

# $\beta$ -Arrestins1 inhibit autophagy induced by hypoxic injury in human pulmonary artery endothelial cells via Akt/mTOR signaling pathway

**Hongjuan Ning**

Jinzhou Medical University

**Jingyu Deng**

Jinzhou Medical University

**Delei Kong**

First Affiliated Hospital of China Medical University

**Feng Chen**

PLA Rocket Force Characteristic Medical Center

**Yongfei Liu**

PLA Rocket Force Characteristic Medical Center

**Lina Shan** (✉ [shanlina321@163.com](mailto:shanlina321@163.com))

First Affiliated Hospital of Jinzhou Medical University <https://orcid.org/0000-0002-5392-5299>

**Zheng Zhang**

PLA Rocket Force Characteristic Medical Center

**Taohong Hu**

PLA Rocket Force Characteristic Medical Center

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## Research

**Keywords:** Hypoxia, Pulmonary artery hypertension (PAH), Human pulmonary artery endothelial cells (hPAECs),  $\beta$ -Arrestins1, VEGFR3, Autophagy

**Posted Date:** March 12th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-16971/v1>

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# Abstract

## Background

To investigate the potential effects of  $\beta$ -Arrestins1 on autophagy and apoptosis in human pulmonary artery endothelial cells (hPAECs) under hypoxic stress.

## Methods

The hPAECs were exposed to normoxic condition and hypoxic injury for 24 h, 48 h, and 72 h, respectively. Then, to explore effects of autophagy on hPAECs with hypoxia, cells were administrated with 3-MA (an inhibitor autophagy).  $\beta$ -Arrestins1 was regulated to explore its effects on autophagy and apoptosis of hPAECs. Transwell cell migration assay and scratch assay were performed to detect the migration by light microscope. Then, CCK-8 assay was used to investigate the proliferation of hPAECs. Meanwhile, TUNEL assay served as apoptosis in hPAECs. In addition, Western blotting assay applied to evaluate protein expressions.

## Results

Hypoxia contributed to hyperproliferation, migration apoptosis resistance of hPAECs. Meanwhile, autophagy was increased in hypoxic hPAECs. Excessive proliferation–migration and apoptosis resistance of hPAECs were reversed after inhibition of autophagy. Then, the  $\beta$ -Arrestins1 and VEGFR3 expression levels were decreased in hypoxic conditions. Moreover, the activity of Akt/mTOR signal pathway was restrained after hypoxic injury. At last,  $\beta$ -Arrestins1+/+ repressed the increased autophagy and apoptosis resistance of hPAECs under hypoxia.

## Conclusions

Our study indicated that  $\beta$ -Arrestins1 mediated VEGFR3 reduced excessive autophagy and apoptosis resistance via Akt/mTOR pathway of hPAECs under hypoxia. It may provide a promising therapeutic target for pulmonary artery hypertension (PAH).

## Background

Pulmonary arterial hypertension (PAH) is a fatal disease characterized by a significant elevation in mean pulmonary artery pressure induced by pulmonary vasoconstriction, vascular inflammation, vascular remodeling<sup>(1–3)</sup>. However, current common anti-vasoconstriction therapies fail to target vascular remodeling, resulting in only modest improvement of morbidity and mortality<sup>(4)</sup>. Pulmonary vascular remodeling refers to the excessive proliferation, migration and apoptosis resistance of vascular endothelial cells and smooth muscle cells in resistant pulmonary artery (PAS) after a variety of pathological factors such as hypoxia, inflammation, stress, resulting in lumen stenosis<sup>(5,6)</sup>. Importantly, the barrier damage, dysfunction, increased proliferation and apoptosis resistance of pulmonary artery endothelial cells (PAECs) was implicated in the occurrence and development of vascular remodeling.

Therefore, the key to inhibit vascular remodeling of PAH is interfering with the pathological processes of PAECs.

Autophagy is an evolutionarily conserved biological process involving the degradation and recycling of cytoplasmic constituents by lysosomes. Autophagy plays an essential role in maintaining cellular homeostasis<sup>(7,8)</sup>. However, persistent pathological stimulation (such as hypoxia, hunger and lack of growth factors) will trigger autophagy-related imbalance, damaging cell structures and functions, inducing apoptosis, autophagy cell death or apoptosis resistance<sup>(9)</sup>. Previous studies suggested hypoxia-induced dysregulation of autophagy involved in the hyperproliferation and migration of endothelial cells<sup>(5,10,11)</sup>. Hypoxia-induced activation of autophagy gained more insight on PAH. Li et al.<sup>(12)</sup> observed that pulmonary vascular remodeling was followed by autophagy activation via downregulation of mTOR in PAH patients and hypoxia-induced PAH mice. Paradoxically, study demonstrated that light chain-3B(LC3B) partially inhibited the proliferation of PAECs<sup>(13)</sup>, suggesting autophagy might suppress vascular remodeling. However, little is known about the specific role of autophagy in the hypoxia-induced PAECs dysfunction, proliferation, migration and apoptosis.

$\beta$ -Arrestins (ARRBs) are multifunctional cytoplasmic proteins originally considered as negative adaptors of G protein-coupled receptors (GPCRs) by regulation of their desensitization and internalization. Studies also indicated that  $\beta$ -arrestins acted as scaffold proteins that could activate intracellular signaling pathway (such as Akt) independently of activation of GPCR<sup>(14)</sup>. In addition, accumulated studies found that  $\beta$ -Arrestins contributed to the regulation of various diseases by autophagy<sup>(15-18)</sup>. Meanwhile, current study has confirmed that  $\beta$ -Arrestins 1 inhibits vascular endothelial growth factor receptor 3 (VEGFR3) internalization and degradation to maintain protective effect on PAH resulted from damaged endothelial cells<sup>(15)</sup>. However, the underlying mechanism of  $\beta$ -Arrestins1 and autophagy was unknown in PAECs.

Previous studies have found that autophagy involved in dysfunction of PAECs. Meanwhile,  $\beta$ -Arrestins1 was also considered as an effective target for PAH. However, the detailed effects of  $\beta$ -Arrestins1 and autophagy on PAECs is still unclear currently. Therefore, the present study was designed to elucidate the potential effects of  $\beta$ -Arrestins1 and autophagy on hypoxia-induced hPAECs, which would be an optimal target for suppressing the development of PAH.

## Methods

### Cell culture and treatment

Human pulmonary artery endothelial cells were purchased from ScienCell (Catalog, #3100) with endothelial cell culture medium (ECM, Cat. #1001) Culture. As mentioned in previous studies, hPAECs in the experimental group were placed in normal pressure, hypoxic incubator (3% O<sub>2</sub>, 5% CO<sub>2</sub>, 92% N, 37 °C) for 24 h, 48 h and 72 h respectively, while those in the control group were placed in normal pressure,

normal incubator (5% CO<sub>2</sub>, 37 °C) for 72 h. <sup>(19-20)</sup> To regulate the autophagy level in hPAECs, 3-methyladenine (3MA, 5 mM, Sigma) were administrated to inhibit autophagy <sup>(21)</sup>.

## The $\beta$ -Arrestin 1 intervention

GenePharma Co., Ltd (GenePharma, Shanghai, China.) synthesized and purified small interfering RNA (siRNA) duplex sequences targeting  $\beta$ -arrestin 1. The sequences of the oligonucleotides were: sense 5'-AAAGCCTTCTGAGAAC-3'. According to the manufacturer's protocol, cells were transfected with Lipofectamine RNAi MAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA). To further determine the role of  $\beta$ -arrestin 1 in hPAECs autophagy and apoptosis, siRNA- $\beta$ -arrestin 1 was obtained to knock down  $\beta$ -arrestin-1 <sup>(22)</sup>. For over-expressions of  $\beta$ -arrestin-1, meanwhile, cells were transfected with plasmids by Lipofectamine 2000 (Invitrogen, Gaithersburg, MD, USA) <sup>(16)</sup>.

## Cell Migration Assay

As prescribed in previous study <sup>(23)</sup>, we suspended hPAECs with different treatments by 100  $\mu$ L Matrigel matrix (BD Bioscience, San Jose, CA, USA) and seeded on the top chamber of the 24-well inserts (8  $\mu$ m pore size; Corning, Tewksbury, MA, USA). Then, we added serum-free medium to the lower compartments and cells were incubated for 0.5 h to hydrate basilar membrane. Furthermore, the cell suspension ( $1 \times 10^5$  cells/100  $\mu$ L serum-free MEM media) was added into the upper compartments. Meanwhile, we added complete culture solution (500  $\mu$ L) into the lower compartments after incubation for 24 h. We removed un-migration cells through the pores. The hPAECs were fixed for 30 min in 4% paraformaldehyde after passed through the filter, stained for 20 min with 0.1% crystal violet. Five random fields (100 $\times$ ) were captured under the microscope (Olympus, Tokyo, Japan). In addition, the confluent hPAECs were wounded by pipette tips in 6-well plates. As mentioned in previous study <sup>(24)</sup>, given rise to one acellular 1-mm-wide lane per well. After washed by PBS, hPAECs were administrated by different treatments. Wounded areas were photographed at zero time. After 48 h of incubation, photos were taken from the same areas as those recorded at zero time. The closure area of wound was calculated as follows: migration area (%) =  $(A_0 - A_1)/A_0 \times 100$ .  $A_0$  represented the area of original wound area,  $A_1$  represented the remaining area of wound at the metering point. The experiments were performed at least three times.

## Cell Proliferation Assay

Cell proliferation assay was performed as described previously <sup>(25)</sup>. In brief, hPAECs were cultured in 96-well plates, the medium of acridinium bromide group was replaced by complete medium containing acridinium bromide (10  $\mu$ M, MedChemExpress Biotechnology Shanghai, China) for 24 h, and 0.1% dimethylsulfoxide (DMSO) was employed as vehicle control. Then, medium was added to 10  $\mu$ L cell counting kit-8 (CCK8, Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) and incubated for another 1.5 h. Furthermore, we dissolved the resulted formazan precipitates in DMSO. At the same time, we read immediately the optical density at 450 nm by a microplate reader.

