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Long noncoding RNA *HOXA-AS2* ameliorates chronic intermittent hypoxia-induced lung inflammation by regulating *miR-17-5p/tipe2* axis

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KEYWORDS

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Abstract

Purpose: The purpose is to confirm whether long noncoding RNA *HOXA-AS2* relieves chronic intermittent hypoxia (CIH)-induced lung inflammation.

Methods: Male Sprague Dawley rats were used to establish a CIH rat model. Hematoxylin and Eosin staining was used on the lung tissue injury to determine the successful construction of CIH animal model. Arterial partial pressure of oxygen (PaO₂) and carbon dioxide (PaCO₂) were measured. *HOXA-AS2* was overexpressed to evaluate its role in the progression and development of CIH. T cell differentiation and cytokine production were determined using flow cytometry. Cell apoptosis was determined using terminal deoxynucleotidyl transferase dUTP nick end labelling assay kit. The target of *HOXA-AS2* and *miR-17-5p* was predicted by the Encyclopedia of RNA Interactomes (ENCORI) and confirmed using luciferase assay.

Results: *HOXA-AS2* was downregulated in CIH rat models. Lung tissue injury was observed in CIH rats, and the injury was attenuated by the overexpression of *HOXA-AS2*. PaO₂ was reduced and PaCO₂ was induced in CIH rats, which was reversed by the overexpression of *HOXA-AS2*. The overexpression of *HOXA-AS2* inhibited CIH-induced cell apoptosis. It also reversed alterations in the levels of interferon gamma (IFN γ), interleukin (IL)-2, IL-6, IL-1 β , tumor necrosis factor alpha (TNF- α), and transforming growth factor beta1 (TGF- β 1) in rats caused by CIH. The overexpression of *HOXA-AS2* prevented the induction in CD4⁺ IFN- γ ⁺ T cells and reduction in CD4⁺ TGF- β 1⁺ T cells. The overexpression of *HOXA-AS2* upregulated tumor necrosis

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factor- α -induced protein 8-like 2 (*tipe2*) key regulator through directly targeting *miR-17-5p*. Further experiments proved that *tipe2* was the direct target of *miR-17-5p*.

Conclusion: This study manifested that *HOXA-AS2* acted as an anti-inflammatory regulator and protected lung tissue injury from CIH in the rat model; this was mediated by upregulation of *tipe2* through directly targeting *miR-17-5p*. *HOXA-AS2* upregulated the expression of *tipe2*, providing new understanding and therapeutic target for CIH.

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Introduction

Long-term chronic intermittent hypoxia (CIH) is usually accompanied by abnormality of cardiopulmonary circuit, including pulmonary hypertension, atherosclerosis, and lung inflammation.^{1,3} CIH-induced lung inflammation is involved in the production of many inflammatory molecules, including tumor necrosis factor α (TNF- α) and interleukins (ILs), such as IL-1 β , IL-6, and IL-2.⁴ During inflammation, CD4⁺ T lymphocytes could differentiate into T helper 1 (Th1, pro-inflammatory) cells, which specifically release interferon gamma (IFN γ),⁵ while regulatory T (Treg, anti-inflammatory) cells are suppressed, demonstrating reduction in CD4⁺ transforming growth factor-beta 1⁺ (TGF- β 1) T cells.⁶ Therefore, inhibition of lung inflammation is an effective path to prevent lung injury from CIH. Aerobic exercise, resistance exercise, and herbal medicine can successfully target inflammatory cytokines in chronic diseases,^{7,9} but there is a need to figure out the mechanism of lung inflammation to provide a new insight to CIH treatment.

Long noncoding RNAs (lncRNAs, >200 nucleotides [nt]) are a group of transcripts which have no coding potential but have the ability to bind to microRNAs (miRs) through base-pairing interactions, regulating protein expression.¹⁰ LncRNAs are dysregulated and involved into the pathophysiological process of CIH.¹¹ miRs are a category of small, conserved RNAs (~22 nucleotides) that are involved in almost all the pathophysiological process through targeting protein-coding transcripts.¹² miRs play an anti-inflammatory role in many diseases, including CIH and asthma.¹³⁻¹⁵ For example, lncRNA *Mirt1* binds to *miR-4661-5p* to regulate target gene expression, demonstrating a critical regulator of myocardial infarction aggravated by CIH.¹⁶ *HOXA cluster antisense RNA 2 (HOXA-AS2)*, a 1048-bp lncRNA, plays an anti-inflammatory role in different inflammatory models.^{17,18} However, no publications has reported the role of *HOXA-AS2* in CIH-induced lung inflammation.

Tumor necrosis factor- α -induced protein 8-like 2 (tipe2) is a key regulator of inflammation and immune homeostasis,¹⁹ and has strong anti-inflammatory properties.²⁰ Loss of *tipe2* is associated with the upregulation of pro-inflammatory cytokines, such as IL-6 and TNF- α , and downregulation of anti-inflammatory factors, such as TGF- β 1, thereby promoting proliferation and differentiation of T lymphocytes.¹⁹ *miR-21* has been revealed to promote T cell apoptosis by targeting *tipe2*.²¹ It is thus hypothesized that *HOXA-AS2/miR-17-5p/tipe2* axis could play a functional role in the progression and development of CIH. However, limited literature has described the role of *tipe2* in the progression of CIH. Therefore, this study was aimed to identify

the anti-inflammatory role of *HOXA-AS2/miR-17-5p/tipe2* in a CIH rat model through a series of experiments.

