RELM-β knockout inhibits the development of hypoxia-induced pulmonary hypertension through PLC-IP3R-Ca2+ signaling pathway

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Abstract

Purpose Pulmonary vascular remodeling (PVR) is an important pathological mechanism of hypoxia-induced pulmonary hypertension (HPH), in which the proliferation of pulmonary artery smooth muscle cells (PASMCs) plays an important role. Resistin-like molecule beta (RELM-β), a secretory protein, can promote the proliferation of PASMCs induced by hypoxia. As an important signaling molecule, Ca^{2+} plays an important role in cell proliferation. RELM-β can regulate cell proliferation by changing the intracellular calcium concentration ($[Ca^{2+}]_i$), but the specific regulatory mechanism of RELM-β on Ca^{2+} and the pathogenesis of HPH has not been fully elucidated.

Methods We employed both in vivo and in vitro RELM-β knockout (RELM-$\beta^{-/-}$) models to examine the effects of RELM-β on pulmonary hemodynamics, PASMCs proliferation, intracellular Ca^{2+} release, and associated mechanisms.

Results The expression of RELM-β increased in rat HPH model and hypoxia treated PASMCs, which led to pulmonary hemodynamic changes (increased mean pulmonary artery pressure (mPAP), right ventricular hypertrophy, pulmonary artery thickening) and PASMCs proliferation. However, knockout of RELM-β had the opposite effect. RELM-β deletion decreased the expression of phospholipase C (PLC), inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R), and $[Ca^{2+}]_i$. In addition, inhibition of PLC and IP_3R can reduce $[Ca^{2+}]_i$.

Conclusion Our research results have confirmed the role of RELM-β as a cytokine-like growth factor in the proliferation of PASMCs and contribute to HPH. This was achieved by upregulating $[Ca^{2+}]_i$ through the PLC/IP_3R pathway.

Introduction

HPH is an important comorbidity of chronic obstructive pulmonary disease (COPD) [1, 2]. Recent epidemiological investigations have shown that HPH is present in approximately 30–70% of patients with moderate to severe COPD[3]. The five-year survival rate for PH patients without lung transplantation is only 38.1%, and it drops to 15% if the condition progresses to cor pulmonale[4, 5]. The well-recognized pathophysiological process involving HPH is characterized by pulmonary vasoconstriction and small artery remodeling, followed by increased pulmonary vascular resistance. The proliferation of PASMCs is the key process[6–8].

RELM-β is a secretory protein belonging to the RELM gene family[9]. Hypoxia-induced mitogenic factor (HIMF/RELM-α) is the first member and has been proven to be involved in PASMCs proliferation, angiogenesis and HPH[10]. However, RELM-α is only expressed in rodents, while its human direct homolog, RELM-β, is expressed in both rodents and humans, which has attracted our attention to its potential clinical value[11]. Recombinant human RELM-β (rhRELM-β) can significantly increase the proportion of human pulmonary artery smooth muscle cells (hPASMCs) in S phase through FAK/survivin and decrease the proportion in G0/G1 phase, thus promoting mitosis[12]. In addition, Han et al. found

that hypoxia can upregulate the expression of RELM-β protein in hPASMCs and promote cell proliferation. Silencing RELM-β inhibits cell proliferation and alleviates the progression of HPH[13]. These results suggest that RELM-β plays an important role in the pathogenesis of HPH. However, there are relatively few studies on the mechanism of RELM-β in HPH, and most of the experiments have been carried out in vitro. Few studies have focused on the overall hemodynamic effects. The specific molecular mechanism of RELM-β in HPH has not been fully elucidated.

Ca^{2+} plays an important role in regulating cell proliferation[14]. In our previous study using a gene silencing model of HPH, we found that RELM-β promotes the proliferation of PASMCs through the Ca^{2+}-dependent downstream pathways PI3K/Akt/mTOR and PKC/MAPK. RELM-β promotes the influx of Ca^{2+} by regulating store-operated Ca^{2+} entry (SOCE) [15]. However, whether the effect of RELM-β on $[Ca^{2+}]_i$ is through direct action or through indirect effect via the intermediate signal pathway is still unknown. HIMF can activate the IP_3R channel of the endoplasmic reticulum (ER) through PLC-IP_3 in a tyrosine-dependent manner, resulting in an increase in $[Ca^{2+}]_i$ and PASMCs proliferation[16]. Since RELM-β is a direct homolog of HIMF in human, we hypothesized that RELM-β may promote Ca^{2+} release from the ER through PLC-IP_3R, thus increasing $[Ca^{2+}]_i$ to promote the development of HPH[17].

Our results confirm that RELM-β promotes PASMC proliferation and HPH development. This was achieved by increasing the concentration of intracytoplasmic Ca^{2+} through the PLC/IP_3R signaling pathway.

