The protective effects of Azilsartan against hypoxia in endometrial stromal cells: an implication in endometriosis

Keywords
endometriotic lesions, HIF-1α, angiotensin II receptor, hypoxia, Endometriosis, Azilsartan (AZL)

Abstract
Introduction
Endometriosis is a reproductive disorder closely associated with hypoxia stress. Increasing evidences have implied the important roles of angiotensin II (ANG II) receptors in the pathophysiology of endometriosis. Thus, we speculated that Azilsartan (AZL), an ANG II receptor blocker, might have an effective function in controlling endometriosis.

Material and methods
The endometriotic animal model was established in female SD rats (6-8 weeks old, 200-220 g). Rats were divided into sham group and endometriosis (EMS) group. Rats in EMS group were anaesthetized by halothane and a mid-ventral incision was performed to expose the bowels. Human endometrial stromal cell line T-HESC was used for the in vitro assays. The T-HESC cells were cultured in DMEM-F12 mediumwith 10% fetal bovine serum (FBS, Hyclone), and 4 mM L-glutamine, 0.25% HEPES plus necessary antibiotics (Sigma-Aldrich, USA), at 37°C in a humidified atmosphere of 5% CO2.

Results
The results show that upregulation of ANG II type 1 (AT1) receptor was observed in the endometriotic rat models. Treatment with AZL prevented the development of endometriotic lesions and suppressed the expressions of HIF-1α and cyclooxygenase 2 (COX-2) in endometriotic rats. In vitro assays proved that hypoxia-induced proliferation, migration, and invasion of T-HESC cells were attenuated by AZL. AZL inhibited the expression levels of hypoxia-inducible factor-1α (HIF-1α), COX-2, and prostaglandin E2 (PGE2) production in hypoxia-induced T-HESC cells. Overexpression of HIF-1α blocked the effects of AZL on T-HESC cells in response to hypoxia.

Conclusions
AZL showed therapeutic function on endometriosis through inhibiting hypoxia-induced cell proliferation, migration, and invasion of T-HESC cells via HIF-1α/COX-2/PGE2 signaling.

Explanation letter
Dear Editorial Board,
Thank you and the reviewers for the generous comments on the manuscript (AMS-14327-2022-01) titled “The protective effects of Azilsartan against hypoxia in endometrial stromal cells: an implication in endometriosis”. We have gone over all the points that were raised and have edited the manuscript to address all the concerns of the editor and the reviewers. I attached an improved version of the manuscript. All the changes made are highlighted in red. Thank you in advance for your continued consideration and support of our manuscript. We truly appreciate the opportunity to submit this revision.
Point by point reply to comments:
Review 1:
This is a very interesting paper by Chen et al. about the effects of Azilsartan, a blocker of ANG II receptor, on controlling endometriosi. Overall the article is well written, the experiments are well done and the conclusions are supported by the experimental data. However, there are a couple of points

that in my opinion need to be addressed before publication:
Major points:
1. In Figure 7A, the authors only examined the levels of COX-2 at the mRNA levels. Protein levels of COX-2 should be measured by western blot analysis and the results should be included in this Figure.
Answer: Thank you. As per recommendation, protein levels of COX-2 were measured with Western blot analysis. The results have been included in Figure 7B.

2. The authors should clarify the rationale of the choice of the concentrations of Azilsartan used in this study.
Answer: Thank you. The concentrations used in this study are based on previous studies. To address your concerns, the following articles have been cited in the revised manuscript.
[11]. Pan B, Zheng L, Fang J, Lin Y, Lai H, Gao J, Pan W, Zhang Y, Ni K, Lou C, He D. Azilsartan Suppresses Osteoclastogenesis and Ameliorates Ovariectomy-Induced Osteoporosis by Inhibiting Reactive Oxygen Species Production and Activating Nrf2 Signaling. Front Pharmacol. 2021;12:774709.
[13]. Dong Q, Li Y, Chen J, Wang N. Azilsartan Suppressed LPS-Induced Inflammation in U937 Macrophages through Suppressing Oxidative Stress and Inhibiting the TLR2/MyD88 Signal Pathway. ACS Omega, 2021;6(1):113-118.

Minor points:
3. English editing needs to be done before publication.
Answer: Thank you. To address your concerns, we have invited a native English speaker in our field to revise the spelling and grammar of this manuscript. All grammatical errors have been eliminated. Corrections are marked in red in the revised manuscript.
4. Scale bars are missing in Figure 1.
Answer: Thank you. Scale bars have been added in Figure 1
5. Molecular weights are missing in Figure 5 and Figure 8.
Answer: Thank you. Molecular weights have been added in Figure 5, Figure 7B, and Figure 8.
Review 2:
The authors showed therapeutic function of Azilsartan on endometriosis through inhibiting hypoxia-induced cell proliferation, migration, and invasion of T-HESC cells via HIF-1α/COX-2/PGE2 signaling The manuscript need improvement in many ways.
- Add clearly the hypothesis, aims and goals of this work to the last paragraph to your introduction.
Answer: Thank you. The following content has been added to the last paragraph of the “Introduction” section to clarify the aims of this work:
“However, whether AZL possesses a protective effect against endometriosis is still unknown. We speculated that AZL might have an effective function in controlling endometriosis. Here, we studied its effects on endometriosis development in vivo and hypoxia-induced metastasis of endometrial stromal cells in vitro”.
- Methods should have appropriate citation of references. Check and revise them properly.
Answer: Thank you. As per recommendation, the following articles have been cited in the “Materials and Methods” section of the revised manuscript.
[11]. Pan B, Zheng L, Fang J, Lin Y, Lai H, Gao J, Pan W, Zhang Y, Ni K, Lou C, He D. Azilsartan Suppresses Osteoclastogenesis and Ameliorates Ovariectomy-Induced Osteoporosis by Inhibiting Reactive Oxygen Species Production and Activating Nrf2 Signaling. Front Pharmacol. 2021;12:774709.
[12] Y. Wang, M. Zhang, R.Bi, et al. ACSL4 deficiency confers protection against ferroptosis-mediated acute kidney injury. Redox Biol, 51(2022):102262.
[13]. Dong Q, Li Y, Chen J, Wang N. Azilsartan Suppressed LPS-Induced Inflammation in U937 Macrophages through Suppressing Oxidative Stress and Inhibiting the TLR2/MyD88 Signal Pathway. ACS Omega, 2021;6(1):113-118.
[14] Y. Han, X. Qian, T. Xu, Y. Shi, Carcinoma-associated fibroblasts release microRNA-331-3p containing extracellular vesicles to exacerbate the development of pancreatic cancer via the SCARA5-FAK axis. Cancer Biol Ther, 23(2022):378-392.

--Materials and methods are poorly written, many details are missed
Answer: Thank you. As per recommendation, more information and references have been added to the “Materials and Methods” section.

