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Original Article

Long noncoding RNA TUG1 promotes airway smooth muscle cells proliferation and migration via sponging miR-590-5p/FGF1 in asthma

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Abstract: The proliferation and migration of airway smooth muscle cells (ASMCs) plays an important role in asthma. Recently, the function of long noncoding RNA (lncRNA) in the ASMCs has been realized. This study tries to investigate the role of lncRNA TUG1 for the ASMCs and focus on the deepgoing mechanism in the proliferation and migration. In the asthma rat model, TUG1 expression level was increased comparing with control. In the cellular assay with gain and loss of functions, lncRNA TUG1 promoted the ASMCs proliferation and migration, and reduces apoptosis. In the mechanical investigation, results unveiled that miR-590-5p acted as the target of TUG1, while FGF1 was targeted by miR-590-5p. Overall, this study reveals the vital regulation of TUG1/miR-590-5p/FGF1 axis for the proliferation and migration of ASMCs.

Keywords: Asthma, airway smooth muscle cells, TUG1, miR-590-5p, FGF1

Introduction

Asthma is a chronic inflammatory pulmonary disease of the bronchial airway and has high incidence in pediatric population, progressively causing increasing morbidity and becoming a critical public health concern [1, 2]. Asthma is characterized by chronic airway hyper-responsiveness, airway inflammation and airway remodeling [3]. The proliferation of airway smooth muscle cell (ASMC) plays an important role in airway remodeling [4]. Besides, various of inflammatory cytokines are highly upregulated in ASMCs of asthma with inflammation, including platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and connective tissue growth factor (CTGF) [5, 6].

The increasing evidence supports the new point of view that noncoding RNA (ncRNAs), a subgroup of epigenetic manners, play vital role in the ASMCs. For example, miR-216a level is considerably lower in the ASMC cells and the overexpression of miR-216a markedly suppressed cell proliferation and promoted cell apoptosis [7]. LncRNA GAS5 is elevated in the ASMCs stimulated by platelet-derived growth factor-BB (PDGF-BB), which promotes the cell proliferation of ASMCs through miR-10a/BDNF [8].

In this study, we demonstrated the regulatory axis of TUG1, miR-590-5p and FGF1 for the ASMCs in the asthma, playing an important role in promoting ASMCs proliferation and migration, thus contributing to asthma. This finding describes a novel TUG1/miR-590-5p/FGF1 pathway in the pathogenesis of asthma.

Materials and methods

Chronic asthmatic rat model establishment

Sprague Dawley rats (SD, female, 60-80 g) were obtained from the Experimental Animal Center of Beijing Chaoyang Hospital affiliated to Capital Medical University. And this assay was approved by the Institutional Animal Care and Use Committee and followed the NIH Guidelines for the Care and Use of Laboratory Animals. At 1, 7 and 15 day, rats were respectively and intraperitoneally injected with ovalbumin antigenic (OVA, 1 ml, 10%) suspension (100 mg ovalbu-

min, 100 mg aluminum hydroxide) for the sensitization as previously described [9].

Isolation of ASMCs

The rat ASMCs in the asthma rats models were isolated and purified as previously described [10]. The tracheas were Tracheas after the rats were anesthetized and sacrificed, and then epithelium fibrous tissue was wiped out. The remaining tissue was stored in the PBS and digested with collagenase I and elastase IV at 37°C. After digestion, the free ASMCs cells were centrifuged to segregation, and cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS).

Oligonucleotides transfection

To silence the lncRNA expression, the specific siRNA targeting TUG1 and negative control were synthesized by RiboBio (Guangzhou, China). Besides, the plasmid for TUG1 was also synthesized by RiboBio to enforce TUG1 expression. ASMCs were transiently transfected with si-TUG1, si-NC with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols.

RNA extraction and real-time PCR

Total RNA was extracted from ASMCs cells through TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. PrimeScript RT Reagent Kit was performed for the cDNA synthesis (Takara, Dalian, China). Real-time PCR analyses were conducted with SYBR Green Premix Ex Taq (Takara, China) and miRcute miRNA qPCR detection kit (Tiangen) at an ABI 7500 system. GAPDH and U6 acted as endogenous controls. Primers sequences were listed in Table S1.

