Acta Biochim Biophys Sin, 2016, 48(9), 840–849
doi: 10.1093/abbs/gmw068
Advance Access Publication Date: 13 July 2016
Original Article

Original Article

MicroRNA-181a-5p enhances cell proliferation in medullary thymic epithelial cells via regulating TGF-β signaling

Dongguang Guo, Yaqiong Ye, Junjie Qi, Lihua Zhang, Lifeng Xu, Xiaotong Tan, Xiaofang Yu, Qihong Liu, Jilong Liu, Yuan Zhang, Yongjiang Ma*, and Yugu Li*

College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, China

*Correspondence address. Tel/Fax: +86-20-85283226; E-mail: yuguli99@163.com (L.Y.)/462700840@qq.com (Y.M.)

Received 14 January 2016; Accepted 14 March 2016

Abstract

The expression profiles of miRNAs in thymus tissues from mice of different age have been demonstrated in our previous study. After an integrated analysis of the miRNA expression profiles, we demonstrated that the expression of miR-181a-5p was significantly decreased in thymic epithelial cells (TECs) from 10- to 19-month-old mice when compared with that in TECs from 1-month-old mice by quantitative reverse transcriptase polymerase chain reaction. We hypothesized that miR-181a-5p in TECs might be associated with the age-related thymus involution through regulating some genes or signaling pathway. To test this hypothesis, the mouse medullary thymic epithelial cells (MTEC1) were used. Transfection with miR-181a-5p mimic promoted the proliferation of MTEC1 cells, but did not affect apoptosis. The effect was reversed when the expression of miR-181a-5p was suppressed in MTEC1 cells. Furthermore, the transforming growth factor beta receptor I (Tgfbr1) was confirmed as a direct target of miR-181a-5p by luciferase assay. Moreover, it was found that overexpression of miR-181a-5p down-regulated the phosphorylation of Smad3 and blocked the activation of the transforming growth factor beta signaling. Nevertheless, an inversely correlation was observed between the expression of *Tgfbr1* and miR-181a-5p in TECs derived from mice of different age. Collectively, we provide evidence that miR-181a-5p may be an important endogenous regulator in the proliferation of TECs, and the expression levels of miR-181a-5p in TECs may be associated with the age-related thymus involution.

Key words: miR-181a-5p, mouse thymic epithelial cell, cell proliferation, Tgfbr1, TGF-β signaling

Introduction

Thymus is a dedicated organ for T cells development and differentiation. However, with aging, thymus undergoes a progressive decline in cellularity, driving a process of severe atrophy, known as thymic involution [1,2]. During the process of thymus involution, the gradually decline in cellularity mainly involves both thymocytes and thymic stromal cells [3], often resulting in a progressive deterioration of the thymic stromal microenvironment. Studies have shown that microenvironmental changes in the thymus and dysfunction of the thymic microenvironmental cells are the underlying cause of age-related thymic involution [3–5]. Thymic epithelial cells (TECs) as an indispensable part of stromal compartment can provide cues and form a microenvironment for thymocyte proliferation, differentiation, and selection through the supply of soluble and membrane-bound molecules [6–9]. Studies have demonstrated that the changes in numeric TECs can account for this physiological regression process of the age-related thymus involution [10,11].

Studies have also revealed that several factors are involved in age-related thymic involution, including the early programming of the thymus, the efficiency of specific T-cell progenitors, the sexual dimorphism, the thymic microenvironment, and the sensitivity of TECs to sex hormones [10,12–14]. However, in recent years, it has been demonstrated that an important role for the epigenetic factors, microRNAs (miRNAs), which is well-adapted to tuning involution in the age-related thymus. For example, Papadopoulou *et al.* [2] demonstrated that in TECs the absence of dicer or the miR-29a cluster could trigger a rapid loss of thymic cellularity. Zuklys *et al.* [15] also observed a significant reduction in thymus size and absolute cell number in mice that lacked of dicer1 expression in TECs. More recently, the study by Khan *et al.* [16] revealed that DGCR8-deficient (a component of the miRNA-specific microprocessor complex) TECs were unable to maintain proper thymic architecture and exhibited a dramatic loss of thymic cellularity. All these studies indicate that the expression of canonical miRNAs in thymus or TECs plays a critical role in supporting thymus overall architecture and function.

MiR-181a as a tumor suppressor can inhibit cell proliferation in different kinds of cancer cells [17–23]. In addition, miR-181a is highly expressed in bone marrow and thymus, particularly enriched at the $CD4^+CD8^+$ (DP) stage of thymocyte development, and plays a crucial role during the T-cell development [24–28]. Furthermore, it was reported that the abnormal low-expression of miR-181a in T cells is associated with different kinds of diseases, such as adult T-cell leukemia, chronic lymphocytic leukemia, and acute myeloid leukemia [29–31]. These results indicate that miR-181a is indispensable for the development and maintenance of the T cell-mediated immunity. However, whether miR-181a can directly regulate TECs function remains unclear.

Transforming growth factor-beta (TGF-β) is a multifunctional growth factor for cell proliferation, differentiation, and cell fate [11,32,33]. The overexpression of TGF-β in thymocytes can induce cell cycle arrest, apoptosis, and immunosuppression [34–37]. It was reported that TGF-β and its receptors could be detected on Day 10.5 during the early embryonic development, and played a negative role in the formation of thymic microenvironment and function [11]. These results indicate that TGF-β signaling plays an important negative role in the development and differentiation of TECs.

Although both the TGF-β and miRNAs are important regulators of TECs function, it is still unknown whether the observed alterations in the age-related thymus involution are the consequence of miRNA-mediated effects on the TGF-β signaling. In this study, we found that the expression of miR-181a-5p was strikingly up-regulated in TECs from young mice when compared with that in TECs from adult and aged mice. We speculate that the significant change of miR-181a-5p in TECs may dramatically regulate gene expressions that are involved in cell proliferation, apoptosis, and differentiation, and therefore, play an important regulatory role in the control of the age-related thymus involution. On the other hand, the target genes analysis revealed that miR-181a-5p regulated transforming growth factor beta receptor I (*Tgfbr1*) expression and the expressions of its downstream genes, suggesting that the TGF-β signaling is modulated by the expression of miR-181a-5p in TECs, which may be a mechanism involved in the age-related thymus involution.

Materials and Methods

Animal and thymus tissues

Specific pathogen-free Balb/c mice (1-, 3-, 8-, 10-, 13-, and 19-month-old) were purchased from the Center of Laboratory Animal Science in Guangdong (Guangzhou, China). Protocols for all animal experiments performed in this study were approved by the Animal Care Committee of the South China Agricultural University (Guangzhou, China) in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Isolation of TECs

TECs from 1-, 3-, 8-, 10-, 13-, and 19-month-old mice were isolated and purified according to a previously described method [38]. Thymic tissues from the 1-, 3-, 8-, 10-, 13-, and 19-month-old mice were harvested and transferred in complete Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, USA) for isolation of TECs. In brief, thymic lobes were finely minced to release the majority of thymocytes. The remaining thymic tissue was further digested with 1.5 mg/ml collagenase D (Roche, Mannheim, Germany), 1.0 mg/ml DNase I (Sigma-Aldrich, St Louis, USA) and 1.25 mg/ml Dispase (Roche) at 37 °C for 20 min with intermittent shaking. This step was repeated for three times. After complete digestion, the enriched TECs were obtained by passing through LS magnetic columns. The isolated TECs were used for miRNA quantitative PCR assays to detect the expression of miR-181a-5p.