## Measurement the autophagy in hPAECs

Autophagosome in hPAECs was detected by transmission electron microscopy (TEM) as described previously <sup>(21)</sup>. Briefly, hPAECs were washed with PBS and dehydrated through the graded ethanol. Then, cells were fixed with 2.5% glutaraldehyde and 1% buffered osmium tetroxide. Moreover, Ultrathin sections were stained with 1% uranyl acetate and 0.2% lead citrate. Autophagosomes and autolysosomes were recorded using a transmission electron microscope (JEM1230; JEOL). Meanwhile, sectional areas of the structures of autophagy were measured by VLCDS image analyzer (Leica, Germany). In addition, the average numbers of the autophagy-related structures were calculated in the cytoplasm.

## Determination of apoptosis in hPAECs

We performed Terminal-deoxynucleotidyl transferase mediated-dUTP nick-end labeling (TUNEL) assay to confirm the apoptosis of hPAECs by an assay kit (In Situ Cell Death Detection Kit; Roche Diagnostics) <sup>(26)</sup>. In a short, after different treatments, cells were incubated with TdT and fluorescein-labeled dUTP for 45 min under 37 °C. Furthermore, 4,6-diamidino-2-phenylindole (DAPI) was invoked as identifying nucleus. Confocal microscopy (Olympus Fluoview 2000) served as taking images. Meanwhile, we calculated the percentage of apoptotic cells. We counted five random fields for analysis in each group. We did all assays blindly.

## Western blot assay

We collected and dissolved hPAECs in protein lysis buffer (Sigma). Then, equivalent protein was separated by electrophoresis on 12% SDS-PAGE gels at 120 V for 1.5 h. Furthermore, they were transferred to PVDF membrane by 100 mV electrophoresis for 1.5 h. After blocked in 5% nonfat dry milk (BD Biosciences) at room temperature for 1 h, cellular membranes were subjected to immune-blotting with primary antibodies overnight at the temperature 4 °C. After incubation with appropriate secondary antibodies binding to horseradish peroxidase, we used an enhanced chemiluminescence system (Amersham Bioscience) to visualize blots bands. Furthermore, we determined densitometric analysis of Western blot with VisionWorks LS, version 6.7.1. <sup>(21)</sup>

We used following antibodies: rabbit anti-mouse LC-3, Beclin-1, ATG12-5, p62,  $\beta$ -arrestin1 and VEGFR3, PCNA, Bcl-2 and Bax (1:500, Cellular Signal Technology), rabbit anti-mouse phosphorylated (Thr172) and rabbit anti-mouse total Akt (1:200, Abcal), rabbit anti-mouse mTOR (1:500, Abcal), rabbit anti-mouse p-mTOR (1:200, Abcal), rabbit anti-mouse p70S6k, S6 (1:500, Abcal), rabbit anti-mouse p-p70S6k, p-S6 (1:200, Abcal), and rabbit anti-mouse  $\beta$ -actin (12,000, Abcal).

## Statistics analysis

Data analysis was performed by using Graph Pad Prism 5.0 (San Diego, CA, USA). All quantitative data are presented as the Mean  $\pm$  SEM. The different groups of our study were compared by the homogeneity tests and one-way analysis of variance (ANOVA).  $P < 0.05$  was considered as a statistical significance.

## Results

# Hypoxia increased the migration and proliferation of hPAECs

The transwell assay was performed for evaluation of the migration in hPAECs. The representative immunofluorescence images revealed that after hypoxia treatment, the migration increased time-dependently compared with control group (Fig. 1a). Meanwhile, the CCK-8 assay was performed to assess the viability of hPAECs. As shown in Fig. 1b, the OD values were increased under hypoxic treatments compared with control group ( $p < 0.05$ ), indicating that hypoxia promoted proliferation of hPAECs. Meanwhile, ELISA analysis was used to detect the paracrine effects of hPAECs. The results showed that hypoxic stress increased the level of VEGF, bFGF, HGF and IGF-1 (Fig. 1c-f). Taken together, our data suggested hypoxic injury contributed to the excessive proliferation, migration and paracrine dysfunction of hPAECs.

# Apoptosis resistance was increased significantly in hPAECs with hypoxic injury

To investigate the effects of hypoxic injury on apoptosis, the apoptosis of hPAECs was detected by TUNEL assay. The representative immunofluorescence images (Fig. 2a) revealed that compared to normoxic condition, TUNEL positive cells were markedly increased with hypoxia for 24 h. However, compared to that of in hypoxia 24 h group, apoptosis positive cells were reduced with hypoxia for 48 h, and 72 h, respectively. Meanwhile, the quantitative analysis revealed that the percentages of TUNEL-positive hPAECs with hypoxia for 24 h, 48 h, and 72 h were  $34.87 \pm 0.90\%$ ,  $22.87 \pm 1.52\%$ , and  $14.50 \pm 1.58\%$  respectively, significantly decreased than that in normoxic group ( $7.33 \pm 0.44\%$ ,  $p < 0.05$ , Fig. 2b). Collectively, our data suggested that apoptosis resistance was increased in hPAECs under hypoxic stress time dependently.

# Autophagy was markedly activated in hPAECs under hypoxic conditions

To explore the effect of hypoxia on autophagy, transmission electron microscope was used for detecting autophagy structures. Meanwhile, western blot and semi-quantitative analysis were performed to evaluate autophagy-related protein expression. Electron micrographs showed that the autophagosome formation was increased under hypoxic injury for 24, 48, and 72 h, respectively ( $p < 0.05$ , Fig. 3a, b). The results of western blot and semi-quantitative analysis demonstrated that the expression levels of LC3-I/LC3-II (mediates vesicle elongation and expansion), Beclin-1 (relates to vesicle nucleation) and ATG12-5 (regulate vesicle elongation and expansion) were dramatically increased under hypoxic group ( $p < 0.05$ , Fig. 3c-f). Thus, these results indicated that hypoxia induced over-activation of autophagy compared with normoxic hPAECs.

## **$\beta$ -Arrestins1 and VEGFR3 expression was reduced in hPAECs with hypoxia**

After giving hypoxic treatment for hPAECs, we found that the expression of  $\beta$ -Arrestins1 was decreased with the prolongation of hypoxia. Surprisingly, VEGFR3 expressions in hypoxia groups were also reduced compared with the control group ( $p < 0.05$ , Fig. 4a-c). In summary, these results demonstrated that hypoxia decreased  $\beta$ -Arrestins1 and VEGFR3 expressions.

## **Hypoxia suppressed the activity of the Akt/mTOR signal pathway in hPAECs**

We further detected the effects of hypoxic stress on the activation of the Akt/mTOR signal pathway in hPAECs by Western blot assay. As the typical Western blot results and semi-quantitative analyses shown, compared with normoxic treatment, the phospho-Akt (Ser473) expression of hPAECs was dramatically reduced under hypoxic stress for 24, 48, and 72 h, respectively ( $p < 0.05$ , Fig. 5a, b). Meanwhile, the expression of phospho-mTOR (Ser2448) decreased in hPAECs with hypoxic treatment ( $p < 0.05$ , Fig. 5a, c). In addition, hypoxia remarkably suppressed the downstream effectors of mTOR signaling pathway, such as the phosphorylation of p70 ribosomal S6 subunit kinase (p70S6K) and ribosomal S6 protein (S6) ( $p < 0.05$ , Fig. 5a, d, and e). To sum up, these results indicated that hypoxia contributed to decrease the activity of the Akt/mTOR signal pathway in hPAECs.

## **Autophagy inhibition contributed to hPAECs reduced migration, proliferation and apoptosis resistance**

To explore effect of autophagy on hPAECs with hypoxic injury, autophagy was suppressed with 3-MA, an inhibitor of phosphatidylinositol 3-kinases (PI3K) that played a vital role in various biological processes, such as controlling the activation of mTOR, a key regulator of autophagy. As representative Western blot and semi-quantitative analyses demonstrated that the increased autophagy-related protein (LC3-II, ATG12-5, Beclin-1) induced by 72 h-hypoxia was decreased by 3-MA ( $p < 0.05$ , Fig. 6a-d). Meanwhile, scratch-wound assay and quantitative analyses suggested that 3-MA restrained excessive migration caused by 72 h-hypoxia stress ( $p < 0.05$ , Fig. 6e and 6f). Furthermore, PCNA expression level was decreased after 3-MA treatment ( $p < 0.05$ , Fig. 6i and 6j). As shown in Fig. 6k, compared with 72 h-hypoxia group, the OD value reduced under 3-MA group ( $p < 0.05$ ). And compared with that of in 72 h-hypoxia condition, anti-apoptosis protein Bcl-2 expression in hPAECs with 3-MA was restrained while Bax expression was increased (Fig. 6g). Semi-quantitative analysis indicated that increased Bcl-2/ Bax was decreased in 3-MA group ( $p < 0.05$ , Fig. 6h). Taken together, autophagy inhibition reduced excessive migration, proliferation and apoptosis resistance.

# Regulation of $\beta$ -Arrestins1 changed the expression of VEGFR3

We intervened  $\beta$ -Arrestins1 to investigate its effect on VEGFR3. Western blot revealed that  $\beta$ -Arrestins1 and VEGFR3 expression levels were promoted in  $\beta$ -Arrestins1<sup>+/+</sup> group vs. pcDNA3.1 group, while that in  $\beta$ -Arrestins1<sup>-/-</sup> group was suppressed (Fig. 7a and 7b). Meanwhile, the representative semi-quantitative analyses suggested that after  $\beta$ -Arrestins1 over-expression, protein expressions of  $\beta$ -Arrestins1 and VEGFR3 were increased ( $p < 0.05$ , Fig. 7c-d). Compared to that in siRNA negative control group (siNC group), however, the expression level in knock-down group was decreased. In short, these data suggested that  $\beta$ -Arrestins1<sup>+/+</sup> upregulated the expression of VEGFR3, but  $\beta$ -Arrestins1<sup>-/-</sup> downregulated the protein expression of VEGFR3.

## Regulating $\beta$ -Arrestins1 effected hPAECs function

To confirm the effect of  $\beta$ -Arrestins1 on function of hPAECs,  $\beta$ -Arrestins1 was regulated. The images in light microscope showed that increased migration was reduced after  $\beta$ -Arrestins1 over-expression (Fig. 8a). Meanwhile, the CCK-8 assay demonstrated that the OD value related to proliferation of hPAECs significantly decreased ( $p < 0.05$ , Fig. 8b). Collectively, these data indicated that  $\beta$ -Arrestins1<sup>+/+</sup> suppressed excessive proliferation and migration of hPAECs with hypoxia. However,  $\beta$ -Arrestins1<sup>-/-</sup> reversed these results of hPAECs.

