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MicroRNA-133a alleviates airway remodeling in asthma through PI3K/AKT/mTOR signaling pathway by targeting IGF1R

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Abstract

Asthma is characterized by chronic inflammation, and long-term chronic inflammation leads to airway remodeling. But the potential regulatory mechanism of airway remodeling is not clearly understood, and there is still no effective way to prevent airway remodeling. Present studies have confirmed the role of microRNAs (miRNAs) in the development of disease, which is known as suppressing translation or degradation of messenger RNA (mRNA) at the posttranscriptional stage. In this study, we described the role of miRNA-133a in asthma and demonstrated it in regulating airway remodeling of asthma through the phosphoinositide 3 kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway by targeting IGF-1 receptor (IGF1R). IGF1R helps in mediating the intracellular signaling cascades. Asthmatic mice models were established by sensitization and Ovalbumin challenge. Adenovirus transfer vector carrying miR-133a or miR-133a sponge sequence was used to build the overexpression or downexpression of miR-133a modeling. Real-time polymerase chain reaction and Western blot were used to determine the alterations in the expression of miR-133a and mRNAs and their corresponding proteins. Results showed that miR-133a was downregulated in asthma. Upregulation of miR-133a expression in airway smooth muscle cells in vivo and in vitro could inhibit the activation of PI3K/AKT/mTOR pathway, and reduce the expression of α -smooth muscle actin (α -SMA), indicating that airway remodeling was inhibited. Functional studies based on luciferase reporter revealed miR-133a as a direct target of IGF1R mRNA. In conclusion, these data suggested that miR-133a regulated the expression of α -SMA through PI3K/AKT/mTOR signaling by targeting IGF1R. miR-133a plays a key role in airway remodeling of asthma and may serve as a potential therapeutic target for managing asthmatic airway remodeling.

KEYWORDS

airway remodeling, asthma, microRNA (miRNA), phosphoinositide 3 kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR)

1 | INTRODUCTION

Asthma is a common chronic respiratory disease, causing serious harm to human health. According to 2017 statistics, there are currently about 300 million asthmatic patients around the world, and the prevalence is still increasing year by year (Becker & Abrams, 2017). Asthma is characterized by airway inflammation, reversible airflow limitation, airway hyperresponsiveness, and airway remodeling (Aubier et al., 2016; Russell & Brightling, 2017; Saglani & Lloyd, 2015). The pathologic changes of airway remodeling include mucus hypersecretion, subepithelial fibrosis, airway smooth muscle (ASM) hypertrophy or hyperplasia, subepithelial basement membrane thickness, angiogenesis, and goblet cell metaplasia (Chauhan, Dash, & Singh, 2017; Fehrenbach, Wagner, & Wegmann, 2017; Oliver & Black, 2016). Airway smooth muscle cells (ASMCs) play a major pathological role in the development of airway remodeling. ASMCs play contractile and immunomodulatory roles in the lungs, and they have been more secretory, synthetic, proliferative, and migratory in chronic asthmatic conditions (Jones, Noble, Elliot, & James, 2016; Samitas, Delimpoura, Zervas, & Gaga, 2015; Zissler, Esser-von Bieren, Jakwerth, Chaker, & Schmidt-Weber, 2016). A better understanding of the cellular and molecular mechanisms of ASMCs in asthma helps us to design new strategies to further prevent and alleviate asthma.

Rapamycin target protein (mammalian target of rapamycin [mTOR]) plays an important biological role in regulating cell cycle, protein synthesis, cell energy metabolism, and other pathways (Wang & Zhang, 2017). Till date, very few reports of whether mTOR participates in asthmatic ASMC growth, differentiation, and proliferation were presented. The activation of mTOR mainly depends on phosphoinositide 3 kinase (PI3K)/protein kinase B (Akt) signal transduction. The PI3K/AKT/mTOR signaling pathway is an independent signaling pathway and plays an important role on proliferation and migration of smooth muscle cells (Tan et al., 2016). So, the mechanism of various components of the PI3K/Akt/mTOR signaling pathway involved in inflammation and airway remodeling of asthma remains to be resolved.

Insulin-like growth factor (IGF)-1 plays a critical role in the pathogenesis of asthma, especially in subepithelial fibrosis, airway inflammation, airway hyperresponsiveness (AHR), and airway smooth hyperplasia. IGF-1 receptor (IGF1R) belongs to a family of transmembrane tyrosine kinase receptors (Lee et al., 2014), and it helps in mediating the intracellular signaling cascades of IGFs (IGF-1 and

IGF-2). Upon ligand binding, IGF1R autophosphorylates and induces the PI3K/Akt pathway, which is an integral process involved in smooth muscle development and growth (Huang, Xu, Xie, Zhou, & Qu, 2011).

MicroRNA (miRNA) is a nonencoding small RNA and is involved in various biological behaviors, including organ formation, hematopoiesis, cell proliferation, differentiation, apoptosis, tumor formation, and progression of the disease (Pua & Ansel, 2015; Zhang et al., 2014). miRNA plays a major role in posttranscriptional activities, and inhibits protein translation or direct cleavage of target genes by identifying specific messenger RNA (mRNA) and interacting with the 3'-untranslated regions (3'-UTR) of target mRNAs (Denli, Tops, Plasterk, Ketting, & Hannon, 2004; Luna et al., 2015). miR-133 has two identical mature seed sequences, miR-133a and miR-133b, the mature sequences of miR-133a and miR-133b obtained from miRBase showed no differences between human and mouse (Table 1).

miR-133a has been reported to improve cardiac function by inhibiting AKT, and regulate cell cycle and cell proliferation in breast cancer cells via the EGFR/AKT signaling pathway (Cui, Zhang, Shan, Zhou, & Zhou, 2013; Sang, Jiang, Zhao, & Xin, 2015). Huang has reported that overexpression of miR-133 in C2C12 cells significantly suppressed IGF1R expression at the posttranscriptional level (Chiba, Tanabe, Goto, Sakai, & Misawa, 2009). Gao reported that miR-133a regulates IGF1R expression and vascular smooth muscle cell proliferation in murine atherosclerosis. So, we speculated whether there was a correlation between miR-133a and airway remodeling of asthma through the IGF1R and PI3K/AKT/mTOR signaling pathway.

In this study, the expression of miR-133a via the PI3K/AKT/mTOR signaling pathway in asthma was observed by replicating the mouse asthma model, and also the interaction between miR-133a and IGF1R were detected. We revealed that miR-133a modulates airway remodeling in chronic asthma through the PI3K/AKT/mTOR signaling pathway by targeting IGF1R, and miRNA can be used as a new therapeutic target for managing asthma.

2 | MATERIALS AND METHODS

2.1 | Animal model

Sixty female BALB/C mice weighing 20 g were obtained from the Animal Center of Wenzhou Medical University (Wenzhou, Zhejiang, China), and randomly divided into six groups: (a) control group;

TABLE 1 Mature sequences of miR-133 in human and mouse

ID	Accession	Sequence
hsa-miR-133a	MIMAT0000427	UUUGGUCCCCUUAACCAGCUG
mmu-miR-133a	MIMAT0000145	UUUGGUCCCCUUAACCAGCUG
hsa-miR-133b	MIMAT0000770	UUUGGUCCCCUUAACCAGCUA
mmu-miR-133b	MIMAT0000769	UUUGGUCCCCUUAACCAGCUA

Note. *has* *Homo sapiens*; *mmu* *Mus musculus*.

There is no difference between human and mouse in the sequence of miR-133. The sequence was from miRBase (<http://www.mirbase.org/>).

(b) asthma group; (c) miR-133a overexpression asthma group (Ad-miR-133a); (d) overexpression negative control (NC) asthma group (Ad-miR-133a NC); (e) miR-133a downexpression asthma group (Ad-miR-133a sponge); and (f) downexpression NC asthma group (Ad-miR-133a sponge NC). Mice were kept in a specific pathogen-free environment, provided standard food and water. Asthmatic mouse model was established based on Lloyd protocol (McMillan & Lloyd, 2004). Ovalbumin (OVA) suspension preparation: 20- μ g OVA and 4-mg aluminum hydroxide were dissolved in 0.2-ml physiological saline (NS), and used it for abdominal injection and sensitization. Mice except the control group were sensitized by intraperitoneal injection with 200- μ l OVA suspension on 1st and 12th days, whereas the control group was intraperitoneally injected with NS instead. After sensitization, mice were exposed to aerosolized 5% OVA/NS for 30 min every day on Days 18–23 and once every two days on Days 26–55, whereas the control group was exposed to NS instead. All animal procedures were approved by the Ethics Committee of Animal Experiments of the Wenzhou Medical University (permit number wydw 2014-0060) and were consistent with the standards established by the Guide for the Care and Use of Laboratory Animals.

