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LncRNA TTTY15 regulates hypoxia-induced vascular endothelial cell injury *via* targeting miR-186-5p in cardiovascular disease

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Abstract. – OBJECTIVE: Dysfunction of vascular endothelial cells was associated with diverse human diseases, including cardiovascular disease. Long noncoding RNAs (LncRNAs) were involved in the regulation of cell injury. We aimed to investigate the role of lncRNA testis-specific transcript, Y-linked 15 (TTTY15) in hypoxia-induced cell injury in human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS: Cell counting kit-8 (CCK8) assay was used to check cell viability. Lactate dehydrogenase (LDH) assay kit and flow cytometry were utilized to evaluate the leakage rate of LDH and cell apoptosis, respectively. The protein levels of Cyclin D1 and B-cell lymphoma-2-Associated X (Bax) in hypoxia-induced HUVECs were measured by Western blot. Quantitative Real-time polymerase chain reaction (qRT-PCR) was conducted to detect the expression levels of TTTY15 and miR-186-5p in hypoxia-induced HUVECs. The starBase was utilized to predict the binding sites between TTTY15 and miR-186-5p and the dual-luciferase reporter assay was performed to verify the interaction.

RESULTS: Hypoxia inhibited cell viability and promoted the release of LDH and cell apoptosis in HUVECs. Besides, hypoxia significantly decreased the protein level of Cyclin D1 and increased the protein level of Bax in HUVECs. In addition, the expression level of TTTY15 was obviously upregulated in hypoxia-induced HUVECs, opposite to the level of miR-186-5p. Meanwhile, knockdown of TTTY15 or upregulation of miR-186-5p mitigated hypoxia-induced cell injury in HUVECs. Further studies suggested that TTTY15 targeted miR-186-5p and regulated hypoxia-induced cell injury via interacting with miR-186-5p in HUVECs.

CONCLUSIONS: Downregulation of TTTY15 ameliorated hypoxia-induced cell injury by targeting miR-186-5p in HUVECs.

Key Words:

Cardiovascular disease, LncRNA TTTY15, Hypoxia, Cell injury, MiR-186-5p, HUVECs.

Introduction

Vascular endothelial cells, directly in contact with blood, line the entire circulatory system and are essential building blocks of angiogenesis[1]. Growing evidence showed that vascular endothelial cells were associated with human cardiovascular disease[2,3]. Singhal et al[4] reported that vascular endothelial cells played an important role in myocardial ischemia-reperfusion injury. A previous study showed that hypoxia could regulate vascular endothelial growth factor gene expression[5]. Therefore, it is still significant and imperative to figure out the underlying mechanism of hypoxia-induced cell injury in vascular endothelial cells in cardiovascular disease.

Long noncoding RNAs (LncRNAs) are a type of RNA molecules (more than 200 nucleotides) and cannot be translated into proteins[6]. LncRNAs were reported to participate in the modulation of cell injury[7-9]. Previous reports demonstrated that lncRNA testis-specific transcript, Y-linked 15 (TTTY15) was involved in the regulation of diverse human diseases, including non-small cell lung cancer[10], prostate cancer[11] and severe burns injury[12]. Recently, lncRNA TTTY15 was found to be correlated with hypoxia-induced cardiomyocytes injury[13]. Yet the role of lncRNA TTTY15 in hypoxia-induced vascular endothelial cell injury is hardly reported and needs to be further investigated.

MicroRNAs (MiRNAs) are short (about 22 nucleotides) noncoding RNAs, which mediate gene expression via guiding Argonaute proteins to target sites in the 3'-untranslated region (3'UTR)[14]. Growing evidence has shed light on the fact that miRNAs function in the regulation of cell injury[15-17]. Jang et al[18] showed that miR-186-5p took part in the modulation of cell injury in Alzheimer's disease and high glucose-induced injury in

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AC16 cardiomyocytes[19]. It was also reported that miR-186-5p was correlated with vascular endothelial cell proliferation and apoptosis. However, the precise regulatory mechanism of miR-186-5p in hypoxia-induced vascular endothelial cell injury remains poorly understood.

In this research, the expression level of lncRNA TTTY15 in hypoxia-induced human umbilical vein endothelial cells (HUVECs) was measured. The function and underlying regulatory mechanism of lncRNA TTTY15 in hypoxia-induced cell injury in HUVECs were further explored by subsequent experiments.

Material and Methods

Cell Culture and Treatment

Human umbilical vein endothelial cells (HUVECs) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Roswell Park Memorial Institute (RPMI) medium (Sigma-Aldrich, St Louis, MO, USA), containing 5% CO_2 and 10% fetal bovine serum (FBS; Sigma-Aldrich) was hired to culture cells. For hypoxia treatment, HUVECs were cultured in Hypoxia Chamber Glove Box (Plas-Labs, Lansing, MI, USA) with 94% N_2, 5% CO_2 and 1% O_2. For the normoxia treatment, the condition was 74% N_2, 5% CO_2 and 21% O_2 according to a previous report[20].

