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miR-217 represses TGF-β1-induced airway smooth muscle cell proliferation and migration through targeting ZEB1

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ABSTRACT

Aberrant proliferation and migration of airway smooth muscle cells (ASMCs) contribute to the pathogenesis of airway remodeling during asthma development. Here, the potential function of microRNA-217 (miR-217) on the cell proliferation and migration of TGF-β1-induced ASMCs and the involved mechanisms were investigated in this study. We found that miR-217 expression was apparently downregulated in a time and dose dependent characteristic in ASMCs exposed to transforming growth factor-β (TGF-β1) stimulation. Overexpression of miR-217 significantly inhibited TGF-β1-induced proliferation, migration, extracellular matrix (ECM) deposition, but promoted apoptosis in ASMCs, whereas, miR-217 inhibitor showed an opposed effect. Bioinformatics analyses revealed that the 3'-untranslated region (UTR) of ZEB1 was a potential target for miR-217, which was further confirmed by luciferase activity, qRT-PCR and western blot assay. In addition, rescue experiment also displayed that restoration of ZEB1 expression partially abrogated the inhibitory effect of miR-217 on TGF-β1-induced proliferation and migration in ASMCs. By chromatin immunoprecipitation (ChIP) assay, we further confirmed that the binding of ZEB1 to the fibronectin promoter in TGF-β1-treated ASMCs was reduced by miR-217 overexpression. Therefore, our findings suggested the potential protective role of miR-217 on the attenuation of cell proliferation and migration was through targeting ZEB1 in TGF-β1-stimulated ASMCs.

1. Introduction

Asthma as a common chronic respiratory disease causes millions of mortality and morbidity among children worldwide [1]. It is a complex syndrome characterized by airway hyper-responsiveness (AHR), airway inflammation, mucus hypersecretion, and airway remodeling [2], of which airway remodeling has been emphasized to mainly contribute to the severity of asthma through increased extracellular matrix (ECM) deposition, abnormal airway smooth muscle cell (ASMC) proliferation and migration [3,4]. It has been demonstrated that increased proliferation and migration of ASMCs disturb the pulmonary functions by enhancing the thickness of airway wall and narrowing airway lumen in patients with chronic airways diseases [5]. Currently, several therapeutic agents such as corticosteroids, β2-agonists, anticholinergics, H1-anti-histamine, and theophylline have been applied for asthma treatment, nevertheless, their efficiencies on airway remodeling are unsatisfied [6]. Therefore, novel therapeutic strategies based on the better understanding of the intrinsic mechanisms of asthma development is urgently needed.

microRNAs (miRNAs) are a class of small (21–25 nucleotides in length), single stranded, noncoding RNAs that post-transcriptionally regulate target gene expressions by complementarily binding to their 3'-untranslated region (3'-UTR) [7,8]. It has been widely reported that miRNAs participate in diverse cellular processes in both normal physiology and pathogenesis, such as differentiation, cell growth, apoptosis, and inflammation [9]. For asthma, several miRNAs including miR-145, miR-25, miR-320, and miR-10a have been shown to be associated with the airway inflammatory response, hyperresponsiveness, remodeling, as well as modulation of ASMC contractility [10,11]. In recent years, miR-217 has been demonstrated to be involved in regulating multiple biological processes including cell growth, apoptosis, differentiation, and metastasis in various cell types [12,13]. However, the effect of miR-217 on ASMC proliferation and migration during asthma development remains largely unknown.

Zinc finger E-box binding homeobox 1 (ZEB1, also named TCF8 or DeltaEF1) belongs to the ZEB transcription factor family and contains a centrally located homeodomain, two zinc finger clusters, and protein binding domains, which facilitate it to be involved in DNA binding and interacting with transcription regulators [14]. It has been demonstrated that ZEB1 promotes tumor invasion and metastasis by inducing

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Table 1
The primer sequences for qRT-PCR.

Gene	Forward	Reverse
miR-217	5'-ACACTCCAGCTGGGTA CTGCATCAGGAACTG-3'	5'-CTCAACTGGTGTCGTGGAGTC GGCAATTCAGTTGAGUCCAAUCA-3'
U6	5′-CGCTTCGGCAGCACATATAC-3′	5′-AAATATGGAACGCTTCACGA-3′
ZEB1	5'-TCGGAAAGAGCTGTTCGCTT-3'	5'-AGGAGGGGGCTGACATACAT-3'
GAPDH	5'-GCAACTAGGATGGTGTGGCT-3'	5'-TCCCATTCCCCAGCTCTCATA-3'

epithelial-to-mesenchymal transition (EMT) in various carcinoma cells [15]. Recent studies have also suggested the essential role of ZEB1 on the modulation of its target genes via feedback loops with different miRNAs. For instance, ZEB/miR-200 feedback loop has been widely verified as the central molecular motor for cancer progression towards metastasis through regulating the state of cancer stem cells [16]. Furthermore, ZEB1 has also been illustrated to participate in the excessive production of ECM proteins through the transforming growth factor-β1 (TGF-β1) signaling pathway in corneal endothelial cells [17]. Therefore, we supposed that ZEB1 might be involved in the ECM deposition, proliferation and migration of ASMC following TGF-β1 exposure.