Western blot analysis

Cultured ASMCs were lysed using RIPA buffer (Thermo Scientific, Waltham, MA, USA) with protease inhibitor cocktail. Protein concentration was tested by by BCA protein assay kit (Beyotime, Shanghai, China). The lysed protein samples were moved by SDS-PAGE and transferred polyvinylidene difluoride membrane. The membrane was incubated with primary anti-FGF1 antibody (Abcam, Hercules, CA, USA) and secondary antibody. Finally, the blots of protein were measured using ECL kit (Pierce, San Diego, CA, USA) and Chemi Doc XRS+ Imager (Bio-Rad).

CCK-8 proliferation

Transfected cells were administrated with Cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan). Cells (2×10^3 cells per well) were seeded into 96-well plates and disposed with f CCK-8 reagent (10 ul) and cultured at 37°C. At different time, the absorbance was detected at 450 nm by spectrophotometric plate reader (Bio-Rad).

Transwell migration assay

The ASMCs cells' migration ability was tested by the 24-well Transwell assay using pre-coated with Matrigel chamber (8 μm pore size, Corning, NY, USA). After transfection, 3×10^4 ASMCs cells were suspended upper floor of the chamber with 200 μl of FBS free DMEM medium. 600 μl DMEM medium with FBS was added to the lower floor of the chamber. The cells were incubated at 37°C and 5% CO_2.

Flow cytometry

ASMCs' apoptosis was measured using the flow cytometry according to the manufacturer's protocols. After being transfected with siRNAs or plasmids, cells were digested and resuspended in binding buffer, and stained with Annexin V (5 μl) and propidium iodide (10 μl, PI, BD Pharmingen, San Diego, CA, USA). All procedures were performed according to the manufacturer's instructions.

Cell cytoplasm/nucleus fraction isolation

The subcellular location of TUG1 in the ASMCs was detected using the Cytoplasmic and Nuclear RNA Purification Kit (Thermo, Scientific, Rockford, IL, USA). The expression levels of GAPDH, U1 acted as the controls. The fractions were detected using qRT-PCR assays.

Dual luciferase reporter assay

Potential binding sites within miR-509-5p and the 3'-UTR of TUG1 and FGF1 were inserted into pGL3-basic vector. pGL3 with pRL-TK Vector (Promoga) were respectively transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen). 48 h after transfection, the luciferase reporter assays were performed using the Dual Luciferase Reporter Assay System.