Methods

Establishment of CIH rat model

Male Sprague Dawley (SD) rats (n = 36, 200-250 g) were purchased from Jiangsu ALF Biotechnology Co. Ltd. (Jiangsu Province, China). Rats were kept under 12-h light/12-h dark cycle at 23 \pm 2°C with a humidity of 40 \pm 5% and provided standard animal food and drinking water. All experiment procedures were carried out in compliance with the ethical standards under a protocol approved by the Institutional Animal Care and Use Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University, and were executed conforming to the Care and Use of Laboratory Animals published by the US National Institutes of Health.²²

The CIH model was established following the study conducted by Zhang et al.³ Briefly, SD rats were moved into sealed chambers away from the normal environment. Compressed air and nitrogen were infused into the chambers at a cycle of 90 s. To make sure a minimum of 5% oxygen concentration, pure nitrogen was infused into each chamber during the first 30 s. For the remaining 60 s, each chamber was infused with compressed air to make oxygen concentration delivery of 21%. Normal air was forced into the chambers of control group at a cycle of 90 s. Rats were randomly assigned into the following six groups (n = 6 rats/group): sham group: chambers were infused with normal air; CIH group: chambers were infused with compressed air and nitrogen; sham+AAV-*HOXA-S2* group: sham rats were injected with adeno-associated virus (AAV) containing *HOXA-S2* gene (AAV-*HOXA-S2*) through tail vein; sham-AAV-NC (negative control) group: sham rats were injected with AAV containing scramble sequence of *HOXA-S2* gene (AAV-NC) through tail vein; CIH+AAV-*HOXA-S2*: CIH rats were injected with AAV-*HOXA-S2* vector; CIH+AAV-NC: CIH rats were injected with AAV-NC vector.

Blood gas analysis

After establishing the CIH rat model, arterial partial pressure of oxygen (PaO₂) and carbon dioxide (PaCO₂) was measured using GEM Premier 4000 & 3500 (Instrumentation Laboratory, Lexington, MA, USA). At the end of the experiments, rats were scarified by an overdose of chloral

hydrate (100 mg/kg). Lung tissues, spleen, and serum were collected for further experiments.

Hematoxylin and Eosin (H&E) staining

Lung tissues were fixed in 4% buffered formalin solution for 48 h at 4°C and embedded in paraffin wax. The fixed lung tissues were then cut into 5-μm sections and stained using the H&E staining kit (Powerful, China) according to the manufacturer's protocols. The stained slides were photographed under a microscope (Nikon, Tokyo, Japan).

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay

Cell apoptosis was determined using TUNEL assay kit (Abcam, Cambridge, UK). Lung tissues were fixed in 4% buffered formalin solution for 48 h at 4°C and embedded in paraffin wax. The fixed lung tissues were then cut into 5-μm sections. Deparaffinization and dehydration were performed using xylene and graded ethanol, respectively. The tissue sections were incubated with 20-μg/mL recombinant Proteinase K solution (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C for 30 min. The sections were washed for at least three times with phosphate buffered saline (PBS). The sections were incubated with DNA-labeling solution for 1 h at 37°C, followed by incubation with antibody solution for 30 min at room temperature. 4',6-Diamidino-2-phenylindole (DAPI; Abcam), 20 μL, was added to all slides, which were gently covered with cover slips. The slides were photographed under a fluorescent microscope (Nikon).

Flow cytometry

Flow cytometry was used to examine the ratio of CD4⁺IFN-γ⁺ T cells and CD4⁺TGF-β1⁺ T cells in the spleen. The spleen tissues were cut into small pieces and digested in 400-μ/mL collagenase III (Abcam) for 20 min at 37°C. Tissues were filtered using 200-mesh sieves and centrifuged at 1000 rpm for 5 min. Cells were resuspended in PBS, fixed using 4% buffered formalin solution for 1 h, and treated with 1% Triton X-100 (Merck KGaA, Darmstadt, Germany) for 5 min at room temperature. The fixed cells were stained with the following antibodies for flow cytometry: CD4 (Abcam), IFN-γ (Invitrogen/Thermo Fisher Scientific), and TGF-β1 (Shanghai Yaji Biotechnology Co. Ltd., Shanghai, China). In

all, 30,000 stained cells were collected and analyzed using Gallios flow cytometer (Beckman Coulter, Fullerton, CA, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Lung tissues were cut into small pieces and lysed using TRIzol RNA isolation reagents (Invitrogen). Tissues were centrifuged at 5000 rpm for 10 min, the supernatant was collected, and Multiskan SkyHigh microplate spectrophotometer (Thermo Fisher Scientific) was used to determine RNA concentration. QuantiTect reverse transcription kit (QIAGEN GmbH, Hilden, Germany) was used to synthesize complementary DNA (cDNA) from 300-ng total RNA. qRT-PCR was performed using SYBR[™] Green PCR Master Mix (Thermo Fisher Scientific) through ProFlex PCR System (Thermo Fisher Scientific). The relative RNA expression was quantified using the 2^{-ΔΔCT} method. Primary sequences (Sigma-Aldrich Corp., Milwaukee, WI, USA) used in this study are listed in Table 1.