## Intervention of $\beta$ -Arrestins1 influenced apoptosis resistance

Then, after giving hPAECs hypoxic treatment, we over-expressed and knocked down  $\beta$ -Arrestins1 to discovery the role of  $\beta$ -Arrestins1 in apoptosis resistance. The images of TUNEL assay (Fig. 9a) showed that compared to hPAECs in pcDNA3.1 group, TUNEL-positive cells were significantly promoted under  $\beta$ -Arrestins1<sup>+/+</sup> (Fig. 9b,  $p < 0.05$ ). However, TUNEL-positive cells were reduced after  $\beta$ -Arrestins1<sup>-/-</sup> compared with that in siNC group. All in all, these data suggested over-expression of  $\beta$ -Arrestins1 suppressed apoptosis resistance, but knock-down of  $\beta$ -Arrestins1 increased resistance of apoptosis in hPAECs.

## $\beta$ -Arrestins1<sup>+/+</sup> inhibited autophagy, while $\beta$ -Arrestins1<sup>-/-</sup> activated autophagy in hPAECs

To confirm the effect of  $\beta$ -Arrestins1 on autophagy, we intervened  $\beta$ -Arrestins1. As these data in Western blot suggested that the protein expression levels of LC3-I/LC3-II, ATG12-5 and Beclin-1 were suppressed, whereas the p62 expression was increased in  $\beta$ -Arrestins1<sup>+/+</sup> group vs. pcDNA3.1 group (Fig. 10a). Differently, compared with that in siNC group, these protein expression levels were increased in  $\beta$ -

Arrestins1<sup>-/-</sup> group. Moreover, semi-quantitative analyses ( $p < 0.05$ , Fig. 10b-e) indicated that these autophagy-related protein expression levels were similar to that of Western blot. Taken together, our results demonstrated that  $\beta$ -Arrestins1<sup>+/+</sup> deregulated the activation of autophagy, but  $\beta$ -Arrestins1<sup>-/-</sup> upregulated activity of autophagy.

## **$\beta$ -Arrestins1 regulated the activity of the Akt/mTOR signal pathway in hPAECs**

Furthermore, we detected the effect of  $\beta$ -Arrestins1 on the activation of the Akt/mTOR signal pathway by Western blot assay. As the typical Western blot results and semi-quantitative analyses showed that compared with  $\beta$ -Arrestins1<sup>-/-</sup>,  $\beta$ -Arrestins1<sup>+/+</sup> significantly increased the phospho-Akt (Ser473) expression of hPAECs ( $p < 0.05$ , Fig. 11a, b). Meanwhile, the expression of phospho-mTOR (Ser2448) increased in hPAECs with  $\beta$ -Arrestins1<sup>+/+</sup> treatment ( $p < 0.05$ , Fig. 11a, c). Meanwhile,  $\beta$ -Arrestins1<sup>-/-</sup> suppressed the downstream effectors of mTOR signal pathway, such as the p70S6K and S6 ( $p < 0.05$ , Fig. 11a, d, and e). In short, these results indicated that  $\beta$ -Arrestins1 contributed to regulate the activity of the Akt/mTOR signal pathway of hPAECs.

## **Discussion**

In present study, we observed exacerbated autophagy in hPAECs with hypoxia for 24 h, 48 h, 72 h with the time dependently. Meanwhile, apoptosis also occurred in hPAECs with hypoxia for 24 h. However, apoptosis was reduced in hPAECs with hypoxia for 48 h, 72 h respectively. In addition, hypoxic injury decreased  $\beta$ -Arrestin 1 and VEGFR3 in hPAECs. Furthermore,  $\beta$ -Arrestin 1 upregulation reduced excessive migration and proliferation induced by hypoxia in hPAECs. Concurrently, regulating  $\beta$ -Arrestin 1 changed the autophagy and apoptosis resistance of hPAECs caused by hypoxic stress. To sum up, our data for the first time suggested that selective intervention of  $\beta$ -Arrestin 1 has the effects on autophagy of hPAECs under hypoxia presumably via activating the VEGFR3 and Akt/mTOR signaling. Together, we identified  $\beta$ -Arrestin 1 as a potential therapeutic target for patients with hypoxia-related PAH (Fig. 12).

Autophagy, as a double-edged sword, not only plays a vital role in maintaining the normal function of homeostasis, but also be significantly induced and over-upregulated by adverse stimulus, such as hypoxia<sup>(26)</sup>. Previous studies found that excessive activation of autophagy induced by hypoxia, which was an important factor that caused increased migration and over-proliferation of PAECs<sup>(5, 10)</sup>. In our study, increased proliferation and migration were shown in hPAECs with hypoxic injury. Meanwhile, over activated autophagy and high expression level of autophagic proteins, such as LC3-I/LC3-II (mediates vesicle elongation and expansion), Beclin-1 (relates to vesicle nucleation) and ATG12-5 (regulates vesicle elongation and expansion), were observed in hypoxia-stress hPAECs by transmission electron microscope and Western blot. However, after given 3-MA, an inhibitor of autophagy, excessive migration and hyperproliferation were suppressed, suggesting autophagy regulated migration and proliferation in

hPAECs. Furthermore, the TUNEL assay demonstrated that apoptosis-resistance in hPAECs was dramatically enhanced under hypoxia time-dependently. Compared with that of hypoxia, pro-apoptosis protein Bax expression in hPAECs with 3-MA was increased while anti-apoptosis protein Bcl-2 expression was restrained, which suggesting inhibition autophagy promoted apoptosis of hPAECs in hypoxic conditions. In summary, our data revealed that autophagy exacerbated migration, proliferation and apoptosis-resistance of hypoxic hPAECs, which suggesting no difference with previous study.

Akt/mTOR signaling pathway contributed to regulating cell survival, and oxidative stress<sup>(27)</sup>. Concurrently, the mammalian target of rapamycin (mTOR), a key regulator in diverse cellular functions, including protein synthesis and apoptosis, inhibited the cellular catabolic pathway, such as negatively regulated autophagy.<sup>(28)</sup> Previous study showed that mTOR repressed the autophagy in PAH<sup>(29)</sup>. Li et al.<sup>(12)</sup> also indicated that mTOR ameliorated hypoxia-triggered PAH by autophagy-pathway. Our results further showed that hypoxia reduced the expressions level of p-Akt and p-mTOR in hPAECs. In addition, hypoxic injury decreased the phosphorylation of mTOR substrates, such as p70S6K and S6, which shows no difference with previous study. Taken together, our data suggested that hypoxia downregulated the activation of Akt/mTOR signal pathways in hPAECs.

Previous study found that  $\beta$ -arrestin1 could activate Akt signal pathway<sup>(10)</sup>. We found that the p-Akt (Ser473) and p-mTOR (Ser2448) expression of hPAECs were dramatically promoted under  $\beta$ -Arrestins1<sup>+/+</sup> condition, but that of  $\beta$ -Arrestins1<sup>-/-</sup> were decreased. Meanwhile,  $\beta$ -Arrestins1 over-expression promoted the downstream factors of mTOR signaling pathway, such as the p70S6K and S6. However, these expression levels were suppressed in knock-down group. Thus, our findings showed that  $\beta$ -Arrestins1 promoted the activity of Akt/mTOR signaling pathway in hPAECs. In addition, previous study demonstrated that VEGFR3 protected hypoxia-induced PAH<sup>(30,31)</sup>. Recent study reported that in human PAH, expression of  $\beta$ -Arrestin 1 was decreased and was associated with a loss of VEGFR3 expression. Fortunately, our results also indicated hypoxia suppressed  $\beta$ -Arrestins1 and VEGFR3 expression, suggesting that it is consistent with the results of previous study. Moreover, study found the ablation of Arrb1 aggravated hypoxia induced PAH, suppressed VEGFR3 and impaired downstream Akt activity in mice. In addition, knock out of  $\beta$ -Arrestin 1 inhibited VEGF-C-mediated cell migration in vitro<sup>(15)</sup>. Similarly, our results of regulating cellular  $\beta$ -Arrestin 1 was consistent with previous study. These findings suggested that  $\beta$ -Arrestin 1 played an essential protective effect on PAH.

In our study, to explore the effect of  $\beta$ -Arrestin1 on autophagy and apoptosis,  $\beta$ -Arrestin1 was regulated to observe changes of autophagy and apoptosis resistance for the first time. We found that compared with hypoxia,  $\beta$ -Arrestins1<sup>+/+</sup> inhibited autophagy and apoptosis resistance, while  $\beta$ -Arrestins1<sup>-/-</sup> activated autophagy and apoptosis resistance in hPAECs.

Present research has some clinical significance, however, this also includes some limitations. The hypoxic cellular model was limited as an artificial experimental model that could not fully simulate the PAH environment in vivo. In addition, the specific function of  $\beta$ -Arrestins1 has been fully unknown. Thus,

it is necessary to determine the exact mechanism to understand the process of hypoxic PAH in future studies.

## Conclusions

Collectively, our current study demonstrated that hypoxic stress increased autophagy and apoptosis resistance by downregulation of  $\beta$ -Arrestins1 and VEGFR3, which inhibiting Akt/mTOR signal pathway in hPAECs, suggesting that promoting  $\beta$ -Arrestins1 mediated inhibition of autophagy may be a potential target for PAH.

## Declarations

### Acknowledgements

Not applicable

### Consent for publication

Not applicable.

### Data Availability of data and materials

All data in this study are available and included in this published article.

### Competing interests

The authors declare that they have no competing interests.

### Funding

This work was supported by the National Nature Science Foundation of China (No.81700050).

### Authors contributions

HJN and JYD have contributed equally to this work. HJN, JYD, ZZ, LNS and THH designed the study, drafted the manuscript, and approved its final version. DLK, FC and YFL acquired data, revised the article's intellectual content, and approved the final version. LNS, THH and ZZ are responsible for the integrity of this work. All authors read and approved the final manuscript.

### Author details

1Department of Respiratory, The First Affiliated Hospital of Jinzhou Medical University, Jinzhou 121001, China.

2 Department of Cardiology, Postgraduate Training Base in PLA Rocket Force Characteristic Medical Center, Jinzhou Medical University, 121001, Liaoning, China.