Cell Transfection

Small interfering RNA against lncRNA TTTY15 (named as si-TTTY15), miR-186-5p mimic (named as miR-186-5p) and miR-186-5p inhibitor (named as Anti-miR-186-5p), as well as their corresponding controls (si-NC, miR-NC, Anti-NC) were obtained from GenePharma (Shanghai, China). Cell transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) referring to the given procedures.

Counting Kit-8 (CCK-8) Assay

After treatment, HUVECs were seeded into 96-well plates and then incubated with 10 μL CCK-8 solution (Beyotime, Shanghai, China) for 2 h. Optical density values were examined at 450 nm wavelength under the microplate reader (Bio-Rad, Hercules, CA, USA).

Lactate Dehydrogenase (LDH) Activity Measurement

The leakage rate of LDH was analyzed to assess the cell injury. The released LDH in the collected medium was detected using LDH assay kit (Beyotime) according to a previous report [21].

Flow Cytometry

Annexin Apoptosis Detection Kit (Sigma-Aldrich, St. Louis, MO, USA) was hired to measure cell apoptosis according to the provided procedures. In a word, cells were resuspended using the binding buffer and then 5 μL Annexin V-fluorescein isothiocyanate (Annexin V-FITC) and 5 μL propidium iodide (PI) were added to the buffer to incubate for 5 min in the dark. The stained cells were analyzed by flow cytometry (Countstar, Shanghai, China).

Western Blot

Proteins from cells were isolated using RIPA buffer (Vazyme, Nanjing, China) and were segregated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Afterwards, the proteins were transferred onto the polyvinylidene difluoride (PVDF) membranes (Vazyme) and then the membranes were blocked by 5% non-fat milk (Vazyme). After being washed by phosphate-buffered saline (PBS), the membranes were incubated with the primary antibodies: anti-Cyclin D1 (1:2000, ab226977, Abcam, Cambridge, United Kingdom), anti-B-cell lymphoma-2-Associated X (Bax) (1:1000, ab199677, Abcam) or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:2500, ab9485, Abcam) overnight. After the rewashing with PBS, the membranes were incubated with the secondary antibody (1:3000, ab205718, Abcam) for 3 h. Finally, the membranes were treated with the ECL kit (Vazyme) and then were analyzed using the ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA).

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using the TRIzol reagent (Vazyme). The PrimeScript™ RT Master Mix kit (TaKaRa, Dalian, China) was used to transcribe RNA to complementary DNA (cDNA). The SYBR Green PCR Master Mix (Vazyme) was employed to conduct the qRT-PCR and the data were analyzed using $2^{-\Delta\Delta Ct}$ method. Beta-actin (β-actin) and U6 were used as the inner controls. Primers used in our research: TTTY15 (forward 5'-TCTATGACCTGGAAGC-3', reverse 5'-ATCTGATGGAACCCTA-3'); miR-186-5p (forward, 5'-GCCAATCTGGTATCGATCTACCGTG-3'; reverse, 5'-GG-

