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MiR-17-92 Cluster Regulates Cell Proliferation and Collagen Synthesis by Targeting TGFB Pathway in Mouse Palatal Mesenchymal Cells

Ling Li,[1,2] Jia-Yu Shi,[1] Gui-Quan Zhu,[1] and Bing Shi[1,2*]

[1] *State Key Laboratory of Oral Disease, West China College of Stomatology, Sichuan University, Chengdu, P.R. China*

[2] *Department of Cleft Lip and Palate Surgery, West China College of Stomatology, Sichuan University, Chengdu, P.R. China*

ABSTRACT

Elongation and elevation of palatal shelves, mainly caused by proliferation and extra-cellular matrix synthesis of palatal mesenchymal cells (PMCs), are essential for normal palatal development. Transforming growth factor beta (TGFB) pathway could induce proliferation inhibition and collagen synthesis in PMCs. Recent studies found that miRNA-17-92 (miR-17-92) cluster, including miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a, expressed in the 1st bronchial arch of mouse embryos during the period of palatal shelf elongation and elevation, and directly targeted TGFB pathway in cancer cell lines. Whether miR-17-92 cluster expresses and targets TGFB pathway in PMCs has not yet been studied. Using quantitative real-time RT-PCR, we found that miR-17-92 expressed in PMCs and decreased from embryonic day (E) 12 to E14 in palatal shelves. MTT assay and Western blot showed that miR-17-92 inhibited TGFB1 induced proliferation inhibition and collagen synthesis in PMCs by decreasing TGFBR2, SMAD2, and SMAD4 protein level. Further luciferase assay showed that miR-17 and miR-20a directly targeted 3′UTR of TGFBR2, and that miR-18a directly targeted 3′UTR of SMAD2 and SMAD4. We thus conclude that miR-17-92 cluster could inhibit TGFB pathway induced proliferation inhibition and collagen synthesis in PMCs by directly targeting TGFBR2, SMAD2, and SMAD4. J. Cell. Biochem. 113: 1235–1244, 2012.

KEY WORDS: TGFB PATHWAY; MiRNA-17-92; PALATAL MESENCHYMAL CELL; PROLIFERATION; COLLAGEN SYNTHESIS

Cleft palate is a common congenital deformity, which is caused by mal-development of secondary palate. The secondary palate primordium extends from the oral surface of the maxillary processes to form palatal shelves. Before palatal fusion, palatal development includes palatal shelf elongation and elevation. The normal processing of these two steps demands normal proliferation and extra-cellular matrix secretion of palatal mesenchymal cells (PMCs) [Meng et al., 2009]. Disturbance of any of the two steps can cause cleft palate.

Palatal development is under the control of a complex network of several pathways, including TGFB pathway [Meng et al., 2009]. TGFB pathway is mainly involved in cell growth arrest, apoptosis, and epithelial–mesenchymal transition [Heldin et al., 2009]. TGFB ligands (TGFB1, TGFB2, and TGFB3) activate the membrane receptor serine/threonine kinase complex composed of type II (TGFBR2) and type I (TGFBR1) receptors. TGFBR1 phosphorylates SMAD2/3. Phosphorylated SMAD2/3 (p-SMAD2/3) subsequently form oligomeric complex with Smad4. The Smad2/3-Smad4 complex then translocates to the nucleus where it can either promote or inhibit transcription of target genes [Heldin et al., 2009].

During palatal development, all the components of TGFB pathway (TGFB1, TGFB2, TGFB3, TGFBR1, TGFBR2, SMAD2, SMAD3, and SMAD4) are differentially expressed in the mesenchyme of palatal shelves [Fitzpatrick et al., 1990; Gehris et al., 1991; Greene et al., 2003; Nawshad et al., 2004; Meng et al., 2009], and are vital for palatal mesenchymal development [Greene et al., 2003; Meng et al., 2009]. Any factor influencing TGFB pathway may disturb palatal mesenchymal development. TGFB1 is involved in palatal development in both mesenchyme [Meng et al., 2009] and epithelium [Iordanskaia and Nawshad, 2011]. TGFB1 could

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*Correspondence to: Bing Shi, Department of Cleft Lip and Palate Surgery, West China College of Stomatology, Sichuan University, No. 14, Section 3, RenMinNan Road, Chengdu, P.R. China. E-mail: shibingcn@vip.sina.com

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obviously promote extra-cellular matrix synthesis in mouse PMCs [D'Angelo and Greene, 1991; D'Angelo et al., 1994]. But the effect of TGFB1 on proliferation of mouse PMCs seems to be unclear [Linask et al., 1991]. Therefore, confirming the function of TGFB1 in mouse PMCs is important when we want to investigate the factors that may influence TGFB pathway.

MiRNA is a ~22 nucleotide RNA that regulates post-transcriptional eukaryotic gene expression during embryonic development. MiRNAs can bind the 3′-UTR of the target mRNAs and thus inhibit their protein translation. Hundreds of miRNAs has been identified, but their specific biological functions in specific cells or tissues are still unclear. MiRNAs could participate in cell signal pathways by targeting certain molecules [Inui et al., 2010]. MiR-17-92 cluster, including miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a, has been found to continuously express in the 1st bronchial arch of mouse embryos during embryonic day (E) 12–14, which is the critical period for palatal shelf elongation and elevation [Mukhopadhyay et al., 2010]. MiR-17-92 has been validated to be a potent inhibitor of TGFB pathway in cancer cell lines [Dews et al., 2010; Mestdagh et al., 2010]. Therefore, it is likely that miR-17-92 cluster might also express in PMCs and regulate TGFB pathway during the period of palatal shelf elongation and elevation.

In this study, we found that the expression level of miR-17-92 cluster decreased from E12 to E14, and inhibited TGFB1 induced proliferation inhibition and collagen synthesis in PMCs. Luciferase assay further found that miR-17/20a directly targeted 3′UTR of TGFBR2 and miR-18a directly targeted 3′UTR of SMAD2 and SMAD4 in PMCs. We thus conclude that miR-17-92 cluster could inhibit TGFB pathway induced proliferation inhibition and collagen synthesis in PMCs by directly targeting TGFBR2, SMAD2, and SMAD4.

MATERIALS AND METHODS

CELL CULTURE

All the animals involved in our study were approved by the Animal Care and Use Committee of Sichuan University. We dissected palatal shelves from C57BL/6J mouse embryos on E13.5, and separated the mesenchyme from the epithelia. To dissociate individual cells, the palatal mesenchyme was incubated in PBS with 0.25% trypsin and 0.02% EDTA for 15 min. Primary culture of PMCs was initiated by seeding 5 or 2 ml DMEM/F12 into 25 ml flask or flat plate. TGFB1 stimulation experiments were performed with recombinant TGFB1 (5 ng/ml; R&D system).