--primer design should be added.
Answer: Thank you. The following content has been added to the "Materials and methods" section:
“The following primers were used:
MMP-2 (forward: 5′ -GATACCCCTTTGACGGTAAGGA-3′, reverse: 5′ -CCTTCTCCCAAGGTCCATAGC-3′); MMP-9 (forward: 5′-ACGCACGACGTCTTCCAGTA-3′, reverse: 5′ -CCACCTGGTTCAACTCACTCC-3′); PCNA (forward: 5′ -CCTGCTGGGATATTAGCTCCA-3′, reverse: 5′ -CAGCGGTAGGTGTCGAAGC-3′); HIF-1α (forward: 5′-TGACTGTGCACCTACTATGTCACTT-3′,
reverse: 5′-GGTCAGCTGTGGGTAATCCACTC-3′);
COX-2 (forward: 5′-TGACTGTGCACCTACTATGTCACTT-3′,
reverse: 5′-GGTCAGCTGTGGGTAATCCACTC-3′);
GADPH (forward: 5′-GCACCGTCAAGGCTGAGAAC-3′,
reverse: 5′-ATGGTGGTGAGACGCCAGT-3′)”.
--catalog number for all antibodies
Answer: Thank you. Catalog numbers for antibodies have been added to the manuscript.
“Membranes were incubated with rabbit anti-HIF-1α diluted in blocking buffer (1: 500; #ab179483, Abcam Cambridge, MA) overnight at 4°C and then incubated with secondary goat-anti-rabbit antibody (1: 3000; #ab150077, Abcam Cambridge, MA) for 1 h at room temperature”.
--kits and reagents should be added
Answer: Thank you. The resources of kits and reagents have been added to the revised manuscript.
--Details of ELISA method.
Answer: Thank you. To address your concern, the following content has been added to the revised “Materials and Methods” section.
“Briefly, 50 μl of each standard or sample was added into the appropriate wells, followed by adding 50 μl Biotin-labeled antibody working solution into each well.
After incubation for 45 min at 37°C, each well was washed 3 times, followed by 0.1 mL of SABC working solution being added into each well for 30 min at 37 □. 90 ml of TMB substrate was then added and incubated at 37°C in the dark for 20 minutes. The reaction was then stopped by adding 50 μL of stop solution to each well. Results were then read at 450 nm within 20 minutes”.

Review 3:
In the study titled “The protective effects of Azilsartan against hypoxia in endometrial stromal cells: an implication in endometriosis”, the authors report the pharmacological function of Azilsartan, a blocker of ANG II receptor, in controlling endometriosis. Firstly, they found that ANG II type 1 receptor (AT1R) was upregulated in endometriotic rat models. Treatment with Azilsartan prevented the development of endometriotic lesions and suppressed the expression of HIF-1α and COX-2 in endometriotic rats. In vitro assays proved that hypoxia-induced proliferation, migration, and invasion of T-HESC cells were attenuated by Azilsartan. Azilsartan inhibited the expression levels of HIF-1α, COX-2, and PGE2 production in hypoxia-induced T-HESC cells. Finally, they report that the therapeutic function of Azilsartan on endometriosis is mediated by the HIF-1α/COX-2/PGE2 signaling pathway.
The topic of the paper is interesting although needs some adjustments.
----Full names of abbreviations should be provided.
Answer: Thank you. All the full names of abbreviations have been shown where they appeared the first time in the revised manuscript:
“angiotensin II (ANG II), Azilsartan (AZL), ANG II type 1 (AT1), cyclooxygenase 2 (COX-2), hypoxia-inducible factor-1α (HIF-1α), prostaglandin E2 (PGE2), Azilsartan (AZL), endometriosis (EMS), proliferating cell nuclear antigen (PCNA), matrix metalloproteinase (MMP), Cell counting kit-8 (CCK-8), Enzyme-linked immunosorbent assay (ELISA), analysis of variance (ANOVA)”...
----English needs to be reviewed to eliminate typos.
Answer: Thank you. To address your concerns, we have invited a native English speaker in our field to revise the spelling and grammar of this manuscript. All grammatical errors have been eliminated. Corrections are marked in red in the revised manuscript.
----References should be updated.
Answer: Thank you. To address your concerns, references have been updated in the revised manuscript. Corrections are marked in red in the References list.
----Introduction on “Azilsartan” is not sufficient.
Answer: Thank you. To address your concerns, the following content has been added to the

“Introduction” section in the revised manuscript to introduce Azilsartan:
“Azilsartan (AZL) is an AT1 receptor blocker that is used for the treatment of hypertension. Recently, several studies have demonstrated the protective benefits of AZL in a wide range of diseases. For example, Liu et al. showed that AZL suppressed inflammatory response by increasing e-NOS phosphorylation [9]. Furthermore, it inhibited hydroperoxide-induced oxidative stress in endothelial cells [10]. However, whether AZL possesses a protective effect against endometriosis is still unknown. We speculated that AZL might have an effective function in controlling endometriosis. Here, we studied the effects of AZL on endometriosis development in vivo and hypoxia-induced metastasis of endometrial stromal cells in vitro”.
----Ethical approval should be mentioned in the “Materials and Methods” section.
Answer: Thank you. Ethical approval has been added to the “Materials and Methods” section in the revised manuscript.
“The protocol of this study was approved by the Ethical Committee of the General Hospital of Ningxia Medical University”.

----It’s unknown how many mice were used in each group of the experiment.
Answer: Thank you. Rats were divided equally into the sham group and the endometriosis (EMS) group (ten rats for each group).

Response letter20220530.docx

Title: The protective effects of Azilsartan against hypoxia in endometrial stromal cells: an implication in endometriosis

Abstract

Endometriosis is a reproductive disorder closely associated with hypoxia stress. Increasing evidences have implied the important roles of angiotensin II (ANG II) receptors in the pathophysiology of endometriosis. Thus, we speculated that Azilsartan (AZL), an ANG II receptor blocker, might have an effective function in controlling endometriosis. We investigated the effects of AZL on endometriosis development *in vivo*. The results show that upregulation of ANG II type 1 (AT1) receptor was observed in the endometriotic rat models. Treatment with AZL prevented the development of endometriotic lesions and suppressed the expressions of HIF-1α and cyclooxygenase 2 (COX-2) in endometriotic rats. *In vitro* assays proved that hypoxia-induced proliferation, migration, and invasion of T-HESC cells were attenuated by AZL. AZL inhibited the expression levels of hypoxia-inducible factor-1α (HIF-1α), COX-2, and prostaglandin E2 (PGE_2) production in hypoxia-induced T-HESC cells. Overexpression of HIF-1α blocked the effects of AZL on T-HESC cells in response to hypoxia. In conclusion, AZL showed therapeutic function on endometriosis through inhibiting hypoxia-induced cell proliferation, migration, and invasion of T-HESC cells via HIF-1α/COX-2/PGE_2 signaling.