Cell culture and cell transfection

Murine thymic epithelial cell line MTEC1 cell was obtained from Peking University Health Science Center (Beijing, China). Human embryonic kidney 293 T (HEK-293T) cell was obtained from the American Type Culture Collection (Manassas, USA). Both MTEC1 and HEK-293T cells were cultured in complete DMEM containing 10% fetal bovine serum (FBS) (Gibco) in a humidified atmosphere containing 5% CO_2 at 37 °C. Briefly, MTEC1 cells were seeded in 24-well plates at a density of 0.5–1 × 10^5 cells per well. After 24 h, oligonucleotides [50 nmol mimic or mimic negative control (mimic-NC), and 100 nmol inhibitor or inhibitor-NC] were transfected into cultured cells using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instruction. The miR-181a-5p mimic, miR-181a-5p inhibitor, and negative control (mimic/inhibitor-NC) were purchased from RiboBio (Guangzhou, China).

qRT-PCR

Total RNAs were extracted from TECs and MTEC1 cells with Trizol reagent (Takara, Kusatsu, Japan). Total RNA concentrations were measured using a NanoDrop ND-1000 fluorospectrometer (NanoDrop Technologies, Wilmington, USA). cDNA was synthesized using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) in accordance with the manufacturer's instructions. qRT-PCR was performed with SYBR Green real-time PCR Master Mix (Toyobo). For measuring the expression of miR-181a-5p in isolated TECs, the bulge-loop miRNA qRT-PCR Primer Sets (including one reverse transcription primer and a pair of quantitative PCR primers for each set) specific for the miR-181a-5p were designed by RiboBio (Guangzhou, China). The relative gene primers for mRNA (**Table 1**) were designed by the Primer Premier 5.0 software according to the published genome sequences, *β-actin*, and *U6* were used to normalize the relative abundance of mRNA and miRNA, respectively. qPCR analysis was performed using Bio-Rad CFX96 Real-Time PCR system (Bio-Rad, Hercules, USA). The relative expression level

Table 1. Primers of mRNA used in qRT-PCR

Gene	Accession No.	Sequence (5′–3′)	Product size (bp)
Tgfbr1	NM_009370	(F) AGCTCCTCATCGTGTTGGTG	137
		(R) GGCCTGTCTCGAGGAATTAGG	
Tgfbr2	NM_009371	(F) TACGGGCTACCAAAAATCAAAGG	131
		(R) AAGGATAATGGCAGGGAACAAAA	
Tgfbrap1	NM_001013025	(F) AAGAGGACGAACTGGGACTG	186
		(R) CACAGCTGTCTGCCCTCTTG	
Tgfbi	NM_009369	(F) CCCGCCAGTTTCTCTCAGTT	169
		(R) CCACGTTTGGAGGGATCGAA	
Tgif2	NM_173396	(F) ATGGGAGGAGAGGGGATCTG	200
		(R) TTAGGGGCTAAGCTGGACCT	
Smad2	NM_001252481	(F) GTCCATCTTGCCATTCACTCCG	118
		(R) ACACCACTTTTCTTCCTGTCCATTC	
Smad3	NM_016769.4	(F) CCACGCAGAACGTGAACACCAAG	189
		(R) CATCCTTCTTCATGTTGAAGGC	
Smad7	NM_001042660	(F) CATCTTCATCAAGTCCGCCACAC	188
		(R) CCTTCACAAAGCTGATCTGCACG	
Cyclin D1	NM_007631.2	(F) AGGCGGATGAGAACAAGCAGAC	176
		(R) CGGTAGCAGGAGAGGAAGTTG	
Cyclin D3	NM_001081635	(F) ACACTCGCTTTGTTTGGGT	106
		(R) TGCAGGACATCTGTTTTTGG	
Cyclin E1	NM_007633	(F) GCGTCTAAGCCCTCTGACCATTG	191
		(R) CAGAAGCAGCGAGGACACCATAG	
Cdk4	NM_009870	(F) CTACATACGCAACACCCG	118
		(R) TCAAAGATTTTCCCCAACT	
Cdk6	NM_009873	(F) GGACATCATTGGACTCCCAGGA	159
		(R) GGATTAAACGTCAGGCATTTCAGA	
C-myc	NM_010849	(F) CTATCACCAGCAACAGCAG	134
		(R) CAACATAGGATGGAGAGCAGAG	
Cyclin B1	NM_172301	(F) CTCCAAGCCCGATGGAAACA	177
		(R) GCTCTTCCTCCAGTTGTCGG	
β-actin	NM_007393	(F) CATCCGTAAAGACCTCTATGCCAC	171
		(R) ATGGAGCCACCGATCCACA	

of each gene was calculated from three different experiments and was determined by using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Cultured MTEC1 cells were lysed in RIPA buffer [50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1% NP40, 0.5% (w/v) sodium deoxycholate, and 0.1% sodium dodecylsulfate] (Beyotime, Nanjing, China) supplemented with protease and phosphatase inhibitor mixture (Sigma-Aldrich) and vortexed briefly. After centrifugation at 15,000 *g* for 15 min at 4°C, the protein sample was collected and the concentration was determined by BCA kit (Beyotime). Sample buffer was used to dilute the lysates, and the proteins (20 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, USA). After being blocked with skimmed milk, the blots were incubated overnight at 4°C with mouse anti-C-myc, mouse anti-Cdk4, mouse anti-Cyclin D1, mouse anti-Cyclin E1, mouse anti-Tgfbr1, rabbit anti-phosphorylated Smad2 (p-Smad2), mouse anti-phosphorylated Smad3 (p-Smad3), and mouse anti-Tubulin monoclonal antibodies. All these antibodies were from Santa Cruz Biotech (Santa Cruz, USA) The membranes were then washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Santa Cruz) at 37°C for 90 min, and then developed with BeyoECL Plus kit (Beyotime).

Cell viability assay

MTEC1 cells were seeded in 96-well plates at a density of 2–5 × 10^3 cells per well, and transfected with miR-181a-5p mimic, miR-181a-5p inhibitor, or miR-NC as described above. Cell viability was analyzed at the indicated time points (24, 48, and 72 h) using the cell-counting kit-8 (CCK-8) regents (Beyotime) according to the manufacturer's instruction.

Cell cycle assay

MTEC1 cells were cultured in DMEM with 10% FBS at 48 h after transfection, and then were fixed with 70% ethanol overnight at −20°C for 24 h. The cell cycle assay was determined using the Cell Cycle Analysis Kit (Beyotime) with a flow cytometer (BD Biosciences, San Jose, USA) and data were analyzed with ModFit Lt 4.1 software (Verity Software House, Topsham, USA).

Cell apoptosis assay

At 48 h after transfection, the cell apoptosis rate was quantified by gating PI (Propidium Iodide) and Annexin V-positive cells on a fluorescence-activated cell-sorting flow cytometer (BD Biosciences) according to the instructions of Apoptosis and Necrosis Assay Kit (Kaiji, Nanjing, China).

Bioinformatics prediction of target genes

In order to identify the target genes of miR-181a-5p in MTEC1, the TargetScan (http://www.targetscan.org), miRNABase (http://www.miRNAbase.org), and miRNA (http://www.microrna.org) were used to predict the targets of miR-181a-5p. The functional analysis and pathway enrichment analysis were performed by the Gene Ontology (GO) database (http://www.gene ontology.org) and KEGG pathway database via the Pathway-Express software, respectively.

Generation of the reporter constructs containing Tgfbr1 3′-untranslated regions

The 3′-untranslated regions (3′UTRs) of *Tgfbr1* that contained putative target sites of miR-181a were amplified by PCR from genomic DNA. The PCR products were cloned between *Sac*I and *Sal*I sites of the luciferase reporter vector pmiRGLO (Promega, Madison, USA). The primer sequences of the *Tgfbr1* 3′UTR (NCBI reference sequence: NM_009370) (positions 416–422 bp and 1164–1170 bp) were as follows: forward, 5′-CGAGCTCCTAGTAGTACATTCT CAGAGG-3′ and reverse, 5′-GCGTCGACCCCTTCCCAAGAT AGAATCC-3′. The mutant *Tgfbr1* 3′UTR plasmids was generated using a Stratagene mutation kit (Stratagene, Heidelberg, Germany) by GENEray Company (Shanghai, China), and both two seed sequences were mutated from 'UGAAUGT' to 'UAACUAA', respectively.