2.2 | Lung function

Mouse pulmonary function test was performed and analyzed by using Lung function instrument (FlexiVent America). The mice were anesthetized with 10% chloral hydrate until they had no spontaneous breathing. Also underwent tracheal intubation with 18G endotracheal intubation needle and connected to a pulmonary function instrument. Then the intermittent positive pressure ventilation was performed with the standard tidal volume of 0.2 ml, respiratory rate of 150 times/min, PEEP 2 ml H₂O. The resistance of airway (Rn) was recorded after stabilization of respiration. Methacholine at various concentrations (10–80 mg/ml) was used for provocative test. The Rn was recorded at intervals of 3 min after each administration, and the percentage of each concentration and baseline was calculated.

2.3 | Primary ASMC culture and identification

Control and asthmatic mice were anesthetized with an intraperitoneal injection of 10% chloral hydrate after the last challenge. Then the researchers took the lungs, removed bronchial vessels and connective tissues, cut trachea and bronchus into 1 $\times$ 1 mm pieces, digested in collagenase type I for 60 min, centrifuged, and removed collagenase type I; then, the cells were cultured in 5% CO₂ at 37°C in Dulbecco modified Eagle medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 50 units/ml penicillin, and 100 g/ml streptomycin. Differential adherence method was used to remove fibroblasts. ASMCs cultured for the third generation showed obvious “hill and valley” phenomenon, and this generation of cells were used for cell identification and subsequent experiments. ASMCs were treated with different protein inhibitors to investigate α -SMA expression: PI3K/AKT was inhibited by LY294002 at 10 μ mol/ml

for 48 hr; mTOR was inhibited by PP242 at various concentrations of 0.5 to 2 μ mol/ml for 24 hr.

Primary ASMCs at third generations were seeded in 12 wells for 24 hr without FBS, next fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked with 5% goat serum in sequence, then anti- α -SMA (Abcam, Cambridge, United Kingdom) was added, which was diluted in 1:200 ratio in 1% bovine serum albumin (BSA)/PBS, and incubated overnight at 4°C. Cells were stained with rhodamine (fluorescein isothiocyanate)-conjugated AffiniPure goat anti-rabbit IgG (Bioworld, Nanjing, China) for α -SMA. Nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI). Olympus BX61 fluorescence microscope was used to collect the images.

2.4 | Adenovirus constructs

We inserted a synthetic miR-133a or miR-133a sponge sequence into Adenovirus (Ad) vector (Hanbio, Shanghai, China), which expressed green fluorescent protein (GFP). The Ad of different sequences was added to reach a multiplicity of infection of 500 for transduction, and virus titration was 1×10^{10} PFU/ml. ASMCs were seeded at 1×10^5 cells/well in a 6-well plate in DMEM without FBS for 24 hr. Then the medium was replaced with 1 ml fresh medium containing 5 μ l Ad vector and then were incubated for next 8 hr. After which, the medium was removed and replaced with fresh DMEM containing 10% FBS, and the cells were further incubated for 72 hr. The cells were designated as Ad-GFP, miR-133a overexpression Ad transfer vector (Ad-miR-133a) that expressed GFP and miR-133a. In contrast, miR-133a sponge Ad transfer vector (Ad-miR-133a sponge) stably expressed both GFP and miR-133a sponge that competitively inhibited miR-133a expression, the NC miR-133a Ad transfer vector (Ad-miR-133a NC), and NC miR-133a sponge Ad transfer vector (Ad-miR-133a sponge NC) that stably expressed GFP only.

The mice were anesthetized with an intraperitoneal injection of 10% chloral hydrate and endotracheally intubated by using intravenous indwelling needle PVC. We used purified Ad at virus titration of 1×10^{11} PFU/mL for animal intratracheal injection to build overexpressed miR-133a or downexpressed miR-133a of asthmatic mice model, and 10 μ l purified Ad transfer vector per mouse on Days 0 and 14 of asthma mode establishment.

2.5 | Lung hematoxylin-eosin staining, immunohistochemistry, and cell immunofluorescence

After anesthetization, the right lung tissue of mice was extracted, then fixed by 4% paraformaldehyde, dehydrated, and embedded in paraffin for lung hematoxylin-eosin (HE) staining and immunohistochemistry.

After deparaffinization and rehydration, lung tissues were stained with hematoxylin (Beyotime, Shanghai, China) for 5 min and eosin (Beyotime) for 5 s for pathological changes.

Immunohistological staining of lung tissue sections underwent antigen retrieval by incubating the slides for 15 min in 0.01 M sodium citrate pH 6.0 at 95°C. Then incubated with 1:200 diluted α -SMA

antibody (anti- α -SMA; Abcam Cat# ab5694 Lot# RRID:AB_2223021) in the PBS solution overnight at 4.0°C, followed by the Polink-2 plus polymer HRP detection system (Biosharp, Shanghai, China) and DAB kit (ZSGS-BIO, Beijing, China). The intensity of positive brown color was determined using Image-PRO PLUS 6.0 software.

Primary ASMCs from control and asthmatic mice were seeded in 12-well plate at a density of 5×10^4 /ml. After 24 hr of serum deprivation, asthmatic ASMCs were stimulated with Ad-miR-133a, Ad-miR-133a sponge, Ad-miR-133a NC, and Ad-miR-133a sponge NC for 8 hr, whereas control ASMCs were stimulated with PBS instead; then the stimulant was removed, fresh DMEM containing 10% FBS was added, and culture for 72 hr was continued before immunofluorescence. Then, ASMCs were fixed in 4% paraformaldehyde, inactivated endogenous peroxidase in 3% H_2O_2 , blocked nonspecific antibody, and incubated with anti- α -SMA (Abcam Cat# ab5694 Lot# RRID:AB_2223021), which was diluted in 1:200 ratio in 1% BSA/PBS overnight at 4°C. Then stained with rhodamine (Tetramethylrhodamine)-conjugated AffiniPure goat anti-rabbit IgG (Biosworld) for α -SMA. The nuclei stained with DAPI were visualized under Olympus BX61 fluorescence microscope to capture the images.

2.6 | Western blot

Cells were collected using cell scrapers and lysed in the radio-immunoprecipitation assay buffer containing proteinase inhibitor and phosphatase inhibitor. The cells were then centrifuged at 12000 rpm for 20 min, and protein supernatant was qualified by the BCA method (Thermo Fisher Scientific, MA). Equal amounts of denatured proteins (20 μ g) were separated by 8% to 16% sodium dodecyl sulfate polyacrylamide gel electrophoresis and subsequently electrotransferred onto polyvinylidene difluoride membranes. The proteins of interest were detected with antibodies specific to phosphorylated AKT (p-AKT; Abcam Cat# ab8805 Lot# RRID:AB_306791), AKT (Abcam Cat# ab74117 Lot# RRID:AB_1523105), phosphorylated mTOR (p-mTOR; Millipore Cat# 09-345 Lot# RRID:AB_11214180), mTOR (Abcam Cat# ab2732 Lot# RRID:AB_303257), p-p70S6k (Thermo Fisher Scientific Cat# A14037 Lot# RRID:AB_2534220), IGF1R (Abcam Cat# ab39522 Lot# RRID:AB_2122268), α -SMA (Abcam Cat# ab5694 Lot# RRID:AB_2223021), and GAPDH (LifeSpan Cat# LS-B7345-50 Lot# RRID:AB_11204972) at 4°C overnight. The secondary antibody conjugated with HRP binds by the chemiluminescent substrate (Thermo Fisher Scientific, MA) and