QUANTITATIVE REAL-TIME RT-PCR

Reverse transcription and qPCR of the components of miR-17-92 cluster and miR-200b were performed using Taqman Small RNA Assay (Applied Biosystems) according to the manufacture's instructions. Briefly, 10 ng total RNA was reverse transcribed with miRNA specific primers in 15 μl reaction volumes. Reverse transcription reactions were diluted and amplified in triplicates by TaqMan qPCR on a 7300 Real Time PCR System (Applied Biosystems). Quantification was performed using the $\Delta\Delta C_t$ method. The RT-PCR fold changes were normalized to snoRNA135 (Applied Biosystems).

For Col1a1, Col1a2, and Col3a1, quantitative real-time RT-PCR were carried out as described previously [Zhu et al., 2010]. Briefly, total RNA was isolated with TRIzol reagent (Invitrogen), and reverse-transcribed using a RevertAid First-Strand cDNA Synthesis Kit (Fermentas). PCR amplification of the cDNA template was done using Thunderbird SYBR qPCR mix (Toyobo Biologics) on ABI PRISM 7300 sequence detection system (Applied Biosystems). Reactions were run in triplicate, and results were averaged. Each value was normalized to β-actin. The relative expression level of the genes was calculated using the $\Delta\Delta C_t$ method. The sequences of PCR primers were as follows: Tgfbr2: F5′-CCGCTGCATATCGTCCTGTG-3′; R5′-AGTGGATGGATGGTCCTATTACA-3′ [Duan et al., 2009]; Col1a1: F5′-CTGCTGGCAAAGATGGAGA-3′; R5′-ACCAGGAAGACCCTGGAATC-3′ [Duan et al., 2009]; Col1a2: F5′-TGGTCTTACTGGGAACTTTGCTGC-3; R5′-ACCCTGTGGTCCAACGACTCCTC-TC-3 [Liu et al., 2010]; Col3a1: F5′-CAAATGGCATCCCAGGAG-3′; R5′-CATCTCGGCCAGGTTCTC-3′ [Duan et al., 2009]; β-actin: F5′-CCAACCGTGAAAAGATGACC-3′; R5′-CCAGAGGCATACAGGGACAG-3′ [Li et al., 2011].

CELL TRANSFECTION

PMCs were transfected with mixed miRNA mimics of miR-17-92 components, mixed miRNA inhibitors of miR-17-92 components, or scrambled miRNAs (Ribobio, Guangzhou, China) at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen) following the manufacture's protocol. Total RNA and protein were isolated from the transfected cells, followed by Real-time PCR and Western blot.

WESTERN BLOT

Western blots of COL1A1, COL1A2, COL3A1, TGFBR1, TGFBR2, SMAD2, SMAD3, p-SMAD2/3, and SMAD4 were carried out as described previously [Zhu et al., 2009]. Briefly, total proteins were isolated from the PMCs. Thirty-microgram proteins from each sample were separated on SDS–PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were incubated with anti-COL1A1 (Santa Cruz), anti-COL1A2 (Santa Cruz), anti-COL3A1 (Santa Cruz), anti-p-SMAD2/3 (Santa Cruz), anti-TGFBR1 (Santa Cruz), anti-TGFBR2 (Santa Cruz), anti-SMAD2 (Santa Cruz), anti-SMAD3 (Santa Cruz), and anti-SMAD4 (Santa Cruz) antibodies. Relative p-SMAD2/3 level was normalized to SMAD2 expression.

MTT ASSAY FOR CELL PROLIFERATION

The cell proliferation was quantified by the colorimetric MTT assay as previous described [Zhu et al., 2010]. In brief, cells were incubated with MTT for 4 h. Then supernatant was removed and DMSO was added. Optical densities at 490 nm were measured using culture medium as a blank.

LUCIFERASE REPORTER CONSTRUCTS AND ASSAYS

The 3′ UTR of Tgfbr1, Tgfbr2, Smad2, Smad3, or Smad4 gene was cloned into the pMIR-Report luciferase vector (Ambion, Inc.). The sense and antisense strands of the oligonucleotides were annealed according to manufacturer's protocol. The annealed oligonucleotides were digested with *Hin*dIII and *Spe*I and ligated into pMIR-Report luciferase vector. A BlpI site was added into each insert to test

for positive clones as described [Cheng et al., 2005]. pMIR-Report β-gal vector was used as a control for transfection efficiency. PMCs were lysed 24 h after transfection, and reporter activity was measured using Luciferase and β-galactosidase assay kits (Beyotime). Each experiment was repeated at least three times. The sequences of Tgfbr1, Tgfbr2, Smad2, Smad3, or Smad4 3′UTR oligonucleotides were as follows:

Tgfbr1 Wide Type Sense: AATGCACTAGT AAGATCTTAAACTAA-CACTTTAAGCTCAGCAAGCTTAATGC
Tgfbr1 wide type antisense: GCATTAAGCTTGCTGAGCTTAAAGT-GTTAGTTTAAGATCTTACTAGTGCATT
Tgfbr1 mutant sense: AATGCACTAGTAAGATCTTAAACTAAGAG-TATAAGCTCAGCAAGCTTAATGC
Tgfbr1 mutant antisense: GCATTAAGCTTGCTGAGCTTATACTCT-TAGTTTAAGATCTTACTAGTGCATT
Tgfbr2 wide type sense: AATGCACTAGTCAGCCAATAACGTTTG-CACTTTAGCTCAGCAAGCTTAATGC
Tgfbr2 wide type antisense: GCATTAAGCTTGCTGAGCTAAAGTG-CAAACGTTATTGGCTGACTAGTGCATT
Tgfbr2 mutant sense: AATGCACTAGTCAGCCAATAACGTTTCCA-GTTAAGCTCAGCAAGCTTAATGC
Tgfbr2 mutant antisense: GCATTAAGCTTGCTGAGCTTAACTG-GAAACGTTATTGGCTGACTAGTGCATT
Smad2 wide type sense: AATGCACTAGTGTCTCATCAGTTAAAG-CACCTTGTGGCTCAGCAAGCTTAATGC
Smad2 wide type antisense: GCATTAAGCTTGCTGAGC CACAA-GGTGCTTTAACTGATGAGAC ACTAGTGCATT
Smad2 mutant sense: AATGCACTAGTGTCTCATCAGTTAAACCT-CGTAGTGGCTCAGCAAGCTTAATGC
Smad2 mutant antisense: CATTAAGCTTGCTGAGCCACTACGAGG-TTTAACTGATGAGACACTAGTGCATT
Smad3 wide type sense: AATGCACTAGTGTAACGGCCTTCACAG-CACCTTC GCTCAGCAAGCTTAATGC
Smad3 wide type antisense: GCATTAAGCTTGCTGAGCGAAGGT-GCTGTGAAGGCCGTTACACTAGTGCATT
Smad3 mutant sense: AATGCACTAGTGTAACGGCCTTCACAC-CAGCTACGCTCAGCAAGCTTAATGC
Smad3 mutant antisense: GCATTAAGCTTGCTGAGCGTAGCTGGT-GTGAAGGCCGTTACACTAGTGCATT
Smad4 wide type sense: AATGCACTAGTCCAAAGGCCGAGCAC-CACCTTAGGGGCTCAGCAAGCTTAATGC
Smad4 wide type antisense: GCATTAAGCTTGCTGAGCCCCTAA-GGTGGTGCTCGGCCTTTGGACTAGTGCATT
Smad4 mutant sense: AATGCACTAGTCCAAAGGCCGAGCACGAG-CATAGGGGCTCAGCAAGCTTAATGC
Smad4 mutant antisense: GCATTAAGCTTGCTGAGCCCCTATGGT-GGTGCTCGGCCTTTGGACTAGTGCATT