Dual luciferase reporter assay

The pmiRGLO-Tgfbr1-3′UTR (WT/MUT1/MUT2/MUT3) plasmid (400 ng) with miR-181a-5p mimic or mimic-NC were co-transfected into HEK-293T cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer's recommendation. Dual-luciferase activity was analyzed at 48 h after transfection using the Dual luciferase Reporter Assay system (Promega, Madison, USA) according to the manufacturer's instructions. Luciferase activity was calculated as the ratio of Firefly to Renilla and each experiment was performed in triplicate.

Statistical analysis

All experiments were performed in triplicate and repeated at least three times. All data were presented as the mean ± SD. The statistical analysis was performed by using Student's *t*-test to determine the significant differences using the commercial software (SPSS 17; SPSS, Chicago, USA). A value of $P < 0.05$ was considered as statistically significant.

Results

The expression of miR-181a-5p in mouse TECs

Our previous study has demonstrated the expression profiles of miRNAs in thymus tissues from mice of the different ages (1-, 10-, and 19-month-old), which suggest that the up-regulated or down-regulated miRNAs in thymus may play a critical role in inducing thymus involution [39]. In this study, we further investigated a potential miRNA, miR-181a-5p, whose expression levels was significantly decreased in the thymus tissue from young mice when compared with that from adult and aged mice.

In order to further explore the role of miR-181a-5p in thymus, TECs were isolated from the 1-, 10-, and 19-month-old mice, and then the expression of miR-181a-5p was detected by qRT-PCR. As shown in **Fig. 1**, the expression of miR-181a-5p was robustly decreased in TECs derived from 10- to 19-month-old mice compared with those derived from 1-month-old mice (**Fig. 1**). This result indicated that the expression levels of miR-181a-5p were dramatically down-regulated during the thymus aging process, suggesting that miR-181a-5p may be an important player in regulating thymus aging.

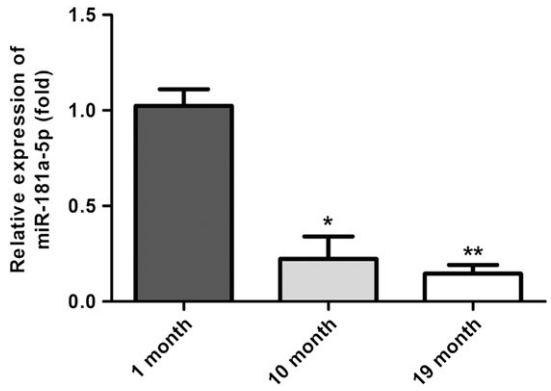

Figure 1. Expression of miR-181a-5p in isolated TECs miR-181a-5p was detected by qRT-PCR in TECs from 1-, 10-, and 19-month-old mice. *U6* was used as a reference. For every group, 6 to 18 thymi were pooled. $*P < 0.05$, $**P < 0.01$.

The effect of miR-181a-5p on cells viability and apoptosis

To understand the function of miR-181a-5p in TECs, the miR-181a-5p mimic, mimic-NC, miR-181a-5p inhibitor, or inhibitor-NC were transfected into MTEC1 for gain- or loss-of-function experiments, respectively. As shown in **Fig. 2A,B**, transfection of MTEC1 with miR-181a-5p mimic or miR-181a-5p inhibitor strikingly increased or decreased the expression levels of miR-181a-5p, when compared with the control-NC group. To further clarify the role of miR-181a-5p in TECs function, the cell viability was determined by CCK-8 assay. Interestingly, it was found that overexpression of miR-181a-5p in MTEC1 significantly enhanced cells viability 24 and 48 h after transfection, as compared with control group (**Fig. 2C**). As expected, the miR-181a-5p inhibitor has an opposite effect on MTEC1 cells viability (**Fig. 2D**). These results indicated that ectopic expression of miR-181a-5p in MTEC1 significantly increased the cell viability.

In addition, to investigate the effect of miR-181a-5p on cell apoptosis, flow cytometric analysis was performed. As shown in **Fig. 2E**, no obvious change was observed in the miR-181a-5p mimic-transfected and miR-181a-5p inhibitor-transfected cells. This result indicated that the expression of miR-181a-5p did not affect the cell apoptosis in MTEC1 cells.

MiR-181a-5p promotes TEC proliferation

In order to understand how MTEC1 cell viability was enhanced by miR-181a-5p, cell cycle analysis was performed by flow cytometry. As shown in **Fig. 3A,B**, the number of cells was significantly reduced in G1 phase and the number of cells was significantly increased in S phase in miR-181a-5p mimic group, when compared with the mimic-NC group. However, the percentage of cells in G1 phase was significantly increased in miR-181a-5p inhibitor group, as compared with the inhibitor-NC group (**Fig. 3A,C**). These data suggested that

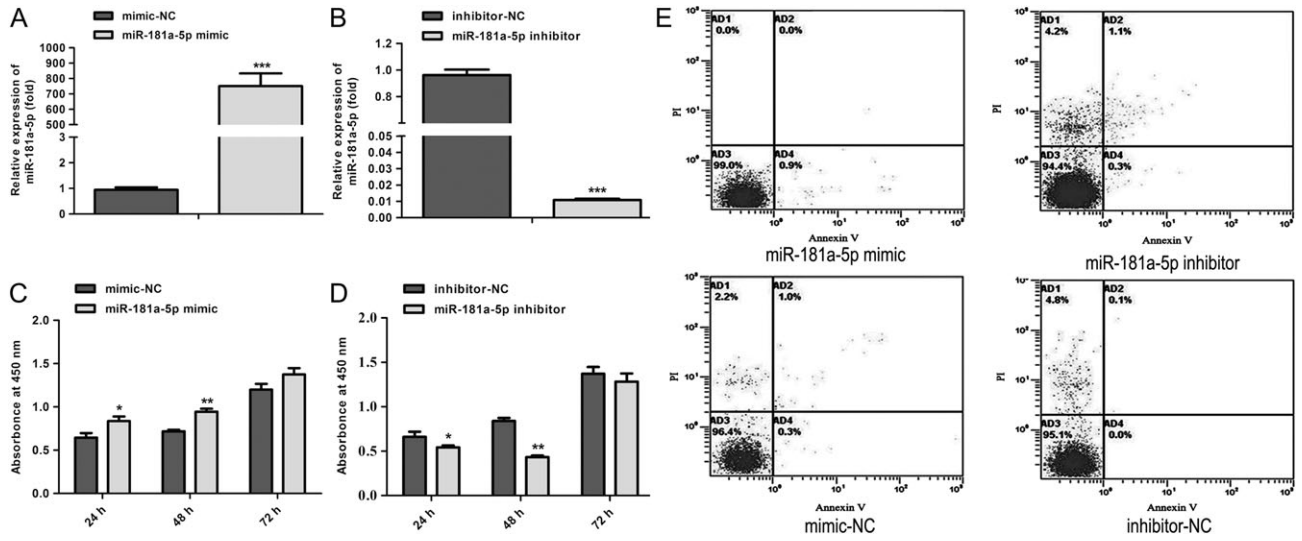

Figure 2. Effect of miR-181a-5p on MTEC1 cell viability and apoptosis (A,B) The transfection efficiency of miR-181a-5p mimic/inhibitor (50/100 nM) into MTEC1 cells was measured at 48 h after transfection. (C,D) Cell viability analysis was performed by CCK-8 at 24, 48, and 72 h after transfection. (E) At 48 h after transfection, cell apoptosis was analyzed by flow cytometry. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