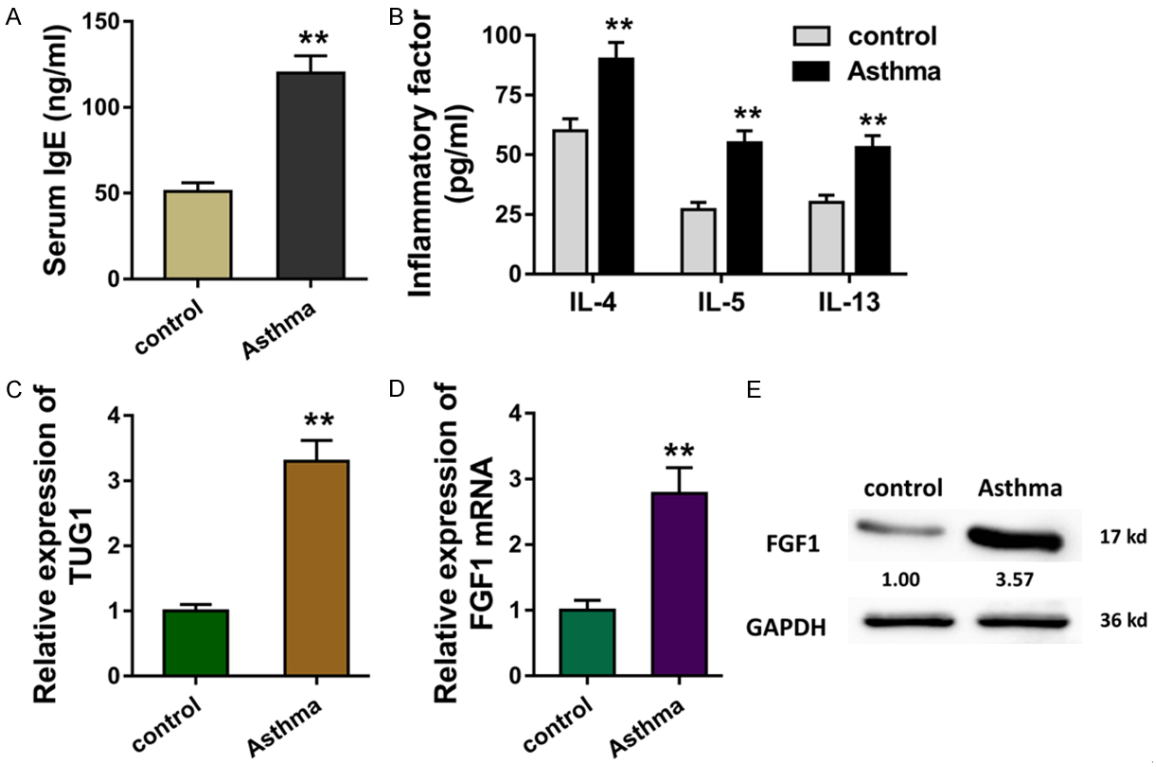


Figure 1. LncRNA TUG1 is up-regulated in the ASMCs asthma cells. A. ELISA showed the level of IgE in the ASMCs isolated from asthma rat models and control models respectively. B. The expression of inflammatory factors in the asthma ASMCs cells, including IL-4, IL-5, IL-13. C. RT-PCR showed the lncRNA TUG1 in the asthma ASMCs and controls. D. FGF1 mRNA in the ASMCs isolated from asthma groups. E. Western blot showed the FGF1 protein in the ASMCs compared to the controls cells. **P less than 0.01.

Statistics analysis

All values and data are calculated and reported as the mean ± SD manner. Independent sample t-test was performed for the two-group comparison. One-way ANOVA analysis was performed for multiple-group comparison. $P<0.05$ was considered to be statistical significance.