RESULTS

MiR-17-92 EXPRESSION DECREASED IN PALATAL SHELVES FROM E12 TO E14

Palatal shelves of mouse embryos elongate from E12 to E13.5, elevated from E13.5 to E14, and contact each other at the medial edge in the horizontal position after E14 [Murray and Schutte, 2004]. To have a quantitative measurement of miR-17-92 cluster expression in palatal shelves during the period of elongation and elevation, we performed real time RT-PCR analysis. We excised palatal shelves from E12, E13, and E14 C57BL/6J mouse embryos, and found that the expression levels of miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a significantly decreased from E12 to E14 (Fig. 1A–F). MiR-200b was served as the positive control (Fig. 1G) because it continuously expressed in palatal shelves from E12 to E14 without significant increase or decrease [Mukhopadhyay et al., 2010]. To further explore whether miR-17-92 expressed in PMCs, we isolated PMCs and carried out RT-PCR 24 h after the primary culture. We found that all the components of miR-17-92 cluster expressed in PMCs in vitro (Fig. 1H), and the expression level of miR-17-92 cluster in mouse palatal shelves and PMCs exhibited similar expression tendencies, which may imply that miR-17-92 expression is comparable between palatal shelves and PMCs (Fig. 1H and Supplementary Fig. S1).

TGFB1 INHIBITED PROLIFERATION AND PROMOTED COLLAGEN SYNTHESIS IN PMCs

Proliferation of PMCs is important for palatal shelf elongation [Murray and Schutte, 2004]. To investigate the effect of TGFB1 on PMC proliferation, we added recombinant TGFB1 into PMC medium, and measured cell proliferation rate by MTT assay. We found that PMCs induced by TGFB1 had a significantly lower cell proliferation rate than control PMCs after culturing for 72 h (Fig. 2A,B).

Collagen synthesis of PMCs is important for palatal shelf elongation and elevation [Murray and Schutte, 2004; Meng et al., 2009]. Collagen types I and III are the predominant collagens in the mesenchyme of palatal shelves [Meng et al., 2009]. The polypeptide composition of collagen types I and III are $[a1(I)]_2a2(I)$ and $[a1(III)]_3$ [Hames and Hooper, 2005], which are encoded by Col1a1, Col1a2, and Col3a1 genes. As the three genes are SMAD target genes, their expression could be promoted by TGFB ligands in fibroblasts [Verrecchia et al., 2001]. TGFB1 was found to promote collagen synthesis in PMCs [D'Angelo et al., 1994]. To confirm the effect of TGFB1 on collagens types I and III synthesis in PMCs, we added recombinant TGFB1 (5 ng/ml) into the medium. The mRNA and protein levels of Col1a1, Col1a2, and Col3a1 were used for detecting the expression of collagen types I and III. Real-time RT-PCR and Western blot showed that both mRNA (Fig. 2C) and protein (Fig. 2D) levels of Col1a1, Col1a2, and Col3a1 were higher in TGFB1 induced PMCs than that in controls after culturing for 24 and 48 h, respectively. This result indicates that TGFB1 promotes collagen types I and III synthesis in PMCs.

MiR-17-92 INHIBITED TGFB1 INDUCED PROLIFERATION INHIBITION AND COLLAGEN SYNTHESIS IN PMCs

To investigate the influence of miR-17-92 cluster on TGFB pathway, PMCs were first cultured in common medium for 72 h to achieve 80% confluence. Then we reseeded PMCs and transfected PMCs with miR-17-92 mimics (the mixture of miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a mimics), miR-17-92 inhibitors (the mixture of miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a inhibitors), or scrambled miRNAs. Twelve hours after