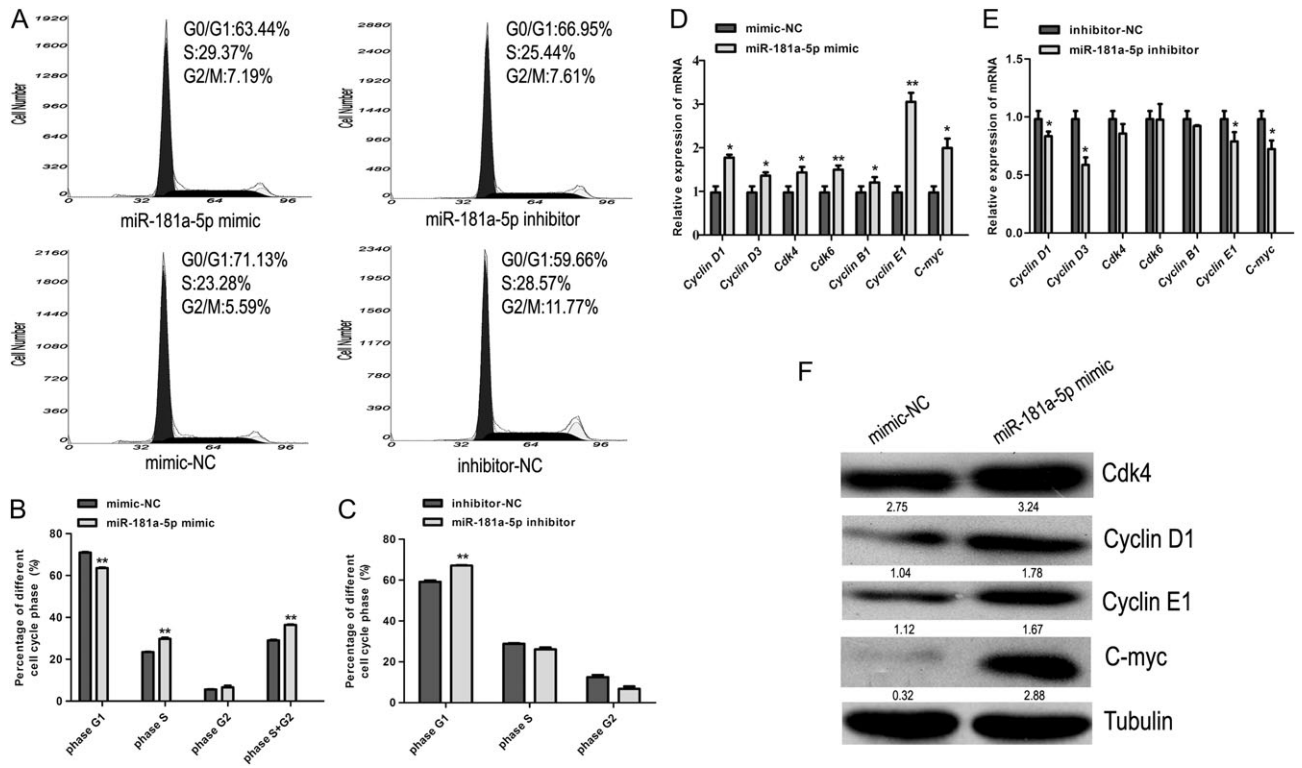

Figure 3. miR-181a-5p promotes MTEC1 cells proliferation (A–C) Cell cycle progression was analyzed by flow cytometry 48 h after transfection with miR-181a-5p mimic/mimic-NC (50 nM) or miR-181a-5p inhibitor/inhibitor-NC (100 nM). (D, E) mRNA expressions of Cyclin D1, Cyclin D3, Cdk4, Cdk6, Cyclin B1, Cyclin E1, and C-myc in miR-181a-5p mimic/mimic-NC (50 nM)- or miR-181a-5p inhibitor/inhibitor-NC (100 nM)-transfected cells. (F) Forty-eight hours after transfection, the protein levels of Cdk4, Cyclin D1, Cyclin E1, and C-myc were measured in miR-181a-5p mimic- and mimic-NC-transfected MTEC1 cells, respectively. Data were normalized to the level of *β-actin* for mRNA and to the level of tubulin for protein in each sample and the qPCR data were presented as $2^{-\Delta\Delta CT}$. $*P < 0.05$, $**P < 0.01$.

elevated expression of miR-181a-5p in MTEC1 could significantly promote cell cycle progression.

To confirm the above results, the cell cycle-related genes were further detected by qPCR and western blot analysis. Firstly, the expression of cell cycle-related genes at mRNA level was measured in miR-181a-5p mimic, miR-181a-5p inhibitor, and their control-NC groups, respectively. As shown in **Fig. 3D**, compared with that in the mimic-NC group, the mRNA levels of Cyclin D1, Cyclin D3,

Cyclin B1, Cyclin E1, Cdk4, Cdk6, and C-myc were significantly up-regulated in miR-181a-5p mimic group. Meanwhile, Cyclin D1, Cyclin D3, Cyclin E1, and C-myc were significantly down-regulated in miR-181a-5p inhibitor group (**Fig. 3E**). Furthermore, the protein expression of Cyclin D1, Cyclin E1, Cdk4, and C-myc were detected by western blot analysis. As shown in **Fig. 3F**, the protein levels of Cyclin D1, Cyclin E1, Cdk4, and C-myc were also up-regulated in miR-181a-5p mimic group, as compared with those in the mimic-NC group. All these data suggested that ectopic expression of miR-181a-5p in MTEC1 up-regulated the cell cycle-related genes, promoted cell cycle progression and increased the cell proliferation.

Putative target genes of miR-181a-5p

To further understand the mechanism that overexpression of miR-181a-5p promotes the proliferation of MTEC1. TargetScan (http://www.targetscan.org), miRNABase (http://www.miRNAbase.org/), and miRNA (http://www.microrna.org) were used to identify the potential target genes. The results showed that 81 genes were identified as the potential targets of miR-181a-5p (**Fig. 4A**). In order to further identify the real targets of miR-181a-5p among these predicated genes, GO term/KEGG pathway enrichment analysis was performed. It was found that the TGF-β signaling had a significantly statistical difference, which indicated that it may be involved in the MTEC1 proliferation (**Supplementary Tables S1 and S2**).

To further confirm the real targets of miR-181a-5p, the mRNA expression levels of Tgfbr1, Tgfbr2, Smad2, Smad7, Tgfbrap1, Tgfbi, and Tgif2 were measured in miR-181a-5p mimic-, mimic-NC-, miR-181a-5p inhibitor-, and inhibitor-NC-transfected cells. As shown in **Fig. 4B,C**, only the expression levels of *Tgfbr1* in MTEC1 were significantly down-regulated after transfection with miR-181a-5p mimic and significantly up-regulated after transfection with miR-181a-5p inhibitor, when compared with their control-NC groups (**Fig. 4B,C**). This result indicated that *Tgfbr1* may be a directly target of miR-181a-5p.

Tgfbr1 is a direct target of miR-181a-5p in MTEC1 cells

To further confirm the above results, the protein levels of Tgfbr1 were further determined by western blot analysis. The results showed that the protein expression of Tgfbr1 was significant down-regulated in miR-181a-5p mimic group, when compared with the mimic-NC group (**Fig. 5A,B**). As expected, the protein level of Tgfbr1 was significantly increased when the expression of miR-181a-5p was suppressed in MTEC1 (**Fig. 5A**). These results further suggested that Tgfbr1 may be a real target of miR-181a-5p.