3 Department of Respiratory Medicine, First Affiliated Hospital of China Medical University, Shenyang 110001, China.

4 Department of critical care medicine, PLA Rocket Force Characteristic Medical Center, Beijing 100088, China.

5 Department of Cardiology, PLA Rocket Force Characteristic Medical Center, Beijing 100088, China.

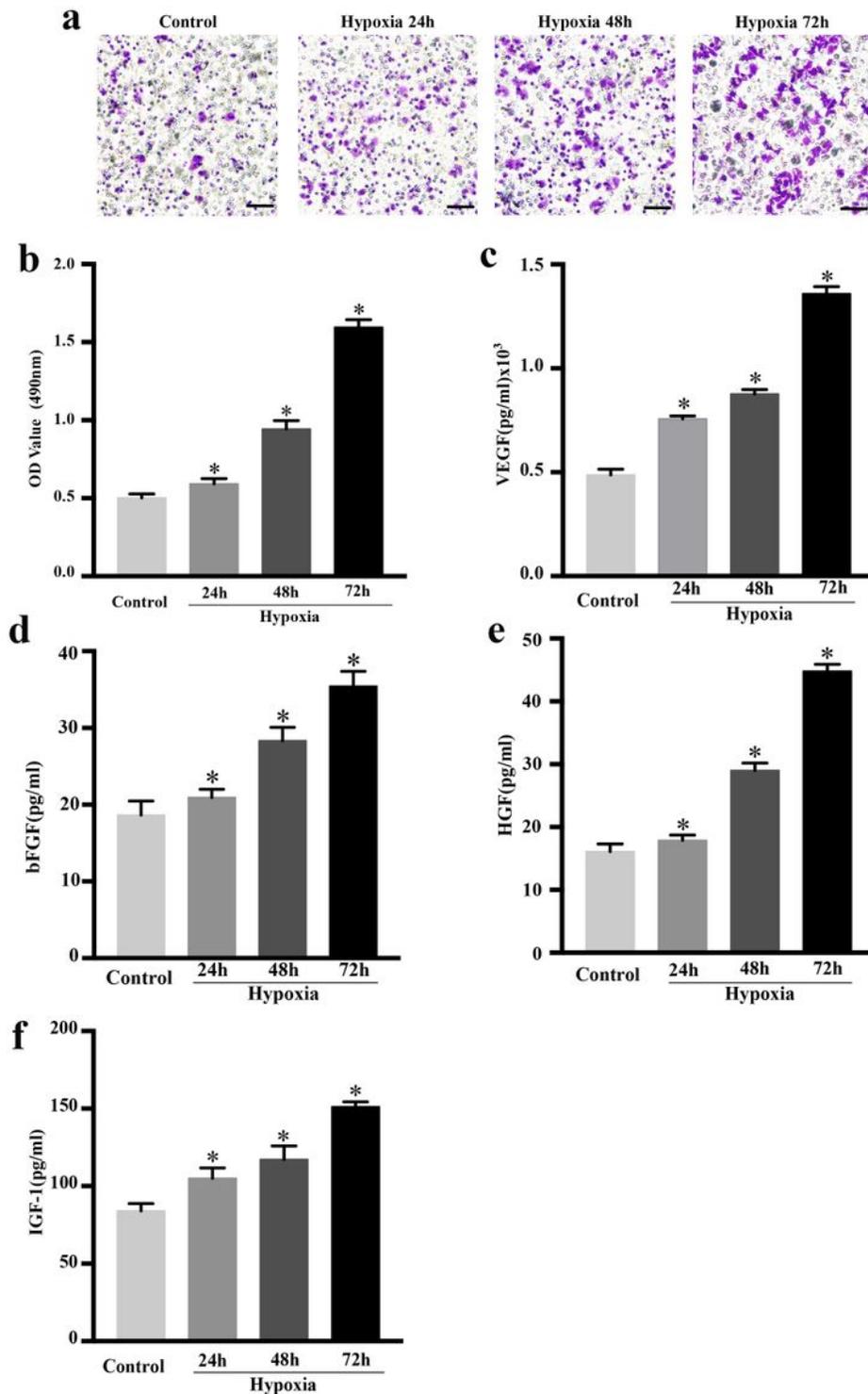
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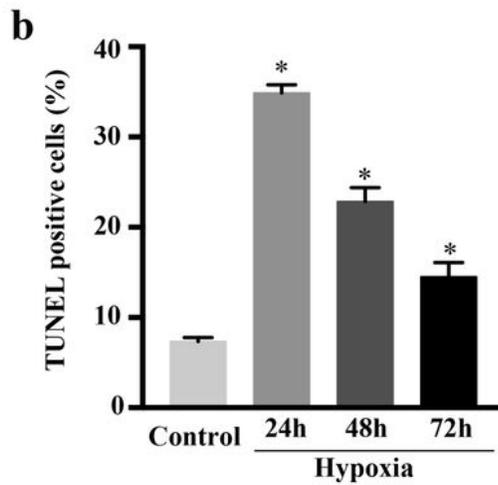
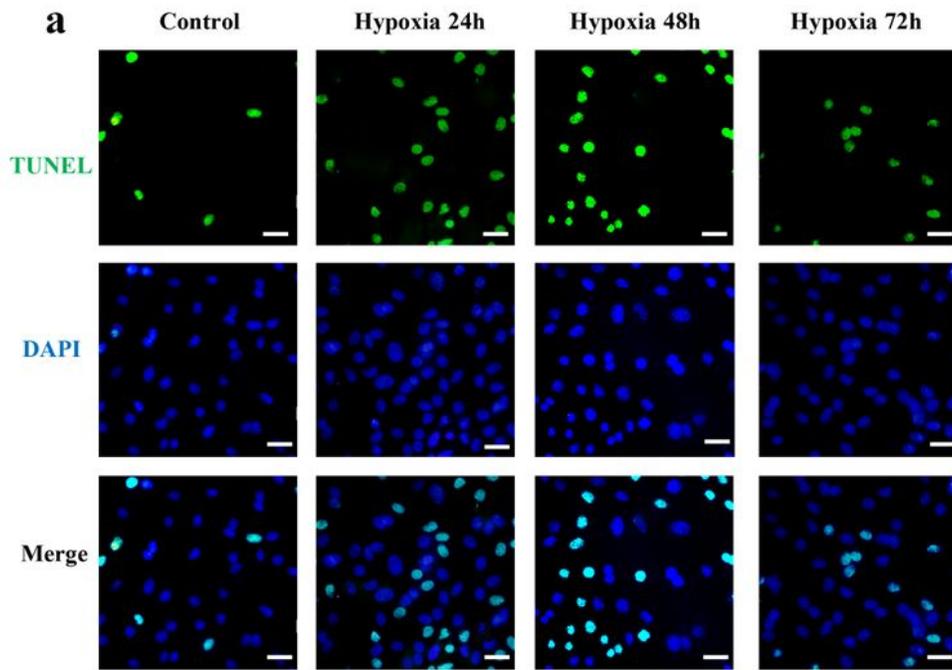
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## Figures



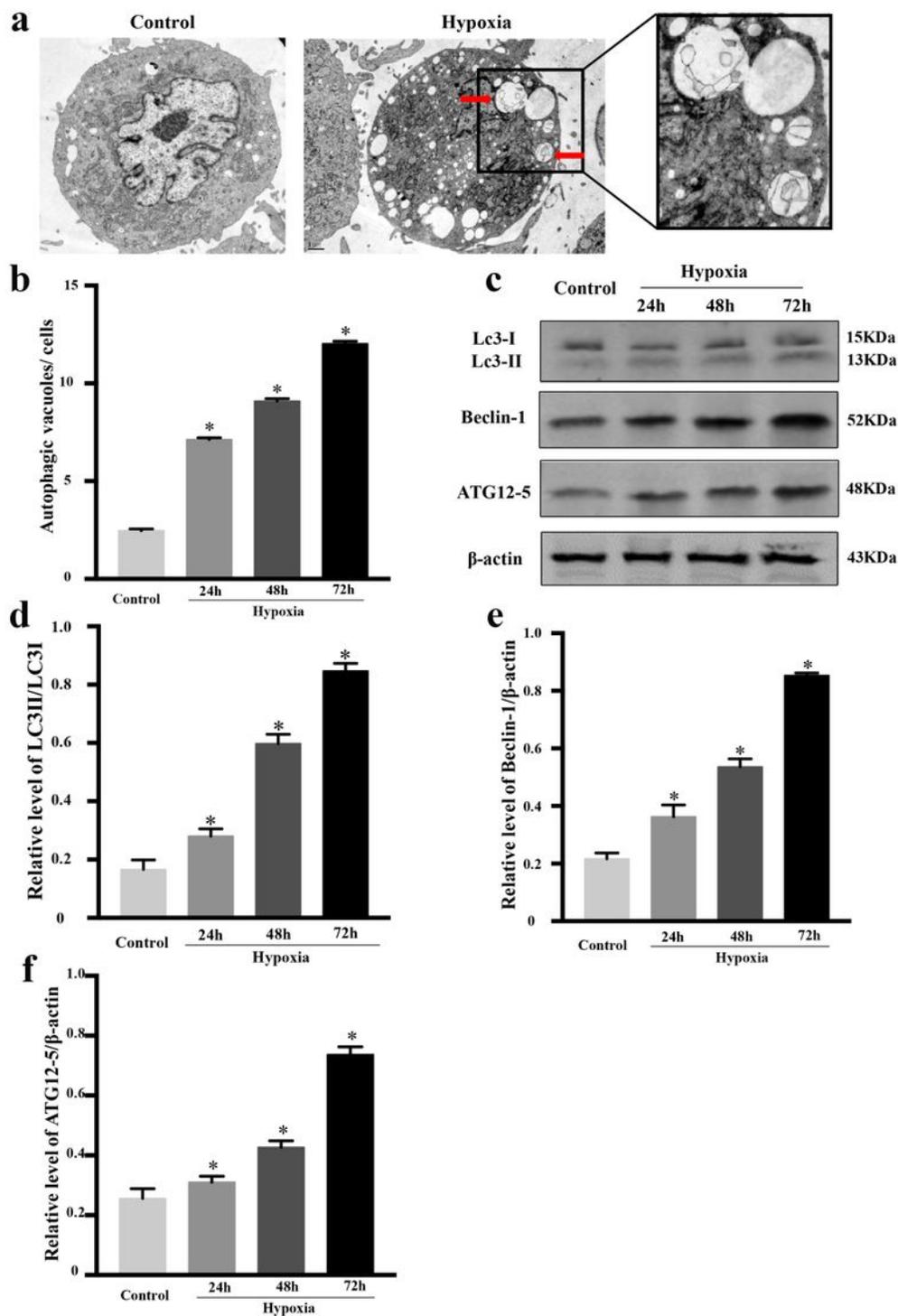
**Figure 1**

Hypoxic stress contributed to migration, proliferation and dysfunction of hPAECs. a Transwell cell migration assay was performed to detect the migration by light microscope (scale bar, 100  $\mu$ m). b The proliferation of hPAECs treated with hypoxia (\* $p < 0.05$ ). Quantification of VEGF (c), bFGF (d), HGF (e) and IGF-1 (f) was presented at the indicated time points ( $n = 5$ , \* $p < 0.05$ ). Data are expressed as the means  $\pm$  SEM;  $n = 5$ ; \* $p < 0.05$