Methods

Construction of RELM-$\beta^{-/-}$ SD rats

RELM-$\beta^{-/-}$ rats were constructed by Cyagen Biotechnology Co., Ltd (Suzhou, China). To create an RELM-$\beta^{-/-}$ rat model (SD) by CRISPR/Cas-mediated genome engineering. The build process is as follows: The rat RELM-β gene (gene ID: 498074; GenBank accession number: NM_001024281.1) is located on rat chromosome 11. A total of 4 exons were identified, of which the ATG start codon was located in exon 2 and the TAA termination codon was located in exon 4. Exon 1 to exon 4 will be selected as the target site. Two pairs of gRNA targeting vectors will be constructed and confirmed by sequencing. Cas9 mRNA and gRNA transcribed in vitro will be coinjected into fertilized eggs for the production of knockout (KO) rats. The pups will be genotyped by polymerase chain reaction and sequence analysis. Germline passages were carried out, and RELM-$\beta^{-/-}$ rats were identified. F2 generation male rats aged 8–10 weeks were selected for the experiment. (Animal Experimental Unit use License NO: SYXK2-19-0009)

Construction of the rat model

Rats aged 8–10 weeks were randomly divided into the normoxic (N) group and chronic hypoxia (CH) group. Normoxic group: normobaric normoxic environment. Chronic hypoxia group: rats were placed in a

special oxygen control box with 10%±0.5% oxygen concentration and 60%±0.5% humidity (Huaxiao Electronic Technology Co., Ltd., Changsha, Hunan Province, China) for 8 hours a day for 21 days. All rats were placed in a specific pathogen-free (SPF) environment. These animals were allowed to receive free access to food and water every day[15].

Determination of Hemodynamics and RVHI

Rats were anesthetized by intraperitoneal injection of 1% pentobarbital sodium (35 mg/kg). The PAP of rats was measured by right cardiac catheterization, and the specific values were measured by connecting transducers and physiological multichannel instruments (BL-420S biological signal acquisition system, Chengdu Tai Meng Co., China). Because right ventricular pressure(RVP) is close to the PAP, this study recorded RVP to reflect PAP, as described previously[18]. After the blood pressure measurement was over, the rats were bled to death. The weights of the right ventricle (RV), left ventricle (LV) and interventricular septum (S) were measured, and then the right ventricular hypertrophy index (RVHI) (RVHI = RV/(LV + S) %) was calculated. As mentioned earlier[17].

Extraction and Cultivation of PASMCs

The medial membrane of the right pulmonary artery was isolated, and the PASMC was extracted. The extracted cells were then cultured at 37 ° C in a 5%CO2 incubator. Cells were passaged at a ratio of 1:2 and primary cultures were completed within 10 passages. As described earlier[19].

Construction of the cell model

The PASMCs of RELM-$\beta^{-/-}$ and wild-type (WT) rats were isolated and cultured. PASMCs were treated with hypoxia and normoxia. Hypoxia treatment conditions: the cells were placed in a three-gas incubator with 1% O2, 94% N2 and 5% CO2 (SQ-80 N, Shanghai Musi Experimental Equipment Co., Ltd., China) for 24 hours. Normoxic treatment: The cells were placed in a cell incubator with 21% O2, 74% N2 and 5% CO2 (Thermo Fisher, model 3111) for 24 hours.

H&E staining

The left lung tissue of rats was stained with HE. The tissue was fixed, trimmed, dewaxed, hydrated, stained, differentiated, transparent, sealed and so on. Finally, the pathological sections were observed and photographed under a microscope (DM2000LED, Leica, Germany). As mentioned earlier[15].

Western blot (WB) analysis

Lung tissue and PASMCs samples needed for the experiment were collected. The protein concentration was determined by the BCA method. Then, Sample loading, electrophoresis, membrane transfer, blocking and primary antibody incubation (RELM-β antibody (1:500, Abcam); PLC antibody (1:1000, Abcam); IP_3R antibody (1:1000, Abcam. membrane washing, and incubation with secondary antibody (Anti-Rabbit IgG (H + L) Antibody 1:50000 KPL). ECLA and B solution were mixed at a 1:1 volume and then dripped evenly on the film to develop. The picture was saved, and the grayscale of the image was analyzed with ImageJ software. As described in a previous experiment[20].

Quantitative real-time PCR (qPCR) analysis

Total RNA of RELM-β and IP_3R was extracted from lung tissue and PASMCs, respectively. Using the PerfectStart Green qPCR SuperMix polymerase chain reaction kit (TransGen Biotech. AQ601-04) for RT—PCR detection. The PCR primers were as follows: Rat RELM-β-149-F, 5'-ATAGTCCCAGGGAACGCGCA-3' and Rat RELM-β-149-R, 5'-ACAACCATCCCAGCAGGACA-3'; Rat IP_3R-107-F, 5'-GAAGCTGGGGAAGATGAGGAAGAG-3' and Rat IP_3R-107-R, 5'-TGTCCCTCTTTAGCATCTTGTGCC-3'; Rat GAPDH-140-F, 5'-GCAAGTTCAACGGCACAG-3' and Rat GAPDH-140-R, 5'-GCCAGTAGACTCCACGACAT-3'. Relative expression was calculated using the 2^−ΔΔCT method. As described in a previous experiment[21].