Keywords: Endometriosis, Azilsartan (AZL), angiotensin II receptor, endometriotic lesions, hypoxia, HIF-1α

1. Introduction

Endometriosis is a reproductive disorder that occurs in women of reproductive age [1]. It is manifested by the formation of endometrial tissues outside the uterus, with the symptoms of infertility, dysmenorrhea, and long-term pelvic pain [2]. It is crucial to fully understand the pathogenesis of endometriosis for developing proper therapeutic approaches. Over the past decades, hypoxia has been shown to play a key role in regulating numerous important processes in the development of endometriosis [3]. In particular, endometrial stromal cells undergo epigenetic modulation under hypoxic conditions, since they normally reside in an oxygen-enriched environment [4]. It is evident that hypoxia is one of the major factors responsible for the metastasis of endometrial stromal cells. Interestingly, the expression of HIF-1α, a crucial responder to hypoxia stress, is upregulated in the ectopic endometrial stromal cells [5]. These findings provide evidence for the participation of hypoxia in the modulation of endometrial stromal cells, which represents a crucial mechanism of endometriosis.

Angiotensin II (ANG II), an octapeptide, is one of the biologically active components of the renin-angiotensin system [6]. Previous studies have shown that ANG II may cause reduced blood flow and lead to hypoxic conditions in several organs, thereby participating in various diseases. Marciante *et al*. [7] reported that ANG II is involved in the development of hypertension and cognitive decline mediated by chronic intermittent hypoxia. It induces hypoxia in the kidney via both non-hemodynamic and hemodynamic mechanisms. ANG II has been found to elicit its multiple actions through its specific receptors, type 1 (AT1) and type 2 (AT2). Therefore, targeting either one is an effective approach for blocking the effects of ANG II.

Increasing evidences have implied that ANG II receptors may participate in the pathophysiology of endometriosis. Several studies have proven that ANG II receptor blockers have the capacity to control the development of endometriosis. For instance, an ANG II receptor blocker, losartan, suppresses the formation of endometriotic lesions in experimental endometriotic rats [8]. Azilsartan (AZL) is an AT1 receptor blocker that is used for the treatment of hypertension. Recently, several studies have

demonstrated the protective benefits of AZL in a wide range of diseases. For example, Liu *et al.* showed that AZL suppressed inflammatory response by increasing e-NOS phosphorylation [9]. Furthermore, it inhibited hydroperoxide-induced oxidative stress in endothelial cells [10]. However, whether AZL possesses a protective effect against endometriosis is still unknown. We speculated that AZL might have an effective function in controlling endometriosis. Therefore, we studied its effects on endometriosis development *in vivo* and hypoxia-induced metastasis of endometrial stromal cells *in vitro*.

2. Methods and materials

2.1 Animal model

The endometriotic animal model was established in female SD rats (6-8 weeks old, 200-220 g). Rats were divided equally into the sham group and endometriosis (EMS) group, and the AZL+EMS group (ten rats for each group). Rats in the EMS group were anaesthetized with halothane and a mid-ventral incision was performed to expose the bowels. The protocol of this study was approved by the Ethical Committee of the General Hospital of Ningxia Medical University. Then, the right uterine horn was removed and longitudinally opened using scissors. After cutting into 4 mm^2 pieces, the fragments were sutured to the intestine mesentery. The incision was closed, and the rats were kept for 4 weeks to induce endometriosis. Rats in the sham group were subjected to a similar surgery without transplantation of the uterine tissue. Rats in the EMS group were randomly divided into 2 groups: in the EMS model group, rats were orally treated with an equal volume of normal saline solution; in the AZL treatment group, rats were orally treated with AZL (3.0 mg/kg body weight) [11] for 21 days after surgery.

2.2 Immunofluorescence

The endometriotic lesions were separated, fixed in 4% paraformaldehyde, embedded in paraffin, and cut into sections for the immunofluorescence assay. The sections were incubated with blocking buffer 5% BSA at room temperature for 2 h, and then incubated with anti-AT1 antibody (diluted in 1:200; Abcam) at 4°C overnight, and then incubated with AlexaFluor 488-conjugated secondary antibody

(1:200; Invitrogen, Carlsbad, CA) for1 h at 37°C. Finally, the immunofluorescence results were analyzed using a confocal fluorescence microscope(Nikon, Japan).

2.3RT-PCR analysis

The mRNA levels of relevant genes proliferating cell nuclear antigen (PCNA), matrix metalloproteinase (MMP)-2, MMP-9, HIF-1α, and COX-2 in total RNAs from endometriotic lesions or cultured cells were assessed using qRT-PCR with a Prime ScriptRT reagent kit (Takara, Japan) and SYBR Green I kit (Takara) on a Bio-Rad IQ5 Real-Time System (Bio-Rad, Hercules, CA). The β-actin was applied as an internal control. The relative expression of target genes was analyzed using the $2^{-\Delta\Delta Ct}$ approach [12]. The following primers were used:

MMP-2 (forward: 5′ -GATACCCCTTTGACGGTAAGGA-3′, reverse: 5′ -CCTTCTCCCAAGGTCCATAGC-3′); MMP-9 (forward: 5′-ACGCACGACGTCTTCCAGTA-3′, reverse: 5′ -CCACCTGGTTCAACTCACTCC-3′); PCNA (forward: 5′ -CCTGCTGGGATATTAGCTCCA-3′, reverse: 5′ -CAGCGGTAGGTGTCGAAGC-3′); HIF-1α (forward: 5′-TGACTGTGCACCTACTATGTCACTT-3′, reverse: 5′-GGTCAGCTGTGGGTAATCCACTC-3′); COX-2 (forward: 5′-TGACTGTGCACCTACTATGTCACTT-3′, reverse: 5′-GGTCAGCTGTGGGTAATCCACTC-3′); GADPH (forward: 5′-GCACCGTCAAGGCTGAGAAC-3′, reverse: 5′-ATGGTGGTGAGACGCCAGT-3′).

2.4 Culture of endometrial stromal cells

Human endometrial stromal cell line T-HESC (ATCC, Manassas, VA) was used for the *in vitro* assays. The T-HESC cells were cultured in DMEM-F12 medium (Hyclone, Logan, UT) with 10% fetal bovine serum (FBS, Hyclone), and 4 mM L-glutamine (Sigma-Aldrich, St. Louis, MO), 0.25% HEPES plus necessary antibiotics (Sigma-Aldrich, USA), at 37°C in a humidified atmosphere of 5% CO_2. Hypoxia exposure was conducted at 1% O_2, and 5% CO_2 using a ProOx C21 nitrogen-induced hypoxia system (BioSpherix, Red Field, NY). For the AZL

treatment group [13], T-HESC cells were pre-treated with 5 μM AZL, followed by hypoxia exposure for 48 h.

2.5 Cell transfection

The full-length cDNA sequence of HIF-1α was linked to the pcDNA3.0 vector to construct the pcDNA3.0-HIF-1α. Afterward, cell transfection of T-HESC cells with pcDNA3.0-HIF-1α or pcDNA3.0 was performed utilizing the lipofectamine3000 reagent (Thermo Fisher Scientific, Massachusetts, USA). Finally, the cells were harvested at 48h after transfection to detect the transfection efficiency.