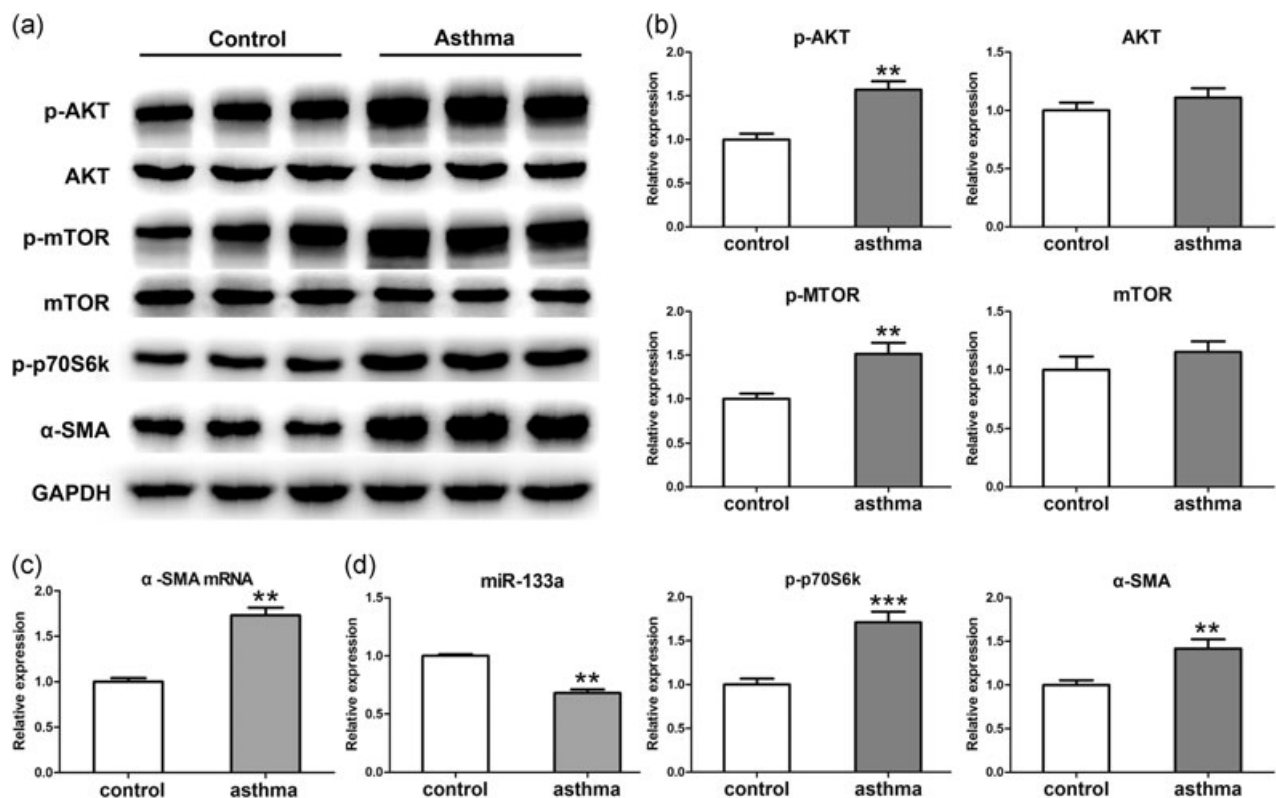


FIGURE 1 Increased expression of p-AKT, p-mTOR, and α -SMA in asthmatic mice compared with the control ones. (a) The protein expressions of p-AKT, p-mTOR, p-p70S6k, and α -SMA were higher in asthmatic mice group. Protein was quantified by means of Western blotting in the lungs of control mice and asthmatic mice. All the experiments were performed in triplicate. (b) Densitometric analyses for p-AKT, p-mTOR, p-p70S6k, and α -SMA. The relative expression of protein was normalized to GAPDH. Bars = mean $\pm$ SD. ** $p < 0.01$ and *** $p < 0.001$. (c) The expression of α -SMA mRNA was higher in the asthma group. α -SMA mRNA was normalized to β -actin. Bars = mean $\pm$ SD. ** $p < 0.01$. (d) The expression of miR-133a was decreased in the asthma group. miR-133a was normalized to U6. Bars = mean $\pm$ SD. ** $p < 0.01$. α -SMA: α smooth muscle actin; mRNA: messenger RNA; p-AKT: phosphorylated protein kinase B; p-mTOR: phosphorylated mammalian target of rapamycin; SD: standard deviation

then the membranes were visualized. Gray levels were detected on AlphaEaseFC Stand Alone 4.0.

2.7 | Total RNA extraction and quantitative real-time quantitative PCR analysis

Total RNA was extracted from mice lung using TRIzol™ reagent (Thermo Fisher Scientific) according to the manufacturer's instructions, and the quality of RNA was estimated by absorbance ratios of 260/280 nm greater than 1.8. Followed by reverse transcription using first strand concentration-dependent downregulation (complementary DNA [cDNA]) synthesis kit (Thermo Scientific), and the total of 1 µg of RNA was used for each reverse transcription reaction in the presence of random primers.

The expression levels of α -SMA mRNA were analyzed using the SYBR Green qPCR Kit (Thermo Fisher Scientific). The reaction mixture contains 10 µl SYBR Green Mix, 0.8 µl forward primers, 0.8 µl reverse primers, and 1 µg diluted cDNA and 5–8 µl H₂O. The relative expression of genes was normalized to β -actin. The melting curve was used to examine the quality of polymerase chain reaction (PCR) amplification of each sample, and quantification was performed with the standard curve method.

The reverse transcription reaction of miR-133a and the expression levels of miR-133a were determined by the Bulge-Loop™ miRNA quantitative real-time quantitative PCR (RT-qPCR) Stater Kit (Ribobio, Guangzhou, China), and the relative expression of genes was normalized to U6.

2.8 | Luciferase reporter assay

The role of miRNA was detected using a specially designed tool, pmiR-RB-Report™. Reporter plasmid pmiR-IGF1R containing the 3'-UTR derived from the mouse IGF1R gene was designed by Ribobio. The 3'-UTR IGF1R was cloned immediately downstream of the Renilla luciferase gene (hRluc). The hRluc was used as a reporter gene and the firefly luciferase gene (hLuc) as a reference gene. The relative changes of luciferase activity of Renilla Luciferase were detected to determine whether miRNA could regulate the gene. Before transfection, ASMCS were cultured in 96-well plates reaching 30% to 50% cell density, then cotransfected with 100 nM miR-133a mimic or NC along with pmiR-3'-UTR IGF1R using Lipofectamine 2000. After 48 hr, luciferase activity was analyzed using a dual luciferase assay system (Promega), where Renilla luciferase activity in the lysates was used to normalize the firefly luciferase activity.

2.9 | Statistical analysis

All experiments were repeated at least three times, and all data were analyzed by using SPSS 22.0 statistical software. Data were presented as mean $\pm$ standard deviation. Comparisons between the two observations were assessed by *t* test and differences among each group were analyzed by using one-way analysis of variance when multiple comparisons were made. *P* < 0.05 was considered statistically significant.

TABLE 2 Comparison of baseline values of total airway resistance (Rn) in each mice group

Group	n	Rn
Control	6	0.661 $\pm$ 0.026
Asthma	6	0.818 $\pm$ 0.015***
Ad-miR-133a	6	0.706 $\pm$ 0.079**
Ad-miR-133a sponge	6	0.898 $\pm$ 0.017*

Note. ****P* < 0.001 (Asthma versus control).

***P* < 0.01 (Ad-miR-133a/asthma versus asthma).

**P* < 0.05 (Ad-miR-133a sponge/asthma versus asthma).

3 | RESULTS

3.1 | OVA-induced allergic airway remodeling and activation of AKT and mTOR signaling pathways in asthmatic mice

To determine the levels of α -SMA in the lungs of control and asthmatic mice, asthmatic group mice were challenged with OVA, whereas the control group with NS. The mice were challenged by OVA solution, and then the lung tissues were removed from the two groups of mice within 24 hr after the last challenge. The expression of α -SMA was detected by qRT-PCR and Western blotting, respectively. Results showed that the baseline expression of α -SMA was higher in asthmatic mice than control ones (Figure 1c), suggesting airway remodeling in asthmatic mice.