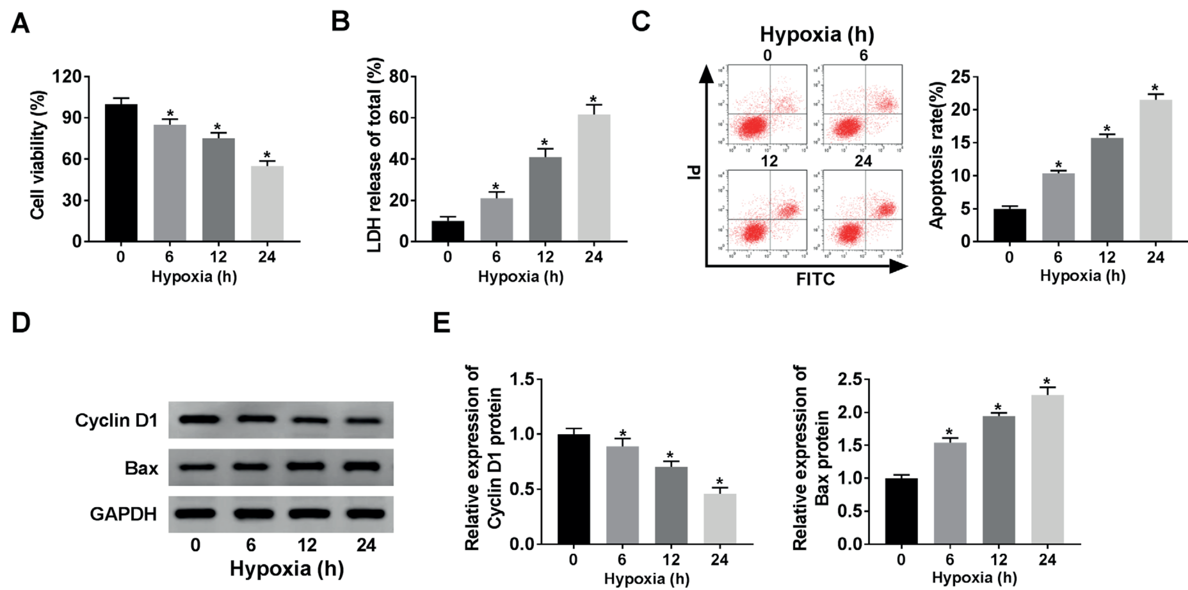

Figure 1. Hypoxia contributed to cell injury in HUVECs. **(A)** The CCK-8 assay was used to check the viability of HUVECs under hypoxia treatment for different time (0, 6, 12 and 24 h). **(B)** LDH assay kit was utilized to evaluated cell injury in different conditions. **(C)** Flow cytometry assay was hired to check cell apoptosis. **(D and E)** The protein levels of Cyclin D1 and Bax in hypoxia-induced HUVECs were measured by Western blot. *$p < 0.05$.

GCAGTGTGACGTTGCGT-3'); β-actin (forward 5'-GCACCACACCTTCTACAATG-3', reverse, 5'-TGCTTGCTGATCCACATCTG-3'); U6 (forward, 5'-TCCGGGTGATGCTTTTCCTAG-3', reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3').

Dual-Luciferase Reporter Assay

The potential target sites between lncRNA TTTY15 and miR-186-5p were predicted by starBase[22]. The wild type sequence of TTTY15 harboring the target sites of miR-186-5p was inserted into the pGL3 vector (Promega, Madison, WI, USA) to construct the luciferase reporter vector wt-TTTY15. Similarly, the mut-TTTY15 reporter vector was built by mutating the potential target sites of miR-186-5p. After that, the vectors with miR-186-5p or miR-NC were cotransfected into HUVECs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The luciferase activity was measured by the Dual-Glo® Luciferase Assay System kit (Promega).

Statistical Analysis

Experimental data were calculated using GraphPad Prism (GraphPad, La Jolla, CA, USA) and the data were presented by mean ± standard deviation (SD). Two independent groups were compared by using Student's *t*-test. The one-way analysis of variance (ANOVA) with post-hoc Tukey's test was utilized to assess the difference for more than two groups. Every experiment was repeated at least three times independently. $p < 0.05$ was considered as statistically significant.

Results

Hypoxia Induced Cell Injury in HUVECs

To explore the effect of hypoxia on cell injury, HUVECs were first cultured in the hypoxia chamber for the different time. Later, cell viability was checked, and the result showed that the viability of HUVECs was gradually declined with the increasing treatment time of hypoxia (Figure 1A). Next, we checked the leakage rate of LDH and found that the rate was positively associated with the treatment time of hypoxia (Figure 1B). Also, apoptosis assay showed that the more treatment time of hypoxia, the higher cell apoptosis rate was observed in HUVECs (Figure 1C). Besides, the protein levels of proliferation-related protein (Cyclin D1) and apoptosis-related protein (Bax) were measured, and the data showed that the level of Cyclin D1 was gradually decreased with the increasing treatment time of hypoxia, whereas the expression of Bax was gradually in-

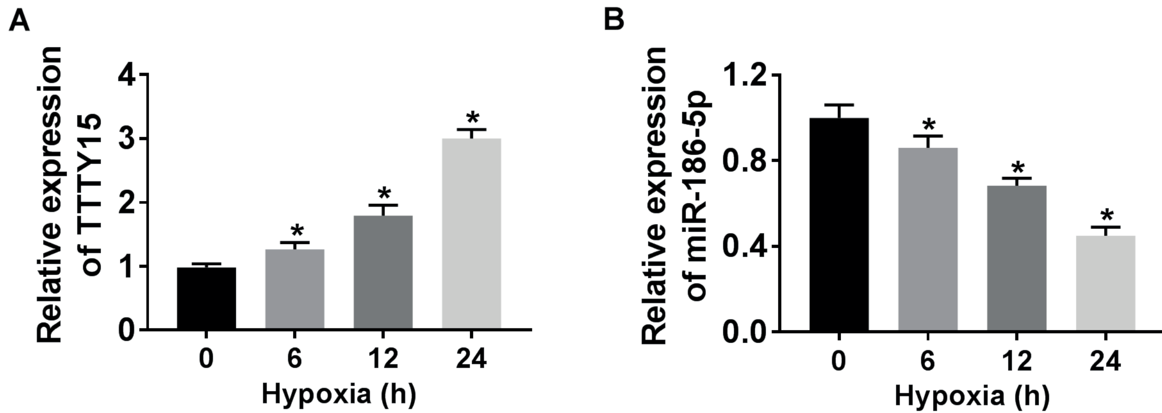

Figure 2. Hypoxia treatment elevated the expression of lncRNA TTTY15 and decreased the level of miR-186-5p. **(A** and **B)** The expression levels of lncRNA TTTY15 and miR-186-5p in HUVECs under hypoxia treatment for different time (0, 6, 12 and 24 h) were detected by qRT-PCR. *$p < 0.05$.

creased (Figure 1D and 1E). Altogether, these results illuminated that hypoxia could induce cell injury in HUVECs in a time-dependent manner.