Detection of $[Ca^{2+}]_i$ by flow cytometry (FCM)

Cells that were not treated or received rhRELM-β or other combined treatment were washed and collected. The calcium ion mother liquor was diluted to 5uM.The cells were incubated at 37°C in the dark for 30 minutes and mixed every 3–5 minutes. Cell precipitation was collected by centrifugation. Wash the cells with serum-free medium. The cells were detected by flow cytometry within 1 hour. The fluorescence of Ca^{2+}-FITC (excitation wavelength Ex = 488 nm, emission wavelength Em = 530 nm) was detected by the FITC channel (FL1). Fluorescence intensity is proportional to free calcium ion concentration. As described in the previous experiment, Liu X et al used FCM to detect the change of intracellular Ca^{2+} concentration after inhibition of IP_3R[22].

Cell proliferation assay

Cell proliferation was measured by the EdU method. PASMCs were collected, logarithmic growth phase cells were taken, digested and washed, and the cells were inoculated into 24-well plates for cell adhesion. Then, the cells were treated with recombinant human RELM-β or other combined treatment. The cells were treated with EdU labeling, cell fixation and permeability. A click reaction mixture was added, and Hoechst 33342 solution was added for DNA staining. Finally, pictures were collected under an inverted fluorescence microscope (Nikon, DS-Fi3). As described in a previous experiment[23].

Statistical analysis

SPSS26.0 statistical software was used to analyze the data. The data are represented by "statistics ± SD". The significant difference between the two independent groups was determined by t test. For multiple groups (all groups are equal in number and are two-way comparisons between multiple means), analysis of variance and Tukey's test were used for statistical analysis. $P < 0.05$ indicates that it is statistically significant.

Results

Gene knockout can completely block the hypoxia-induced upregulation of RELM-β

We used CRISPR/cas-mediated genomic engineering to construct the RELM-$\beta^{-/-}$ rat model (Fig. 1a). The successful construction of the RELM-$\beta^{-/-}$ rat model was verified by qPCR and WB. The specific manifestations were as follows: mRNA (Fig. 1b, c) and protein (Fig. 1d, e) of RELM-β were highly expressed in lung tissue and PASMCs of rats in the CH + WT group, but no expression of RELM-β was detected in lung tissue and PASMCs of the CH + RELM-$\beta^{-/-}$ group.

RELM-$\beta^{-/-}$ significantly reduces the development of HPH

RELM-$\beta^{-/-}$ can significantly reduce hypoxia-induced PVR, mPAP and RVHI(RV/(LV + S) %). In this experiment, we measured the outer diameter of small arteries (45–100μm), and the degree of PVR was indicated by the percentage of midmembrane thickness (MT%) estimated from H&E-stained tissue(Fig. 2a, c). The mPAP was measured by right cardiac catheterization (Fig. 2b, d). The RVHI was measured by weighing (Fig. 2e). The results showed that PVR, mPAP, and RVHI were significantly lower in the RELM-$\beta^{-/-}$ group than in the WT group under chronic hypoxic conditions($P < 0.05$), but there was no significant difference between RELM-$\beta^{-/-}$ group and WT group when exposed to normoxia ($P > 0.05$) (Fig. 2a-e).

RELM-β activates PLC-IP_3R

RELM-β, as the upstream molecule of the PLC and IP_3R pathways, regulates the expression of PLC and IP_3R. We detected the expression of PLC and IP_3R by qPCR and WB. The results showed that under normoxia or chronic hypoxia, knockout of RELM-β in lung tissue and PASMCs decreased the expression of PLC protein (Fig. 3a, b) and the expression of IP_3R mRNA (Fig. 3c, d) and protein (Fig. 3e, f). Addition of U73122 (PLC inhibitor) to PASMCs resulted in lower IP_3R protein expression than that of the control group (Fig. 3g). Addition of rhRELM-β activated PLC phosphorylation ($P < 0.05$) (Fig. 3h).

RELM-β increases $[Ca^{2+}]_i$ through the PLC-IP_3R pathway

RELM-β is capable of enhancing $[Ca^{2+}]_i$ via the PLC-IP_3R pathway. In the calcium-free Buffer solution, we used FCM technology to detect the concentration of Ca^{2+} in PASMCs. The fluorescence intensity is proportional to the concentration of intracellular free calcium ion. Knockout of RELM-β gene could significantly reduce the average fluorescence intensity of Ca^{2+} induced by hypoxia ($P < 0.05$). Nonetheless, under normoxic conditions, the knockout of the RELM-β gene did not significantly alter the average fluorescence intensity of Ca^{2+} ($P > 0.05$) (Fig. 4a, c). The average fluorescence intensity of Ca^{2+} in the rhRELM-β group was notably elevated compared to the control group ($P < 0.05$) (Fig. 4b, d). The

average fluorescence intensity of Ca^{2+} was significantly reduced in the U73122 + rhRELM-β group or xestospongin C + rhRELM-β group compared to the rhRELM-β group ($P < 0.05$) (Fig. 4b, d).