Western blotting analysis

The lung tissues were cut into small pieces and homogenized for 10 s for three times in ice-cold radioimmunoprecipitation assay (RIPA) lysis and extraction buffer (Thermo Fisher Scientific). After stewing for 15 min on ice, the tissues were centrifuged at 5000 rpm for 5 min at 4°C, and the supernatant was collected. Protein concentration was tested using bicinchoninic acid (BCA) protein assay kit (Abcam); 30-μg total protein was loaded and separated using 10% ExpressPlus[™] polyacrylamide gel electrophoresis (PAGE; GenScript Corporation, Piscataway, NJ, USA) at 100 V. Proteins were transferred from gels to polyvinylidene fluoride (PVDF) membranes (Merck KGaA) at 100 V for 1 h. The membranes were blocked using 5% fat-free milk for 2 h at room temperature. The target protein was probed with the corresponding primary antibodies at 4°C overnight and incubated with secondary antibodies for 2 h at room temperature. The protein signal was developed using Pierce[™] ECL Western blotting substrate (Thermo Fisher Scientific) and imaged using GelView 6000Plus Imaging Systems (Btflux, China). The protein bands were quantified using the ImageJ software (Softonic, Spain). Primary antibodies used were as follows: *tipe2* (ab110389, 1:1000 dilution) and *β-actin* (ab8227, 1:5000 dilution).

Table 1 The primary sequences used in this study.

Gene name	Forward (5'-3')	Reverse (5'-3')
<i>HOXA-AS2</i>	AACCCATCTTTGCCTTCTGC	CGGAGGAGTTTGGAGTTGG
<i>Tipe2</i>	GTGACTGACCACATACCCCA	AGTGTTAGTGCCAGGTGAGC
<i>miR-17-5p</i>	GGCAAAGTGCTTACAGTGC	GTGCAGGGTCCGAGG
<i>U6</i>	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
<i>β-actin</i>	GTCTGCCTTGGTAGTGGATAATG	TCGAGGACGCCCTATCATGG

Enzyme-linked immunosorbent serologic assay (ELISA)

ELISA was used to measure the serum levels of IFN- γ , TNF- α , IL-2, IL-6, IL-1 β , and TGF- β 1. ELISA was performed using commercial kits (R&D Systems, Minneapolis, MN., USA) according to the manufacturer's instruction.

Cell culture and transfection

Normal human bronchial epithelial (NHBE) cells and human embryonic kidney (HEK) 293T cells were obtained from Ningbo Mingzhou Biotechnology Co. Ltd. (Mingzhou, Zhejiang Province, China). NHBE cells were cultured in Bronchial Epithelial Cell Growth Medium (BEGMTM; Lonza G and T AG, Switzerland) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1% penicillin-streptomycin solution (100 \times ; Procell Life Science & Technology, Wuhan, Hubei Province, China), and SingleQuots[®] kit (Lonza G and T AG) at 37°C with 5% CO₂. In all, 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% FBS, and 1% penicillin-streptomycin solution at 37°C with 5% CO₂.

Short hairpin of *HOXA-AS2* (sh*HOXA-AS2*) and its negative control (sh*HOXA-AS2*-NC), Adeno-associated virus containing *HOXA-S2* gene (AAV-*HOXA-S2*) and its negative control (AAV-NC), and *miR-17-5p* mimic and its negative control (NC-mimic) were purchased from Hanbio Biotechnology Co. Ltd. (Shanghai, China). They were dissolved into nanopure water at a stock concentration of 10 μ g/mL and mixed with lipofectamine 2000 (Invitrogen) to form transfection mixture (1 ng/ μ L) according to manufacturer's instructions. Cells were cultured with FBS-free medium into a sterile six-well plate; 200-ng transfection mixture was added to each well at 37°C. After 8 h, FBS-free medium was replaced with normal culture medium for another 24 h.

Plasmid and luciferase reporter assay

The full sequence of *HOXA-AS2* (*HOXA-AS2*-WT) and its mutant sequence (*HOXA-AS2*-MUT), or the full sequence of *tipe2* (*tipe2*-WT) and its mutant sequence (*tipe2*-MUT) were cloned into the downstream of the firefly luciferase gene in psiCHECK2 (Promega, USA). The constructed psiCHECK2 vector was co-transfected with *miR-17-5p* mimic or NC-mimic into 293T cells using lipofectamine 2000. After cultured for 24 h, luciferase activity was performed using dual luciferase reporter assay Kit (Vazyme, Jiangsu Province, China).

Statistical analysis

All results were analyzed using GraphPad Prism 8.1.0 (GraphPad, San Diego, CA, USA). All data were performed as mean \pm standard error of mean (SEM). Statistical differences between two groups were analyzed using Student's *t*-test under non-normal conditions. Statistical differences among more than two groups were performed using one-way ANOVA, followed by the Bonferroni's post hoc tests. Normality of data was assessed using Shapiro-Wilk

normality tests. Homogeneity of data was analyzed using Bartlett's test. $P < 0.05$ was considered as statistically significant.

Results

HOXA-AS2 was downregulated in lung tissues from CIH rat model

The expression of *HOXA-AS2* was determined to investigate the potential role of *HOXA-AS2* in the progression of CIH. In the lung tissues of CIH rat model, granulocyte and lymphocyte infiltration, edema, and bleeding were observed, and the thickness of alveolar wall and blood vessels was increased in alveolar chambers, compared to that from sham group (Figure 1A). PaO₂ level was reduced and PaCO₂ was increased in CIH rat model, compared to that in sham rats (Figures 1B and C). The relative expression of *HOXA-AS2* was downregulated in CIH rat model, compared to that in sham rats (Figure 1D).