LncRNA TTTY15 was Significantly Upregulated in Hypoxia-Induced HUVECs, Opposite to the Expression Level of miR-186-5p

To probe the role of lncRNA TTTY15 and miR-186-5p in hypoxia-induced HUVECs, we measured their expression levels. The data showed that lncRNA TTTY15 was significantly upregulated in hypoxia-induced HUVECs (Figure 2A), while miR-186-5p was markedly downregulated in hypoxia-induced HUVECs (Figure 2B). From these results, it could be concluded that lncRNA TTTY15 and miR-186-5p might be associated with the regulation of hypoxia-induced cell injury in HUVECs.

Knockdown of lncRNA TTTY15 Attenuated Hypoxia-Induced Cell Injury in HUVECs

To further explore the function of lncRNA TTTY15 in hypoxia-induced cell injury, HUVECs were first transfected with si-TTTY15 or si-NC and the knockdown efficiency of si-TTTY15 was verified (Figure 3A). Hereafter, the viability of hypoxia-induced HUVECs transfected with si-TTTY15 or si-NC was checked, and the result showed that downregulation of lncRNA TTTY15 apparently promoted cell viability (Figure 3B). In addition, knockdown of lncRNA TTTY15 clearly inhibited the release of LDH in hypoxia-induced HUVECs (Figure 3C). Moreover, lncRNA TTTY15 silencing remarkedly suppressed the apoptosis of hypoxia-induced HUVECs (Figure 3D). Further analysis indicated that the silence of lncRNA TTTY15 strikingly elevated the protein level of Cyclin D1 and notably decreased the protein level of Bax in hypoxia-induced HUVECs (Figure 3E and 3F). To sum up, these results demonstrated that downregulation of TTTY15 mitigated hypoxia-induced cell injury in HUVECs.

Upregulation of miR-186-5p Mitigated Hypoxia-Induced Cell Injury in HUVECs

To further dissect the function of miR-186-5p in hypoxia-induced cell injury, miR-186-5p and miR-NC were introduced into HUVECs and the overexpression efficiency of miR-186-5p was detected (Figure 4A). Afterwards, the viability of hypoxia-induced HUVECs transfected with miR-186-5p or miR-NC was evaluated, and the data showed that upregulation of miR-186-5p significantly boosted cell viability (Figure 4B). Besides, miR-186-5p mimic strikingly reduced the leakage rate of LDH in hypoxia-induced HUVECs (Figure 4C). In addition, enforced expression of miR-186-5p conspicuously restrained the apoptosis of hypoxia-induced HUVECs (Figure 4D). Meanwhile, upregulating miR-186-5p clearly increased the protein level of Cyclin D1 and significantly decreased the protein level of Bax in hypoxia-induced HUVECs (Figure 4E and 4F). All in all, these results manifested that upregulation of miR-186-5p attenuated hypoxia-induced cell injury in HUVECs.

LncRNA TTTY15 Targeted miR-186-5p and Negatively Regulated miR-186-5p Expression

The interaction between lncRNAs and miRNAs was documented in many cell injury-relat-

