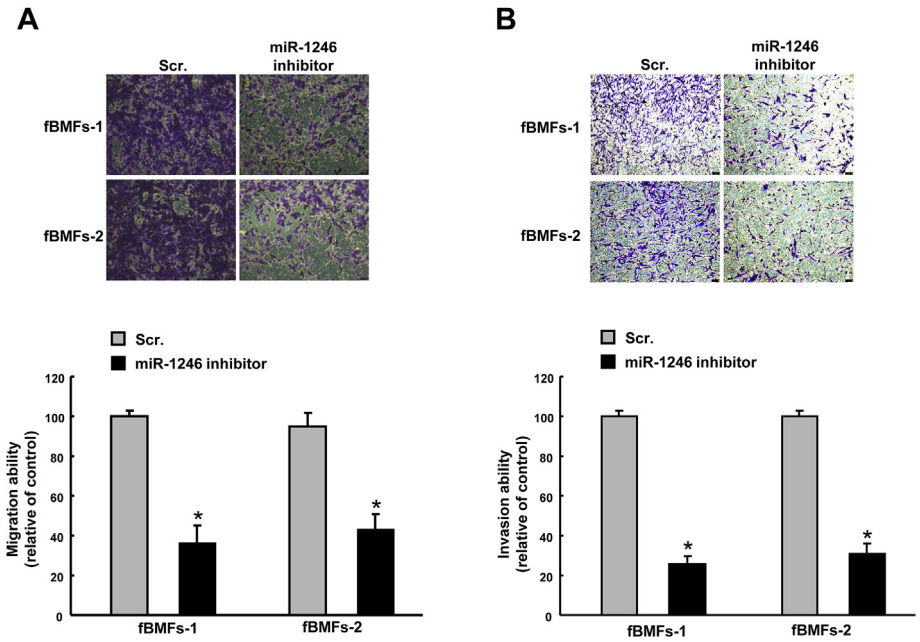

Figure 3 Downregulation of miR-1246 reduces the migration capacity of fBMFs. The migration (A) and invasion (B) ability of fBMFs was assessed by transwell migration assay and the quantification result was presented as the relative value of scramble control. *$p < 0.05$ compared with scramble control.

α2(I) chains was about 3:1 in OSF cells instead of 2:1 in the normal fibroblasts for type I collagen.[13] Our results showed that miR-1246 was positively correlated with type I collagen α1 (COL1A1), 2 (COL1A2), and 3 (COL1A3) chain from oral cancer tissues using TCGA dataset, indicating that miR-1246 may interact with type I collagen. Furthermore, inhibition of miR-1246 eliminated the expression of type I collagen α1 in two fBMFs, suggesting that downregulation of miR-1246