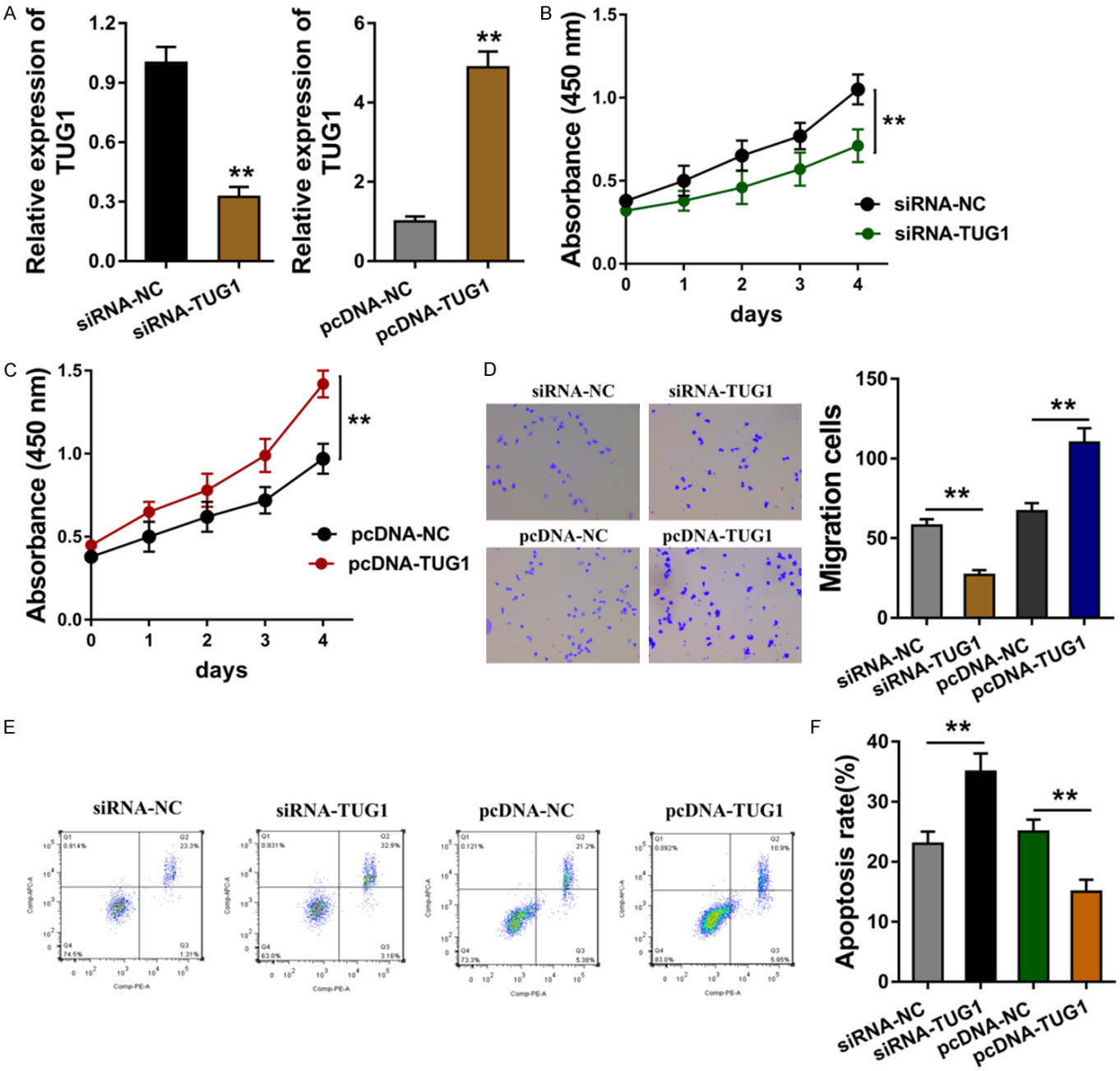
Results

LncRNA TUG1 is up-regulated in the ASMCs of rat asthma models

Firstly, we constructed the rat asthma and the ASMCs were extracted from the rats. ELISA showed that the level of IgE was significantly up-regulated in the ASMCs cells isolated from asthma groups (**Figure 1A**). Besides, the representative inflammatory factors for the asthma were measured, presenting the over-expression of IL-4, IL-5, IL-13 in the ASMCs cells isolated from asthma groups (**Figure 1B**). RT-PCR showed that lncRNA TUG1 was significantly up-regulated in the asthma ASMCs (**Figure 1C**). Moreover, the FGF1 mRNA was found to be up-regulated in the ASMCs isolated from asthma groups (**Figure 1D**). Western blot showed that FGF1 protein was up-regulated in the ASMCs compared to the controls cells (**Figure 1E**). Therefore, our research finds that lncRNA TUG1 is up-regulated in the ASMCs asthma cells.

LncRNA TUG1 promotes the ASMCs proliferation and migration, and reduces apoptosis

In the ASMCs cells isolated from the asthma rats, we had found that TUG1 was increased. Thus, in order to discover the roles of TUG1 for the ASMCs, silencing and overexpression of TUG1 was constructed using the siRNA and plasmid transfection (**Figure 2A**). CCK-8 assay indicated that silencing of TUG1 inhibited the proliferation ability, and the overexpression of TUG1 promoted the proliferation ability (**Figure 2B**, **2C**). Invasive ability of ASMCs by Transwell showed that silencing of TUG1 inhibited the invasion, and the overexpression of TUG1 pro-


A
Relative expression of TUG1
1.2
0.9
0.6
0.3
0.0
**
siRNA-NC
siRNA-TUG1
Relative expression of TUG1
6
4
2
0
**
pcDNA-NC
pcDNA-TUG1
B
Absorbance (450 nm)
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siRNA-NC
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pcDNA-NC
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pcDNA-TUG1
Migration cells
150
100
50
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**
**
siRNA-NC
siRNA-TUG1
pcDNA-NC
pcDNA-TUG1
E
siRNA-NC
siRNA-TUG1
pcDNA-NC
pcDNA-TUG1
F
Apoptosis rate(%)
40
30
20
10
0
**
**
siRNA-NC
siRNA-TUG1
pcDNA-NC
pcDNA-TUG1

Figure 2. LncRNA TUG1 promotes the ASMCs proliferation and migration, and reduces apoptosis. A. Silencing and overexpression of TUG1 was constructed using the siRNA and plasmid transfection. B, C. CCK-8 assay indicated the proliferation ability of ASMCs after transfection. D. Invasive ability of ASMCs by Transwell showed the invasion of ASMCs after transfection. E, F. Apoptotic rate of ASMCs by flow cytometry showed the apoptosis of ASMCs after transfection. **P less than 0.01.