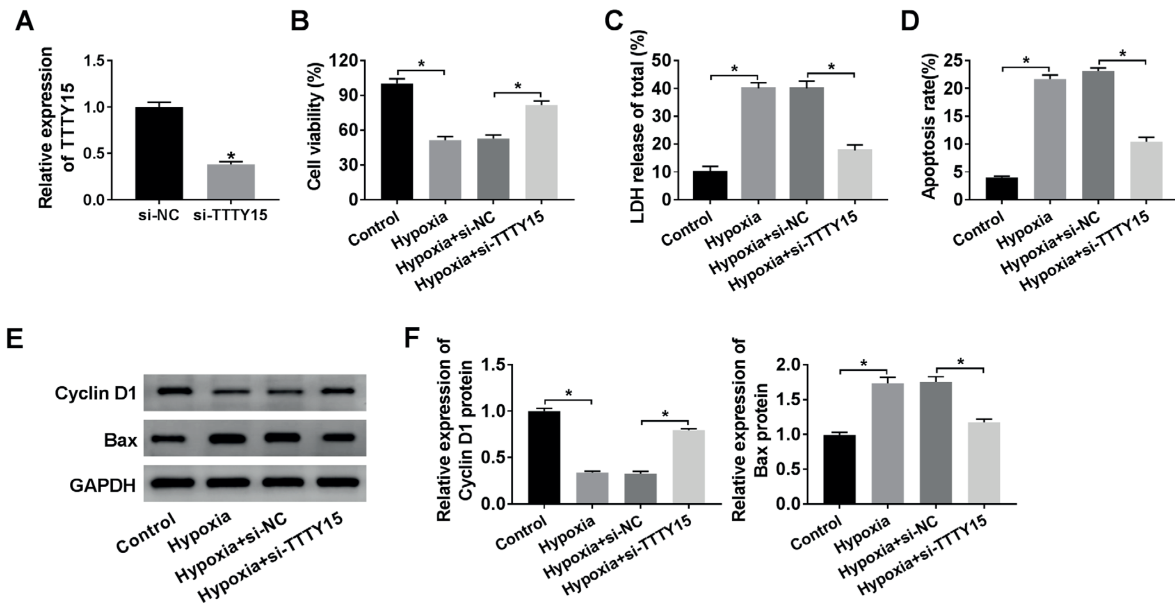

Figure 3. Knockdown of lncRNA TTTY15 extenuated hypoxia-induced cell injury in HUVECs. **(A)** The level of lncRNA TTTY15 in HUVECs transfected with si-TTTY15 or si-NC was assessed by qRT-PCR. **(B)** The viability of HUVECs treated with Hypoxia or Hypoxia + si-TTTY15, as well as matched controls, was checked by CCK8 assay. **(C)** The leakage rates of LDH in Control and treated HUVECs were estimated by the LDH assay kit. **(D)** The apoptosis rates in Control and treated HUVECs were checked by flow cytometry. **(E** and **F)** The protein levels of Cyclin D1 and Bax in samples were measured by Western blot. *$p < 0.05$.

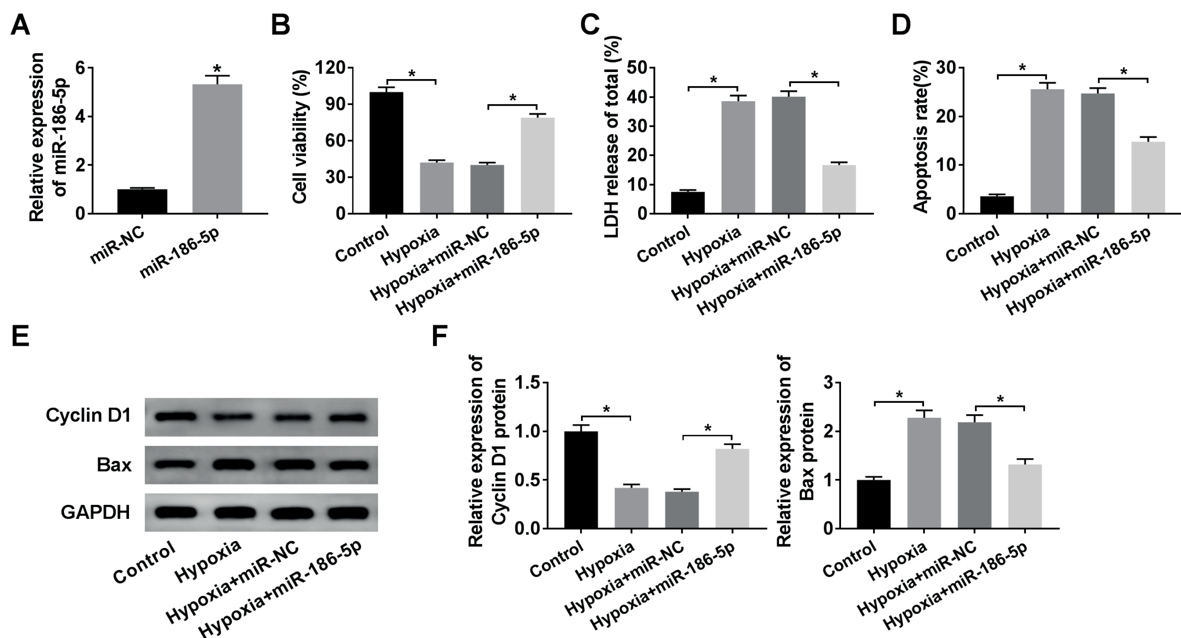

Figure 4. MiR-186-5p mimic eased hypoxia-induced cell injury in HUVECs. **(A)** The level of miR-186-5p in HUVECs transfected with miR-186-5p or miR-NC was checked by qRT-PCR. **(B)** The viability of HUVECs treated with Hypoxia or Hypoxia + miR-186-5p, as well as matched controls, was checked by CCK8 assay. **(C)** The leakage rates of LDH in Control and treated HUVECs were evaluated by LDH assay kit. **(D)** Cell apoptosis was detected by flow cytometry. **(E** and **F)** The protein levels of Cyclin D1 and Bax in Control and treated HUVECs were measured by Western blot. *$p < 0.05$.