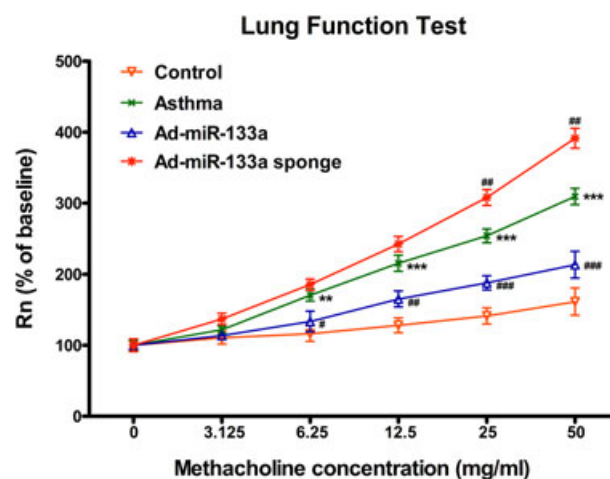


FIGURE 2 The resistance of airway (Rn) was alleviated when the asthmatic mice transfected with Ad-miR-133a. Comparison of Rn values after methacholine challenge in each mice group. The ratio of Rn value to baseline value was increased gradually with the increased concentration of methacholine in each group. Control group and Ad-miR-133a group were increased gently. The ascending range of asthma group was significantly higher than that of Ad-miR-133a group, and the amplitude of increase in Ad-miR-133a sponge group was also significantly higher than that of asthma group, and the difference was statistically significant. All the values were shown as mean $\pm$ SD; ***p* < .01, ****p* < .001 versus control, #*p* < .05, ##*p* < .01, ###*p* < .001 versus asthma. SD: standard deviation [Color figure can be viewed at wileyonlinelibrary.com]

To identify the underlying mechanism of AKT, mTOR in OVA-induced allergic airway disease, p-AKT, p-mTOR, phosphorylated ribosome protein subunit 6 kinase 1 (p-p70S6k) were assessed using Western blotting in mice of controls and asthmatic groups. The p-AKT in asthmatic mice was significantly increased compared with

the control ones (Figure 1a); a comparable increase of p-mTOR and p-p70S6k in asthmatic mice was also observed (Figure 1a), suggesting the activation of AKT and mTOR signaling pathway. The grayscale value of protein expression was determined using Western blotting, which was summarized in the corresponding bar charts (Figure 1b)

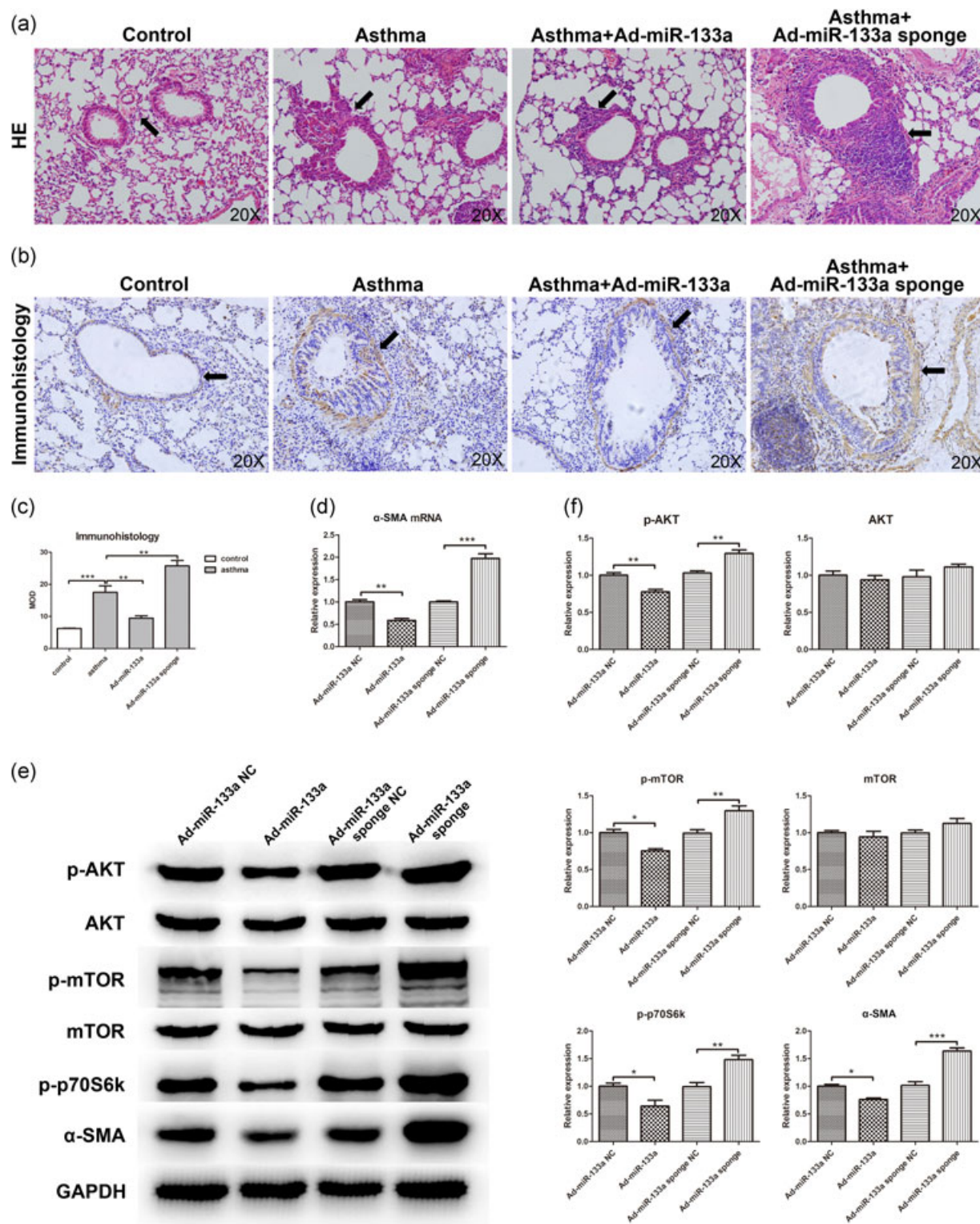


FIGURE 3 Continued.

3.2 | miR-133a was downregulated in the lung section of asthmatic mice

To investigate the expression of miR-133a in asthmatic mice, we compared the changes of miR-133a between three asthmatic groups and three control groups by using qRT-PCR. Results showed that miR-133a was downregulated in the lung section of asthmatic mice compared to control mice, with U6 housekeeping gene as a loading control (Figure 1d).

3.3 | miR-133a alleviated airway resistance in asthmatic mice

Lung function was detected using Lung function instrument. The data showed a total Rn of Ad-miR-133a sponge group was higher than that of asthma group, otherwise the total Rn of Ad-miR-133a group was lower than the asthma group, and the differences were statistically significant ($P < 0.05$; Table 2).

With the increased concentration of methacholine, the ratio of Rn to a baseline value of each group was increased gradually. Rn value of the control group and Ad-miR-133a group was increased slowly, Rn ratio of the asthmatic group was significantly increased compared with the control group. The ascending range of asthma group was significantly higher than that of Ad-miR-133a group, and the amplitude of increase in Ad-miR-133a sponge group was also significantly higher than that of asthma group (Figure 2).

3.4 | miR-133a inhibited airway remodeling in asthmatic mice

To investigate the effect of miR-133a on ASM, we transfected asthmatic mice with Ad-miR-133a and Ad-miR-133a sponge, respectively. Mice were killed within 24 hr after the last OVA challenge, and lung tissues underwent HE staining. Inflammatory cell infiltration was mostly pronounced in the Ad-miR-133a sponge group, followed by asthma group, and the Ad-miR-133a and control groups showed the least (Figure 3a).

The thickness of smooth muscle layer and the mean optical density of α -SMA expression in lung sections were measured by

immunohistochemical analysis. The smooth muscle layer was thickest in asthmatic mice transfected with Ad-miR-133a sponge, and then was asthmatic mice, followed by the ones transfected with Ad-miR-133a, finally the control mice, as shown in Figures 2c and 3b.

The protein expression levels of α -SMA were measured by Western blotting. As shown in Figure 3, we observed that the levels of α -SMA were increased in asthmatic mice transfected with Ad-miR-133a sponge compared with the asthmatic group (Figure 3e,f). In contrast, treatment with Ad-miR-133a decreased the expression of α -SMA (Figure 3e,f). These results suggested that miR-133a negatively regulated the expression of α -SMA. The α -SMA mRNA levels were valued by qRT-PCR, and the expression of α -SMA mRNA in Ad-miR-133a sponge group showed the highest (Figure 3d).