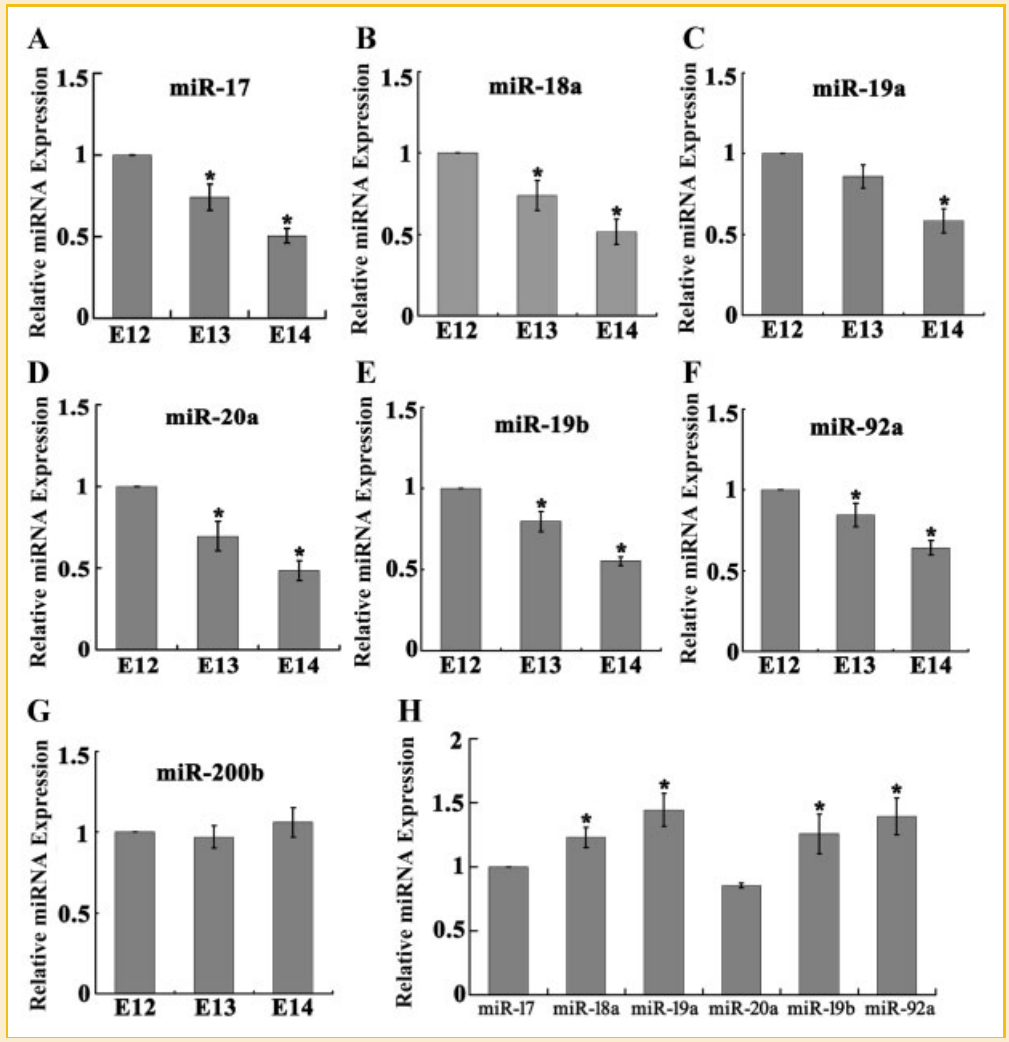

Fig. 1. Expression of miRNAs in miR-17-92 cluster in palatal shelves and PMCs. A–F: Relative expression of mature miR-17-92 cluster (miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a) in palatal shelves on E12, E13 and E14. G: miR-200b, served as the positive control, is continuously expressed in palatal shelves with no decrease or increase from E12 to E14. H: Relative expression of mature miR-17-92 cluster in cultured PMCs. Quantification was performed using the $\Delta\Delta C_t$ method. The RT-PCR fold changes were normalized to snoRNA135. The results were expressed as mean ± SE, n = 3; $^*P < 0.05$ versus expression level on E12 (A–G) or expression level of miR-17 (H); (E) embryonic day.

transfection, we added recombinant TGFB1 into the medium. The proliferation rate was measured for up to 72 h after TGFB1 treatment. The mRNA and protein levels of Col1a1, Col1a2, and Col3a1 were tested 24 and 48 h after TGFB1 treatment, respectively.

Using MTT assay, we found that PMCs transfected with miR-17-92 mimics had a significantly higher cell proliferation rate than that transfected with scrambled miRNAs or miR-17-92 inhibitors (Fig. 3A,B). PMCs transfected with miR-17-92 inhibitors had a significantly lower cell proliferation rate than that transfected with scrambled miRNAs. These results indicate that miR-17-92 cluster disturbed TGFB1 induced proliferation inhibition in PMC (Fig. 3A,B).

Real-time RT-PCR and Western blot showed that the mRNA and protein levels of Col1a1, Col1a2, and Col3a1 were lower in PMCs transfected with miR-17-92 mimics than that in PMCs transfected with miR-17-92 inhibitors or scrambled miRNAs. The mRNA and protein levels of Col1a1, Col1a2, and Col3a1 in PMCs transfected with miR-17-92 inhibitors were higher than that in PMCs transfected with scrambled miRNAs (Fig. 3C,D). These results suggest that miR-17-92 cluster inhibit TGFB1 induced collagen type I and III synthesis in PMCs.

MiR-17-92 INHIBIT ENDOGENOUS Tgfbr2, Smad2, AND Smad4 IN PMCs

As miR-17-92 cluster inhibited TGFB1-induced proliferation inhibition and collagen synthesis in PMCs, we want to know whether the phosphorylation of SMAD2/3 induced by TGFB signaling could be inhibited by miR-17-92 cluster. Western blot was used to evaluate the expression level of p-SMAD2/3 1 h after TGFB1 stimulation in PMCs. We found both the expression levels of p-SMAD2/3 and SMAD2 were inhibited in PMCs transfected with miR-17-92 mimics (Fig. 4A). Further quantitative analysis