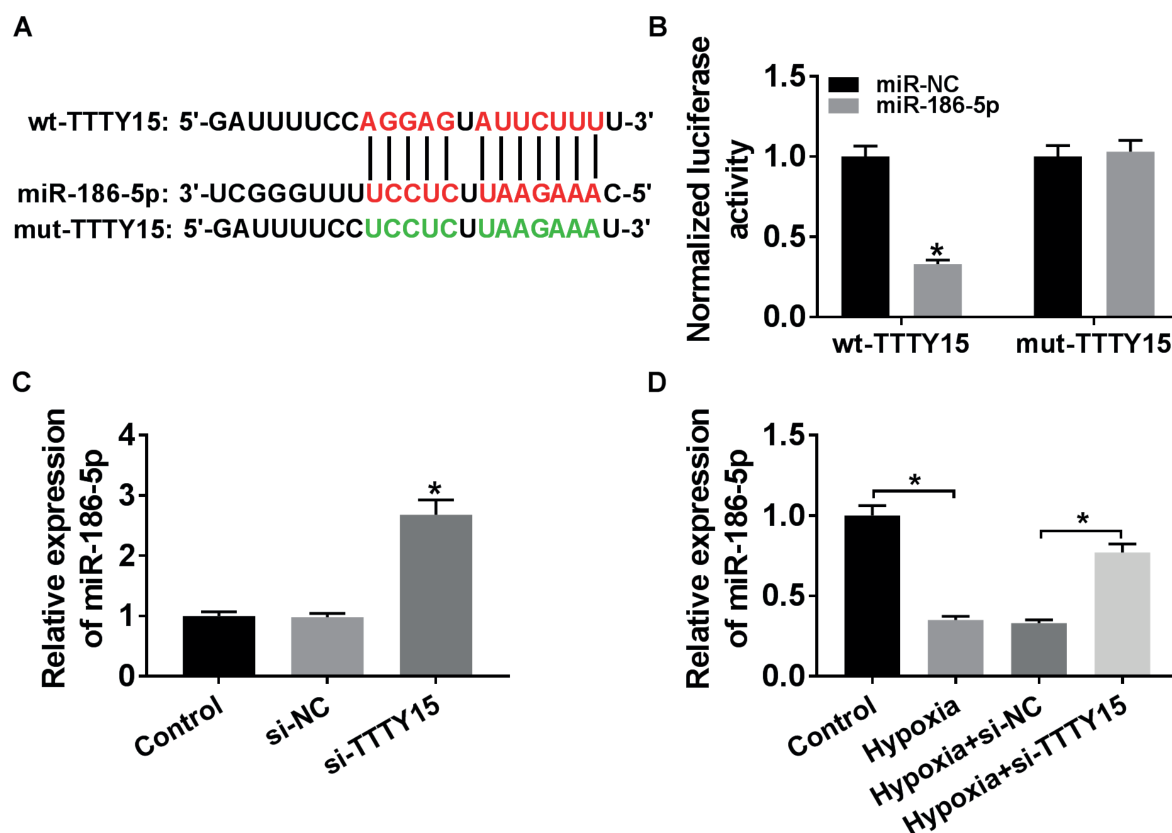

Figure 5. LncRNA TTTY15 targeted miR-186-5p and negatively modulated the expression of miR-186-5p. **(A)** The possible binding sites between lncRNA TTTY15 and miR-186-5p were predicted by starBase. **(B)** The dual-luciferase reporter assay was performed to verify the interaction between lncRNA TTTY15 and miR-186-5p. **(C)** The expression level of miR-186-5p in Control and HUVECs transfected with si-TTTY15 or si-NC was measured by qRT-PCR. **(D)** The expression level of miR-186-5p in HUVECs treated with Hypoxia or Hypoxia + si-TTTY15, as well as corresponding controls was determined by qRT-PCR. $*p < 0.05$.

ed papers[7,9]. In this research, miR-186-5p was predicted to be a target of lncRNA TTTY15 by starBase (Figure 5A) and the dual-luciferase reporter assay indicated that miR-186-5p remarkedly reduced the luciferase activity of wt-TTTY15 in HUVECs, rather than mut-TTTY15 (Figure 5B). Subsequently, we measured the expression level of miR-186-5p and found that knockdown of TTTY15 notably elevated the expression level of miR-186-5p compared with corresponding controls in HUVECs (Figure 5C). Similarly, miR-186-5p was also upregulated in hypoxia-induced HUVECs transfected with si-TTTY15 compared with the transfection with si-NC (Figure 5D). In summary, our results illustrated that lncRNA TTTY15 could interact with miR-186-5p and negatively modulated the expression of miR-186-5p.