Intracellular Ca^{2+} release through PLC-IP_3R promotes PASMCs proliferation

RELM-β increased $[Ca^{2+}]_i$ via the PLC-IP_3R signaling pathway, thus promoting the proliferation of hypoxia-induced PASMCs. I In our study, we used the EdU method to determine the newly synthesized DNA of PASMC, and the DNA synthesis rate was used to indicate PASMC proliferatio (ratio of the number of green fluorescent to blue fluorescent cells). PASMCs were sustained in a calcium-free medium. Our findings demonstrate that the knockout of the RELM-β gene markedly diminished the hypoxia-induced DNA synthesis rate of PASMCs ($P < 0.05$) (Fig. 5a, c). However, under normoxic conditions, the knockout of the RELM-β gene did not significantly influence the DNA synthesis rate ($P > 0.05$) (Fig. 5a, c). The DNA synthesis rate of PASMCs in the rhRELM-β group was significantly elevated compared to the control group ($P < 0.05$) (Fig. 5b, d). The DNA synthesis rate was significantly lower in the U73122 + rhRELM-β, Xestospongin C + rhRELM-β, or BAPTA-AM + rhRELM-β groups compared to the rhRELM-β group($P < 0.05$) (Fig. 5b, d).

Discussion

HPH is an important complication in moderate to severe COPD[3]. The main pathological changes in HPH are PASMCs proliferation and PVR, but its specific pathogenesis has not been fully elucidated. Currently, there is no specific drug for HPH to effectively block its progression, and it is worth clarifying the possible pathogenesis of HPH to achieve effective treatment. In our previous articles, we found that RELM-β can regulate Ca^{2+}-dependent downstream molecules to participate in the development of HPH, but the specific molecular mechanism between RELM-β and Ca^{2+} is still unclear. To further clarify the regulatory mechanism of RELM-β on Ca^{2+}, we utilized RELM-$\beta^{-/-}$ rats for our study. We found that during long-term exposure to hypoxia, RELM-β could promote the release of Ca^{2+} from ER through the PLC-IP_3R pathway, thus upregulating the concentration of Ca^{2+} in PASMCs. The results showed that the increase of $[Ca^{2+}]_i$ could significantly upregulate PASMCs proliferation, vascular remodeling, PAP and right ventricular remodeling and promote the development of HPH.

RELM-β belongs to a highly conserved family of RELM secretory proteins. RELMs, also known as those found in the inflammatory zone (FIZZ), have four members: RELM-α/FIZZ1/HIMF, RELM-β/FIZZ2, resistin/FIZZ3 and RELM-γ/FIZZ4. Currently, RELM-α and RELM-β are the most concerning subtypes. However, because RELM-α is only expressed in rodents, the focus of our research has shifted to RELM-β, which is expressed in both rodents and humans. RELM-β is located at 3q13.1 with a molecular size of 9.003 kDa[24]. It shares 58.6% amino acid sequence homology with RELM-α, and the homology of the

protein is as high as 69%[10]. The expression patterns and functions of the two molecules are also very similar, so RELM-β is considered to be a direct functional homolog of RELM-α in humans[10].

RELM-β can be expressed in vascular smooth muscle cells, endothelial cells, fibroblasts and inflammatory cells and participates in cell proliferation, collagen deposition, fibrosis, inflammation and other pathological changes[25, 26]. Angelini DJ et al found that RELM-β expression was significantly increased in human PASMCs with scleroderma-associated PH and induced PASMCs proliferation, which was a key molecule involved in pulmonary hypertension (PH)[25]. In addition, it has been found that hypoxia can promote the proliferation of PASMC by increasing the expression of RELM-β[27]. In our previous study, we used an animal model of HPH to confirm this conclusion. In addition, we further found that RELM-β expression gradually increased with prolonged exposure to hypoxia and reached stability on Day 21. At the same time, HPH-related PVR and mPAP also peaked. It is suggested that the expression level of RELM-β may be related to the development of HPH[15]. Therefore, in this experiment, we specifically chose 21 days as the exposure time in vivo.

Previous studies have found that RELM-α plays a key role in mouse lung development, and knockout of RELM-α leads to the death of experimental animals in the embryonic stage[28]. Therefore, the silencing method is commonly used to study it in vivo. Considering the homology of RELM-β and RELM-α, we chose to silence RELM-β in previous experiments to explore its role in HPH. Lin et al used gene modification to conduct conditional knockout of the HIMF gene in vivo and proved that it could significantly reduce pathological changes such as PASMCs proliferation and PVR in mice[29]. In the follow-up of our preliminary experiment, we also found that conditional knockout of RELM-β alone did not cause early death of fetal rats. Subsequent studies have also shown that knockout of the HIMF gene in vitro can significantly reduce the concentration of Ca^{2+} in mouse cardiomyocytes, prevent cardiomyocyte hypertrophy, and significantly reduce cardiac remodeling[30]. Therefore, in this experiment, we used a gene knockout animal model to further verify the role of RELM-β in HPH. Our current results showed that knockout of the RELM-β gene could also significantly inhibit the proliferation of PASMCs, pulmonary vascular/right ventricular remodeling and mPAP induced by hypoxia, thus significantly inhibiting the development of HPH. According to this evidence, we consider RELM-β, compared to RELM-α (only expressed in rodents), to be of more clinical significance.