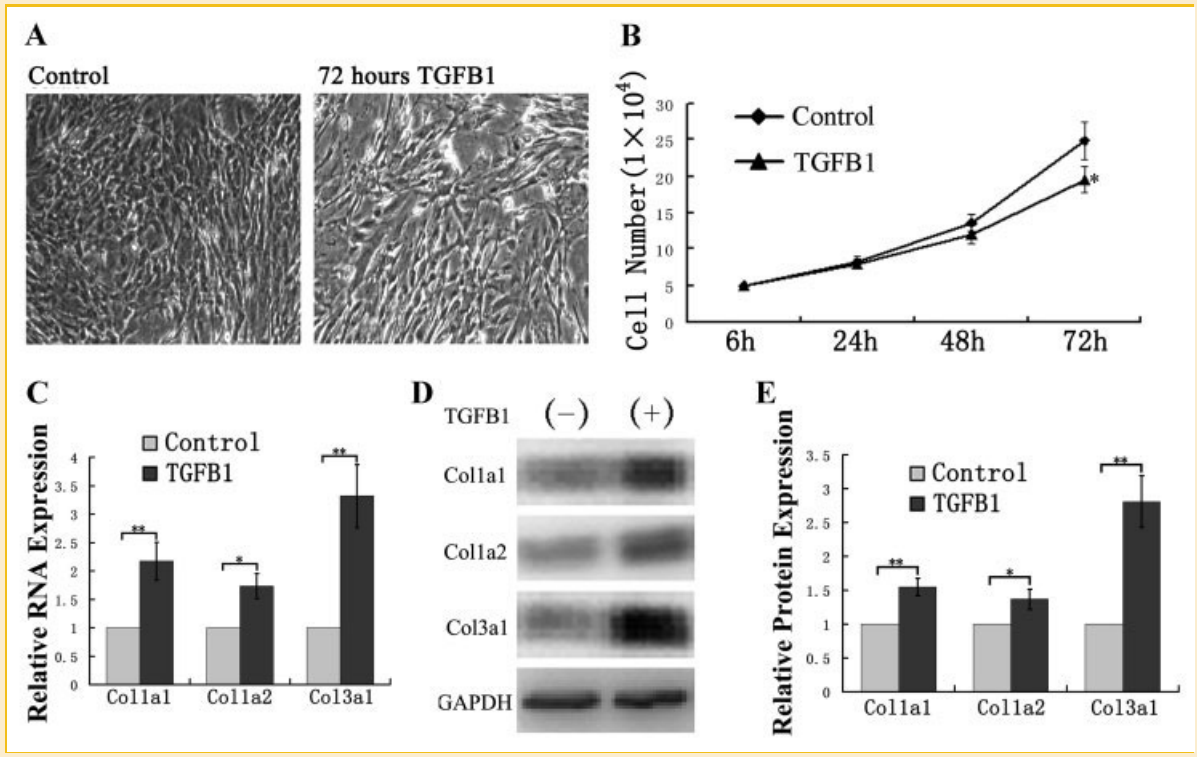

Fig. 2. TGFB1 induced proliferation inhibition and collagen synthesis in PMCs. A: Phase contrast microscopy of PMCs treated with TGF-β1 for 72 h. B: The growth curve of PMC in different groups. TGFB1 induced group had a significantly lower proliferation rate than the control group. Values are mean ± SD of triplicate experiments in each group; $^{*}P < 0.05$. C: Changes in Col1a1, Col1a2, and Col3a1 mRNA levels in PMCs after 24 h of TGFB1 treatment, as measured by real-time RT-PCR. Quantification was performed using the $\Delta\Delta C_t$ method. The fold changes were normalized to β-actin. The results were expressed as mean ± SE, n = 3; $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. D: Changes in Col1a1, Col1a2, and Col3a1 protein levels in PMCs after 48 h of TGFB1 treatment, as measured by Western blot. E: Quantitative analysis of Western blot. The results were expressed as mean ± SE, n = 3; $^{*}P < 0.05$, $^{**}P < 0.01$ versus control.

showed that the relative p-SMAD2/3 level normalized to SMAD2 in TGFB1 + miR-17-92 mimics group was lower than that in TGFB1 + scrambled miRNA group, (Supplementary Fig. S2A), which indicates that relative p-SMAD2/3 level could be inhibited by miR-17-92 in PMCs.

As inhibition of relative p-SMAD2/3 maybe either caused by reduced TGFB receptor activity or reduced SMAD2/3 expression, we quantified TGFBR1, TGFBR2, SMAD2, and SMAD3 protein level in the presence of miR-17-92 mimics and inhibitors in PMCs. As p-SMAD2/3 must first form complexes with SMAD4, and then could be translocated to the nucleus, we also quantified SMAD4 protein level. Western blot revealed that TGFBR2, SMAD2, and SMAD4 levels were appreciably lower in miR-17-92 mimics transfected PMCs and higher in miR-17-92 inhibitors transfected PMCs than that in scrambled miRNA transfected PMCs, whereas TGFBR1 and SMAD3 level was not significantly affected (Fig. 4B; Supplementary Fig. S2B). These results indicate that inhibition of p-SMAD2 is probably caused by inhibition of TGFBR2 and SMAD2 by miR-17-92 mimics, inhibition of p-SMAD3 is probably caused by inhibition of TGFBR2 by miR-17-92 mimics, and that Smad4 is probably repressed by miR-17-92 mimics.

To investigate which specific miRNA represses endogenous TGFBR2, SMAD2, and/or SMAD4, we over-expressed each miRNA from miR-17-92 cluster, respectively, and measured the expression of TGFBR2, SMAD2, and SMAD4 in PMCs. We found that miR-17 and miR-20a mimics appreciably decreased TGFBR2, and miR-17 and miR-20a inhibitors appreciably increased TGFBR2 in PMCs (Fig. 4C,D; Supplementary Fig. S2C,D). MiR-18a mimics significantly decreased SMAD2 and SMAD4, and miR-18a inhibitors significantly increased SMAD2 and SMAD4 in PMCs (Fig. 4E; Supplementary Fig. S2E). MiR-18a mimics also modestly decreased TGFBR2 (Fig. 4E; Supplementary Fig. S2E). However, as miR-18a does not have predicted target sites in 3′UTR of mouse TGFBR2 and miR-18a inhibitors did not increase TGFBR2 in PMCs (Fig. 4E; Supplementary Fig. S2E), we suggest TGFBR2 may not be the target of miR-18a. MiR-19a, miR-19b, and miR-92a mimics or inhibitors did not influence TGFBR2, SMAD2, or SMAD4 (Fig. 4 F–H; Supplementary Fig. S2F–H). These results indicate that miR-17 and miR-20a might target TGFBR2, and that miR-18a might target SMAD2 and SMAD4 in PMCs.

MiR-17/20A TARGETS Tgfbr2 AND miR-18A TARGETS Smad2 AND Smad4 IN PMCs

As miR-17 and miR-20a have the same seed sequences and the same predicted target sites in 3′UTR of mouse Tgfbr1, Tgfbr2, Smad2, and Smad4, we generated pMIR-Report luciferase vectors of Tgfbr1, Tgfbr2, Smad2, and Smad4 for miR-17/20a. As miR-18a has predicted target sites in 3′UTR of mouse Smad2, Smad3, and Smad4,