MiR-186-5p Depletion Rescued lncRNA TTTY15 Silencing-Mediated Effect on Hypoxia-Induced Cell Injury in HUVECs

To figure out the potential regulatory mechanism of miR-186-5p and lncRNA TTTY15 in hypoxia-induced cell injury, Anti-miR-186-5p and Anti-NC were employed to transfect HUVECs and the knockdown efficiency of Anti-miR-186-5p was corroborated (Figure 6A). Thereafter, the viability of hypoxia-induced HUVECs transfected with si-TTTY15 or si-TTTY15 + Anti-miR-186-5p, as well as corresponding controls, were checked and the result showed that miR-186-5p inhibitor inverted lncRNA TTTY15 silencing-mediated promoted effect on the viability of hypoxia-induced HUVECs (Figure 6B). Simultaneously, the reduced leakage rate of LDH in Hypoxia + si-TTTY15 group was reversed following the transfection with miR-186-5p

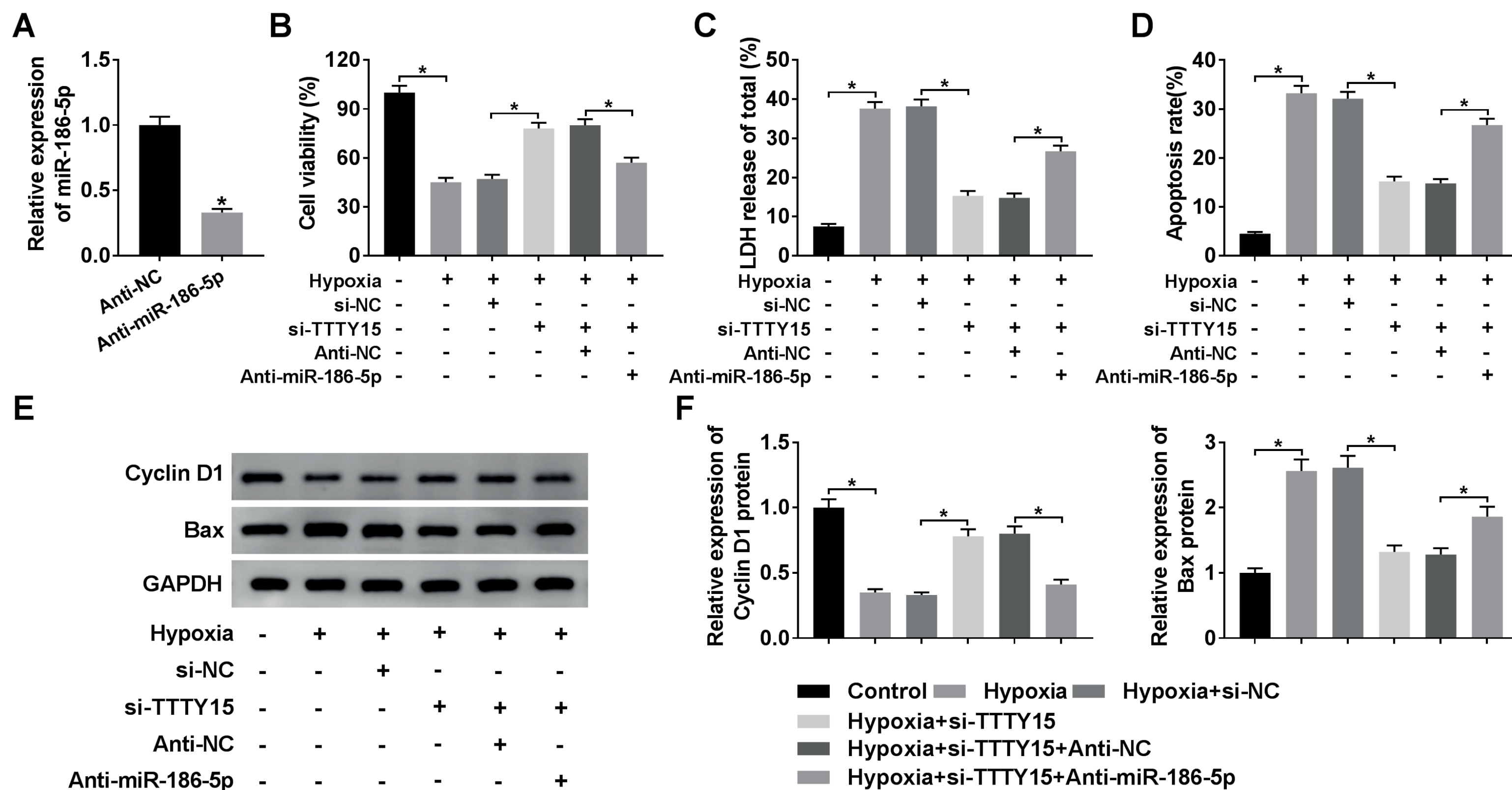

Figure 6. MiR-186-5p inhibitor inversed lncRNA TTTY15 silencing-mediated impact on hypoxia-induced cell injury in HUVECs. **(A)** The expression level of miR-186-5p in HUVECs transfected with Anti-miR-186-5p or Anti-NC was checked by qRT-PCR. **(B)** The viability of HUVECs treated with Hypoxia, Hypoxia + si-TTTY15, Hypoxia + si-TTTY15 + Anti-miR-186-5p or matched controls was measured by CCK8 assay. **(C)** The leakage rates of LDH in treated HUVECs were evaluated by LDH assay kit. **(D)** The apoptosis of treated HUVECs was detected by flow cytometry. **(E and F)** The protein levels of Cyclin D1 and Bax in treated HUVECs were measured by Western blot. *$p < 0.05$.

inhibitor (Figure 6C). In addition, downregulation of miR-186-5p overturned lncRNA TTTY15 silencing-mediated repressive impact on the apoptosis of hypoxia-induced HUVECs (Figure 6D). Also, the increased protein level of Cyclin D1 and the declined protein level of Bax in Hypoxia + si-TTTY15 group were transposed after the additional transfection with Anti-miR-186-5p (Figure 6E and 6F). Taken together, these results suggested that lncRNA TTTY15 mediated hypoxia-induced cell injury via interacting with miR-186-5p in HUVECs.

Discussion

Vascular endothelial cells play a pivotal role in circulatory system and angiogenesis[1]. The dysfunction of vascular endothelial cells was associated with the pathogenesis and initiation of diverse cardiovascular diseases[23,24]. Hypoxia is a vital regulator of blood vessel tone and structure and is also a potent stimulus of angiogenesis[5,25]. In this research, we found that hypoxia induced cell injury in HUVECs. Hence, understanding the hidden regulatory mechanism of hypoxia-induced cell injury in vascular endothelial cells will contribute to the improvement of therapeutic approaches for some cardiovascular diseases.