In a previous study, we found that $[Ca^{2+}]_i$ was related to the regulation of extracellular Ca^{2+} influx induced by SOCE .which was in turn, induced by RELM-β[15]. Previous studies have shown that the promotion of SOCE is closely related to the depletion of calcium stores in the ER. The specific manifestations are as follows: PLC-IP_3 acts on IP_3R Ca^{2+} channels, and IP_3R activates and opens, which releases a large amount of Ca^{2+} in the ER into the cytoplasm. When perceiving ER Ca^{2+} store depletion, it will promote SOCE[31]. This arouses our interest in exploring the mechanism by which RELM-β regulates the release of intracellular Ca^{2+} stores. Fan C et al found that HIMF can upregulate the concentration of Ca^{2+} in PASMCs exposed to hypoxia through PLC-IP_3, resulting in PASMCs proliferation and increased PAP[16]. In our study, RELM-β induced Ca^{2+} store release by PLC-IP_3R, upregulated the hypoxia-induced increase in

$[Ca^{2+}]_i$, and promoted the development of HPH. The results of Fan C et al are consistent with our experimental results, which further confirms that RELM-β and HIMF have high homology in function.

As the second messenger in the cell, Ca^{2+} regulates many different cellular physiological functions and plays an important role in driving gene transcription and cell proliferation. The Ca^{2+} involved in cell mitosis mainly comes from the ER[32], and IP_3R acts as the main channel for the release of Ca^{2+} in the ER[33]. The $[Ca^{2+}]_i$ regulated by RELM-β through the PLC-IP_3R pathway is an important source of Ca^{2+} for PASMCs proliferation. It is worth noting that in this experiment, under normoxic conditions, we found that knockdout of RELM-β decreased the expression of PLC and IP_3R ($P > 0.05$), however, did not cause significant changes in $[Ca^{2+}]_i$. For this result, we consider the following possible reasons. On the one hand, studies have shown that a large amount of Ca^{2+} in the ER is released when the concentration of IP_3 reaches a certain limit. When the level of IP_3 is low, IP_3 rarely binds to IP_3R, resulting in a small number of open IP_3R channels and only local Ca^{2+} signals, while when the level of IP_3 is high, it binds with more IP_3R and induces a large amount of Ca^{2+} release to the cytoplasm through a calcium-induced calcium release mechanism[34–36]. Therefore, we speculate that under normoxic conditions, the expression of RELM-β is relatively lower and the concentrations of PLC and IP_3 are lower than those under hypoxic conditions, which cannot effectively promote the mechanism of calcium-induced calcium release, so the trace expression of RELM-β under normoxic conditions has no significant effect on the change in $[Ca^{2+}]_i$. On the other hand, other studies have shown that the release of stored Ca^{2+} is not completely regulated by the concentration level of IP_3, and the mutual regulatory mechanism between IP_3Rs and Ca^{2+} depends partly on their spatial distribution in cells[37–39]. The specific mechanism needs further study in the future.

Advantages and limitations of this study: Currently, this is the first study using the RELM-$\beta^{-/-}$ method in an SD rat HPH model. On the one hand, it can be used as a further supplement and explanation of the gene silencing model in the previous experiment. The expression of RELM-β can be completely excluded, which is more advantageous to confirm that RELM-β plays an important role in the pathogenesis of HPH. Through the knockout of RELM-β in the embryonic stage and the breeding of RELM-$\beta^{-/-}$ adult rats, it was further confirmed that RELM-β is a nonfatal gene, which provides a strong basis for follow-up gene therapy, but it is still necessary to guard against the occurrence of some recessive potential diseases. On the other hand, it was further verified that RELM-β could regulate both intracellular and extracellular Ca^{2+}. However, there are still some shortcomings in this experiment: on the one hand, we have not further verified that RELM-β regulates SOCE through PLC-IP_3R, and further research is needed. On the other hand, RELM-β is a secretory protein, while PLC is an intracellular molecule. RELM-β needs to act on relevant receptors on the cell membrane to indirectly regulate intracellular PLC molecules, but the specific receptor of RELM-β is still unclear. Recently, a study found that HIMF, a homolog of RELM-β, can bind to the extracellular calcium sensitive receptor (CaSR) of PASMCs to mediate hypoxia-induced smooth muscle

cell proliferation, pulmonary vascular remodeling and pulmonary hypertension[40]. This inspired us to study the regulation of downstream signaling molecules by RELM-β through CaSR.

Notably, most laboratory studies have shown that RELM-β promotes inflammation, cell proliferation and tumor progression, but some studies have found that the deletion of RELM-β can promote airway inflammation, gastrointestinal inflammation and tumor progression[41, 42]. RELM-β plays different roles in different tissues, cells and different stages of disease development. These results suggest that we need to be prepared for the risk tendencies that may arise in later targeted therapy studies.