HOXA-AS2 reduced lung injury in CIH rat model

Overexpression of *HOXA-AS2* was established by transfection of AAV-*HOXA-AS2* to evaluate the involvement of *HOXA-AS2* in lung injury in CIH rat model. The expression of *HOXA-AS2* was increased in both sham and CIH rats after transfection of AAV-*HOXA-AS2* (Figure 2A). The overexpression of *HOXA-AS2* relieved CIH-induced lung tissue injury in rats (Figure 2B). The number of TUNEL-positive cells was also reduced after being transfected with AAV-*HOXA-AS2* in CIH rats (Figure 2C). Cell apoptosis was significantly induced in CIH rats (Figure 2D). Although the overexpression of *HOXA-AS2* caused no significant alterations in cell

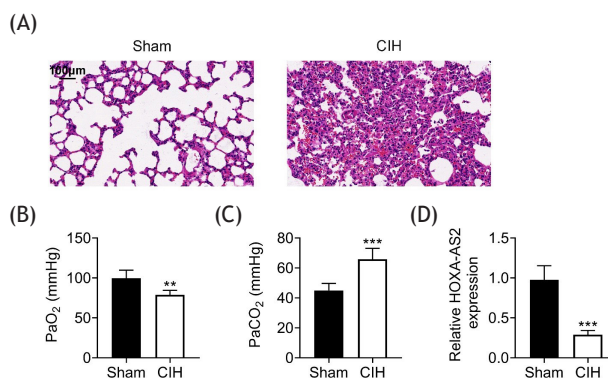


Figure 1 *HOXA-AS2* was downregulated in lung tissues from CIH rat model. (A) H & E staining results demonstrated lung tissue injury in CIH rat model. (B) PaO₂ was reduced in CIH rats. (C) PaCO₂ was elevated in CIH rats. (D) The expression of *HOXA-AS2* was downregulated in CIH rats. ** $P < 0.01$ versus sham; *** $P < 0.005$ versus sham. CIH: chronic intermittent hypoxia; *HOXA-AS2*: *HOXA* cluster antisense RNA 2; H & E staining: hematoxylin and eosin staining; PaO₂: arterial partial pressure of oxygen; PaCO₂: arterial partial pressure of carbon dioxide.

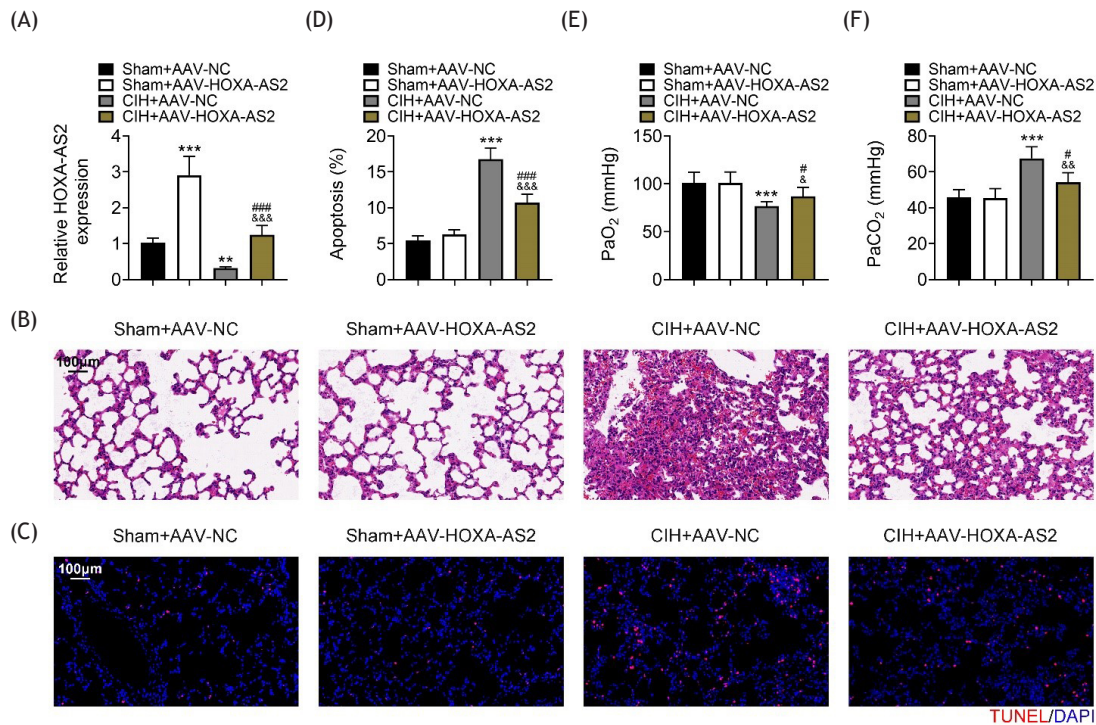


Figure 2 *HOXA-AS2* reduced lung injury in CIH rat model. (A) AAV-*HOXA-AS2* transfection increased the expression of *HOXA-AS2* in CIH rats. (B) AAV-*HOXA-AS2* ameliorated lung injury in CIH rats. (C) AAV-*HOXA-AS2* reduced the number of TUNEL-positive cells in CIH rats. ***P* < 0.01 versus sham + AAV-NC; ****P* < 0.005 versus sham + AAV-NC; #*P* < 0.05 versus sham + AAV-*HOXA-AS2*; ###*P* < 0.005 versus sham + AAV-*HOXA-AS2*; &#i>P < 0.05 versus CIH + AAV-NC; &&#i>P < 0.01 versus CIH + AAV-NC; &&&#i>P < 0.005 versus CIH + AAV-NC. AAV-*HOXA-AS2*: adeno-associated virus containing *HOXA-AS2* gene; AAV-NC: negative control of AAV-*HOXA-AS2*.

apoptosis in sham rats, cell apoptosis in CIH group was reduced (Figure 2D). The overexpression of *HOXA-AS2* influenced no significant alterations in PaO₂ and PaCO₂ levels in sham rats, but significantly increased PaO₂ and decreased PaCO₂ levels in CIH rats (Figures 2E and F). These data demonstrated that *HOXA-AS2* reduced both lung injury and cell apoptosis in CIH rats.