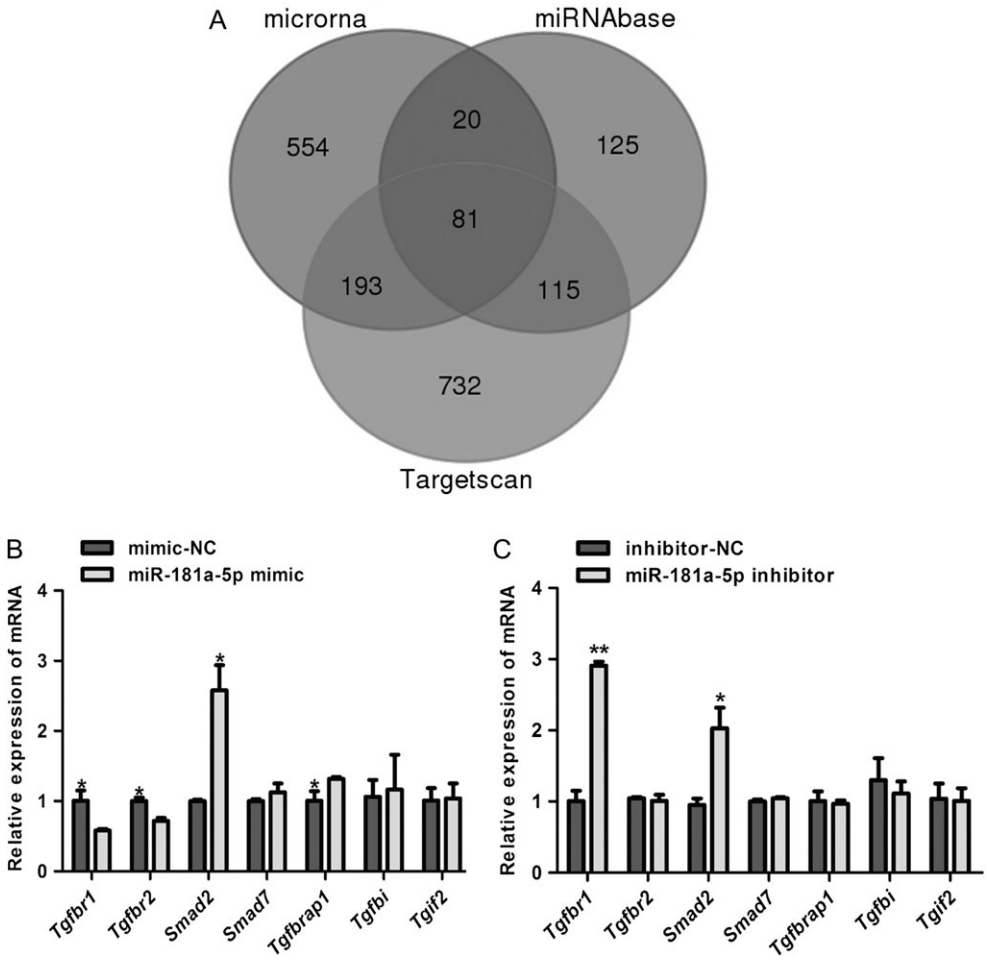

Figure 4. mRNA expression levels of the potential target genes of miR181a-5p (A) The potential targets of miR181a-5p were predicted by the online softwares (TargetScan, miRNABase, and miRNA). (B,C) The levels of *Tgfbr1, Tgfbr2, Smad2, Smad7, Tgfbrap1, Tgfbi*, and *Tgif2* in MTEC1 cells were measured by qPCR 48 h after transfection. Data were normalized to the level of *β-actin* in each sample and the data were presented as $2^{-\Delta\Delta CT}$. $^{*}P < 0.05$, $^{**}P < 0.01$.

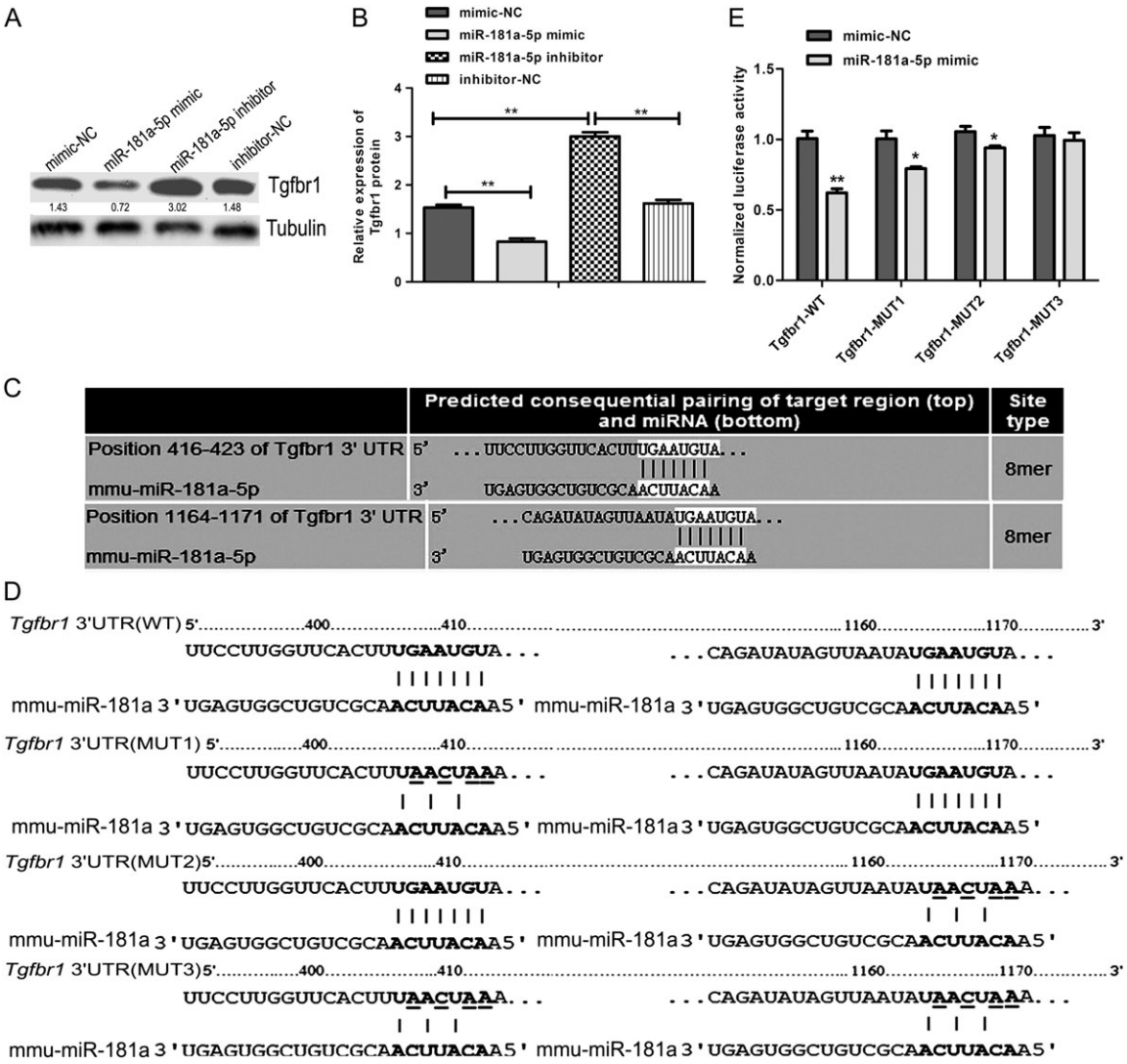

Figure 5. Tgfbr1 is a direct target gene of miR-181a-5p (A,B) The protein expression levels of Tgfbr1 in miR-181a-5p mimic (50 nM)-, mimic-NC (50 nM)-, miR-181a-5p inhibitor (100 nM)-, and inhibitor-NC (100 nM)-transfected MTEC1 cells. Tubulin was used as the loading control. (C) Two potential binding seed sequences in mouse *Tgfbr1* 3′UTR for miR-181a-5p were predicted by TargetScan. The first one is conserved among mammals, and the second one is non-conserved. (D) Four kinds of pmiRGLO vectors were constructed and the four underlined nucleotides in the seed region of *Tgfbr1* 3′UTR were mutated to abolish the interaction between miR-181a-5p and *Tgfbr1* 3′UTR. (E) Luciferase activity was measured in HEK-293T cells after co-transfected with miR-181a-5p mimic (50 nM) or mimic-NC (50 nM) and the pmiRGLO-Tgfbr1-MUT1/ MUT2/ MUT3 (400 ng) or pmiRGLO-Tgfbr1-WT (400 ng). $^{*}P < 0.05$, $^{**}P < 0.01$.