The purpose of this study was to determine the functional role of miR-217 on ASMC proliferation and migration when exposed to TGF-β1, which mimicked the pathological conditions of airway remodeling in patients with asthma, and its underlying regulatory mechanisms were elucidated as well.

2. Materials and methods

2.1. Human airway smooth muscle cell collection and culture

Human airway smooth muscle cells (ASMCs) were obtained from patients without asthma undergoing lung resection surgery at the First Affiliated Hospital of Xi'an Jiaotong University. Briefly, the smooth muscle layer was isolated from the main bronchus of subjects via dissecting microscope. The tissues were washed with ice-cold PBS solution containing penicillin-streptomycin solution for three times, and then were cut into small pieces and digested in Hanks' balanced salt solution (HBSS) containing 0.1% collagenase solution (Sigma Chemical Co., St. Louis, MO, USA) at 37 °C for 30 min. After centrifugation, the isolated cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin solution (Invitrogen, Carlsbad, CA, USA) at 37℃ in a humidified 5% CO_2 atmosphere. Cells cultured from 3 to 5 passages were used for subsequent experiments. Informed consent was obtained from all patients. This study was approved by the Ethics Committee of Xi'an Jiaotong University and in accordance with the Code of Ethics of Declaration of Helsinki. Cells were starved in serum-free for 24 h, and then treated with human recombinant TGF-β1 (R&D Systems, Minneapolis, MN) at the varying concentrations of 0, 1, 5, 10, 20, and 30 ng/ml for 24 h; In another experiment, cells were incubated with TGF-β1 (10 ng/ml) for indicated time points.

2.2. Cell transfection

The miR-217 mimic, miR-217 inhibitor, three scramble miRNAs, ZEB1 siRNA (siZEB1), and siRNA negative control (siNC) (Sequence listed in Supplementary Table S1) were obtained from Genepharma (Shanghai, China). The pcDNA3.1-ZEB1 overexpressing plasmid and empty vector were also purchased from Genepharma Company. ASMCs were transiently transfected with miRNAs (at a final concentration of 50 nM) or plasmids (0.5 μg) by using the Lipofectamine 2000 (Invitrogen) following the manufacturer's protocols. After 24 h transfection, cells were harvested for the following experiments.

2.3. Quantitative RT-PCR

Total RNA was isolated from ASMCs using TRIzol reagent (Invitrogen). For mRNAs detection, RNA was reversely transcribed into cDNA by PrimeScript RT Master Mix Kit (Takara, Dalian, China) according to the manufacturer's instructions. For miRNAs detection, RNA was reversely transcribed into cDNA by TaqMan microRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed using SYBR Green qPCR Master Mix Kit (Takara) on a 7500 Real-time PCR system (Applied Biosystems, Shanghai, China). U6 was used for normalization of miR-217, and GAPDH was used for normalization of ZEB1. The relative gene expression was measured by the $2^{-\triangle\triangle Ct}$ method. The primers were listed in Table 1.

2.4. Cell proliferation assay

Cell proliferation was measured by using 3-(4,5-dimethylthiazol-2 yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated into 96-well plates at the density of 3×10^5 cells per well. After transfection with scramble miRNA, miR-217 mimic, or miR-217 inhibitor for 24 h, following by TGF-β1 treatment for 24 h. 20 μl MTT solution (5 mg/mL) was added to each well and incubated for 4 h at 37℃. Then 100 μl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. The OD value at the wavelength of 490 nm was evaluated using a microplate reader (Bio-Rad, CA, USA). All experiments were performed at least in triplicate.

2.5. Cell apoptosis assay

Cell apoptosis was assessed by Annexin V-FITC-PI Apoptosis Detection kit (Vazyme Biotech, Nanjing, China). Briefly, after transfection for 24 h and following by TGF-β1 treatment for 24 h, ASMCs were washed with PBS and centrifuged at 2000 rpm for 5 min at 4 °C, and then were resuspended with 100 μl of binding buffer at the concentration of 2×10^5 cells/ml. 5 μl of FITC-conjugated Annexin V and 5 μl propidium iodide (PI) were added to the cells and incubated for 15 min in the dark at room temperature. Cell apoptosis was quantified by flow cytometry analysis (BD Biosciences, CA, USA).

2.6. Western blot

Total proteins were extracted from ASMCs using RIPA buffer (Beyotime, Nanjing, China), and the concentrations of proteins were measured by BCA protein assay kit (Pierce, Rockford, USA). Equal amount of proteins were separated by 12% sulfate-polyacrylamide -polyacrylamide (SDS-PAGE) gels, and then were transferred to polyvinylidene difluoide (PVDF) membranes (Millipore, Boston, MA, USA). The membranes were blocked with 5% non-fat milk at room temperature for 1 h. after rinsing, the membranes were incubated with primary antibodies against ZEB1 (ab203829, Abcam, CA, USA), cleaved caspase-3 (ab2302), p21 (ab109520), cyclin D1 (ab134175), Bax (ab32503), Bcl2 (ab32124), collagen I (ab34710), collagen III (ab7778), fibronectin (ab2413), JAK2 (ab108596), phosphorylated (p)-JAK2 (ab32101), STAT3 (ab119352), p-STAT3 (ab76315), and GAPDH (ab181602,) at 4℃ overnight, and then incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG antibody (sc-2768,