The Ad-miR-133a caused a decreased expression of α -SMA; however, the Ad-miR-133a sponge promoted the expression of α -SMA in asthmatic mice. These data explained that overexpression of miR-133a significantly decreased the airway remodeling of asthma.

3.5 | Regulation of α -SMA through PI3K/AKT/mTOR signaling

Primary ASMCs were extracted from control and asthmatic mice, and immunofluorescence test showed that ASMCs accounted for more than 98% (Figure 4). We investigated the role of PI3K/AKT signaling in the regulation of α -SMA in ASMCs by using PI3K inhibitor, LY294002. Asthmatic ASMCs were stimulated using 10 ng/mL LY294002 for 48 hr. In ASMCs from asthmatic mouse, the baseline expression of α -SMA was higher, compared with that in control mice ASMCs. Interestingly, incubation with LY294002 significantly reduced the expression of α -SMA in ASMCs from asthmatic mice, and so did p-AKT, p-mTOR, and p-p70S6k (Figure 5a,b).

To examine whether inhibition of mTOR activation decreases α -SMA expression in asthmatic ASMCs, we stimulated ASMCs with mTOR inhibitor, PP242, of various concentrations for 24 hr. A significant concentration-dependent downregulation of p-mTOR, p-p70S6k, and α -SMA was observed (Figure 6a,b). The inhibition of

FIGURE 3 Activation of AKT, mTOR, and the expression of α -SMA were negatively regulated by miR-133a in asthmatic mice. (a) HE stain (20 $\times$): Mice were killed within 24 hr after the last OVA challenge, HE staining was performed for lung tissues. Inflammatory cell infiltrates were most severe in Ad-miR-133a sponge group, then asthma group, whereas the Ad-miR-133a group, and the control group showed the lightest. (b) Smooth muscle layer was thickest in asthmatic mouse transfected with Ad-miR-133a sponge, and the layer of asthmatic group transfected with Ad-miR-133a was thinner than the asthma group. (c) Baseline thickness of smooth muscle layer from control (control, $n = 6$), asthma (asthma, $n = 6$), asthmatic mice transfected with Ad-miR-133a (Ad-miR-133a, $n = 6$) and asthmatic mice transfected with Ad-miR-133a sponge (Ad-miR-133a sponge, $n = 6$). $**p < 0.01$, $***p < 0.001$. (d) The expression of α -SMA mRNA in Ad-miR-133a sponge was highest. α -SMA mRNA was normalized to β -actin. Bar = mean $\pm$ SD. $**p < 0.01$, $***p < 0.001$. (e) An increased expression of p-AKT, p-mTOR, and α -SMA in mice transfected with Ad-miR-133a sponge compared to Ad-miR-133a sponge NC was observed. In contrast, a decreased expression of protein in Ad-miR-133a transfected group compared to Ad-miR-133a NC group was observed. Proteins were quantified by means of Western blotting in the lungs of Ad-miR-133a transfected asthmatic mice (Ad-miR-133a, A, $n = 6$), Ad-miR-133a sponge transfected asthmatic mice (Ad-miR-133a sponge; $n = 6$), Ad-miR-133a NC transfected asthmatic mice (Ad-miR-133a NC, $n = 6$) and Ad-miR-133a sponge NC transfected asthmatic mice (Ad-miR-133a sponge NC, $n = 6$). (f) Densitometric analyses for p-AKT, p-mTOR, p-p70S6k, and α -SMA. Bar = mean $\pm$ SD. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. α -SMA: α smooth muscle actin; HE: hematoxylin-eosin; mRNA: messenger RNA; NC: hematoxylin-eosin; p-AKT: phosphorylated protein kinase B; p-mTOR: phosphorylated mammalian target of rapamycin; SD: standard deviation; α -SMA; OVA: ovalbumin [Color figure can be viewed at wileyonlinelibrary.com]

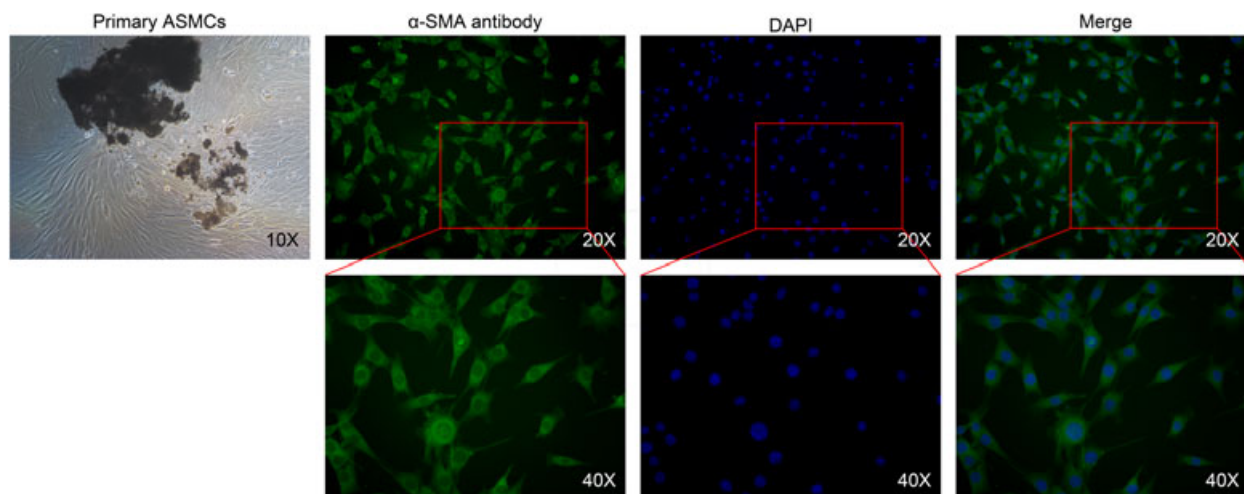


FIGURE 4 Primary ASMCS identification. “Hill and valley” phenomenon of ASMCS was observed by microscope on the 15th day of primary ASMCS culture. Immunofluorescence staining was used to examine the specific action of ASMCS. More than 98% cells showed strong positive staining, and a large amount of green fluorescence could be found in the cytoplasm. The nucleus was stained using DAPI. Original magnification, 10 $\times$, 20 $\times$, and 40 $\times$. ASMC: airway smooth muscle cell; DAPI: 4',6-diamidino-2-phenylindole [Color figure can be viewed at wileyonlinelibrary.com]

mTOR activity by PP242 significantly reduced the expression of α -SMA. Densitometric analysis of all Western blot analyses was summarized in Figure 6b.

3.6 | miR-133a negatively regulated α -SMA expression in ASMCS through targeting PI3K/AKT/mTOR signaling

We next examined whether miR-133a regulates the expression of α -SMA through PI3K/AKT/mTOR signaling in ASMCS. Primary ASMCS from asthmatic mice were transfected with Ad-miR-133a, Ad-miR-133a NC, Ad-miR-133a sponge and Ad-miR-133a sponge NC. Green fluorescence could be observed under a fluorescence microscope after ASMCS were transfected with Ad transfer vector. We confirmed the regulatory effects of miR-133a on α -SMA by using immunofluorescence staining (Figure 7a). Downregulation of miR-133a in the primary asthmatic cells increased the amount of α -SMA, and vice versa when miR-133a was overexpressed. Furthermore, we detected the protein expression of p-AKT, p-mTOR, p-p70S6k, and α -SMA by Western blotting. As shown in Figure 6c, the expression of p-AKT, p-mTOR, p-p70S6k, and α -SMA were shown significantly higher in the Ad-miR-133a sponge group than Ad-miR-133a sponge NC group, and lower in Ad-miR-133a group than Ad-miR-133a NC group (Figure 7b,c). The proteins were measured by Western blotting, and mRNA was measured by RT-qPCR. Densitometric analysis of all Western blot analyses is displayed on a bar chart in Figure 7d. These data suggested that miR-133a negatively regulated the α -SMA expression through PI3K/AKT/mTOR signaling

3.7 | miR-133a directly targets IGF1R mRNA

The protein data of IGF1R expression were detected in Ad-miR-133a, Ad-miR-133a NC, Ad-miR-133a sponge, and Ad-miR-133a sponge NC

of asthmatic ASMCS. As shown in Figure 8a,b, the expression of IGF1R protein was lower in Ad-miR-133a group, but higher in Ad-miR-133a sponge group.