In short, RELM-β plays an important role in the development of HPH. It upregulates the concentration of Ca^{2+} in PAMSCs through the PLC-IP_3R pathwa, thus promoting cell proliferation, vascular remodeling, mPAP elevation and right ventricular remodeling. As a nonlethal gene, RELM-β can provide an effective and feasible basis for later targeted therapy of HPH.

Declarations

Ethical Approval Examination of Hunan University of Chinese Medicine (XMBH-201910080005). And relative animal experiments are permitted.

Availability of data and material The data and materials in the current study are available from the corresponding author on reasonable request.

Conflict of interest The authors declare no conflict of interest that could prejudice the impartiality of the present research.

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Author's contributions AGD and HST conceived the original idea for the study. AGD, GYL, HST, YL, and YX participated in the design and supervision of the study. GYL wrote the first draft of the manuscript, and the final content was developed in collaboration with all authors. GYL, HST, YL, and YX contributed to acquisition of data. GYL, HST, YL, YX, YW, LL, YDF, HLC, and CZ contributed to data analysis and interpretation. AGD, GYL, HST, YL, and YX contributed to the conception, design of the study, and revision of the manuscript. All authors read and approved the final manuscript.

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Figures

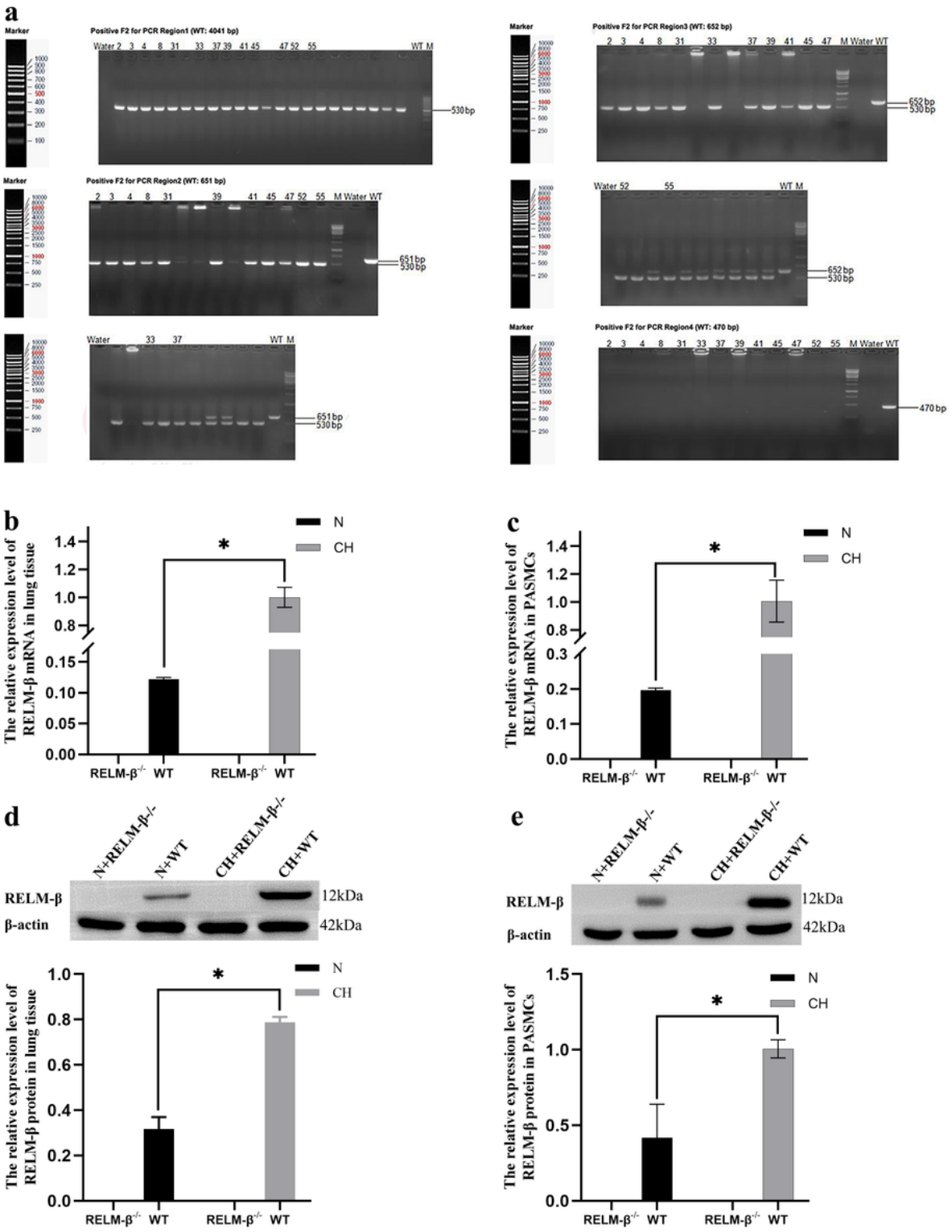

Figure 1

Gene knockout can completely block hypoxia-induced upregulation of RELM-β. **a** We used CRISPR/cas-mediated genomic engineering technology to construct a Retnlb (RELM-β) knockout rat model. Then, genotyping and sequence analysis were carried out by PCR to screen out homozygous gene knockouts. **b, c** qPCR was used to detect the relative expression of RELM-β mRNA in lung tissue and PASMCs of rats in the N+RELM-β$^{-/-}$, N+WT, CH+RELM-β$^{-/-}$, and CH+WT groups. (n=3,*P 0.05). **d, e** WB was used to detect the