LncRNAs have been proved to regulate hypoxia-induced cell injury. Wu et al[7] found that lncRNA TUG1 was associated with hypoxia-induced myocardial cell injury. Teng et al[26] reported that lncRNA RMRP accelerated hypoxia-induced injury in H9c2 cells. Chen et al[27] reported that lncRNA H19 silencing protected PC-12 cells against hypoxia-induced cell damage. Also, Huang et al[13] reported that the suppression of lncRNA TTTY15 attenuated hypoxia-induced cardiomyocytes injury. In our research, we checked the expression level of lncRNA TTTY15 and found that lncRNA TTTY15 was significantly upregulated in hypoxia-induced HUVECs. Next, the function of lncRNA TTTY15 in hypoxia-induced cell injury was explored and the results indicated that downregulation of lncRNA TTTY15 markedly promoted cell viability and suppressed the release of LDH and cell apoptosis in hypoxia-induced HUVECs. Besides, the increased protein level of Cyclin D1 and the declined protein level of Bax were observed in HUVECs treated with Hypoxia + TTTY15. Together, these results demonstrated that downregulation of lncRNA TTTY15 could relief hypoxia-induced cell injury in HUVECs.

Increasing reports showed that lncRNAs could target miRNAs to regulate cell injury[7,9]. In this study, miR-186-5p was verified to be a target of lncRNA TTTY15 and was negatively modulated by lncRNA TTTY15 in hypoxia-induced HUVECs. MiR-186-5p was reported to be associated with cell injury in various diseases. Wu et al[18] reported that miR-186-5p was involved in the regulation of oxidative stress injury of neuron in Alzheimer's disease. Jiang et al[19] found that downregulation of miR-186-5p led to high glucose-induced injury in AC16 cardiomyocytes. Our research showed miR-186-5p depletion attenuated hypoxia-induced cell injury in HUVECs. Further investigation demonstrated that downregulation of miR-186-5p rescued lncRNA TTTY15 silencing-mediated promoted effect on cell viability and the suppressive impact on LDH release and cell apoptosis in hypoxia-induced HUVECs. Taken together, our results suggested that lncRNA TTTY15 mediated hypoxia-induced cell injury via targeting miR-186-5p in HUVECs.

Conclusions

The findings of this demonstrated that lncRNA TTTY15 silencing ameliorated hypoxia-induced cell injury via interacting with miR-186-5p in HUVECs and lncRNA TTTY15 might be an effective diagnostic marker and therapeutic target for hypoxia-induced cell injury.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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