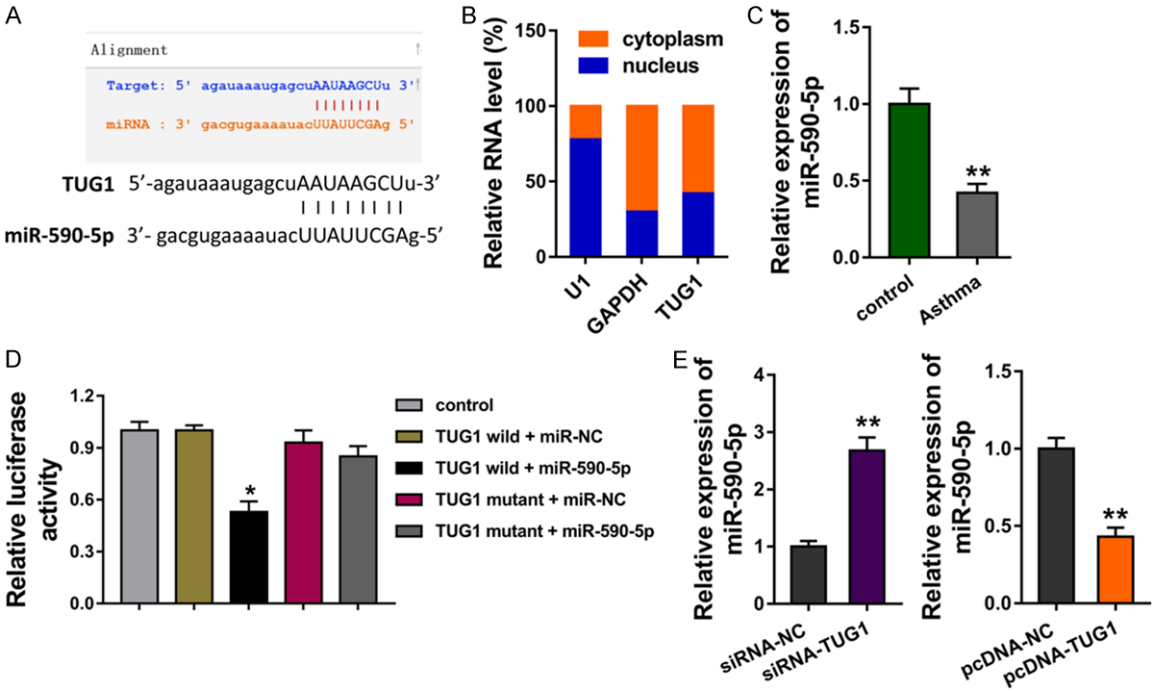

Figure 3. miR-590-5p acts as the target of TUG1 in the ASMCs. A. Bioinformatics analysis for the possible binding sites within miR-590-5p and TUG1. B. The nuclear or cytoplasmic location of TUG1 was tested using the subcellular fraction analysis by RT-PCR. C. RT-PCR showed the -expression of miR-590-5p in the asthma ASMCs and controls. D. Luciferase gene reporter assay showed the co-transfection of miR-590-5p and TUG1. E. The miR-590-5p expression in the ASMCs with transfection of TUG1 siRNA and TUG1 overexpression plasmid. **P less than 0.01. *P less than 0.05.

moted the invasion (**Figure 2D**). Apoptotic rate of ASMCs by flow cytometry showed that silencing of TUG1 increased the apoptosis, and the overexpression of TUG1 inhibited the apoptosis (**Figure 2E**, **2F**). Therefore, the above results indicated that lncRNA TUG1 promotes the ASMCs proliferation and migration, and reduces apoptosis.