To validate the above results, the association between *Tgfbr1* and miR-181a-5p was analyzed using the miRNA target analysis tools. As shown in **Fig. 5**C, a conserved sequence and a non-conserved binding seed sequence were identified at the position of 416–423 bp and 1164–1171 bp in *Tgfbr1* 3′UTR, respectively. In order to confirm the target of miR-181a-5p, four different kinds of recombinant reporter plasmids were generated, including the wild-type (WT), MUT1 containing the mutations at the 416–423 bp sequence, MUT2 containing the mutations at the 1164–1171 bp sequence and MUT3 containing the mutations at the 416–423 bp sequence and the 1164–1171 bp sequence at the same time (**Fig. 5**D).

Subsequently, miR-181a-5p mimic or mimic-NC was co-transfected into HEK-293T cells with the reporter constructs. As shown in **Fig. 5**E, in the *Tgfbr1* 3′UTR-WT group, miR-181a-5p mimic induced a significant reduction of luciferase activity, as compared with mimic-NC group. While in the *Tgfbr1* 3′UTR MUT groups, a significant reduction of luciferase activity was also observed in both the MUT1 and MUT2 group, when compared with their corresponding controls. And no significant reduction of luciferase activity was observed in MUT3 group (**Fig. 5**E). These results indicated that miR-181a-5p directly targets *Tgfbr1* in MTEC1 cells.

miR-181a-5p regulates TGF-β signaling through *Tgfbr1* and *Smad3*

In TGF-β signaling, *Smad2* and *Smad3* are critical downstream mediators of the *Tgfbr1*, and play a central role in activating the TGF-β signaling [40]. Therefore, we detected the levels of p-Smad2 and p-Smad3 by western blot analysis. As shown in **Fig. 6A,B**, compared with the mimic-NC group, overexpression of miR-181a-5p

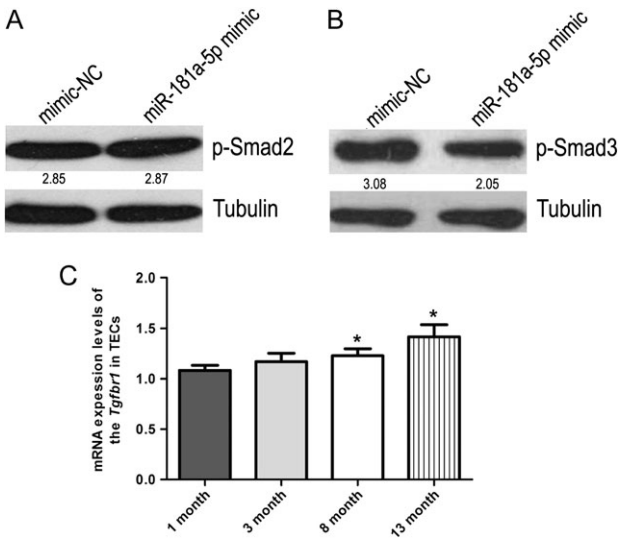

Figure 6. miR-181a-5p regulates the TGF-β signaling in MTEC1 cells (A,B) The protein levels of p-Smad2 and p-Smad3 were measured in the miR-181a-5p mimic- and mimic-NC-transfected MTEC1 cells. (C) The mRNA expression levels of *Tgfbr1* were measured by qPCR in isolated TECs derived from 1-, 3-, 8-, and 13-month-old mice. Data were normalized to the level of β-actin for mRNA and to the level of tubulin for protein in each sample and the qPCR data were presented as $2^{-\Delta\Delta CT}$. $^*P < 0.05$.

significantly down-regulated the protein level of p-Smad3 but not p-Smad2. These results also suggested that miR-181a-5p-regulated MTEC1 cells proliferation is through regulating the TGF-β signaling.

Tgfbr1 expression in TECs from mice of different age

In addition, the expressions of *Tgfbr1* were measured in TECs from 1-, 3-, 8-, and 13-month-old mice. As shown in **Fig. 6**C, when compared with the 1-month-old mice, the expression levels of *Tgfbr1* were gradually increased in TECs from 3-, 8-, and 13-month-old mice. Significant difference was found in both the 8-month-old mice and 13-month-old mice, but not in the 3-month-old mice. These results indicated that the expression levels of miR-181a-5p and *Tgfbr1* in TECs were related to the age-related thymus involution.

Discussion

The almost complete attrition of TECs is an intriguing feature during the process of thymus aging or involution. miRNAs are important modulators of gene expression, whose fundamental function is to fine-tune cellular phenotypes in responding to intrinsic signals or environmental stress [41]. Several studies have shown that the roles of miRNAs in thymus are indispensable via global interference with their biogenesis, particularly the expression of miRNAs in the subpopulations of TECs is essential for the maintenance of thymic microenvironment [2,42,16]. Specific miRNAs expression in TECs can also create a cell type-specific signature to reinforce cell fate specifications [42]. For example, a previous study has indicated that miR-29a cluster deficiency could trigger a rapid loss of thymic cellularity by increasing the amount of IFN-α receptor expressed in the thymic epithelium [2]. In a recent study, Rippo *et al.* [43] demonstrated that miR-181a was involved in cell aging and inflammaging, suggesting that miR-181a was related to important cell functions and age-related diseases. In the current study, we analyzed the expression of miR-181a-5p in TECs derived from 1-, 10-, and 19-month-old mice. The results showed that the expression of miR-181a-5p was dramatically decreased in TECs during the process of thymus aging, thus we speculate that the expression of miR-181a-5p in TECs may play a role in age-related thymus involution and atrophy.

To explore the role of miR-181a-5p in TECs, MTEC1 cells were used in our study. Overexpression of miR-181a-5p in MTEC1 cells promoted cell proliferation, and the effect was reversed when the expression of miR-181a-5p was suppressed. This may support our hypothesis that the expression of miR-181a-5p in TECs is related to the thymic involution, and also suggest that the expression of miR-181a-5p in TECs may directly or indirectly control some genes or signaling pathway related to the TECs proliferation, apoptosis, and differentiation. Previous studies have shown that several pathways appear to be affected in the miRNA-deficient TECs, such as the wingless, bone morphogenetic protein, and TGF-β, which are involved in cell pluripotency, differentiation, and survival [11,42,44,45]. In this study, target gene analysis and pathway enrichment analysis indicated that TGF-β signaling may be regulated by the expression of miR-181a-5p. Furthermore, *Tgfbr1* was confirmed as a direct target gene of miR-181a-5p by using luciferase assay. Our data suggest that the functions of miR-181a-5p in MTEC1 cells may be associated with the TGF-β signaling.