***HOXA-AS2* reduced inflammatory response in CIH rat model**

T cell differentiation and cytokine production were determined using flow cytometry to investigate the role of *HOXA-AS2* to inflammatory response in CIH rats. The number of CD4⁺IFN γ ⁺ T cells was elevated in the lung tissues of CIH rats, compared to that of sham rats, while this elevation was ameliorated by the overexpression of *HOXA-AS2* (Figure 3A). The number of CD4⁺TGF- β 1⁺ T cells was lower in CIH rats than that in sham rats, and this reduction was increased in CIH rats injected with *HOXA-AS2* plasmid (Figure 3B). Serum levels of IFN γ and IL-2 were increased, and serum TGF- β 1 was reduced in CIH rats, and these alterations were reversed by the overexpression of *HOXA-AS2* (Figure 3C). Serum levels of TNF- α , IL-6, and IL-1 β were significantly stimulated in CIH rats, and these enhancements were repressed by the overexpression of *HOXA-AS2* (Figure 3D). It is deduced that *HOXA-AS2* could reduce inflammatory response in CIH rats.

***HOXA-AS2* regulated the expressions of miR-17-5p and *tipe2* in CIH rat model**

In order to confirm the relationship between *HOXA-AS2*, miR-17-5p, and *tipe2*, expressions of miR-17-5p and *tipe2* were determined after the overexpression of *HOXA-AS2* in 293T cells. The overexpression of *HOXA-AS2* suppressed the mRNA expression of miR-17-5p but increased the mRNA expression of *tipe2* in both sham and CIH rats (Figure 4A). The protein expression of *tipe2* was upregulated by the overexpression of *HOXA-AS2* in both sham and CIH rats (Figure 4B). The predictive results from the Encyclopedia of RNA Interactomes (ENCORI; <https://starbase.sysu.edu.cn/>) demonstrated that there was a complementary sequence between *HOXA-AS2* and miR-17-5p (Figure 5A). Luciferase activity was decreased in cells co-transfected with *HOXA-AS2*-WT and miR-17-5p mimic, compared to that with *HOXA-AS2*-WT and NC mimic (Figure 5B). The expression of *HOXA-AS2* was increased by *HOXA-AS2* plasmid and decreased by sh*HOXA-AS2* (Figure 5C), while the expression of miR-17-5p was downregulated by *HOXA-AS2* and upregulated by sh*HOXA-AS2* (Figure 5D) in NHBE cells. These data supported the hypothesis that miR-17-5p was the direct target of *HOXA-AS2*.

***HOXA-AS2* positively regulated *tipe2* by sponging miR-17-5p**

ENCORI and luciferase assay were used to confirm the direct target of *HOXA-AS2* and miR-17-5p. The predictive