miR-590-5p acts as the target of TUG1 in the ASMCs

To identify the deepgoing mechanism by which TUG1 regulated the ASMCs' proliferation and migration, we performed the bioinformatics analysis for the possible miR-590-5p for TUG1 (**Figure 3A**). The location of TUG1 was tested using the subcellular fraction analysis by RT-PCR, unveiling the cytoplasmic distribution in the ASMCs (**Figure 3B**). RT-PCR showed the under-expression of miR-590-5p in the asthma ASMCs comparing controls (**Figure 3C**). Luciferase gene reporter assay showed that the co-transfection of miR-590-5p and TUG1 decreased the activity of luciferase (**Figure 3D**). In ASMCs, the transfection of TUG1 siRNA could enhance the miR-590-5p expression, while the transfection of TUG1 overexpression plasmid could decrease the miR-590-5p expression (**Figure 3E**). Thus, these data suggests that miR-590-5p acts as the target of TUG1 in the ASMCs.

FGF1 acts as the target of miR-590-5p in the ASMCs

Subsequent investigation for the mechanism showed that fibroblast growth factor 1 (FGF1)

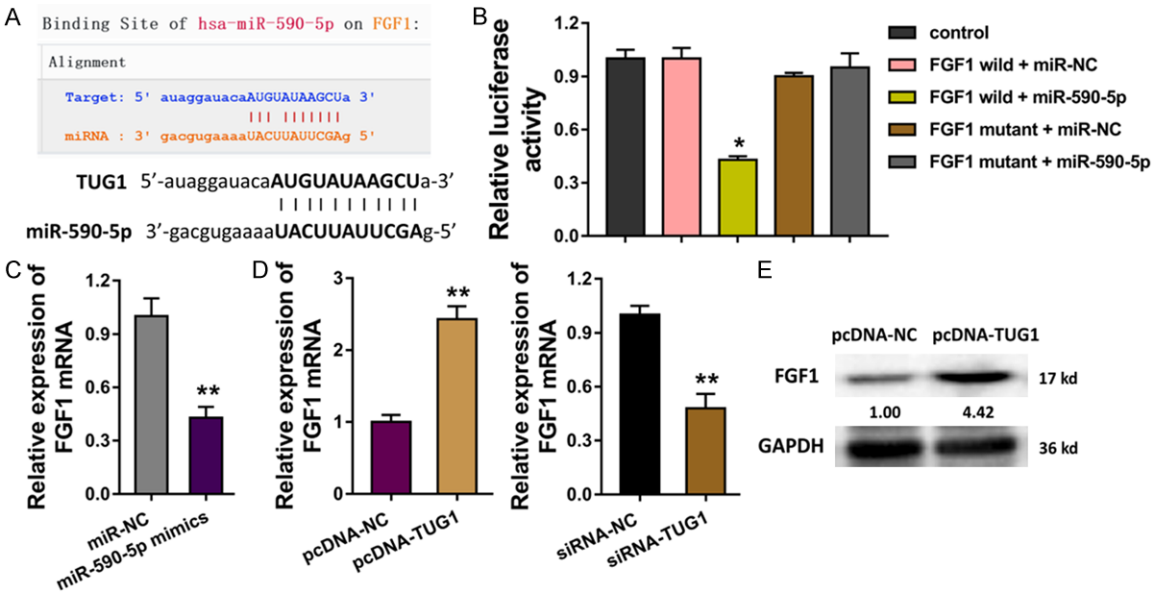

Figure 4. FGF1 acts as the target of miR-590-5p in the ASMCs. A. Bioinformatics tools showed the target of miR-590-5p with fibroblast growth factor 1 (FGF1). B. Luciferase gene reporter assay showed the activity of luciferase after the co-transfection of miR-590-5p and FGF1 wild type. C. FGF1 mRNA in the ASMCs transfected with miR-590-5p mimics. D. The TUG1 overexpression plasmid and TUG1 siRNA were transfected into the ASMCs. E. Western blot showed the FGF1 protein expression with TUG1 overexpression plasmid. **P less than 0.01. *P less than 0.05.

could act as the target of miR-590-5p (**Figure 4A**). Luciferase gene reporter assay showed that the co-transfection of miR-590-5p and FGF1 wild type decreased the activity of luciferase (**Figure 4B**). In the ASMCs transfected with miR-590-5p mimics, FGF1 mRNA was decreased (**Figure 4C**). Moreover, the TUG1 overexpression plasmid increased the FGF1 mRNA expression, while TUG1 siRNA decreased the FGF1 mRNA expression (**Figure 4D**). Western blot showed that TUG1 overexpression plasmid increased the FGF1 protein expression (**Figure 4E**). Based on the results, we find that FGF1 acts as the target of miR-590-5p in the ASMCs.