**Figure 2**

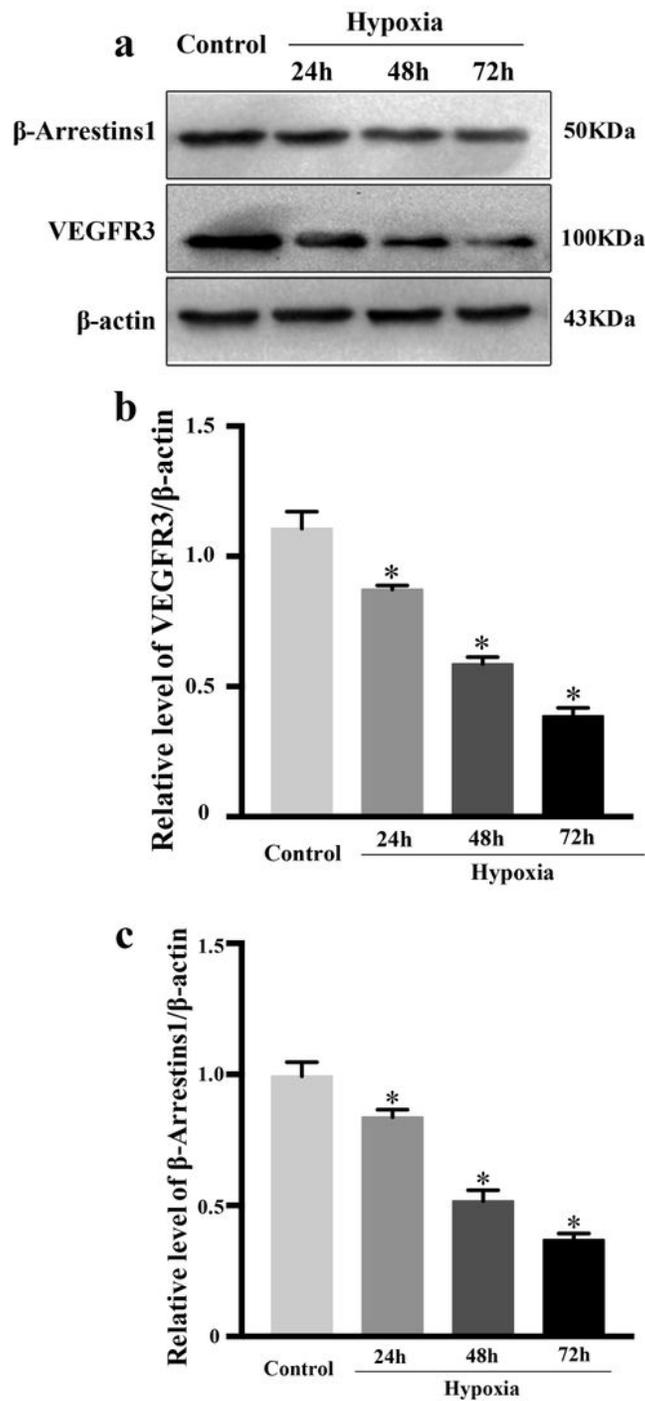
Hypoxia induced apoptosis resistance of hPAECs. a Representative TUNEL images of hPAECs with normoxic and hypoxic treatments (scale bars, 20  $\mu$ m). b Quantification of the apoptotic hPAECs was presented as the percentage of apoptotic cells (n = 5, \*p < 0.05)



**Figure 3**

Hypoxic injury increased autophagy. **a** Electron micrographs showed the autophagic vacuole formation in hPAECs (scale bar, 1  $\mu$ m). The red arrows indicated the double-membraned autophagosome accumulated in cytoplasm. **b** Representative quantification of the average numbers of the autophagy-related structures in the cytoplasm. **c** Representative Western blot of LC3-I/LC3-II, Beclin-1, and ATG12-5 in hPAECs subjected to normal and hypoxic conditions. Semi-quantification of the protein expression levels of LC3-

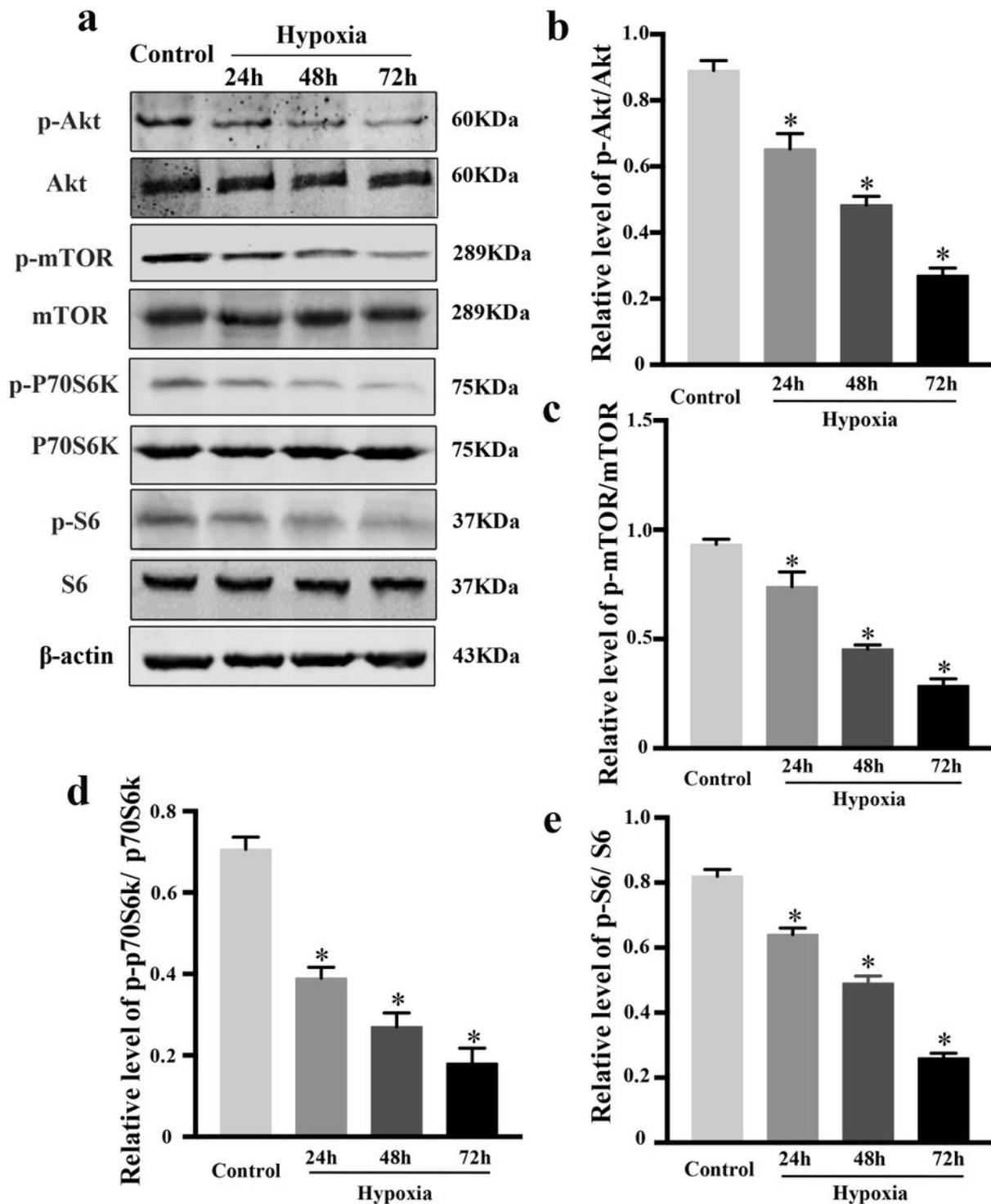
II/ LC3-I (d), Beclin-1 (e) and ATG12-5 (f) at the indicated time points. Data are expressed as the means  $\pm$  SEM; n = 5; \*p < 0.05



**Figure 4**

Hypoxic treatment restrained  $\beta$ -Arrestins1 and VEGFR3 expression levels. a Representative Western blot of  $\beta$ -Arrestins1 and VEGFR3 in hPAECs under normal and hypoxic conditions. Semi-quantification of the