2.6 Western blot

Western blot was carried out to assess protein expression of HIF-1α in T-HESC cells after transfection. The cellular lysates were loaded in SDS-PAGE to separate the target protein, followed by transfer to the polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with rabbit anti-HIF-1α diluted in blocking buffer (1: 500; #ab179483, Abcam Cambridge, MA) overnight at 4°C and then incubated with secondary goat-anti-rabbit antibody (1: 3000; #ab150077, Abcam Cambridge, MA) for 1 h at room temperature. Finally, protein bands were detected with ECL Plus reagent (Thermo Fisher Scientific) and analyzed using Image J software.

2.7 Cell counting kit-8 (CCK-8) assay

Cell viability of T-HESC cells was evaluated by employing a CCK-8 kit (#C0037, Beyotime Biotechnology, Shanghai, China). Briefly, T-HESC cells (5000 cells per well) were maintained in a 96-well plate and subjected to hypoxia exposure for 0, 24, 48, and 72 h with or without 5 μM AZL. Then CCK-8 reagent (10μl) was added to each well and maintained for another 4 h at 37°C. Finally, the detection of absorbance at 450nm was performed using a Microplate Reader (Bio-Rad).

2.8 Transwell assay

The migrative and invasive capacity of T-HESCs were measured with the transwell assay. Briefly, T-HESCs (2×10^4 cells/well) were seeded in the upper chambers in a serum-free medium and subjected to hypoxia exposure for 0, 24, 48, and 72 h with or without 5 μM AZL. Meanwhile, a serum-containing medium was

added to the lower chambers. The inserts were coated with or without the Matrigel. The non-migrating or non-invading cells on inserts were cleansed with a cotton swab after a 24-hour incubation period. The cells that had migrated or invaded through the inserts were stained with DAPI. The images from 5 random fields were obtained with an Inversion Microscope (Zeiss, Germany) [14].

2.9 Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to examine the PGE_2 level in the supernatant of T-HESC cells using a commercial kit (#ab176480, Abcam Cambridge, MA). Briefly, 50 µl of each standard or sample was added into the appropriate wells, followed by adding 50 µl of biotin-labeled antibody working solution into each well. After incubation for 45 min at 37°C, each well was washed 3 times, followed by 0.1 mL of streptavidin conjugate (SABC) working solution being added into each well for 30 min at 37 ℃. 90 ml oftetramethylbenzidine (TMB) substrate was then added and incubated at 37°C in dark for 20 minutes. The reaction was then stopped by adding 50 µL of stop solution to each well. Results were then read at 450 nm within 20 minutes.

The absorbance at 450 nm was detected using the spectrophotometer (Bio-Rad).

2.10 Statistical analysis

All statistical testing was performed using GraphPad Prism 5 software. The data are expressed as the mean ±standard errors of mean (S.E.M.)with three repeats. Statistically significant differences were determined using a one-way analysis of variance (ANOVA).

3. Results

3.1 Up-regulation of the AT1 receptor in endometriotic rat models

Through the IFC assay, there was a significant increase in AT1 expression in the endometriotic lesions from the EMS group compared to the endometrial tissues from the control group (Figure 1A). Consistent with the IFC results, the mRNA levels of AT1 were upregulated in the endometriotic lesions from the endometriosis rats (Figure 1B).

3.2 The impact of AZL on endometriotic lesions formation in experimental endometriosis rats

As shown in figure 2, AZL treatment caused a significant reduction in the size of endometriotic lesions compared to that in the EMS group models, implying that it prevented the development of endometriotic lesions.

3.3 AZL regulated the proliferation- and metastasis-related genes expression in endometriosis models

As illustrated by RT-PCR, rats from the EMS group exhibited significant PCNA increases in endometriotic lesions, which was attenuated by AZL treatment. The increased mRNA levels of MMP-2 and MMP-9 in the EMS group were also reduced after AZL administration (Figures 3).

3.4 AZL suppressed the expressions of HIF-1α and COX-2 in endometriosis models

We detected significant HIF-1α and COX-2 mRNA levels elevations in endometriotic lesions from rats in the EMS group (Figures 4A and 4B). Administration of AZL attenuated the upregulation of both HIF-1α and COX-2 in the endometriotic lesions.

3.5 AZL inhibited cell proliferation of T-HESC cells in response to hypoxia

The protein and mRNA expression levels of AT1 were elevated in T-HESC cells after hypoxia exposure in a time-dependent manner (Figures 5A and 5B). Cell proliferation of T-HESC cells was dramatically increased after hypoxia exposure for 48 h. AZL treatment attenuated the hypoxia-induced cell proliferation of T-HESC cells (Figure 5C).

3.6 AZL modulated the cell migration and invasion of T-HESC cells in response to hypoxia

In Figure 6A, we confirm that hypoxia induced the migration capacity of T-HESC cells, which could be attenuated by AZL. Meanwhile, the enhanced invasive capacity of T-HESC cells was also found to be alleviated after AZL treatment (Figure 6B).

3.7 AZL suppressed COX-2 expression and PGE_2 production in T-HESC cells in response to hypoxia

We next evaluated changes in the COX-2 expression, and the results show that the COX-2 mRNA level was upregulated after hypoxia exposure. Treatment with AZL effectively blocked the elevated mRNA (Figure 7A) and protein levels (Figure

7B) of COX-2 in hypoxia-induced T-HESC cells. In addition, ELISA showed that hypoxia exposure also caused increased PGE_2 production. However, the increased PGE_2 level was attenuated by AZL treatment (Figure 7C).

3.8 HIF-1α mediated the effects of AZL in T-HESC cells in response to hypoxia

HIF-1α expression was also upregulated in T-HESC cells exposed to hypoxia. AZL reduced the expression of HIF-1α against hypoxia induction, as shown by Western blot (Figure 8A). To further confirm the role of HIF-1α, HIF-1α-overexpressing T-HESC cells were constructed through transfection with pcDNA3.0-HIF-1α. As confirmed by Western blot, transfection efficiency with pcDNA3.0-HIF-1α in T-HESC cells was successful with a 4.5-fold increase in HIF-1α expression (Figure 8B).

Transfection with pcDNA3.0-HIF-1α elevated the AZL-caused decrease in proliferation of T-HESC cells (Figure 8C). The inhibitory effects of AZL on migration and invasive capacities were reversed by HIF-1α overexpression (Figures 8D and 8E). In addition, the decreased COX-2 mRNA and PGE_2 levels in AZL-treated T-HESC cells were increased after transfection with pcDNA3.0-HIF-1α (Figures 8F and 8G).