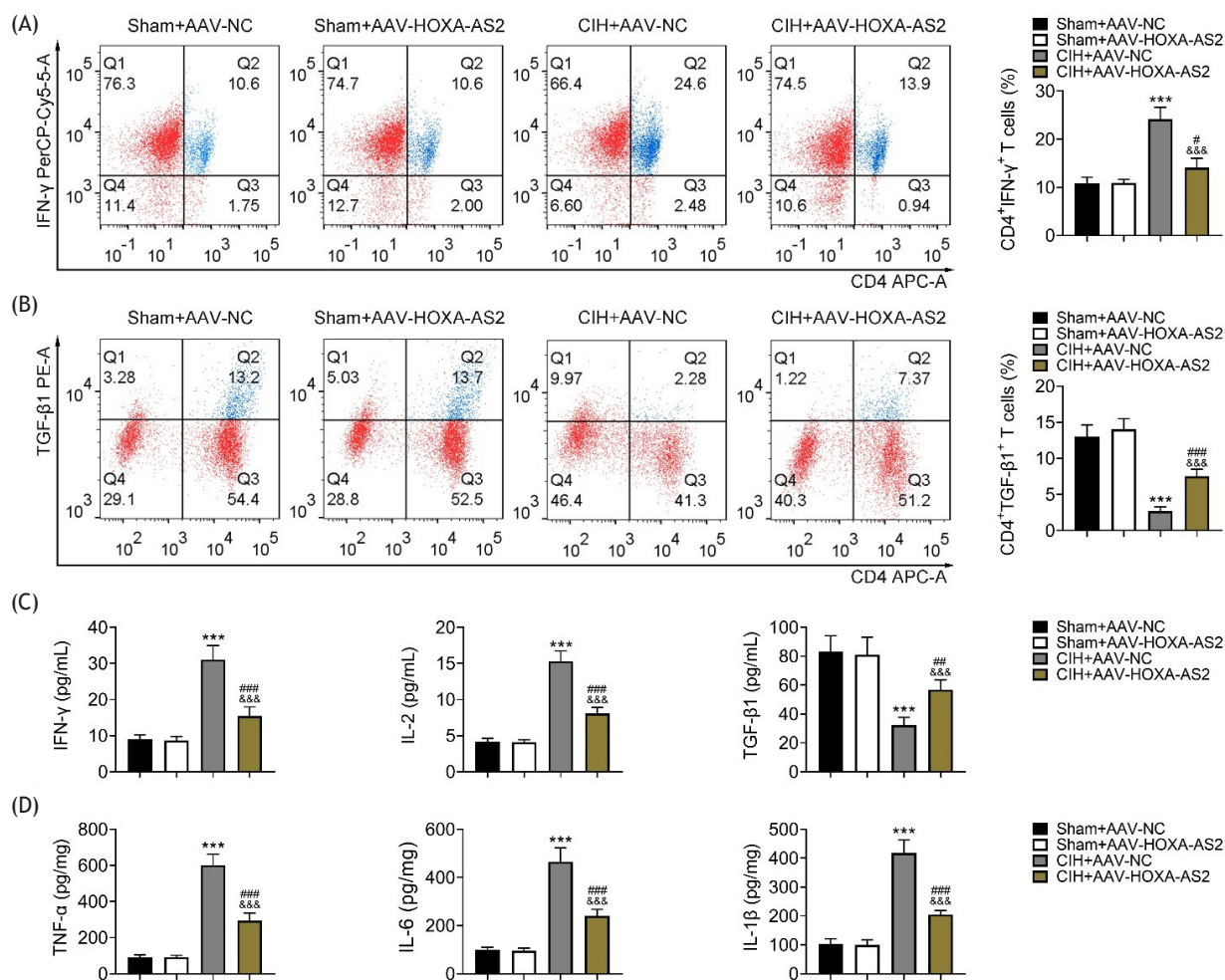


Figure 3 HOXA-AS2 reduced inflammatory response in CIH rat model. (A) AVV-HOXA-AS2 inhibited the induction of CD4⁺ IFN γ ⁺ T cells in CIH rats. (B) AVV-HOXA-AS2 reversed the suppression of CD4⁺TGF- β 1⁺ T cells in CIH rats. (C) AVV-HOXA-AS2 inhibited the induction of IFN γ and IL-2, and the reduction of TGF- β 1 in CIH rats. (D) AVV-HOXA-AS2 ameliorated the induction of TNF- α , IL-6, and IL-1 β in CIH rats. *** P < 0.005 versus sham + AVV-NC; ## P < 0.01 versus sham + AVV-HOXA-AS2; ### P < 0.005 versus sham + AVV-HOXA-AS2; &#&P < 0.005 versus CIH + AVV-NC. IFN γ : interferon gamma; TGF- β 1: transforming growth factor beta1; IL-2: interleukin-2; IL-6: interleukin-6; IL-1 β : interleukin-1 β ; TNF- α : tumor necrosis factor alpha.

results from ENCORI showed that there was a complementary sequence between *miR-17-5p* and 3'-UTR of *tipe2* (Figure 6A). Luciferase activity was reduced in 293T cells co-transfected with *miR-17-5p* mimic and *tipe2*-WT, compared to that with NC mimic and *tipe2*-WT (Figure 6B). The expression of *miR-17-5p* was elicited by *miR-17-5p* mimic and suppressed by *miR-17-5p* inhibitor, while the mRNA expression of *tipe2* was decreased by *miR-17-5p* mimic and increased by *miR-17-5p* inhibitor in NHBE cells (Figure 6C). The protein expression of *tipe2* was downregulated by *miR-17-5p* mimic and upregulated by *miR-17-5p* inhibitor in NHBE cells (Figure 6D). The mRNA and protein expressions of *tipe2* were overexpressed by upregulation of HOXA-AS2 and ameliorated after cotransfection of HOXA-AS2 and *miR-17-5p* mimic in NHBE cells (Figures 6E and F).

Discussion

Chronic intermittent hypoxia is the main feature of obstructive sleep apnea (OSA).¹⁶ CIH could induce lung

injury, causing lung fibrosis and lung cancer.^{23,24} Therefore, it is necessary to prevent CIH-induced lung injury in the early stage. In this study, lung tissue injury was observed in CIH rat model accompanying the downregulation of HOXA-AS2. The overexpression of HOXA-AS2-inhibited cell apoptosis increased the number of anti-inflammatory T lymphocytes and enhanced serum levels of anti-inflammatory molecules, thereby ameliorating lung tissue injury. Further experiments proved that HOXA-AS2 acted as an anti-inflammatory regulator through sponging *miR-17-5p* to upregulate the expression of *tipe2*, providing a new mechanism for CIH, and building up a new insight on the prevention and treatment for CIH.