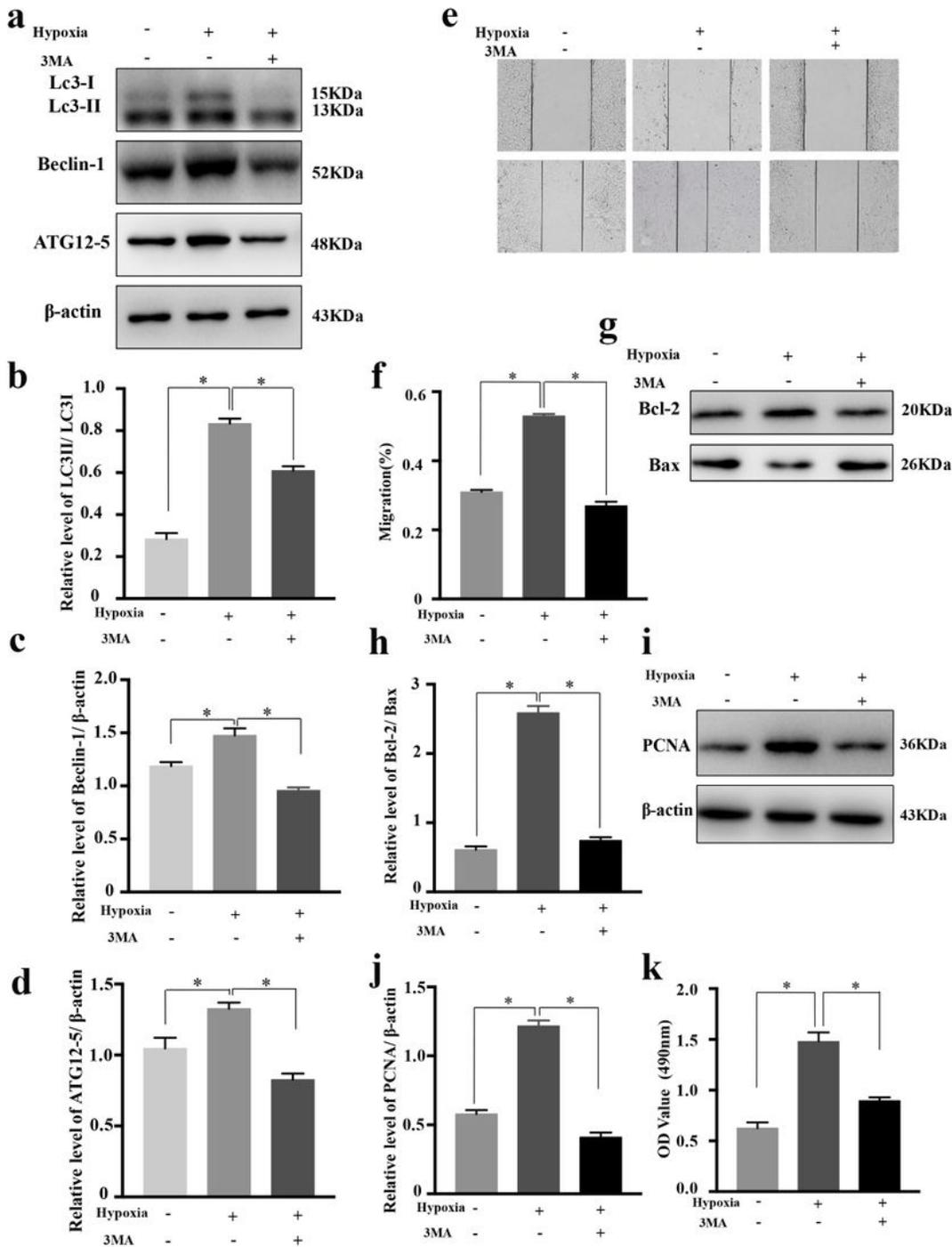
protein expression levels of VEGFR3 (b) and  $\beta$ -Arrestins1 (c) at the indicated time points. Data are expressed as the means  $\pm$  SEM; n = 5; \*p < 0.05



**Figure 5**

Hypoxic stress suppressed the activity of the Akt/mTOR signal pathway. a Representative Western blots of p-Akt/Ak, p-mTOR/mTOR, p-p70S6 K/p70S6 K, p-S6/S6, and  $\beta$ -actin in hPAECs subjected to normal and hypoxic condition for 24, 48, and 72 h, respectively. Semi-quantification of the protein expression

levels of p-Akt (b), p-mTOR (c), p-p70S6K/p70S6K (d), and p-S6/S6 (e). Data are expressed as the means  $\pm$  SEM; n = 5; \*p < 0.05



**Figure 6**

3-MA contributed the change of autophagy, migration, proliferation and apoptosis resistance. a Western blot of autophagy-related proteins in hPAECs under hypoxic and 3-MA conditions. Semi-quantification of the protein expression levels of autophagic protein(b-d). e Representative scratch-wound assay (scale bar,

50  $\mu$ m). f Quantitative analyses of migration (\* $p < 0.05$ ). g Western blot of Bcl-2 and Bax in hPAECs. h Representative quantification of the protein expression levels of Bcl-2/Bax. i Western blot of PCNA in hPAECs. Semi-quantification of the protein expression levels of PCNA (j). k The proliferation of hPAECs treated with hypoxia and 3-MA (\* $p < 0.05$ ). Data are expressed as the means  $\pm$  SEM;  $n = 5$ ; \* indicates  $p < 0.05$

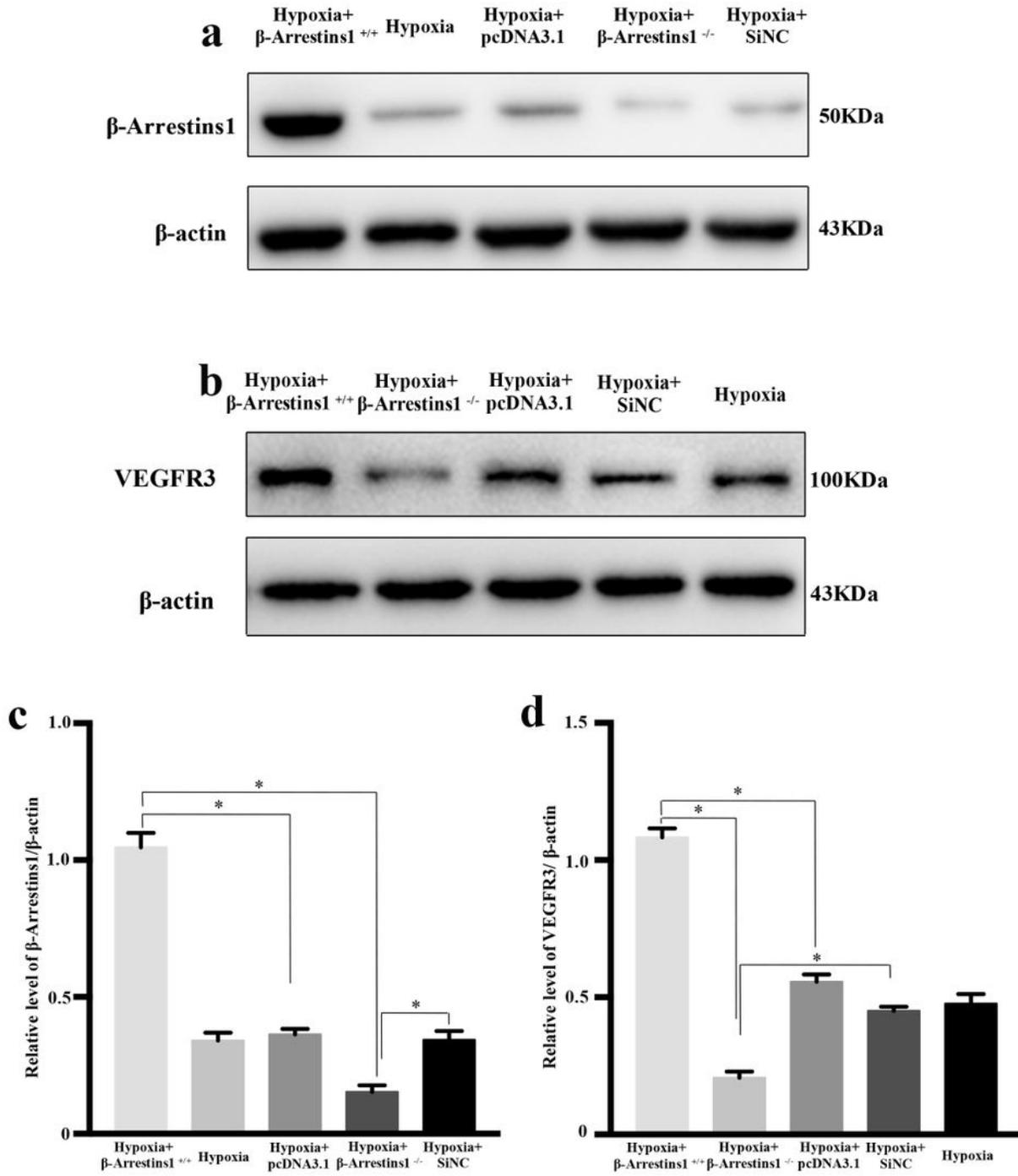
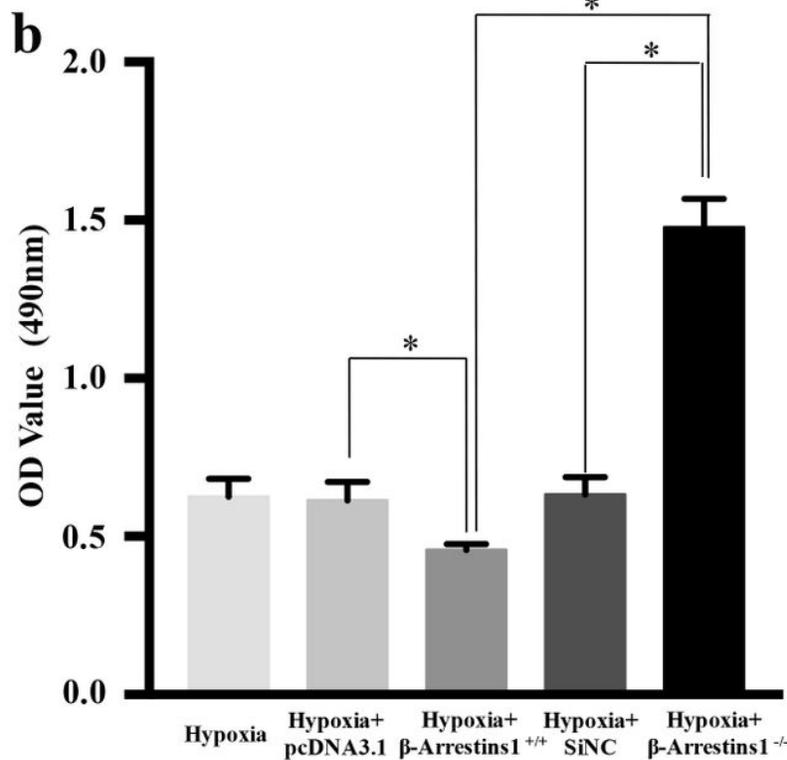
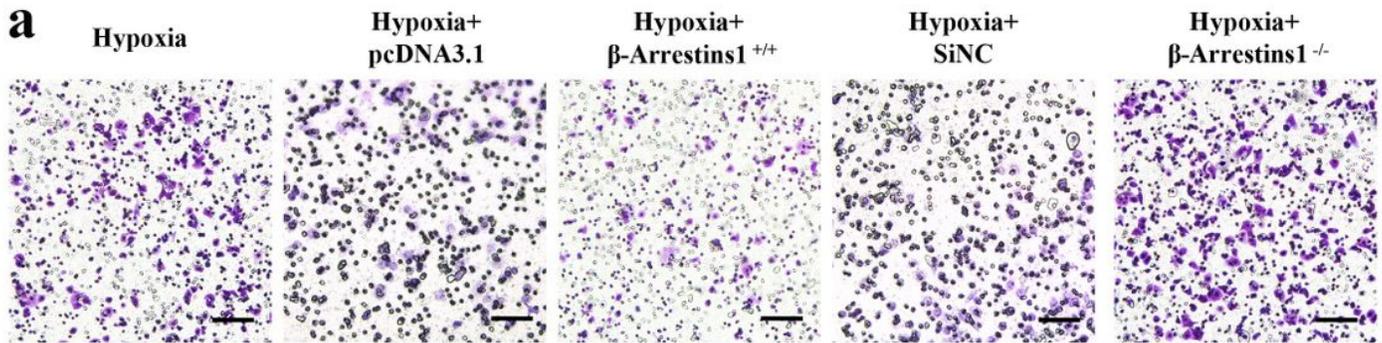