relative expression of RELM-β protein in lung tissue and PASMCs of rats in the N+RELM-β$^{-/-}$, N+WT, CH+RELM-β$^{-/-}$, and CH+WT groups. (n=3,*P 0.05).

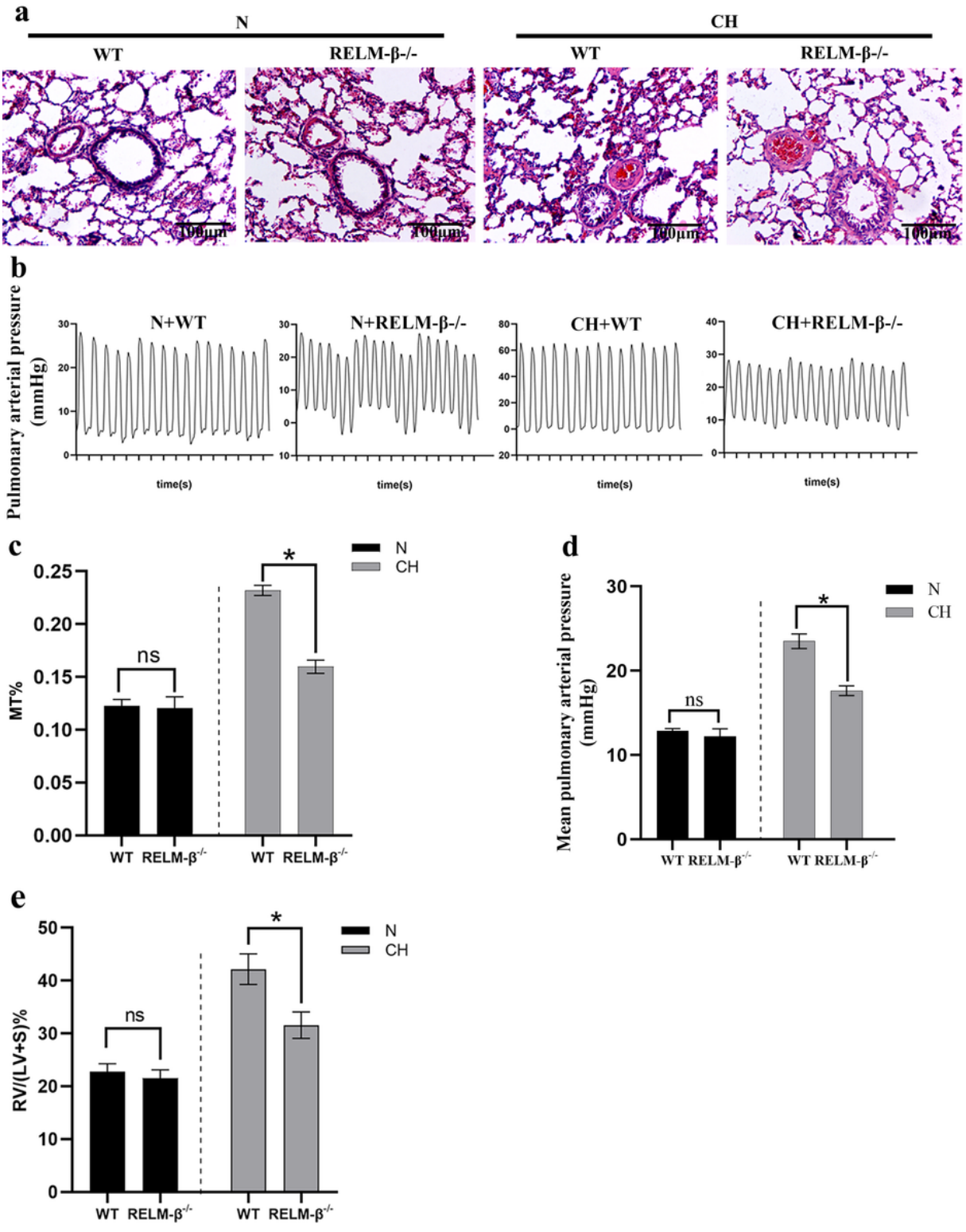

Figure 2

Knockout of RELM-β can significantly reduce the development of HPH. **a, c** H&E-stained sections and corresponding MT% values of lung tissues of the N+WT, N+RELM-β$^{-/-}$, CH+WT, and CH+RELM-β$^{-/-}$ groups.