To better understand the underlying molecular mechanism of miR-133a suppressing the expression of α -SMA, bioinformatic analysis of three publicly available algorithms (starBase, TargetScan, and miRBase) was performed to explore the target gene of miR-133a. Results revealed complementarity between IGF1R 3'-UTR and miR-133a (Figure 8c).

To verify whether there was any interaction between the predicted binding sites of miR-133a within IGF1R mRNA, we cotransfected the luciferase reporter plasmids containing the 3'-UTR of IGF1R and miR-133a mimic in ASMCS cells. Luciferase activity was measured by dual luciferase assays. In the presence of miR-133a mimic, we observed a significant decrease in relative luciferase activity compared with cells transfected with NC (Figure 8d). These results demonstrate that miR-133a binds directly to the 3'-UTR of IGF1R.

4 | DISCUSSION

MiRNAs are noncoding small RNAs that target the 3'-UTR of mRNA, and regulate target gene expression through the degradation of target gene mRNA or translational inhibition of target gene expression. These were involved in all kinds of physiological and pathological processes (Fisher & Lin, 2015; Kai, Xu, & Wu, 2015; Tay et al., 2014). In recent years, many studies have shown the involvement of miRNAs in the proliferative phase of ASMCS (Hu et al., 2014). Our study revealed that the expression of miRNA-133a in asthmatic mice was decreased, and inhibition of endogenous miR-133a by Ad-miR-133a sponge caused an upregulation of IGF1R, p-AKT, p-mTOR, and α -SMA in asthma. In contrast, overexpression of miR-133a by transfection with Ad-miR-133a

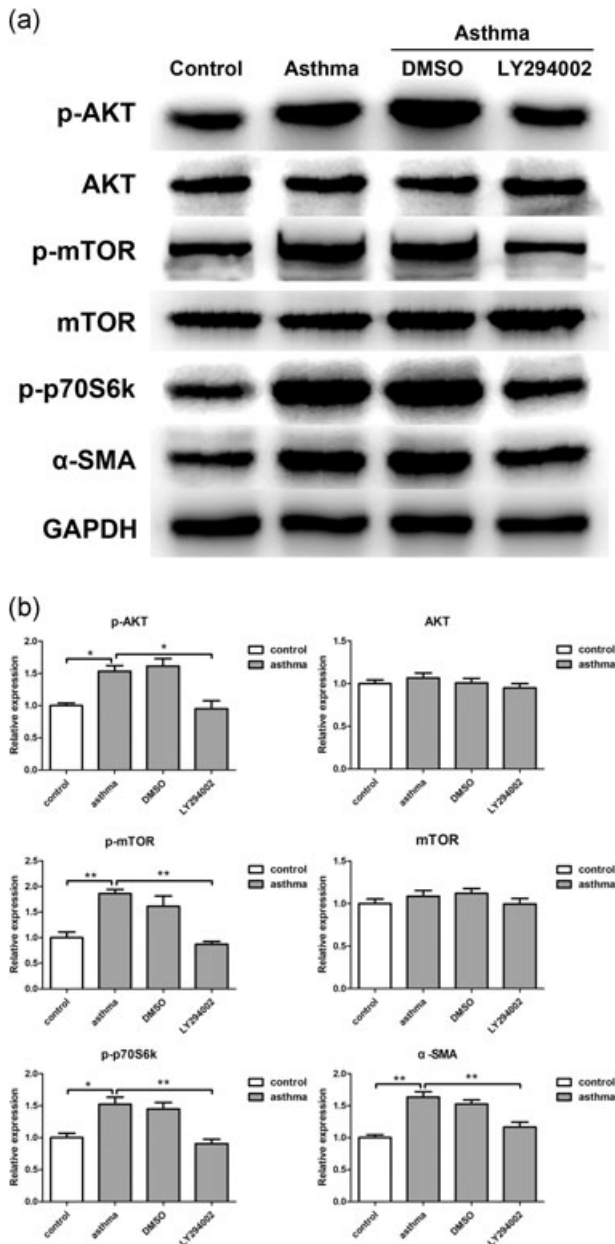


FIGURE 5 α -SMA was regulated by PI3K/AKT in primary ASMCs. (a) Primary ASMC lines from control and asthmatic mice were incubated with or without LY294002 (LY294002: 10 μ mol/L). The proteins of p-AKT, p-mTOR, p-p70S6k, and α -SMA were decreased in LY294002 treated group. Representative results from independent experiments ($n = 3$) were shown below. Protein was quantified by means of Western blotting. (b) Expressions of p-AKT, AKT, p-mTOR, mTOR, p-p70S6k, and α -SMA were detected and analyzed by using densitometry. Bar = mean $\pm$ SD ($n = 3$). * $p < 0.05$ and ** $p < 0.01$. α -SMA: α smooth muscle actin; ASMC: airway smooth muscle cell; PI3K: phosphoinositide 3 kinase; p-AKT: phosphorylated protein kinase B; p-mTOR: phosphorylated mammalian target of rapamycin; SD: standard deviation

decreased the levels of IGF1R, p-AKT, p-mTOR, and α -SMA. In addition, ASMCs treated with PI3K inhibitor, LY294002 and mTOR inhibitor, PP242 caused downregulation of α -SMA protein. These data revealed that the expression of α -SMA was regulated by the PI3K/AKT/mTOR pathway. It is well-known that the phosphorylated

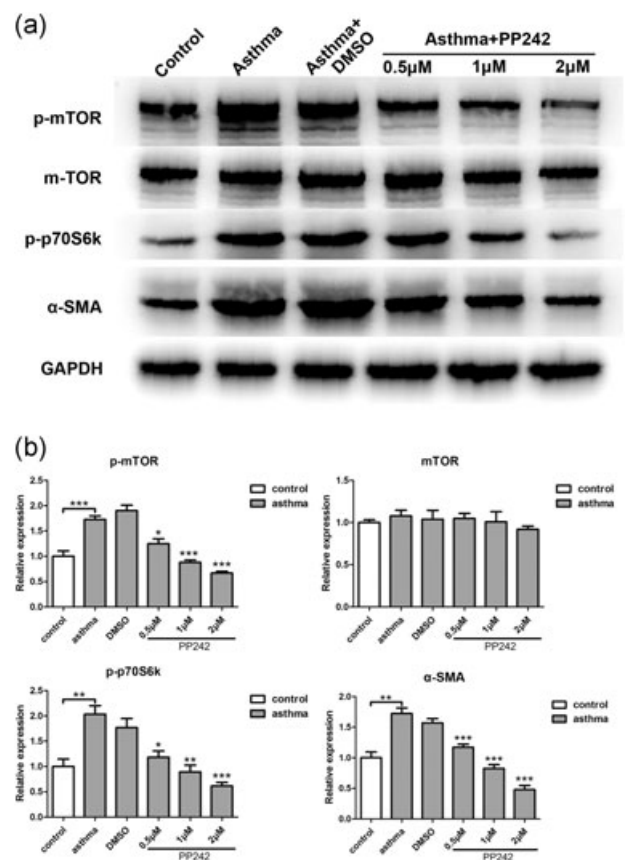


FIGURE 6 Concentration-dependent downregulation of protein of p-mTOR, p-p70S6k, and α -SMA were observed in primary ASMCs. (a) Primary asthmatic ASMC were incubated with or without PP242 (PP242: 0.5–2 μ mol/L), and expression of p-mTOR, mTOR, p-p70S6k, and α -SMA was detected. A significant decrease of p-mTOR, p-p70S6k, and α -SMA was presented in PP242 treated group. (b) The proteins were analyzed using densitometry. The experiment was repeated at least three times. Bar = mean $\pm$ SD. Asthma versus control: ** $p < 0.01$ and *** $p < 0.001$; asthma + PP242 versus asthma: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. α -SMA: α smooth muscle actin; ASMC: airway smooth muscle cell; p-mTOR: phosphorylated mammalian target of rapamycin; SD: standard deviation

IGF1R induced the activation of PI3K/Akt pathway, and so, the expression of IGF1R contributed to α -SMA expression. Dual luciferase reporter assay showed that IGF1R as the direct target of miR-133a. These findings suggested that miR-133a decreased the expression of α -SMA by inhibiting the activation of PI3K/AKT/mTOR signaling pathways via targeting IGF1R. Our studies showed that miR-133a played an important role in airway remodeling.