TGF-β signaling initiated in cells needs a cascade of protein–protein interactions. The first step is that a ligand of TGF-β superfamily binds to its receptor complex, then the Tgfbr2 phosphorylates and activates the Tgfbr1. The activated Tgfbr1 phosphorylates the receptor-specific Smads (R-Smads: Smad2 and Smad3) at two C-terminal Serine residues, which forms heteromeric complexes with Smad4 and translocates into the nucleus to trigger the cell signal transduction [46]. The reaction chains of TGF-β-Tgfbr2-Tgfbr1-Smad2/3 pathway play a crucial role in activating the TGF-β signaling. The inactivation of either one in the reaction chains will lead to impairment of the TGF-β-mediated cell growth and proliferation. This indicates that down-regulated *Tgfbr1* expression or *Smad2/3* phosphorylation will block the activation of latent TGF-β [47–49]. To further identify whether the TGF-β signaling is affected by the expression of miR-181a-5p in MTEC1 cells, the protein levels of p-Smad2 and p-Smad3 were measured in MTEC1 cells. Our results indicated that only the protein levels of p-Smad3 were significantly down-regulated in miR-181a-5p mimic group as compared with the mimic-NC group. This result indicated that the interaction between Tgfbr1 and Smad3 was impaired by overexpressing miR-181a-5p in MTEC1, which resulted in the loss of its ability in responding to TGF-β. In line with our findings, Alliston *et al.* [50] also demonstrated that TGF-β can inhibit the osteoblastic differentiation by suppressing the expression of Smad3. Additionally, Liu *et al.* [49] indicated that the levels of p-Smad2 were also down-regulated by overexpression of miR-181a in mesenchymal stem cells (MSCs). In our study, we also detected the protein levels of p-Smad2 in MTEC1 cells, but no change was observed between the mimic and mimic-NC groups. This may be explained by the limited roles of miRNAs in fine-tuning gene expression or the different expression of miR-181a-5p in different types of cells. All these data suggested that in MTEC1 the TGF-β signaling was regulated by miR-181a-5p which directly targeted *Tgfbr1*, and also indicated that miR-181a-5p affected the cell proliferation in MTEC1 via the activation of TGF-β signaling. Our results are consistent with that of the previous study which demonstrated that the cell proliferation

was reduced in both medullary and cortical TECs lines when exposed to TGF-β *in vitro* [51]. Similar to our findings, study by Liu *et al.* [49] and Raghu *et al.* [52] also demonstrated that miR-181a can inhibit TGF-β signaling by directly targeting *Tgfbr1* in C2C12 mesenchymal precursor cells, MC3T3 preosteoblasts and MSCs, further confirming that the regulatory role of miR-181a-5p in TECs proliferation is tightly linked to TGF-β.

The negative role in the formation of thymic microenvironment and the antiproliferative role in cells of epithelial organ have been well defined for TGF-β signaling [11,53]. A previous study has revealed that increased *Tgfbr3* expression in dicer-deficient TECs may promote thymic involution through enhancing stimulation of Tgfbr2 signaling [11]. It has been suggested that *Tgfbr2* plays an age-dependent negative role in controlling thymic weight and cellularity [54]. Moreover, it has also been demonstrated that the age-related thymus involution in aged mice is mitigated in *Tgfbr2*-deficient TECs [11]. Although the specific role of TGF-β in TECs function and thymus involution has been well described, it is still unknown whether the expression of miR-181a-5p and *Tgfbr1* in TECs affects the TECs-related thymus involution. In our study, we detected the mRNA expression levels of *Tgfbr1* in TECs from 1-, 3-, 8-, and 13-month-old mice, and the results indicated that the levels of Tgfbr1 was gradually up-regulated and inversely correlated with the expression of miR-181a-5p. This may further support our hypothesis that the expression of miR-181a-5p in TECs is associated with the regulation of thymus aging.

In conclusion, our study confirmed that the expression of miR-181a-5p in TECs was strikingly down-regulated during the thymus involution. Overexpression of miR-181a-5p in MTECl cells promoted the cell proliferation through attenuating the activation of TGF-β signaling. Additionally, the expression of miR-181a-5p was opposite to the expression of *Tgfbr1* in TECs from young, adult, and aged mice. Our data indicated that age-related thymus involution in mice may be associated with the dysregulation of miR-181a-5p in TECs. These findings will improve our understanding of the miRNA function in TECs and may provide potential novel therapies to thymus involution.

Supplementary Data

Supplementary data is available at *ABBS* online.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (Nos. 31272519 and 31572475).

References

1. Fu Y, Pau RD, Wang Y, Lopez DM. Thymic involution and thymocyte phenotypic alterations induced by murine mammary adenocarcinomas. *J Immunol* 1989, 143: 4300–4307.
2. Papadopoulou AS, Dooley J, Linterman MA, Pierson W, Ucar O, Kyewski B, Zuklys S, *et al.* The thymic epithelial microRNA network elevates the threshold for infection-associated thymic involution via miR-29a mediated suppression of the IFN-alpha receptor. *Nat Immunol* 2012, 13: 181–187.
3. Zhu X, Gui J, Dohkan J, Cheng L, Barnes PF, Su DM. Lymphohematopoietic progenitors do not have a synchronized defect with age-related thymic involution. *Aging Cell* 2007, 6: 663–672.
4. Aspinall R, Andrew D. Thymic atrophy in the mouse is a soluble problem of the thymic environment. *Vaccine* 2000, 18: 1629–1637.
5. Aspinall R, Andrew D. Age-associated thymic atrophy is not associated with a deficiency in the CD44(+)CD25(–)CD3(–)CD4(–)CD8(–) thymocyte population. *Cell Immunol* 2001, 212: 150–157.
6. Boyd RL, Tucek CL, Godfrey DI, Izon DJ, Wilson TJ, Davidson NJ, Bean AG, *et al.* The thymic microenvironment. *Immunol Today* 1993, 14: 445–459.
7. Ritter MA, Palmer DB. The human thymic microenvironment: new approaches to functional analysis. *Semin Immunol* 1999, 11: 13–21.
8. Palmer DB, Viney JL, Ritter MA, Hayday AC, Owen MJ. Expression of the alpha beta T-cell receptor is necessary for the generation of the thymic medulla. *Dev Immunol* 1993, 3: 175–179.
9. Anderson G, Jenkinson EJ. Lymphostromal interactions in thymic development and function. *Nat Rev Immunol* 2001, 1: 31–40.
10. Gui J, Mustachio LM, Su DM, Craig RW. Thymus size and age-related thymic involution: early programming, sexual dimorphism, progenitors and stroma. *Aging Dis* 2012, 3: 280–290.
11. Hauri-Hohl MM, Zuklys S, Keller MP, Jeker LT, Barthlott T, Moon AM, Roes J, *et al.* TGF-beta signaling in thymic epithelial cells regulates thymic involution and postirradiation reconstitution. *Blood* 2008, 112: 626–634.
12. Olsen NJ, Olson G, Viselli SM, Gu X, Kovacs WJ. Androgen receptors in thymic epithelium modulate thymus size and thymocyte development. *Endocrinology* 2001, 142: 1278–1283.
13. Sutherland JS, Goldberg GL, Hammett MV, Uldrich AP, Berzins SP, Heng TS, Blazar BR, *et al.* Activation of thymic regeneration in mouse and humans following androgen blockade. *J Immunol* 2005, 175: 2741–2753.
14. Dominguez-Gerpe L, Rey-Mendez M. Time-course of the murine lymphoid tissue involution during and following stressor exposure. *Life Sci* 1997, 66: 1019–1027.
15. Zuklys S, Mayer CE, Zhanybekova S, Stefanski HE, Nusspaumer G, Gill J, Barthlott T, *et al.* MicroRNAs control the maintenance of thymic epithelia and their competence for T lineage commitment and thymocyte selection. *J Immunol* 2012, 189: 3894–3904.
16. Khan IS, Taniguchi RT, Fasano KJ, Anderson MS, Jeker LT. Canonical microRNAs in thymic epithelial cells promote central tolerance. *Eur J Immunol* 2014, 44: 1313–1319.
17. Gao W, Yu Y, Cao H, Shen H, Li X, Pan S, Shu Y. Deregulated expression of miR-21, miR-143 and miR-181a in non small cell lung cancer is related to clinicopathologic characteristics or patient prognosis. *Biomed Pharmacother* 2010, 64: 399–408.
18. Taylor MA, Sossey-Alaoui K, Thompson CL, Danielpour D, Schiemann WP. TGF-β upregulates miR-181a expression to promote breast cancer metastasis. *J Clin Invest* 2013, 123: 150–163.
19. Meng F, Glaser SS, Francis H, DeMorrow S, Han Y, Passarini JD, Stokes A, *et al.* Functional analysis of microRNAs in human hepatocellular cancer stem cells. *J Cell Mol Med* 2012, 16: 160–173.
20. Zhang X, Nie Y, Du Y, Cao J, Shen B, Li Y. MicroRNA-181a promotes gastric cancer by negatively regulating tumor suppressor KLF6. *Tumor Biol* 2012, 33: 1589–1597.
21. Wei Z, Cui L, Mei Z, Liu M, Zhang D. miR-181a mediates metabolic shift in colon cancer cells via the PTEN/AKT pathway. *FEBS Lett* 2014, 588: 1773–1779.
22. Shi L, Cheng Z, Zhang J, Li R, Zhao P, Fu Z, You Y. hsa-mir-181a and hsa-mir-181b function as tumor suppressors in human glioma cells. *Brain Res* 2008, 236: 185–193.
23. Shin KH, Bae SD, Hong HS, Kim RH, Kang MK, Park NH. miR-181a shows tumor suppressive effect against oral squamous cell carcinoma cells by downregulating Kras. *Biochem Biophys Res Commun* 2011, 404: 896–902.
24. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. *Science* 2004, 303: 83–86.
25. Neilson JR, Zheng GX, Burge CB, Sharp PA. Dynamic regulation of miRNA expression in ordered stages of cellular development. *Genes Dev* 2007, 21: 578–589.
26. Li QJ, Chau J, Ebert PJ, Sylvester G, Min H, Liu G, Braich R, *et al.* MiR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell* 2007, 129: 147–161.