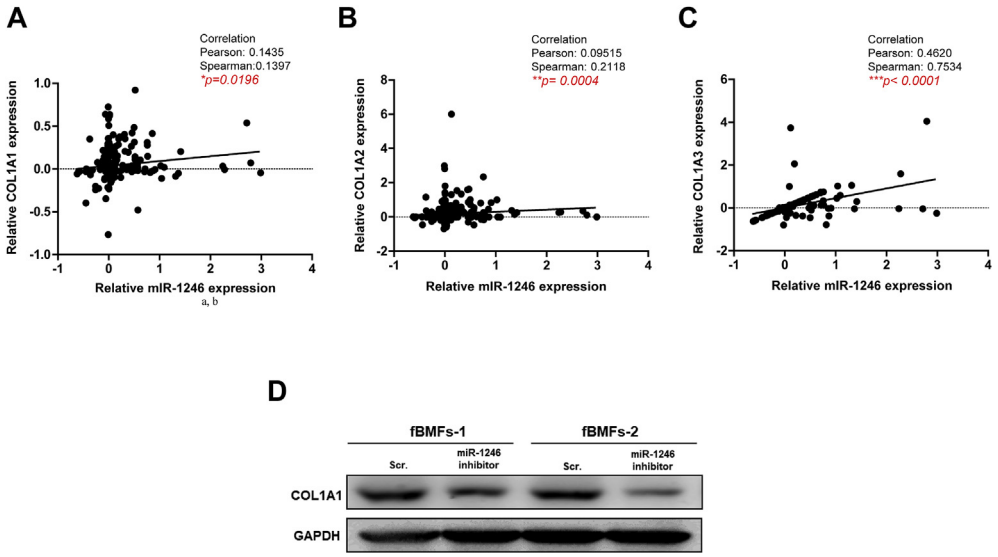

Figure 4 MiR-1246 is associated with type I collagen in oral cancer and fBMFs. Data from The Cancer Genome Atlas suggested that there is a positive correlation between miR-1246 and type I collagen α1 (COL1A1) (A), α2 (COL1A2) (B), and α3 (COL1A3) (C) chains in oral cancer. Pearson and Spearman correlation coefficients were both used to show the relationship; (D) Administration of miR-1246 inhibitor in fBMFs blocked the expression of type I collagen α1.

may be able to impede the abnormally-regulated production of type I collagen. Moreover, it has been shown that type I collagen increases the transdifferentiation and proliferation of myofibroblasts by lowering $\alpha 2\beta 1$ integrin in cardiac fibrosis.[22] Our findings revealed that miR-1246 may alter the myofibroblast activation via modulation of type I collagen. It has been shown that miR-1246 could activate Wnt/β-catenin signaling,[17] which has shown to be important in the TGF-β1-induced type I collagen expression.[23] Therefore, it is possible that miR-1246 affects type I collagen in fBMFs via Wnt/β-catenin. Further studies are required to verify whether miR-1246 could directly binds to type I collagen to affect its expression or via other pathways, such as Wnt/β-catenin.

Collectively, this study showed that miR-1246 contributes to fibrogenesis in the oral cavity through the regulation of type I collagen. Suppression of the increased miR-1246 in fBMFs mitigated the activated myofibroblast features and the expression of type I collagen. Further studies are required to verify that miR-1246 directly binds to type I collagen and maintain the myofibroblast activation.

Conflicts of interest

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

Acknowledgment

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References

1. Lee CH, Ko YC, Huang HL, Chao YY, Tsai CC, Shieh TY, et al. The precancer risk of betel quid chewing, tobacco use and alcohol consumption in oral leukoplakia and oral submucous fibrosis in southern Taiwan. *Br J Cancer* 2003;**88**:366–72.
2. Tilakaratne WM, Klinikowski MF, Saku T, Peters TJ, Warnakulasuriya S. Oral submucous fibrosis: review on aetiology and pathogenesis. *Oral Oncol* 2006;**42**:561–8.
3. Harvey W, Scutt A, Meghji S, Canniff JP. Stimulation of human buccal mucosa fibroblasts in vitro by betel-nut alkaloids. *Arch Oral Biol* 1986;**31**:45–9.
4. Jiang X, Tsitsiou E, Herrick SE, Lindsay MA. MicroRNAs and the regulation of fibrosis. *FEBS J* 2010;**277**:2015–21.
5. Liao YW, Yu CC, Hsieh PL, Chang YC. miR-200b ameliorates myofibroblast transdifferentiation in precancerous oral submucous fibrosis through targeting ZEB2. *J Cell Mol Med* 2018;**22**:4130–8.
6. Lu MY, Yu CC, Chen PY, Hsieh PL, Peng CY, Liao YW, et al. miR-200c inhibits the arecoline-associated myofibroblastic transdifferentiation in buccal mucosal fibroblasts. *J Formos Med Assoc* 2018;**117**:791–7.
7. Pfeffer SR, Yang CH, Pfeffer LM. The role of miR-21 in cancer. *Drug Dev Res* 2015;**76**(6):270–7.
8. Huang Y, He Y, Li J. MicroRNA-21: a central regulator of fibrotic diseases via various targets. *Curr Pharm Des* 2015;**21**:2236–42.
9. Dong Y, Zhao J, Wu CW, Zhang L, Liu X, Kang W, et al. Tumor suppressor functions of miR-133a in colorectal cancer. *Mol Cancer Res* 2013;**11**:1051–60.
10. Roderburg C, Luedde M, Vargas Cardenas D, Vucur M, Mollnow T, Zimmermann HW, et al. miR-133a mediates TGF-beta-dependent derepression of collagen synthesis in hepatic stellate cells during liver fibrosis. *J Hepatol* 2013;**58**:736–42.
11. Lin SS, Peng CY, Liao YW, Chou MY, Hsieh PL, Yu CC. miR-1246 targets CCNG2 to enhance cancer stemness and chemoresistance in oral carcinomas. *Cancers* 2018;**10**:E272.
12. Lin CY, Liao YW, Hsieh PL, Lu MY, Peng CY, Chu PM, et al. LncRNA GAS5-AS1 inhibits myofibroblasts activities in oral submucous fibrosis. *J Formos Med Assoc* 2018;**117**:727–33.