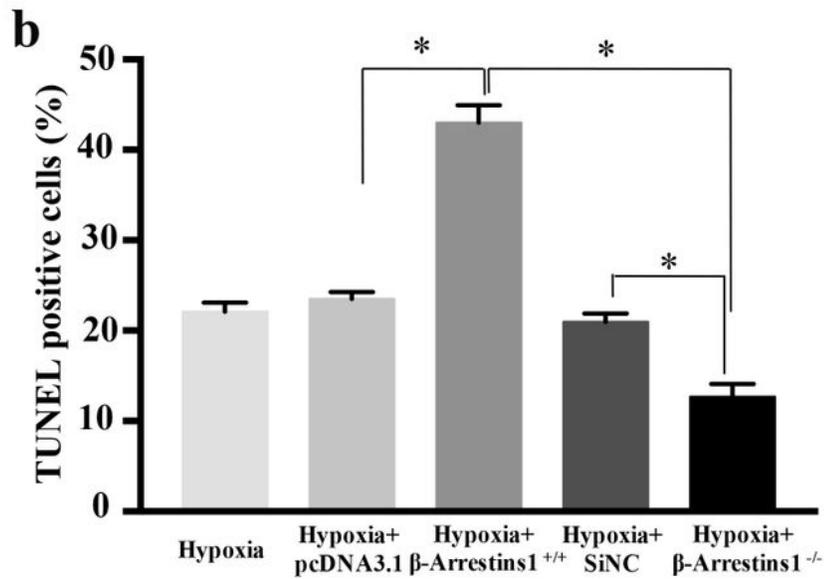
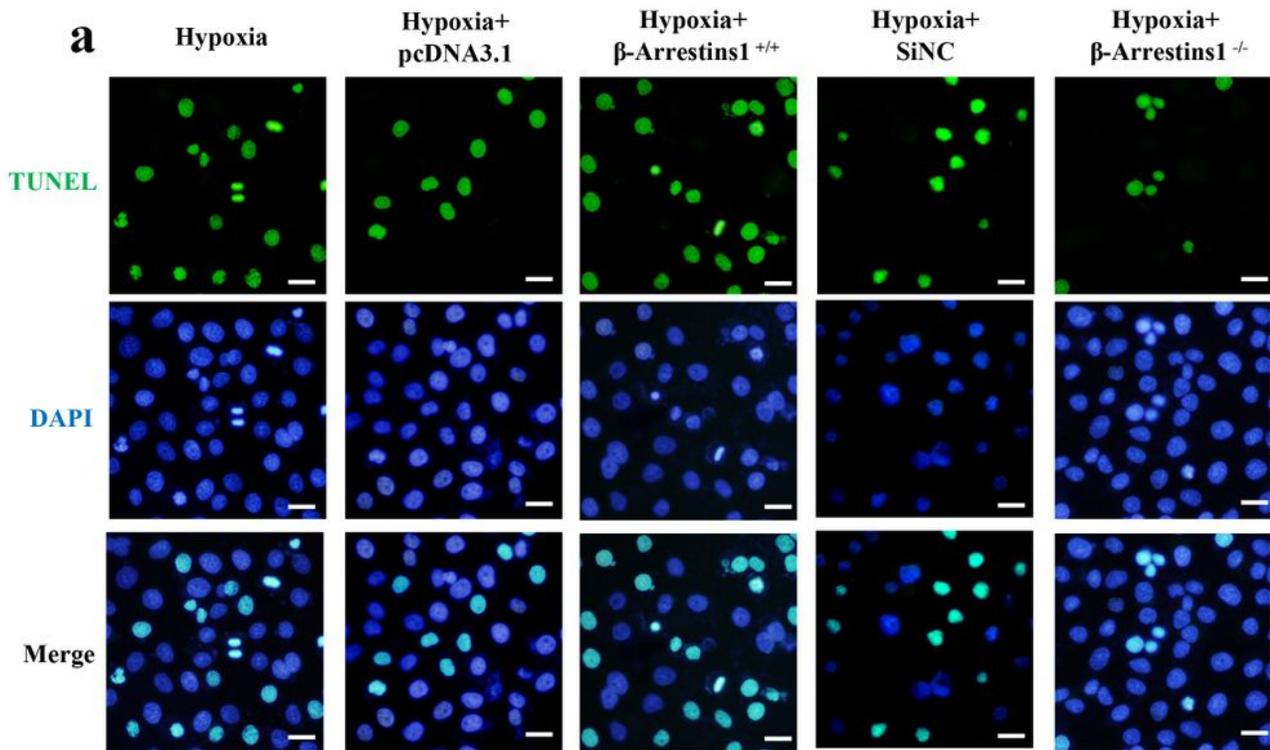
Figure 7

$\beta$ -Arrestins1 contributed the change of VEGFR3 expression levels. a Western blot of  $\beta$ -Arrestins1 in hPAECs under  $\beta$ -Arrestins1<sup>+/+</sup> and  $\beta$ -Arrestins1<sup>-/-</sup> conditions. Quantification of the protein expression levels of  $\beta$ -Arrestins1 (c). b Western blot of VEGFR3 in hPAECs under  $\beta$ -Arrestins1<sup>+/+</sup> and  $\beta$ -Arrestins1<sup>-/-</sup> conditions. Semi-quantification of the protein expression levels of VEGFR3 (d). Data are expressed as the means  $\pm$  SEM; n = 5; \* indicates p<0.05



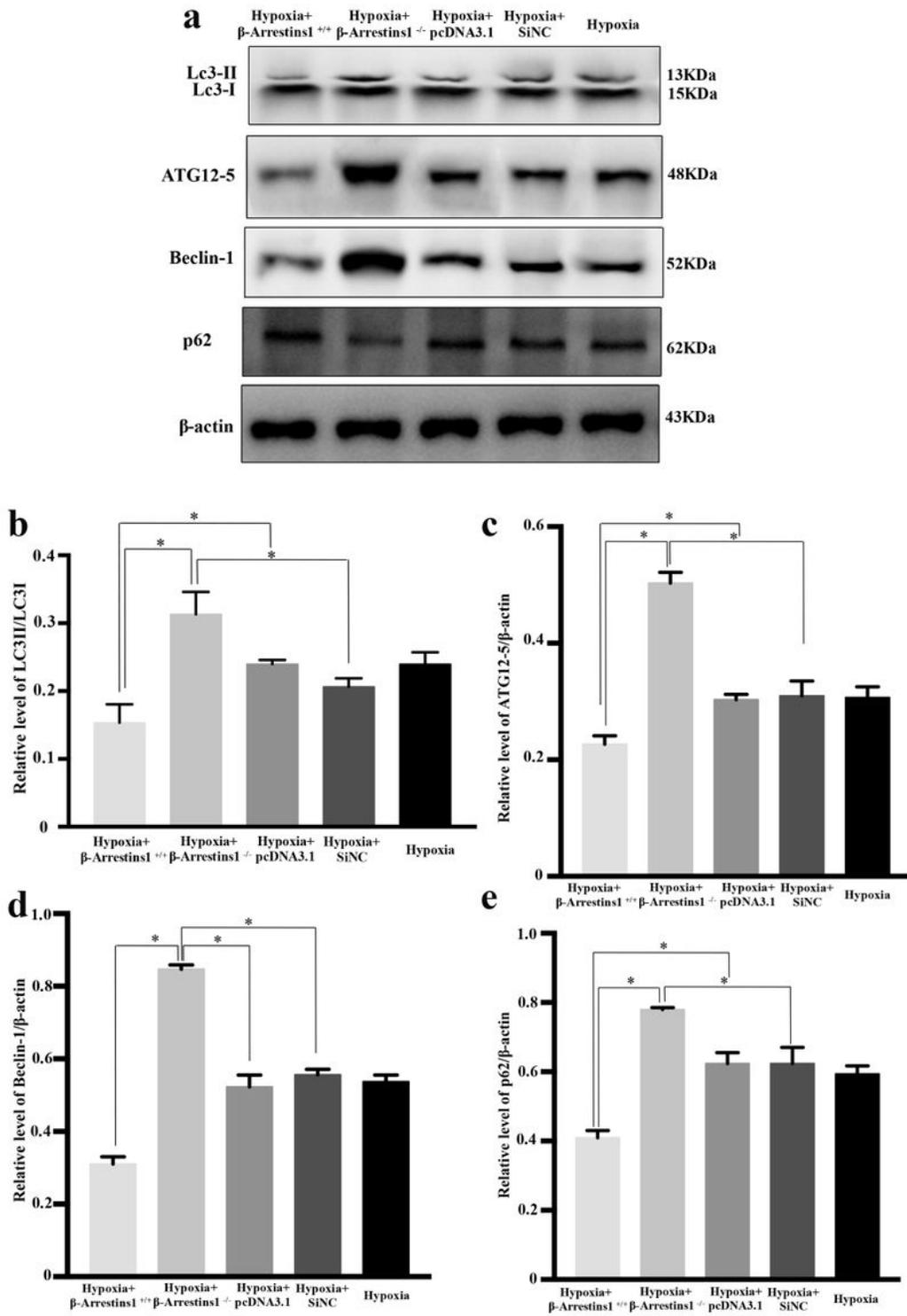
**Figure 8**

The change of migration and proliferation in hPAECs after intervention of  $\beta$ -Arrestins1. a Cell migration assay was used for observing the migration by light microscope (scale bar, 100  $\mu$ m). b The proliferation of hPAECs treated with  $\beta$ -Arrestins1<sup>+/+</sup> and  $\beta$ -Arrestins1<sup>-/-</sup>. Data are expressed as the means  $\pm$  SEM; n = 5; \*p < 0.05