27. Liu G, Min H, Yue S, Chen CZ. Pre-miRNA loop nucleotides control the distinct activities of mir-181a-1 and mir-181c in early T cell development. *PLoS One* 2008, 3: e3592.
28. Ebert PJ, Jiang S, Xie J, Li QJ, Davis MM. An endogenous positively selecting peptide enhances mature T cell responses and becomes an autoantigen in the absence of microRNA miR-181a. *Nat Immunol* 2009, 10: 1162–1169.
29. Debernardi S, Skoulakis S, Molloy G, Chaplin T, Dixon-McIver A, Young BD. MicroRNA miR-181a correlates with morphological subclass of acute myeloid leukaemia and the expression of its target genes in global genome-wide analysis. *Leukemia* 2007, 21: 912–916.
30. Zhu DX, Miao KR, Fang C, Fan L, Zhu W, Zhu HY, Zhuang Y, *et al.* Aberrant microRNA expression in Chinese patients with chronic lymphocytic leukemia. *Leuk Res* 2011, 35: 730–734.
31. Bellon M, Lepelletier Y, Hermine O, Nicot C. Deregulation of microRNA involved in hematopoiesis and the immune response in HTLV-I adult T-cell leukemia. *Blood* 2009, 113: 4914–4917.
32. Govinden R, Bhoola KD. Genealogy, expression, and cellular function of transforming growth factor-beta. *Pharmacol Ther* 2003, 98: 257–265.
33. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, *et al.* Targeted disruption of the mouse transforming growth factorbeta 1 gene results in multifocal inflammatory disease. *Nature* 1992, 359: 693–699.
34. Chen W, Jin W, Tian H, Sicurello P, Frank M, Orenstein JM, Wahl SM. Requirement for transforming growth factor beta1 in controlling T cell apoptosis. *J Exp Med* 2001, 194: 439–453.
35. Ehrhart EJ, Segarini P, Tsang ML, Carroll AG, Barcellos-Hoff MH. Latent transforming growth factor beta1 activation in situ: quantitative and functional evidence after low-dose gamma irradiation. *FASEB J* 1997, 11: 991–1002.
36. Chen W, Frank ME, Jin W, Wahl SM. TGF-beta released by apoptotic T cells contributes to an immunosuppressive milieu. *Immunity* 2001, 14: 715–725.
37. Ewan KB, Henshall-Powell RL, Ravani SA, Pajares MJ, Arteaga C, Warters R, Akhurst RJ, *et al.* Transforming growth factor-beta1 mediates cellular response to DNA damage in situ. *Cancer Res* 2002, 62: 5627–5631.
38. Guo ZB, Chi F, Song Y, Wang CS, Yu RX, Wei TL, Gui JG, *et al.* Transcriptome analysis of murine thymic epithelial cells reveals age-associated changes in microRNA expression. *Int J Mol Med* 2013, 32: 835–842.
39. Ye YQ, Li DT, Ouyang D, Deng L, Zhang Y, Ma YJ, Li YG. MicroRNA expression in the aging mouse thymus. *Gene* 2014, 547: 218–225.
40. Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, *et al.* The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. *Cell* 1997, 89: 1165–1173.
41. Sevignani C, Calin GA, Siracusa LD, Croce CM. Mammalian microRNAs: a small world for fine-tuning gene expression. *Mamm Genome* 2006, 7: 189–202.
42. Zuklys S, Mayer CE, Zhanybekova S, Stefanski HE, Nusspaumer G, Gill J, Barthlott T, *et al.* MicroRNAs control the maintenance of thymic epithelia and their competence for T lineage commitment and thymocyte selection. *J Immunol* 2012, 189: 3894–3904.
43. Rippo MR, Olivieri F, Monsurrò V, Prattichizzo F, Albertini MC, Procopio AD. MitomiRs in human inflamm-aging: a hypothesis involving miR-181a, miR-34a and miR-146a. *Exp Gerontol* 2014, 56: 154–163.
44. Zuklys S, Gill J, Keller MP, Hauri-Hohl M, Zhanybekova S, Balciunaite G, Na KJ, *et al.* Stabilized beta-catenin in thymic epithelial cells blocks thymus development and function. *J Immunol* 2009, 182: 2997–3007.
45. Bleul CC, Boehm T. BMP signaling is required for normal thymus development. *J Immunol* 2005, 175: 5213–5221.
46. Massague´ J. How cells read TGF-beta signals. *Nat Rev Mol Cell Biol* 2000, 1: 169–178.
47. Thompson K, Murphy-Marshman H, Leask A. ALK5 inhibition blocks TGFβ-induced CCN1 expression in human foreskin fibroblasts. *J Cell Commun Signal* 2014, 8: 59–63.
48. Bandyopadhyay B, Han A, Dai J, Fan J, Li Y, Chen M, Woodley DT, *et al.* TbetaRI/Alk5-independent TbetaRII signaling to ERK1/2 in human skin cells according to distinct levels of TbetaRII expression. *J Cell Sci* 2011, 124: 19–24.
49. Liu L, Wang Y, Fan H, Zhao X, Liu D, Hu Y, Kidd AR 3rd, *et al.* MicroRNA-181a regulates local immune balance by inhibiting proliferation and immunosuppressive properties of mesenchymal stem cells. *Stem Cells* 2012, 30: 1756–1770.
50. Alliston T, Choy L, Ducy P, Karsenty G, Derynck R. TGF-beta-induced repression of CBFA1 by Smad3 decreases cbfa1 and osteocalcin expression and inhibits osteoblast differentiation. *EMBO J* 2001, 20: 2254–2272.
51. Kasai M, Hirokawa K, Kajino K, Ogasawara K, Tatsumi M, Hermel E, Monaco JJ, *et al.* Difference in antigen presentation pathways between cortical and medullary thymic epithelial cells. *Eur J Immunol* 1996, 26: 2101–2107.
52. Bhushan R, Grünhagen J, Becker J, Robinson PN, Ott CE, Knaus P. miR-181a promotes osteoblastic differentiation through repression of TGF-β signaling molecules. *Int J Biochem Cell Biol* 2013, 45: 696–705.
53. Miettinen PJ, Ebner R, Lopez AR, Derynck R. TGF-beta induced transdifferentiation of mammary epithelial cells to mesenchymal cells involvement of type I receptors. *J Cell Biol* 1994, 127: 2021–2036.
54. Bravo-Nuevo A, O'Donnell R, Rosendahl A, Chung JH, Benjamin LE, Odaka C. RhoB deficiency in thymic medullary epithelium leads to early thymic atrophy. *Int Immunol* 2011, 23: 593–600.