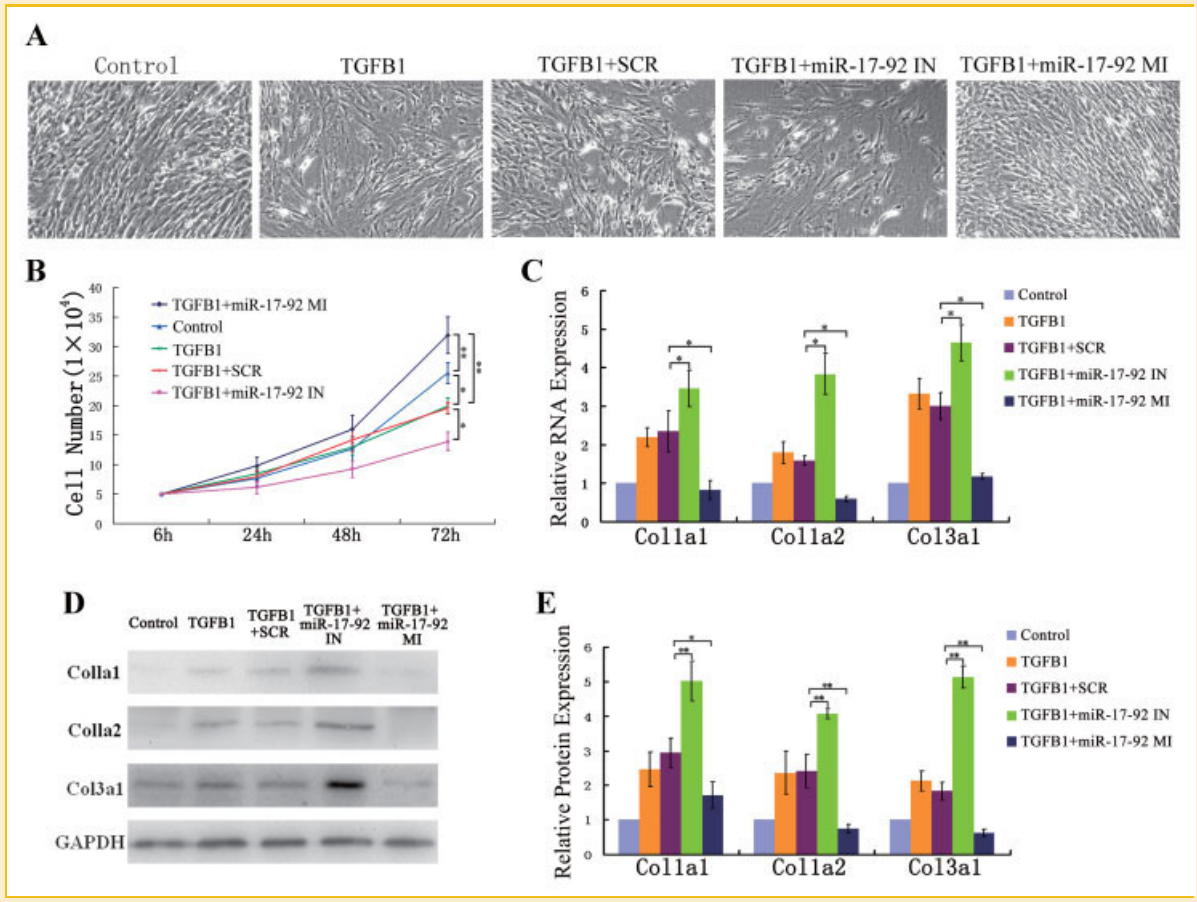

Fig. 3. MiR-17-92 disturbed TGFB1 induced proliferation inhibition and collagen synthesis in PMCs. A: Phase contrast microscopy of PMCs treated with TGFB1, TGFB1 + scrambled miRNAs (SCR), TGFB1 + miR-17-92 inhibitors (IN), and TGFB1 + miR-17-92 mimics (MI) for 72 h. B: The growth curve of PMC in different groups. After 72 h treatment, TGFB1 + miR-17-92 MI group had a significantly higher proliferation rate than TGFB1 group; TGFB1 + miR-17-92 IN group had a significantly lower proliferation rate than TGFB1 group; TGFB1 + SCR group had a similar proliferation rate with TGFB1 group. Values are mean ± SD of triplicate experiments in each group; $^{*}P < 0.05$, $^{**}P < 0.01$. C: Changes in Col1a1, Col1a2, and Col3a1 mRNA levels in the TGFB1, TGFB1 + SCR, TGFB1 + miR-17-92 IN, and TGFB1 + miR-17-92 MI treated PMCs, as measured by real-time RT-PCR after 24 h of TGFB1 treatment. Quantification was performed using the $\Delta\Delta C_t$ method. The fold changes were normalized to β-actin. The results were expressed as mean ± SE, n = 3; $^{*}P < 0.05$, $^{**}P < 0.01$ versus TGFB1 + SCR group. D: Changes in Col1a1, Col1a2, and Col3a1 protein levels in the TGFB1, TGFB1 + miR-17-92 MI, TGFB1 + miR-18-92 IN, and TGFB1 + SCR treated PMCs, as measured by Western blot after 48 h of TGFB1 treatment. E: Quantitative analysis of Western blot. The results were expressed as mean ± SE, n = 3; $^{*}P < 0.05$, $^{**}P < 0.01$ versus TGFB1 + SCR group. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

we generated pMIR-Report luciferase vectors of Smad2, Smad3, and Smad4 for miR-18a. The coding sequence of the firefly luciferase is followed by ~100 nucleotide synthetic DNA fragments encompassing the predicted miRNA binding site from predicted target gene 3′-UTR in either wild-type (WT) or seed mutant constructs.

The 3′UTR recombinant constructs of Tgfbr1, Tgfbr2, Smad2, and Smad4 were transfected into PMCs along with miR-17/20a or scrambled miRNAs. The luciferase activity in PMCs transfected with Tgfbr2 WT constructs plus miR-17/20a mimics was significantly lower than that in PMCs transfected with either Tgfbr2 WT or mutant constructs alone, and the scrambled miRNA did not affect the luciferase activity in either WT or mutant constructs transfected PMCs (Fig. 5B). However, miR-17/20a mimics did not significantly influence the luciferase activity of Tgfbr1, Smad2, and Smad4 constructs (Fig. 5A,C,D). These results indicate that Tgfbr2 is a direct target of miR-17/20a in PMCs.

The 3′UTR recombinant constructs of Smad2, Smad3, and Smad4 were transfected into PMCs along with miR-18a or control mimics. The luciferase activity in PMCs transfected with Smad2 and Smad4 WT constructs plus miR-18a mimics was significantly lower than that in PMCs transfected with either WT or mutant constructs alone, and the scrambled miRNA did not affect the luciferase activity in either WT or mutant constructs transfected PMCs (Fig. 5E,G). However, miR-18a mimics did not significantly influence the luciferase activity of Smad3 constructs (Fig. 5F). These results indicate that Smad2 and Smad4 are direct targets of miR-18a in PMCs.

DISCUSSION

TGFB pathway is one of the most important signaling pathways for palatal development. In this study, we focus on the effects of