Consistent with the previous studies,^{25,26} lung injury and increased levels of PaO₂ were observed in the present study also. The overexpression of HOXA-AS2 reversed CIH-induced lung injury, demonstrating a protective effect on CIH. T cell differentiation was a key procedure to defense the inflammatory injury.^{27,28} During CIH, CD4⁺ T cell differentiated into pro-inflammatory subtype,

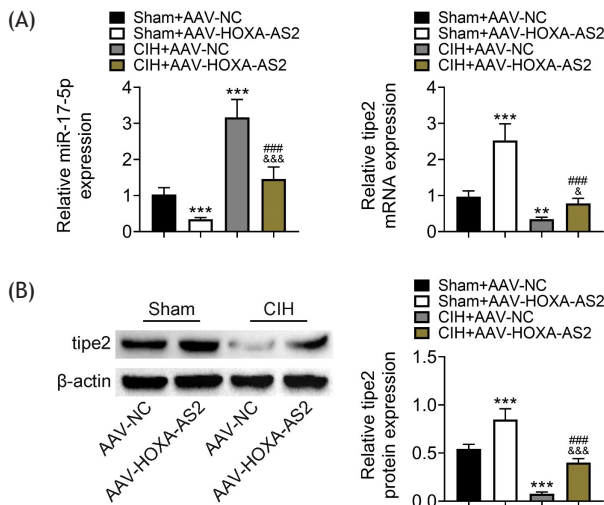


Figure 4 *HOXA-AS2* regulated the expressions of *miR-17-5p* and *tpe2* in CIH rat model. (A) AAV-*HOXA-AS2* downregulated the expression of *miR-17-5p* and upregulated the mRNA of *tpe2* in CIH rats. (B) AAV-*HOXA-AS2* upregulated the protein expression of *tpe2* in CIH rats. ** $P < 0.01$ versus sham + AAV-NC; *** $P < 0.005$ versus sham + AAV-NC; ### $P < 0.005$ versus sham + AAV-*HOXA-AS2*; * $P < 0.05$ versus CIH + AAV-NC; *** $P < 0.005$ versus CIH + AAV-NC. *Tipe2*: tumor necrosis factor- α -induced protein 8-like 2.

CD4⁺IFN γ ⁺ T cells, but not anti-inflammatory subtype, CD4⁺TGF- β 1⁺ T cells, which were ameliorated by the over-expression of *HOXA-AS2*; this demonstrated that *HOXA-AS2* played an anti-inflammatory role in CIH. Upregulation of pro-inflammatory molecules and downregulation of anti-inflammatory molecules were also observed in the present

study, and *HOXA-AS2* reversed inflammatory response in the lung tissues of CIH rat model. This was the first study to describe that *HOXA-AS2* inhibited lung inflammatory response in CIH rat model. The anti-inflammatory effects of *HOXA-AS2* were consistent with the conclusions of previous findings.^{17,29}

LncRNAs can regulate transcriptional process through two different ways: (1) recruiting regulatory factors to modulate the expression of neighboring genes; and (2) acting as competing endogenous RNAs (ceRNAs) to upregulate target proteins through directly sponging miRNAs.¹⁰ In this study, ENCORI prediction and luciferase assay proved that *miR-17-5p* was the target miRNA of *HOXA-AS2*. It is well known that miRNAs suppressed translational process by binding to 3'-untranslated regions (3'-UTR) of target mRNA,¹² and some studies have reported that miRNA played a crucial role in lung diseases.^{30,31} ENCORI predictive assay demonstrated that there was a complementary sequence between *miR-17-5p* and 3'UTR of *tpe2*, suggesting that *tpe2* was the direct target of *miR-17-5p*. Results of luciferase assay, qRT-PCR, and Western blotting analysis further confirmed this conclusion.

Tipe2 is an anti-inflammatory regulator in many biological processes.²⁰ In the lungs, *tipe2* can regulate inflammation and maintain immune homeostasis in asthma.³² The functional role of *tipe2* in CIH-induced lung inflammation has been described for the first time. The mRNA and protein expression of *tipe2* were elevated by *HOXA-AS2*. The expression level of *tipe2* was synchronized with *HOXA-AS2* but negatively correlated with *miR-17-5p*. Therefore, it can be summarized that *HOXA-AS2* exerted its anti-inflammatory effect through *HOXA-AS2/miR-17-5p/tipe2* axis, demonstrating a new mechanism of CIH and providing new therapeutic targets for CIH.

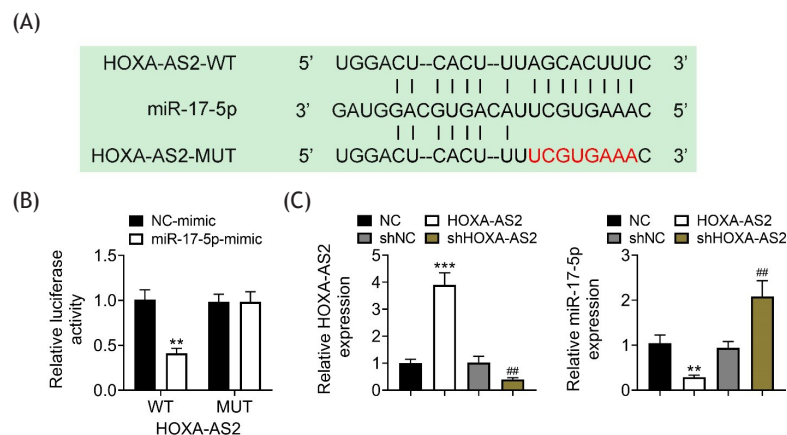


Figure 5 *miR-17-5p* was the direct target of *HOXA-AS2*. (A) There was a complementary sequence between *HOXA-AS2* and *miR-17-5p*. (B) Luciferase activity was decreased in 293T cells co-transfected with *HOXA-AS2*-WT and *miR-17-5p* mimic, compared to that with *HOXA-AS2*-WT and NC mimic. (C) Repression of *HOXA-AS2* reduced the expression of *HOXA-AS2* but increased the expression of *miR-17-5p* normal human bronchial epithelial (NHBE) cells. ** $P < 0.01$ versus NC; *** $P < 0.005$ versus NC; ## $P < 0.01$ versus shNC. *HOXA-AS2*-WT: psiCHECK2 contained full sequence of *HOXA-AS2*; *HOXA-AS2*-MUT: psiCHECK2 contained mutant sequence of *HOXA-AS2*; *HOXA-AS2*: adeno-associated virus containing *HOXA-AS2* gene; NC: negative control of *HOXA-AS2*; sh*HOXA-AS2*: short hairpin of *HOXA-AS2*; shNC: negative control of sh*HOXA-AS2*.