4. Discussion

It has been reported that the renin-angiotensin system (RAS) participates in endometriosis progression. For instance, ANG II regulates COX-2 expression, thus promoting the proliferation of endometrial tissue in endometriosis rats. AT1 and AT2 receptors are located in endometrial stromal cells and their protein levels are increased in endometriotic lesions. The AT1 receptor regulates the development of endometriosis by promoting the cell proliferative and migration capacities of stromal cells and preventing stromal cells from undergoing apoptosis [15]. Tanshinone IIA was reported to contribute to regulating endometriosis progression by decreasing the expressions of estradiol (E2), ANG II, and the AT2 receptor [16]. Particularly, several studies have found that the ANG II receptor blockers have the capacity to repress the development of endometriosis. Losartan, an ANG II receptor blocker, was found to suppress the implant growth of experimental endometriosis rats [8]. Here, we found

that the AT1 receptor expression was upregulated in the endometriotic lesions from endometriosis rats. Treatment with AZL, an AT1 receptor blocker, caused significant reductions in the sizes of the endometriotic lesions.

Currently, endometriosis is widely accepted to be associated with an implantation theory [17]. Ectopic implantation of endometrial tissues may be initiated during the menstrual cycle through the exoteric fallopian tube. Furthermore, the uncontrolled metastasis and cell proliferation of endometrial cells facilitate the development of endometriotic lesions [18]. It is generally accepted that preventing the metastasis of endometrial stromal cells may ameliorate endometriosis [19-21]. In this study, we found that the rats from the EMS group exhibited significant increases in PCNA (for proliferation), MMP-2, and MMP-9 (for metastasis) levels, which could be attenuated by AZL. It is well established that hypoxia acts as a potent risk factor for epigenetic regulation of certain genes involved in differentiation, proliferation, survival, migration, and angiogenesis in endometrial cells. It thereby facilitates the implantation and progression of ectopic endometriotic lesions [22, 23]. Here we used a hypoxia-induced *in vitro* model of endometriosis in endometrial stromal T-HESC cells. We found that AT1 receptor expression was upregulated in T-HESC cells after hypoxia exposure in a time-dependent manner. Treatment with AZL alleviated the hypoxia-induced increase in proliferative, migration, and invasive capacities of T-HESC cells.

Researchers have increasingly discovered that hypoxic stress is one of the most critical driving forces for the development of ectopic endometriotic tissues. Multiple studies over the past years have found that there is a crucial association between the aberrant expression of HIF-1α and endometriosis. Higher expression levels of HIF-1α in endometriosis patients relative to those in the ectopic endometria of women without endometriosis were observed [5]. Compared to stage I/II endometriosis, elevated serum HIF-1α levels were observed in stage III/IV endometriosis, indicating that HIF-1α may be a biomarker for patients with severe endometriosis [24]. It is well validated that HIF-1α led to the increased COX-2 expression, and thus PGE_2 over-production [25]. It has been demonstrated that PGE_2 stimulates the dysregulation

of steroidogenic acute regulatory proteins, vascular endothelial growth factors, and fibroblast growth factors in endometriotic stromal cells. In turn, endometrial and endothelial cell proliferation is induced and this results in estrogen production in the endometriotic tissue [26]. Considering their multipotent effects, the HIF-1α/COX-2/PGE_2 pathway is considered a master regulator of endometriosis. Therefore, we evaluated the overall hypothesis that the augmentation of HIF-1α/COX-2/PGE_2 could be involved in endometriosis. We found that the upregulated expression levels of HIF-1α and COX-2, and PGE_2 production in endometriotic lesions and/or hypoxia-induced endometriotic stromal cells, were repressed by AZL. Overexpression of HIF-1α blocked the effects of AZL on hypoxia-induced endometriotic stromal cells.

In conclusion, we provide evidence to show the therapeutic function of AZL on endometriosis with a novel mechanism of inhibiting hypoxia-induced cell proliferative, migration, and invasive capacities of endometriotic stromal cells. Furthermore, all these phenomena were attributed to the inhibition of HIF-1α/COX-2/PGE_2 signaling.

Acknowledgement

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Figure legends

Figure 1 Upregulation of angiotensin II type 1 (AT1) in endometriotic rat model. (A) IFC for AT1 expression; Scale bar, 100 μm; (B) RT-PCR for mRNA level of AT1 (***, P<0.001 vs. vehicle group).

Figure 2 The impact of AZL on endometriotic lesions in experimental rats. Size of endometrioticlesions (***, P<0.001 vs. vehicle group; ##, P<0.01 vs. EMS group).

Figure 3 The regulation of AZL on related-genes expression in rat endometriosis model. RT-PCR analysis for the mRNA levels of PCNA, MMP-2, and MMP-9 in endometriotic lesions (***, P<0.001 vs. vehicle group; ##, P<0.01 vs. EMS group).

Figure 4 The inhibitory effects of AZL on HIF-1α and COX-2 expression in rat endometriosis model. RT-PCR analysis for the mRNA levels of HIF-1α and COX-2 in endometriotic lesions (***, P<0.001 vs. vehicle group; ##, P<0.01 vs. EMS group).

Figure 5 The effects of AZL on cell proliferation of endometrial stromal cells in response to hypoxia for 12, 24, and 48 hours. (A) Western blot for AT1 expression; (B) RT-PCR for mRNA level of AT1; (C) CCK-8 assay for cell proliferation of T-HESC cells (*, **, ***, P<0.05, 0.01, 0.001 vs. vehicle group; ##, P<0.01 vs. Hypoxia group).

Figure 6 The regulatory effects of AZL on cell migration and invasion of endometrial stromal cells in response to hypoxia. (A) Transwell assay for cell

migration of T-HESC cells (migrated cells number per field) .(B) Transwell assay for cell invasion of T-HESC cells (invaded cells number per field) (***, P<0.001 vs. vehicle group; ##, P<0.01 vs. Hypoxia group).

Figure 7 The inhibitory effects of AZL on COX-2 and PGE_2in endometrial stromal cells in response to hypoxia. (A) RT-PCR analysis for the mRNA level of COX-2 in T-HESC cells. (B) Western blot analysis for the protein level of COX-2 in T-HESC cells. (C) ELISA kits to determine levels of PGE_2 in the supernatant samples (***, P<0.001 vs. vehicle group; ##, P<0.01 vs. Hypoxia group).

Figure 8 HIF-1α mediated the effects of AZL in endometrial stromal cells in response to hypoxia. (A) Western blot for the expression of HIF-1α in T-HESC cells. (B) Western blot for the determination of transfection efficiency with pcDNA3.0-HIF-1α. (C) CCK-8 assay for cell proliferation of T-HESC cells. (D) Transwell assay for cell migration of T-HESC cells (migrated cells number per field). (E) Transwell assay for cell invasion of T-HESC cells (invaded cells number per field) (F) RT-PCR analysis for the mRNA level of COX-2 in T-HESC cells. (G) ELISA kits to determine levels of PGE_2in the supernatant samples(***, P<0.001 vs. vehicle group; ##, P<0.01 vs. hypoxia group; &&, P<0.001 vs. hypoxia+AZL group).