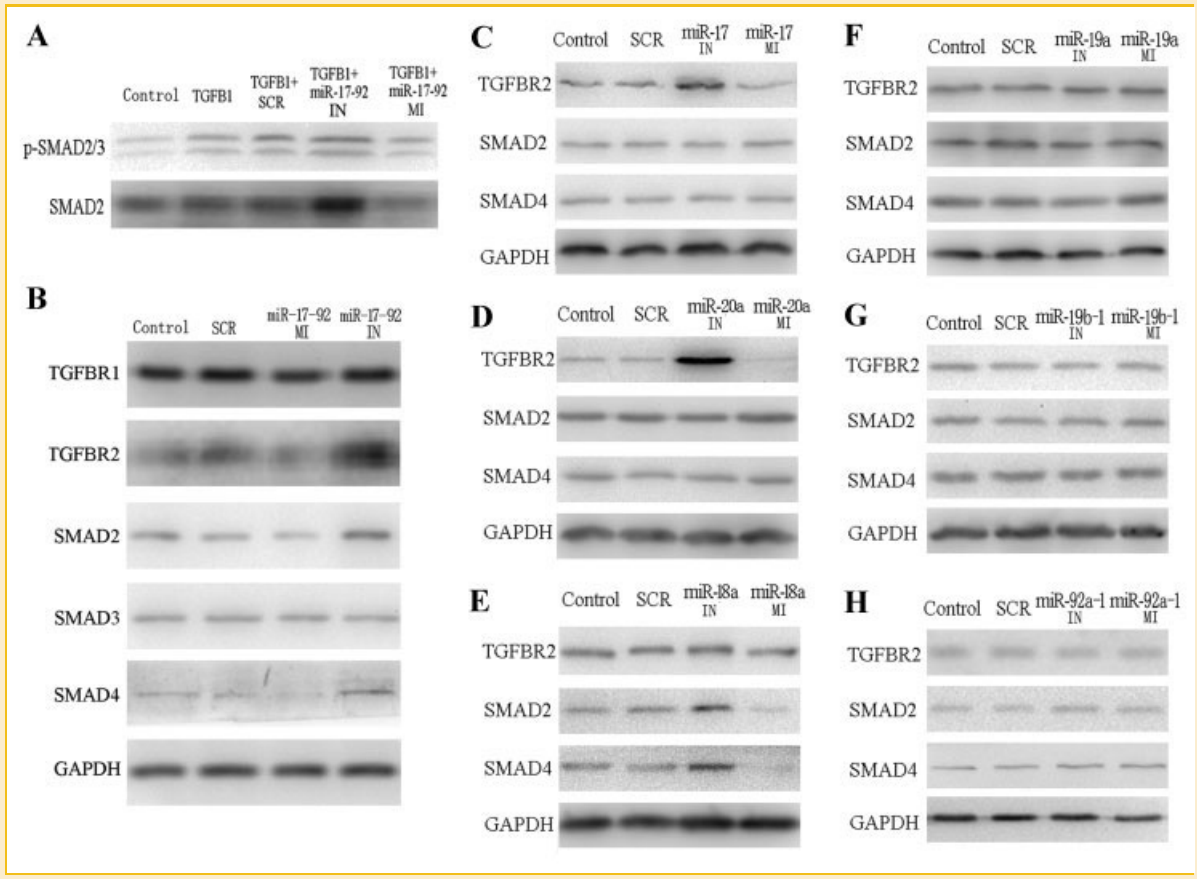

Fig. 4. MiR-17-92 cluster inhibits expression of p-SMAD2/3, TGFBR2, SMAD2, and SMAD4 in PMCs. A: Western blot shows both p-SMAD2/3 and SMAD2 levels are significantly lower in TGFB1 + miR-17-92 MI group than that in TGFB1 + SCR group. B: Western blot shows protein levels of TGFBR2, SMAD2, and SMAD4 are lower in TGFB1 + miR-17-92 MI group than that in TGFB1 + SCR group, whereas protein levels of TGFBR1 and SMAD3 are similar in TGFB1 + SCR, TGFB1 + miR-17-92 MI, and TGFB1 + miR-17-92 IN groups. C: Western blot shows TGFBR2 level is lower in miR-17 MI group than that in SCR group. D: Western blot shows TGFBR2 level is lower in miR-20a MI group than that in SCR group. E: Western blot shows SMAD2 and SMAD4 levels are lower in miR-18a MI group than that in SCR group. F: Protein levels of TGFBR2, SMAD2, and SMAD4 are similar in SCR, miR-19a IN, and miR-19a MI groups. G: Protein levels of TGFBR2, SMAD2, and SMAD4 are similar in SCR, miR-19b IN, and miR-19b MI groups. H: Protein levels of TGFBR2, SMAD2, and SMAD4 are similar in SCR, miR-92a IN, and miR-92a MI groups. MI, mimics; SCR, scrambled miRNA.

miR-17-92 cluster on TGFB pathway in PMCs, and found that miR-17-92 cluster decreased from E12 to E14, and targeted several components of TGFB pathway (TGFBR1, SMAD2, and SMAD4) to inhibit TGFB1 induced proliferation inhibition and collagen synthesis in PMCs. Therefore, we suggest that miR-17-92 cluster may regulate palatal shelf elongation and elevation by regulating TGFB pathway induced proliferation inhibition and collagen synthesis in PMCs.

In our study, TGFB1 was found to decrease proliferation rate and increase collagen synthesis in PMCs, which is similar with most of the cell types [Lyons and Moses, 1990; Heldin et al., 2009]. MiR-17-92 was found to promote PMC proliferation by down-regulating several components of TGFB pathway, which is also similar with several studies focusing on cancer cell lines [Dews et al., 2010; Mestdagh et al., 2010]. We did not investigate the effect of miR-17-92 on down-stream molecules of TGFB signaling, which should be further explored in PMCs because miR-17-92 was found to target several TGFB-responsive [Dews et al., 2010; Mestdagh et al., 2010] and TGFB-nonresponsive genes [Mestdagh et al., 2010].

MiR-17-92 cluster was found to decrease from E12 to E14 here, which is similar with the study of Mukhopadhyay et al. [2010]. They found all the components of miR-17-92 cluster significantly decreased from E12 to E14 in murine embryonic orofacial tissues, which is mainly composed of palatal shelves. As the expression level of miR-17-92 cluster decreased from E12 to E14 in palatal shelves, expression levels of TGFBR2, SMAD2, and SMAD4 may increase from E12 to E14. As palatal shelves of mouse embryos elongate from E12 to E13.5 and elevate from E13.5 to E14 [Murray and Schutte, 2004], we suggest that miR-17-92 cluster may promote proliferation of PMCs by inhibiting TGFB pathway during palatal shelf elongation period, and that decrease of miR-17-92 cluster may promote collagen synthesis in PMCs by increasing TGFB pathway during palatal shelf elevation period.

We found that miR-17/20a directly target Tgfbr2, and that miR-18a directly target Smad2 and Smad4. These results are in accordance with Mestdagh et al. [2010]. But Dews et al. [2010] found that Smad2 is not a direct target of miR-18a or any other components of miR-17-92 cluster. These differences are probably