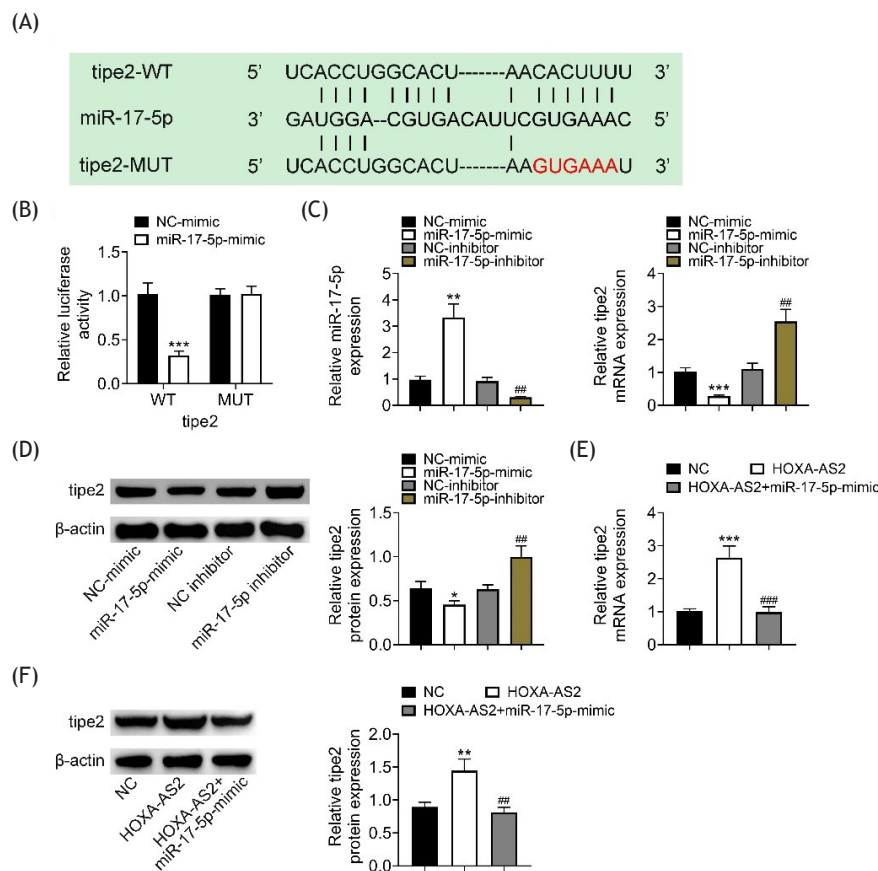


Figure 6 *Tipe2* was the direct target of *miR-17-5p*. (A) There was a complementary sequence between 3'-UTR of *tipe2* and *miR-17-5p*. (B) Luciferase activity was decreased in 293T cells co-transfected with *tipe2*-WT and *miR-17-5p* mimic, compared to that with *tipe2*-WT and NC mimic. (C) *miR-17-5p* mimic increased the expression of *miR-17-5p* and decreased the expression of *tipe2* mRNA in NHBE cells. (D) The protein expression was decreased by *miR-17-5p* mimic and increased by *miR-17-5p* inhibitor in NHBE cells. (E) *miR-17-5p* mimic reversed *HOXA-AS2*-induced upregulation of *tipe2* mRNA in NHBE cells. (F) *miR-17-5p* mimic reversed *HOXA-AS2*-induced upregulation of *tipe2* protein in NHBE cells. * $P < 0.05$ versus NC mimic; ** $P < 0.01$ versus NC mimic; *** $P < 0.005$ versus NC mimic; ## $P < 0.01$ versus NC inhibitor or *HOXA-AS2*; ### $P < 0.005$ versus NC inhibitor or *HOXA-AS2*. NC: negative control.

Conclusion

This study manifested that *HOXA-AS2* acted as an anti-inflammatory regulator and protected lung tissue injury from CIH in a rat model. By directly targeting *miR-17-5p*, *HOXA-AS2* positively regulated the expression of *tipe2*, which played an anti-inflammatory role in CIH, thus preventing lung injury from CIH. The findings of this study provided new understanding and therapeutic targets for CIH to facilitate a new drug discovery for CIH treatment.

Availability of Data and Materials

All data generated and analyzed during this study are included in this article.

Competing interests

The authors stated that there was no conflict of interest to declare.

Author Contributions

Kun Gao and Aiai Lv did conceptualization, methodology, and writing of the original draft of this paper. Formal analysis, resources, and investigation were done by Qiang Zhang. Formal analysis, visualization, and data curation were performed by Yanzhong Li. Project administration, supervision, and validation was executed by Zhiyong Yue. Validation, supervision, and writing, including review and editing, was done by Shuai Xu. All authors read and approved the final manuscript.

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