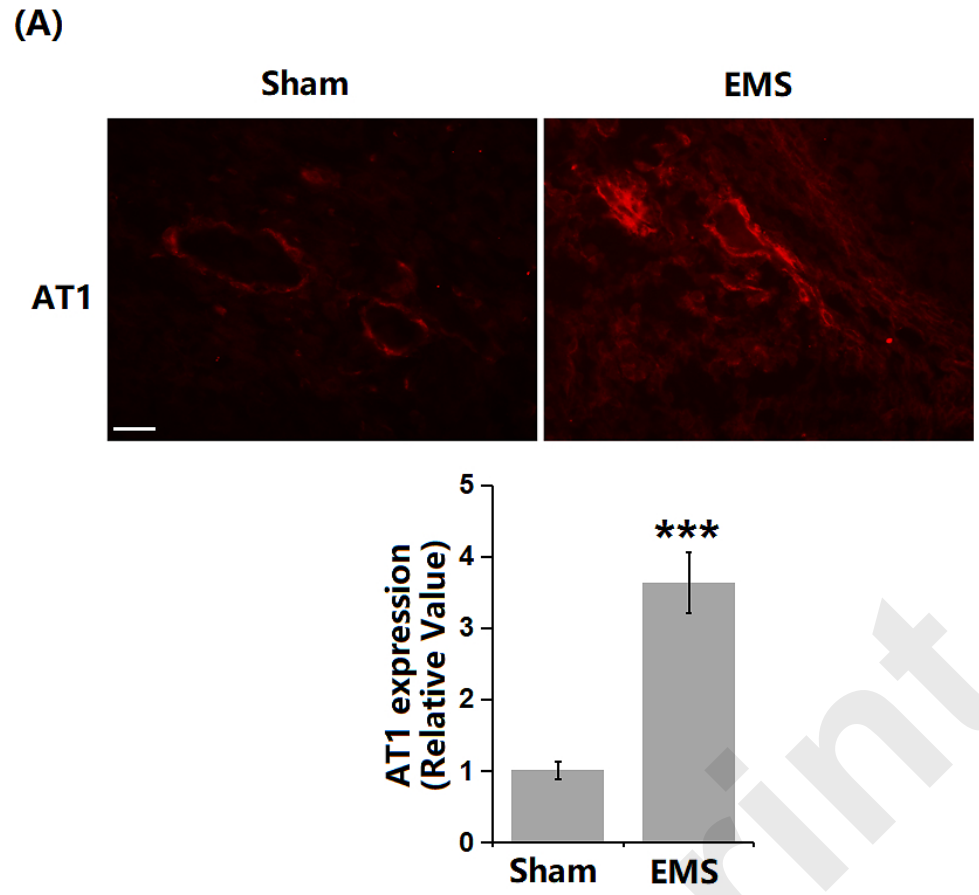
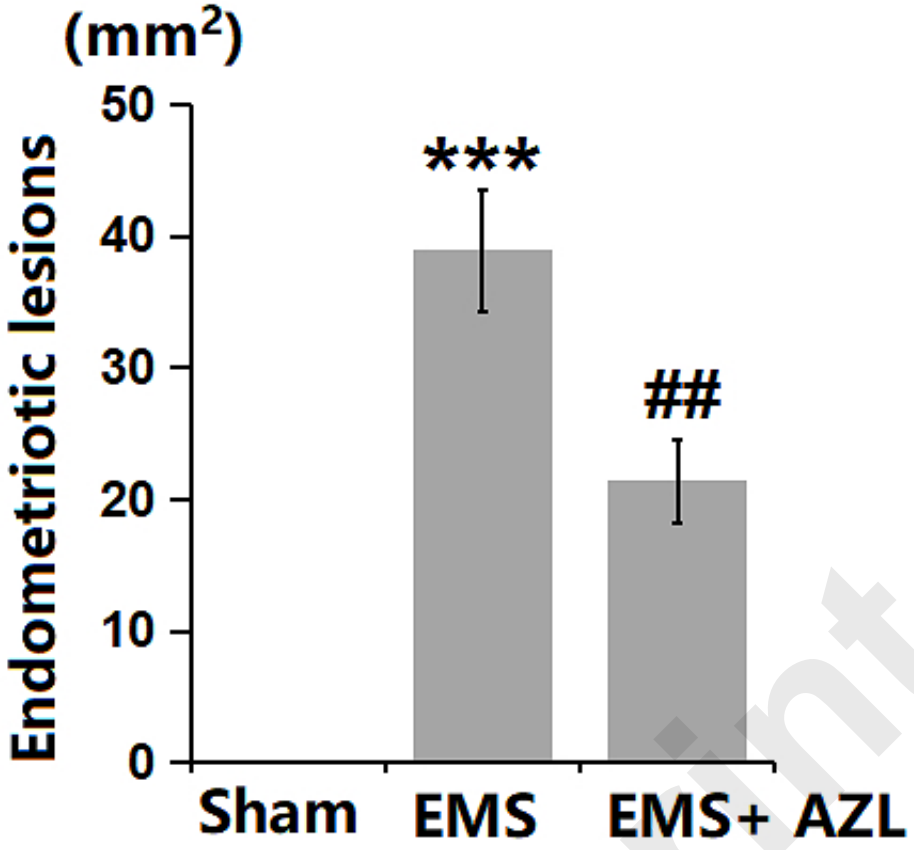


Figure 1



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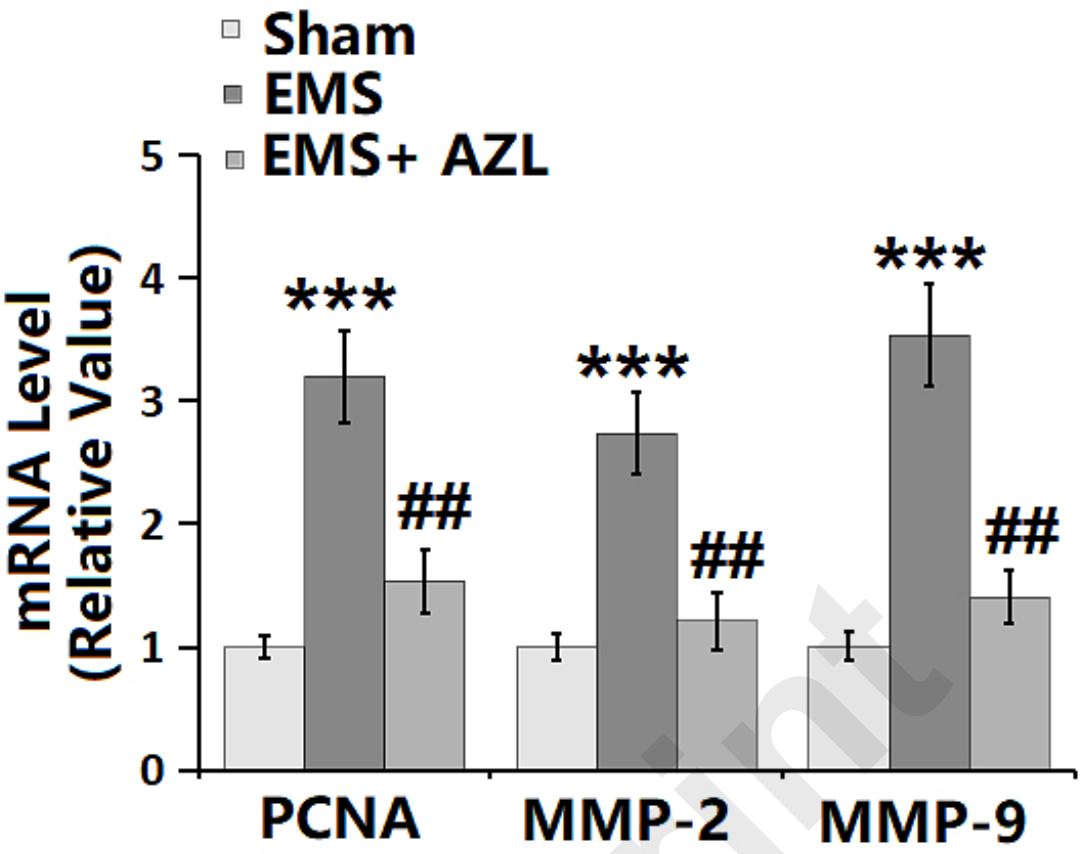