13. Kuo MY, Chen HM, Hahn LJ, Hsieh CC, Chiang CP. Collagen biosynthesis in human oral submucous fibrosis fibroblast cultures. *J Dent Res* 1995;**74**:1783—8.
14. Zhang WC, Chin TM, Yang H, Nga ME, Lunny DP, Lim EK, et al. Tumour-initiating cell-specific miR-1246 and miR-1290 expression converge to promote non-small cell lung cancer progression. *Nat Commun* 2016;**7**:11702.
15. Kim G, An HJ, Lee MJ, Song JY, Jeong JY, Lee JH, et al. Hsa-miR-1246 and hsa-miR-1290 are associated with stemness and invasiveness of non-small cell lung cancer. *Lung cancer* 2016; **91**:15—22.
16. Bhagirath D, Yang TL, Bucay N, Sekhon K, Majid S, Shahryari V, et al. microRNA-1246 is an exosomal biomarker for aggressive prostate cancer. *Cancer Res* 2018;**78**:1833—44.
17. Chai S, Ng KY, Tong M, Lau EY, Lee TK, Chan KW, et al. Octamer 4/microRNA-1246 signaling axis drives Wnt/beta-catenin activation in liver cancer stem cells. *Hepatology* 2016;**64**: 2062—76.
18. Li XJ, Ren ZJ, Tang JH, Yu Q. Exosomal microRNA miR-1246 promotes cell proliferation, invasion and drug resistance by targeting CCNG2 in breast cancer. *Cell Physiol Biochem* 2017; **44**:1741—8.
19. Sakha S, Muramatsu T, Ueda K, Inazawa J. Exosomal microRNA miR-1246 induces cell motility and invasion through the regulation of DENND2D in oral squamous cell carcinoma. *Sci Rep* 2016;**6**:38750.
20. Cooks T, Pateras IS, Jenkins LM, Patel KM, Robles AI, Morris J, et al. Mutant p53 cancers reprogram macrophages to tumor supporting macrophages via exosomal miR-1246. *Nat Commun* 2018;**9**:771.
21. Rajalalitha P, Vali S. Molecular pathogenesis of oral submucous fibrosis–a collagen metabolic disorder. *J Oral Pathol Med* 2005; **34**:321—8.
22. Hong J, Chu M, Qian L, Wang J, Guo Y, Xu D. Fibrillar type I collagen enhances the differentiation and proliferation of myofibroblasts by lowering alpha2beta1 integrin expression in cardiac fibrosis. *BioMed Res Int* 2017;**2017**:1790808.
23. Xu L, Cui WH, Zhou WC, Li DL, Li LC, Zhao P, et al. Activation of Wnt/β-catenin signalling is required for TGF-β/Smad2/3 signalling during myofibroblast proliferation. *J Cell Mol Med* 2017; **21**:1545—54.