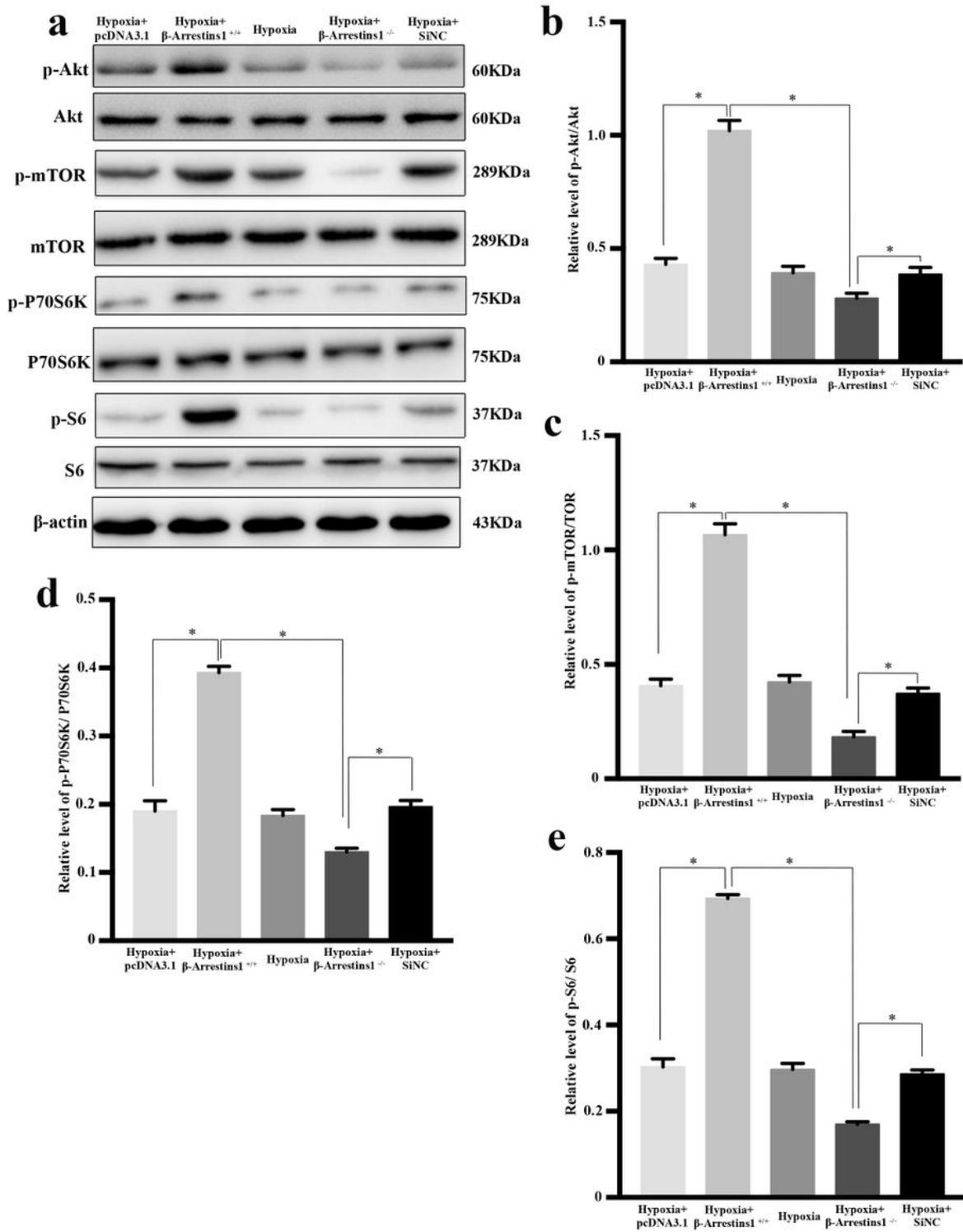
**Figure 9**

The intervention of  $\beta$ -Arrestins1 changed resistance of apoptosis in hPAECs. a Representative TUNEL images of hPAECs with regulating  $\beta$ -Arrestins1 (scale bars, 20  $\mu$ m). b Quantification of the apoptotic hPAECs was presented as the percentage of apoptotic cells (n = 5, \* indicates p<0.05).



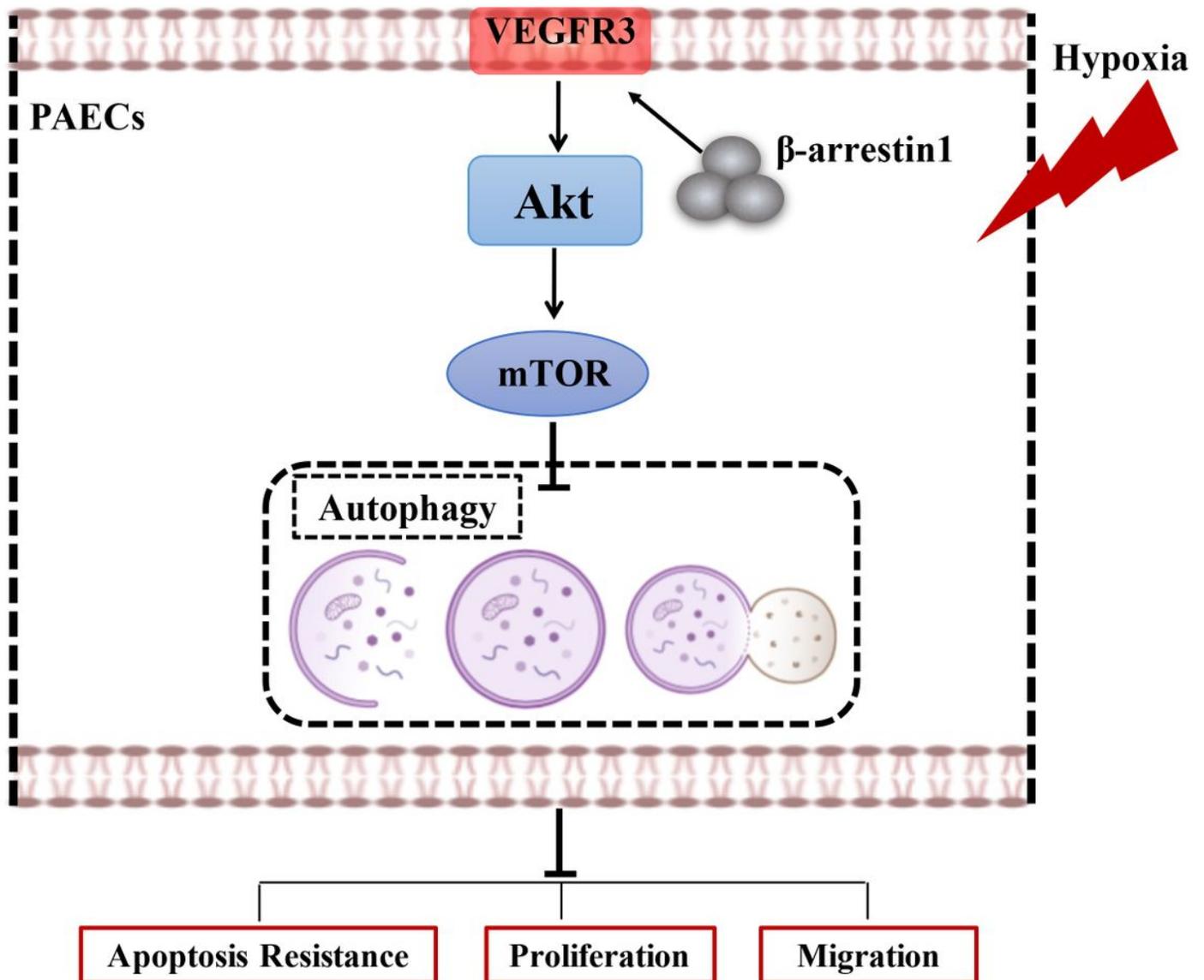
**Figure 10**

The changes of autophagy-related protein expression level. a Representative Western blotting of LC3-I/LC3-II, Beclin-1, p62 and ATG12-5 in hPAECs after regulation of  $\beta$ -Arrestins1. Semi-quantification of the protein expression levels of LC3-II/ LC3-I (b), ATG12-5 (c), Beclin-1 (d) and p62 (e). Data are expressed as the means  $\pm$  SEM; n = 5; \*p < 0.05



**Figure 11**

The regulation of  $\beta$ -Arrestins1 changed the activity of the Akt/mTOR signal pathway. a Western blots of p-Akt/Ak, p-mTOR/mTOR, p-p70S6 K/p70S6 K, p-S6/S6, and  $\beta$ -actin in hPAECs. Representative quantification of the protein expression levels of p-Akt (b), p-mTOR (c), p-p70S6K/p70S6K (d), and p-S6/S6 (e). Data are expressed as the means  $\pm$  SEM; n = 5; \*p < 0.05



**Figure 12**

Proposed mechanism of  $\beta$ -Arrestins1 regulated autophagy of hPAECs caused by hypoxic stress. Hypoxia decreases the protein expression of  $\beta$ -Arrestins1, which inhibits activation of Akt and mTOR. Moreover, repressed mTOR directly enhances autophagy. Therefore,  $\beta$ -Arrestins1 mediating VEGFR3 regulates migration, proliferation and apoptosis resistance by autophagy via Akt/mTOR signal pathway in hypoxic hPAECs.