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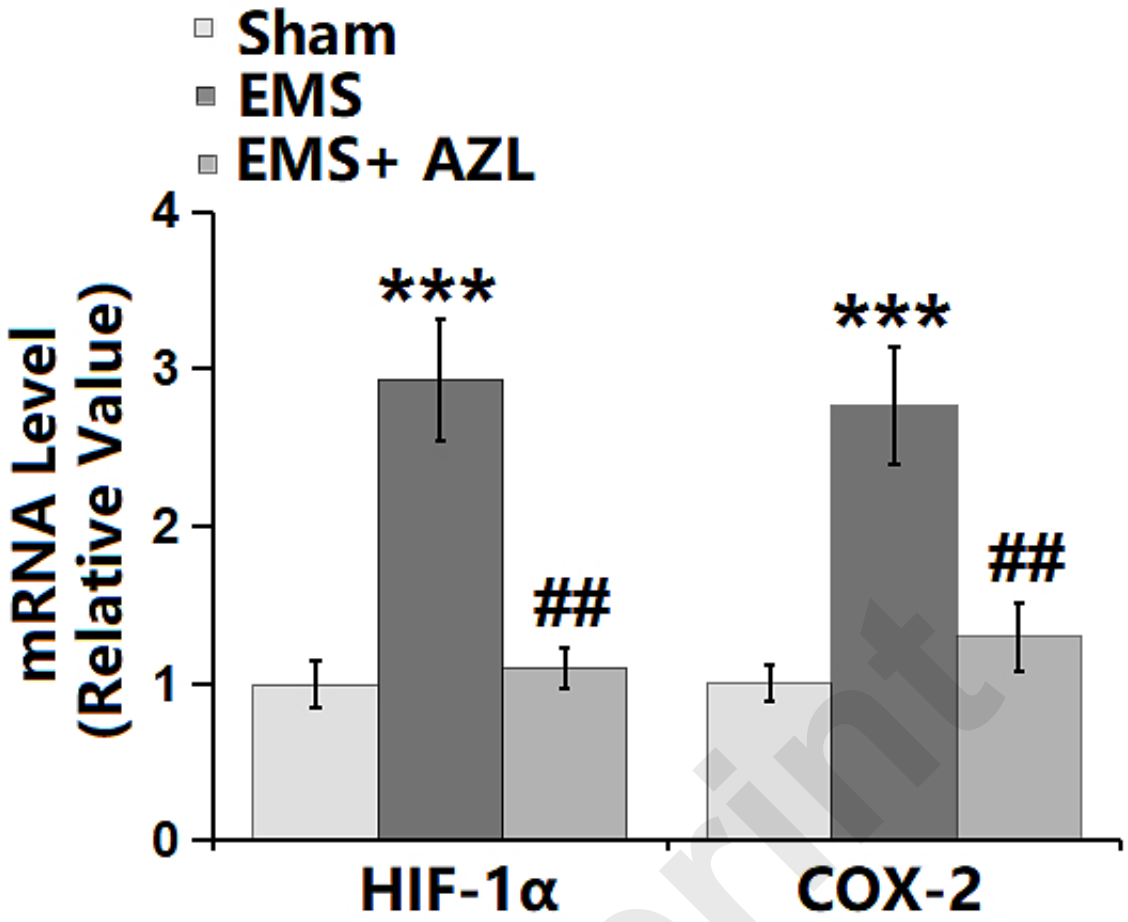