The miR-133 family has two members, miR-133a and miR-133b, and they share many common targets but have different cellular functions. miR-133a is commonly expressed in all muscle cells, while miR-133b is expressed in most of the muscle types, except cardiac muscle (Mitchelson & Qin, 2015). miR-133a plays a regulatory effect in the early muscle-derived myogenic stem cells to differentiate into myoblasts, and the effect lasted in the whole muscle growth. Furthermore, miR-133a could regulate muscle progenitor cells to differentiate into mature muscle cells during

stress or damaged conditions. miR-133b maintains skeletal muscle homeostasis during the early development of skeletal muscle cells. Mature miR-133a is generated by processing its pre-miRNAs, such as miR-133a-1, and miR-133a-2. miR-133a has been identified as muscle-specific miRNA, and was necessary for the development

and function of muscle cells (Yu, Lu, Li, & Wang, 2014). Although its expression in ASM was rarely reported, it has been revealed that expression of mature miR-133a in human airway smooth muscle cells (hASMCs) and mouse ASM tissues by using real-time PCR analyses. Yoshihiko verified that overexpression of miR-133a

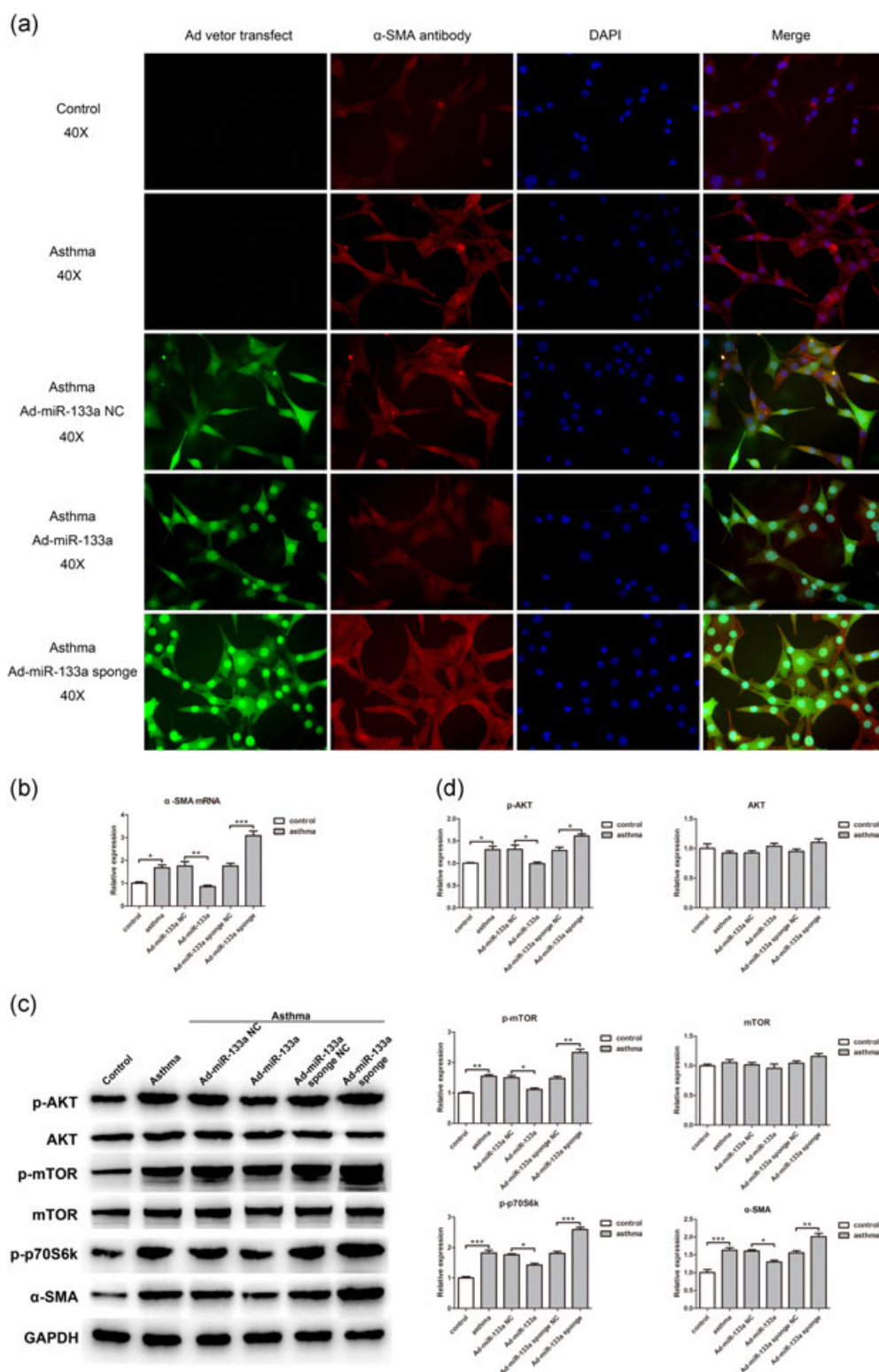


FIGURE 7 Continued.

alleviated hyperresponsiveness of bronchial smooth muscle cells in asthma (Chiba & Misawa, 2010).

In *in vivo* study, lung function test showed worse airway resistance in Ad-miR-133a sponge treated group, but was better in Ad-miR-133a group compared to the asthmatic group. The characteristics of hypertrophied lung smooth muscle cells and thickening of airway walls were found in asthma group, but not in the control group. Interestingly, these pathological features were alleviated with Ad-miR-133a treatment and aggravated with Ad-miR-133a sponge treatment. We also found that α -SMA was highly expressed in asthmatic mice compared to control ones. Moreover, α -SMA was regulated by miR-133a. α -SMA was highly expressed in Ad-miR-133a sponge transfected asthmatic mice, followed by asthmatic mice, and then was Ad-miR-133a transfected asthmatic mice. Similar results were observed in *in vitro* experiments.

PI3K/AKT/mTOR signaling regulates cell growth, proliferation, and differentiation. It plays an important role in lung physiology and pathophysiology. mTOR is an evolutionarily conserved serine/threonine protein kinase, belonging to the PI3K related kinase (PIKK) superfamily, and acts as a center control point in cell proliferation, growth and differentiation processes. Studies found that mTOR can regulate cell growth by increasing the synthesis of transcription proteins, and inhibits cell active death by suppressing autophagy. mTOR has two different complexes in the organism, mTORC1 and mTORC2, which have shared unique components, but differed in their function (Palavra, Robalo, & Reis, 2017).

LY294002 is a selective, permeable cell-specific PI3K inhibitor that acts on the enzyme's ATP binding site. It inhibits cell proliferation and induces apoptosis through inactivating Akt/PKB (Cheng et al., 2014). Chen et al. demonstrated that LY294002 could abrogate the positive effect of miR-21 in accelerating bone marrow mesenchymal stem cell migration and upregulating the expression of MMP-2/MMP-9 via inhibiting the phosphorylation of Akt (Lv et al., 2017). Several pharmacological inhibitors of mTOR have been identified, where PP242 was the earliest discovered mTORC1/2 kinase inhibitor that directly acts on the activation site, and inhibits the activity of mTORC1 and mTORC2 simultaneously. Matthew et al. demonstrated that PP242 caused death of leukemia cells through blocking of both TORC1 and TORC2 (Janes et al., 2010). Pena et al. (2017) also confirmed the suppression of both mTORC1 and mTORC2 in small pulmonary arteries

(PAs) by PP242. Meanwhile, pulmonary vascular remodeling could be reversed, and neointimal occlusion of small PAs could be prevented by using PP242. Qu, Liu, and He (2017) proved that PP242 inhibited both mTORC1 and mTORC2, and delayed the progression of AML by inducing apoptosis in leukemia cells and enabling more leukemic cells in the G0 phase of the cell cycle.

In our study, the protein levels of p-Akt, p-mTOR, and p-p70S6k in the asthmatic mice were significantly higher than those in the control group, and could be inhibited by treatment with PI3K inhibitor, LY294002. In addition, ASMCs were stimulated with various concentrations of mTOR inhibitors for 24 hr. Results showed a concentration-dependent significant downregulation of p-mTOR, p-p70S6k, and α -SMA starting at 0.5 to 2 μ mol/ml. Therefore, these data revealed that the expression of α -SMA was regulated by the PI3K/Akt/mTOR pathway in asthma.