Discussion

Asthma is a chronic inflammatory pulmonary disease of the bronchial airway and has high incidence in pediatric population [11]. The proliferation and migration of airway smooth muscle cells (ASMCs) plays an important role in asthma [12]. Recently, the function of long noncoding RNA (lncRNA) in asthma has been re-recognized.

LncRNA TUG1 has been identified to be oncogenic factor in the human diseases and cancers [13-15]. For example, TUG1 expression and YAP expression are both up-regulated in renal cell carcinoma, and TUG1 promotes cell proliferation and migration via YAP by targeting miR-9 [16]. In osteosarcoma cells, TUG1 is overexpressed in osteosarcoma cells and TUG1 knockdown inhibited glucose consumption, lactate production and cell viability via markedly affecting the expression of hexokinase-2 (HK2) [17]. In prostate cancer, TUG1 promotes tumor cell migration, invasion, and proliferation by negatively regulating the miR-26a [18].

This study tries to investigate the role of lncRNA TUG1 for the ASMCs and focus on the deepgoing mechanism in the proliferation and migration. In the asthma rat model, expression level of TUG1 was increased comparing with control. Moreover, the gain and loss of functions of cellular assay showed that lncRNA TUG1 promoted the ASMCs proliferation and migration, and reduces apoptosis. Therefore, the cellular phenotype of ASMCs could be regulated by the TUG1 in the asthma models' cells. This finding supports the vital role of TUG1 on the asthma physiopathologic mechanism. In vascular smooth muscle cell, TUG1 promotes the proliferation and migration in hypertensive via miR-145-5p/FGF10 axis and Wnt/β-catenin pathway [19].

The bioinformatics prediction revealed putative miR-590-5p-binding sites within TUG1 transcripts 3'-UTR. In the mechanical investigation, results unveiled that miR-590-5p acted as the target of TUG1, while FGF1 was targeted by miR-590-5p. Fibroblast growth factor 1 (FGF1) acts as the vital member for the pro-angiogenic growth factors, as well as vascular endothelial growth factor (VEGF), participating the VEGF-mediated vascular remodeling [20, 21]. In the vascular smooth muscle cells, TUG1 expression is up-regulated induced by ox-LDL, and FGF1 was identified as a direct target of miR-133a [22]. In airway smooth muscle, miR-590-5p expression was significantly down-regulated and overexpression of miR-590-5p inhibited cell proliferation with PDGF combination [23].

This research investigates and identifies the epigenetics regulation of TUG1 on the ASMCs via the competing endogenous RNA (ceRNA) mechanism. This pathway of ceRNA provides an excellent regulating manner for the lncRNA. Via this pathway, TUG1 could promote the proliferation and migration of ASMCs in the asthma. Such as lncRNA-PVT1, which promotes the expression of E2F3 by acting as a ceRNA by competitively sponging miR-203a [24].

Overall, this study reveals the vital regulation of TUG1/miR-590-5p/FGF1 axis for the proliferation and migration of ASMCs. This finding cold unveil the mechanism by which TUG1 modulates the asthma progression.

Disclosure of conflict of interest

None.

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Table S1. Primers sequences for qRT-PCR and sequences of siRNA

	Sequences
TUG1	Forward, 5'-GGACACAATTCGCCACGACTT-3'
	Reverse, 5'-GCGCAGTCCCAGATTCCA-3'
miR-590-5p	Forward, 5'-TGAAAGACGTGATGGCACAC-3'
	Reverse, 5'-CTTCCATTTTGGGGTTTTTGG-3'
FGF1	Forward, 5'-CCCCGTCAGATAATCTGTG-3'
	Reverse, 5'-CTTGTCAGATACGGGAGG-3'
TUG1 siRNA	5'-GTTCATAAAGCACTAAAGTTT-3'
GAPDH	Forward, 5'-AGAAGGCTGGGGCTCATTTG-3'
	Reverse, 5'-AGGGGCCATCCACAGTCTTC-3'