Figure 4

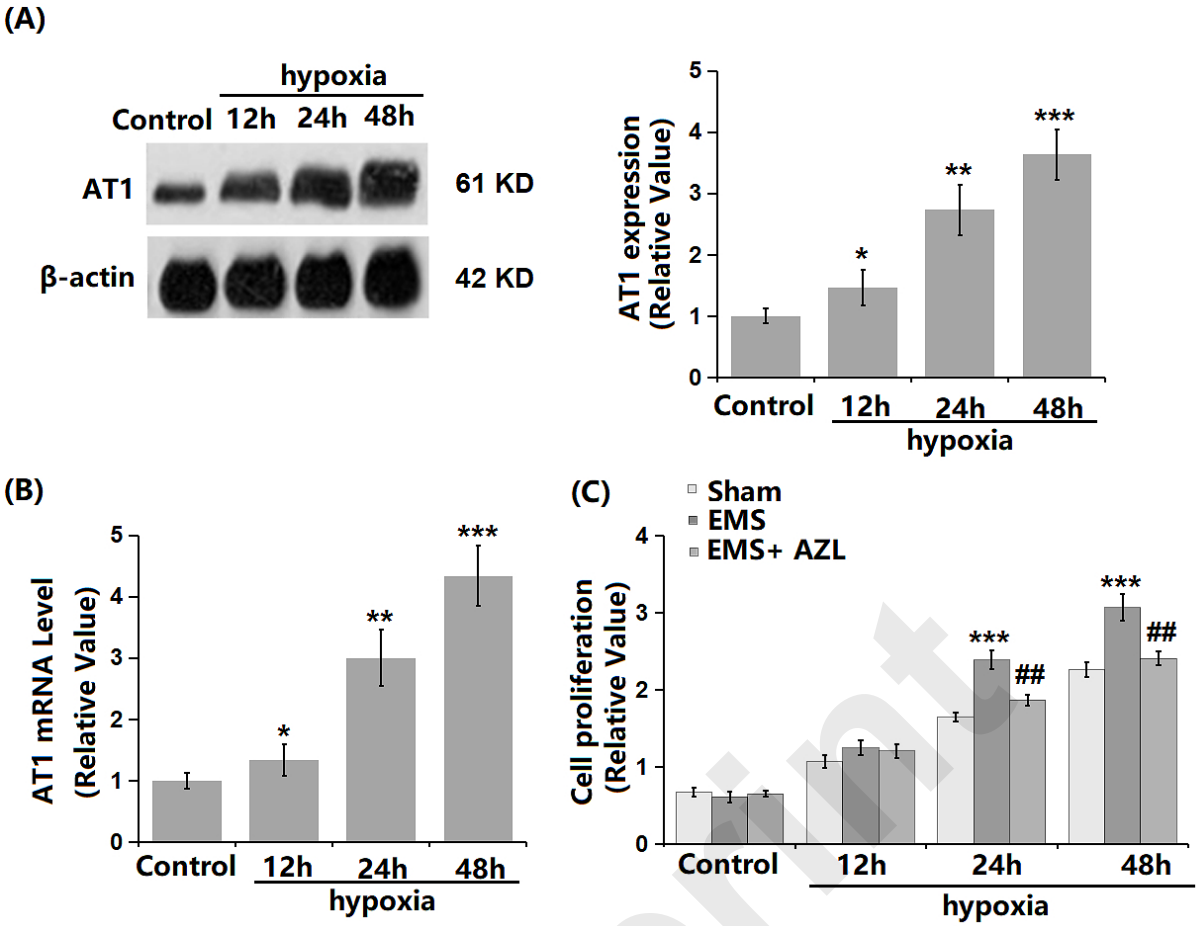

Figure 5

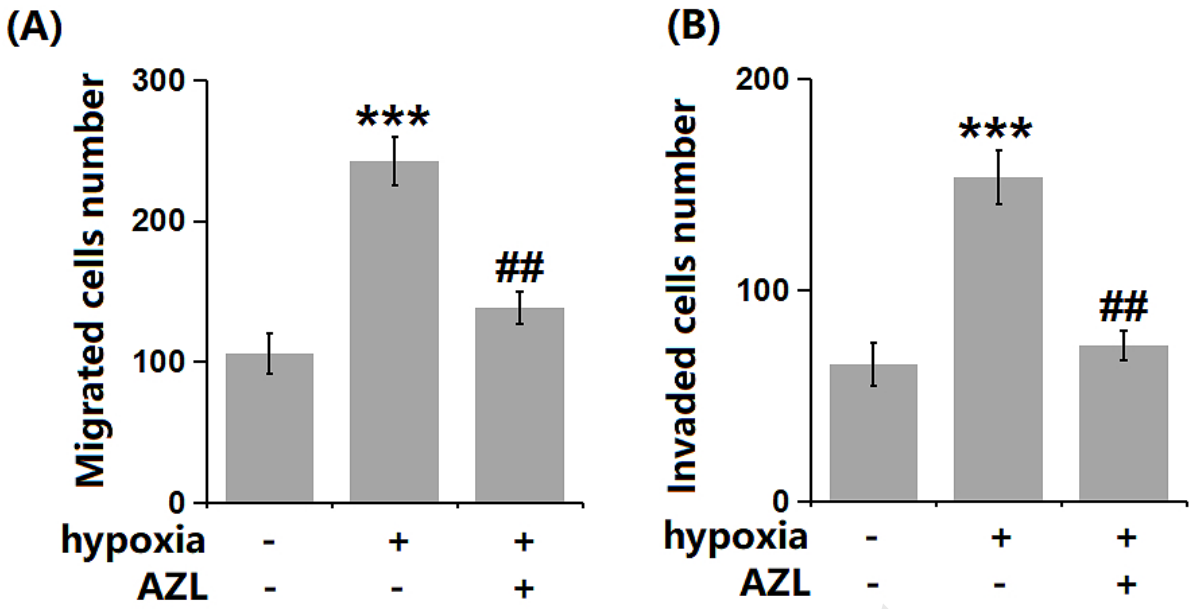

Figure 6

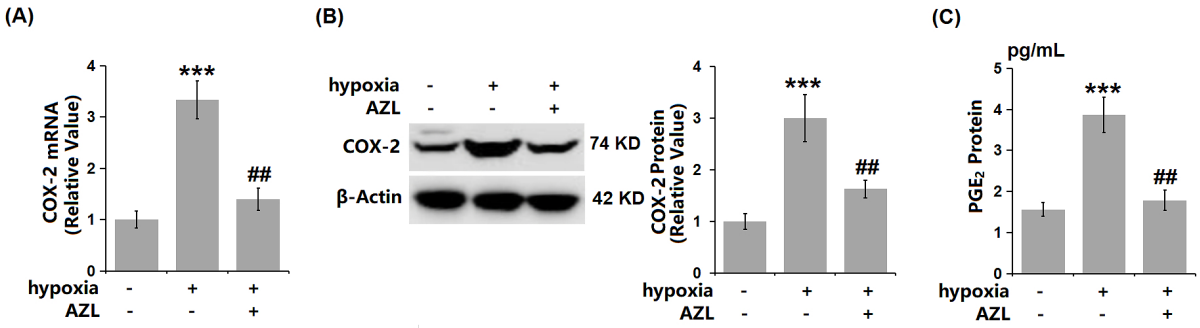

Figure 7

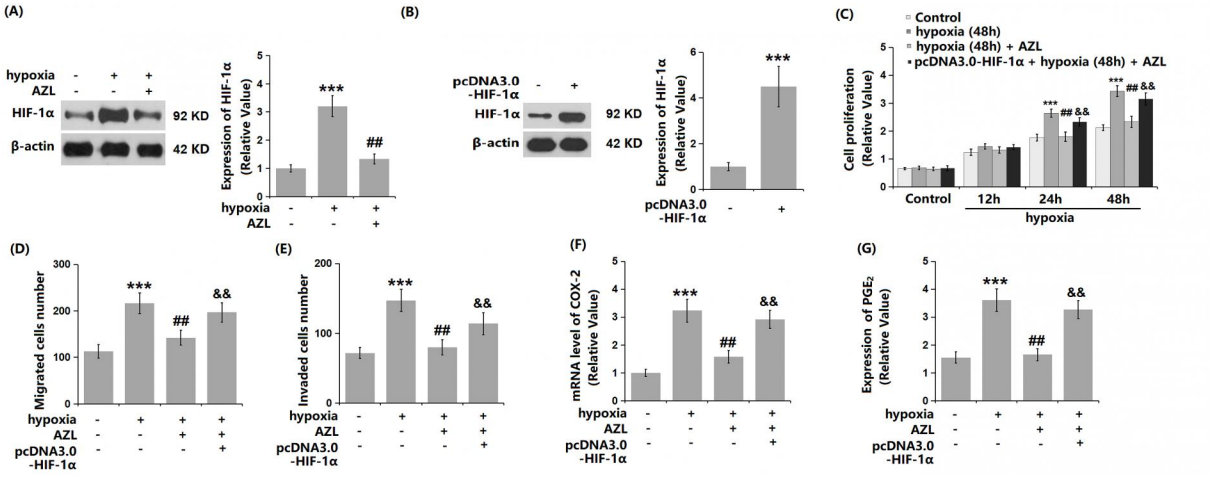

Figure 8