In the current study, we transfected asthmatic mice with Ad-miR-133a and Ad-miR-133a sponge by intratracheal instillation, and found that downregulation of miR-133a promoted airway remodeling, upregulation of miR-133a alleviated remodeling, adversely. In addition, the protein levels of p-AKT, p-mTOR, p-p70S6k, and α -SMA were much higher in Ad-miR-133a sponge group than Ad-miR-133a sponge NC group, and the expression in Ad-miR-133a group was lower than Ad-miR-133a NC group. Similar results were obtained in *in vitro* experiments. Therefore, we considered that upregulation of miR-133a may contribute to the inhibition of activation of PI3K/AKT/mTOR signaling, and ameliorate airway remodeling. These results suggest that miR-133a could regulate PI3K/AKT/mTOR signaling.

To further investigate the specific role of miR-133a in airway remodeling, it was necessary to identify the target gene of miR-133a. We found that the protein expression of IGF1R in Ad-miR-133a sponge treated ASMCs was significantly increased and obviously decreased in Ad-miR-133a treated ASMCs compared to asthmatic ASMCs. We used the dual luciferase reporter system to explore the relationship between miR-133a and IGF1R. Results showed that miR-133a regulated the expression of IGF1R gene through potential seed regions located in its 3'-UTR regions. Our data revealed one of the mechanisms of airway remodeling, where miR-133a controlled the activity of intracellular transduction pathway by targeting IGF1R, and PI3K/AKT/mTOR signaling was the downstream of IGF1R. These results suggested that

FIGURE 7 miR-133a alleviated α -SMA expression through inhibiting the activation of PI3K/AKT/mTOR signaling. (a) Asthmatic ASMCs seeded in 12-well plates were transfected with Ad-miR-133a, Ad-miR-133a sponge, and Ad-miR-133a NC. The effect of miR-133a on α -SMA was visualized by immunofluorescence microscopy. The successful transfected cells were presented in green fluorescence. Representative results of immunofluorescent staining with α -SMA antibodies were presented as red fluorescence. Transfection of Ad-miR-133a markedly inhibited the expression of α -SMA, whereas transfection of Ad-miR-133a sponge increased the expression of α -SMA. The nucleus was stained using DAPI. Original magnification is 40 $\times$. All the experiments were repeated at least three times. (b) RNA was extracted and synthesized to cDNA by qRT-PCR assay to detect the value of α -SMA. α -SMA mRNA was normalized to β -actin. Bar = mean $\pm$ SD. * p < 0.05, ** p < 0.01, and *** p < 0.001. (c) The activation of AKT, mTOR, and the expression of α -SMA were negatively regulated by miR-133a in primary ASMCs. Primary ASMCs from asthmatic mice were transfected with Ad-miR-133a (Ad-miR-133a), Ad-miR-133a sponge (Ad-miR-133a sponge), Ad-miR-133a NC (Ad-miR-133a NC), and Ad-miR-133a sponge NC (Ad-miR-133a sponge NC). The expression levels of p-AKT, p-mTOR, and α -SMA were higher in the asthma group than control ones, and miR-133a negatively regulates the expression levels of p-AKT, p-mTOR, and α -SMA. (d) The experiments were performed in triplicate, expression levels of p-AKT, AKT, p-mTOR, mTOR, p-p70S6k, and α -SMA were detected and analyzed using densitometry. Results were normalized with GAPDH. Bar = mean $\pm$ SD. * p < 0.05, ** p < 0.01, and *** p < 0.001. α -SMA: α smooth muscle actin; ASMC: airway smooth muscle cell; Akt: protein kinase B; cDNA: complementary DNA; DAPI: 4'-6-diamidino-2-phenylindole; mRNA: messenger RNA; mTOR: mammalian target of rapamycin; NC: negative control; PI3K: phosphoinositide 3 kinase; qRT-PCR: quantitative real-time polymerase chain reaction [Color figure can be viewed at wileyonlinelibrary.com]

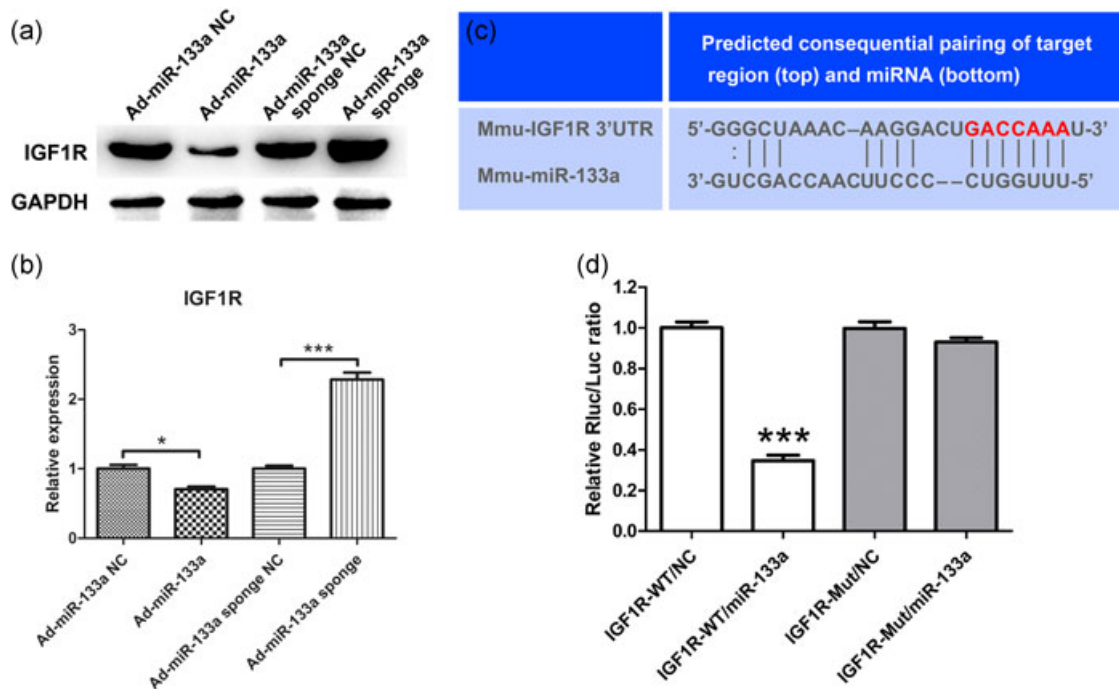


FIGURE 8 miR-133a directly targeted IGF1R. (a) Asthmatic ASMCs were transfected with Ad-miR-133a and Ad-miR-133a sponge, and the protein expression of IGF1R was performed. The expression of IGF1R was decreased after ASMCs being transfected with Ad-miR-133a, but increased after being transfected with Ad-miR-133a sponge. (b) Densitometric analyses of IGF1R. Results were normalized with GAPDH. * $p < 0.05$, *** $p < 0.001$. (c) The binding site of miR-133a to the 3'-UTR of IGF1R. (d) Dual luciferase assays showed a decrease after transfection with miR-133a mimic. Luciferase reporter assays were used for analyzing 3'-UTR sequence. ASMCs seeded in 96-well plates were transfected with reporter plasmid, pmiR-IGF1R containing 3'-UTR cloned downstream of the Renilla luciferase gene, the firefly luciferase gene was used as a reference gene, and miR-133a mimic was cotransfected using Lipofectamine 2000. Luciferase activity was analyzed 48 hr later using a dual luciferase assay. Bar = mean $\pm$ SD. *** $p < 0.01$. Each experiment was performed in triplicate. (Becker & Abrams, 2017). ASMC: airway smooth muscle cell; SD: standard deviation; UTR: untranslated region [Color figure can be viewed at wileyonlinelibrary.com]

miR-133a improved airway remodeling of asthma by targeting IGF1R through inhibition of PI3K/AKT/mTOR signaling pathways.

5 | CONCLUSION

Our study showed the declined expression of miR-133a in asthma. Moreover, overexpression of miR-133a alleviated airway remodeling by downregulating the activation of PI3K/AKT/mTOR signaling via binding to IGF1R 3'-UTR, and down-expression of miR-133a exacerbated airway remodeling. These data suggested that miR-133a may serve as potential therapeutic targets for managing asthmatic airway remodeling.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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