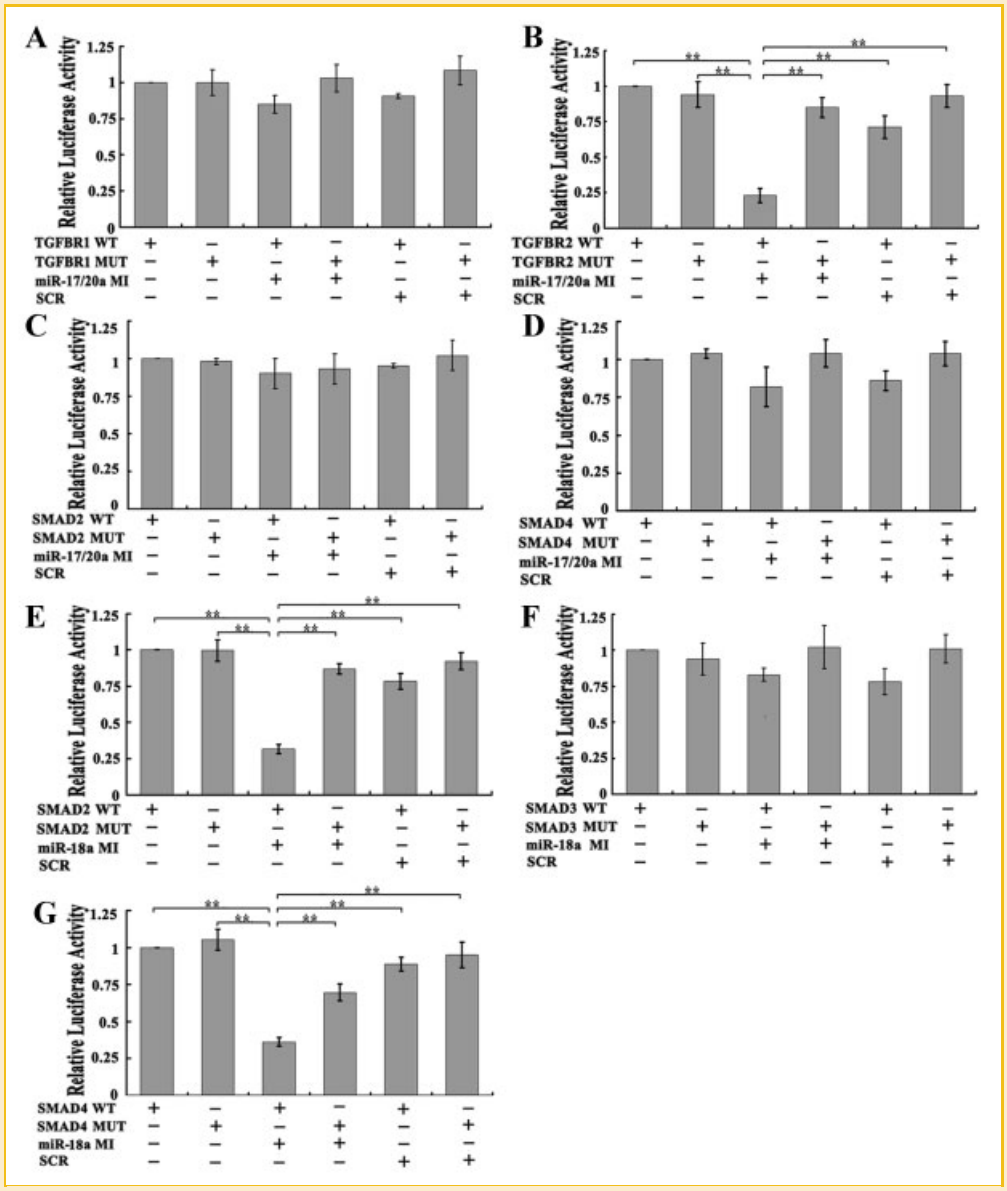

Fig. 5. MiR-17/20a targeted Tgfbr2 and miR-18a targeted Smad2 and Smad4 in PMCs. Luciferase assay is used to validate that the predicted target sites on 3′UTR of Tgfbr1, Tgfbr2, Smad2, Smad3, or Smad4 are targeted by miR-17/20a or miR-18a. PMCs were transfected in triplicate with pMIR-Report β-gal, luciferase reporter constructs that contained wide-type or mutant target site for miRNAs and either miRNA mimics or scrambled miRNAs. Data represent three independent experiments in triplicate. A: miR-17/20a has no effect on Tgfbr1. B: Tgfbr2 is directly targeted by miR-17/20a. C,D: miR-17/20a has no effect on Smad2 and Smad4. E: Smad2 is directly targeted by miR-18a. F: miR-18a has no effect on Smad3. G: Smad4 is directly targeted by miR-18a. Luciferase activity is normalized to β-gal expression. MI, mimics; SCR, scrambled miRNA; WT, wide type construct; MUT, mutant construct; $^{**}P < 0.01$.

due to different cells used in different studies. MiR-18a was found to modestly decrease TGFBR2 protein level in our study, which is in accordance with another study of Ras cells [Dews et al., 2010]. However, as mouse Tgfbr2 does not have predicted binding site for miR-18a in 3′UTR, we suggest that miR-18a may affect TGFBR2 expression indirectly. Here, we did not find direct targets for miR-19a, miR-19b, and miR-92a in the components of TGFB pathway. But Mestdagh et al. [2010] found that these miRNAs could down-regulate several SMAD target genes, and thus influence the function of TGFB pathway in neuroblastoma cells. The function of miR-19a, miR-19b, and miR-92a in PMCs needs further exploration.

During TGFB signal transduction, TGFB ligands first bind to TGFBR2 with very high affinity, only after this binding has been established can TGFBR1 dock into the receptor complex [Heldin et al., 2009]. SMAD2 is phosphorylated by TGFBR1, and then forms oligomeric complex with SMAD4. SMAD4 activate SMAD target genes [Heldin et al., 2009]. Previous studies found that conditional ablation of TGFBR2 in mouse craniofacial neural crest derived PMCs causes cleft palate [Ito et al., 2003], SMAD2 and SMAD 4 are

involved in extra-cellular matrix synthesis in PMCs [Greene et al., 2003], and that conditional ablation of Smad4 in mouse PMCs results in delayed elevation and defective contact of palatal shelves [Chi-Young Yoon et al., 2010]. Therefore, Tgfbr2, Smad2, and Smad4 are critical for palatal mesenchymal development. Regulation of TGFBR2, SMAD2, and SMAD4 by miR-17/20a and miR-18a may play important roles for the normal development of palatal mesenchyme.

MTT assay was used to test cell proliferation rate in our study. Notably, as MTT assay tested the activity of the mitochondrial enzyme, succinyl dehydrogenase, which is expressed only in living cells [Mosmann, 1983], it actually measures total viable cell number. Thus the increased cell number of PMC measured by MTT assay may reflect an increased rate of proliferation and/or a decreased rate of apoptosis. The exact effect of miR-17-92 on PMC proliferation and/or apoptosis needs further exploration.

Interactions between signaling pathways and miRNAs are complex. Besides TGFB pathway, miR-17-92 has been found to regulate several other pathways, including SHH [Uziel et al., 2009] and BMP pathways [Brock et al., 2009]. These pathways are also involved in palatogenesis [Meng et al., 2009]. In addition, TGFB pathway has been found to be regulated by several other miRNAs [Rai et al., 2010; Zhong et al., 2010; Leeper et al., 2011]. Therefore, further studies about interactions between miRNAs and pathways involved in palatogenesis should be taken into consideration to clarify the miRNA regulating mechanisms during palatal development.

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