(n=3, *P<0.05, nsP>0.05). **b, d** Waveform diagram of pulmonary artery pressure(PAP) in four groups and statistical results of mPAP in corresponding groups. (n=5, *P<0.05, nsP>0.05). **e** Statistics of RVHI in each group by weighing method (n=5, *P<0.05, nsP>0.05).

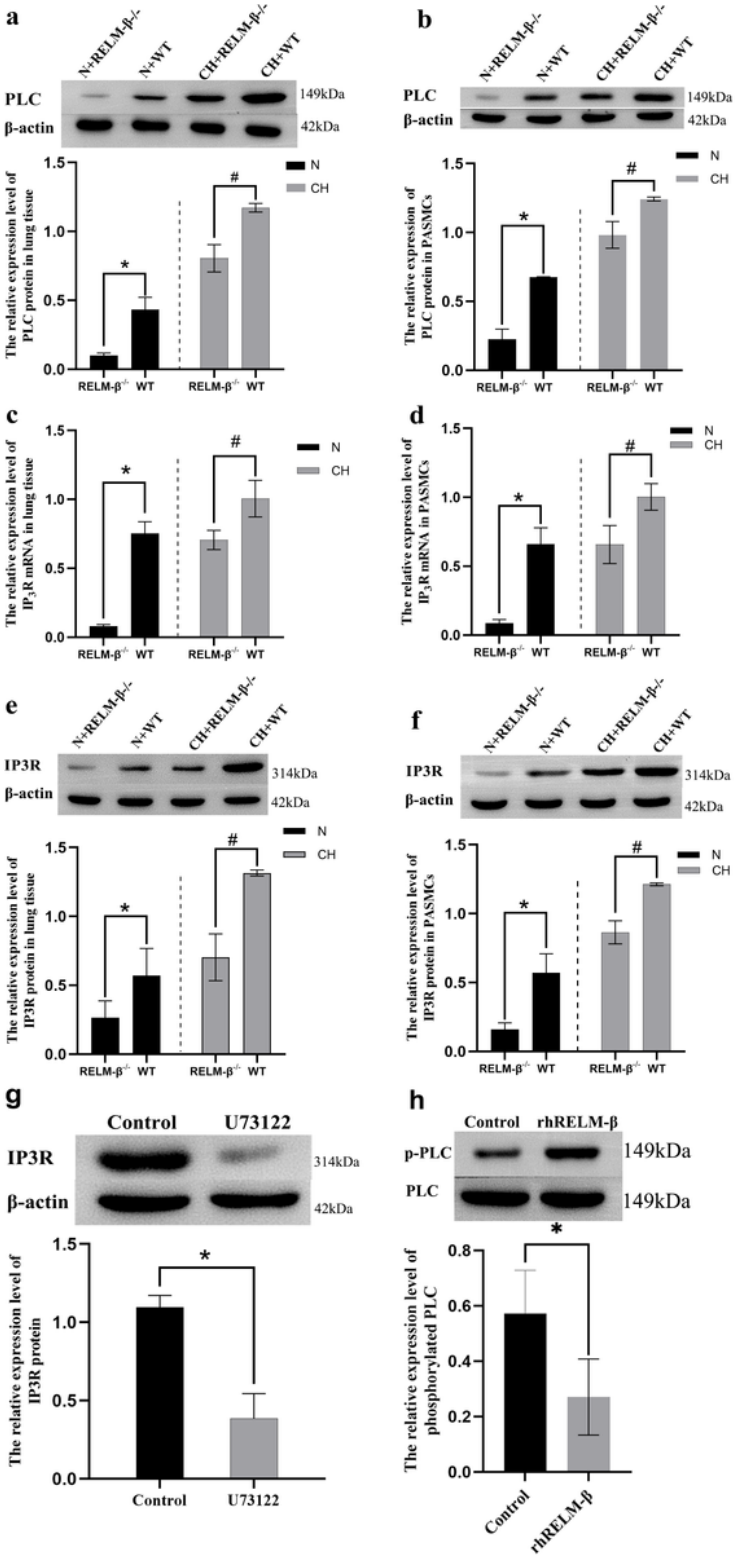

Figure 3

RELM-β regulates the PLC-IP_3R pathway. **a, b** The relative expression of PLC protein in lung tissue and PASMCs of the N+RELM-$β^{-/-}$, N+WT, CH+RELM-$β^{-/-}$, and CH+WT groups. (n=3, *P<0.05, #P<0.05). **c, d** The relative expression of IP_3R mRNA in lung tissue and PASMCs of the four groups mentioned above. (n=3, *P<0.05, #P<0.05). **e, f** The relative expression of IP_3R protein in lung tissue and PASMCs of the above four groups. (n=3, *P<0.05, #P<0.05). **g** The relative expression of IP_3R protein after adding U73122(20umol/l, An inhibitor of PLC). (n=3, *P<0.05). **h** The relative expression level of p-PLC in PASMCs after rhRELM-β(100ng/ml) treatment. (n=3, *P<0.05).

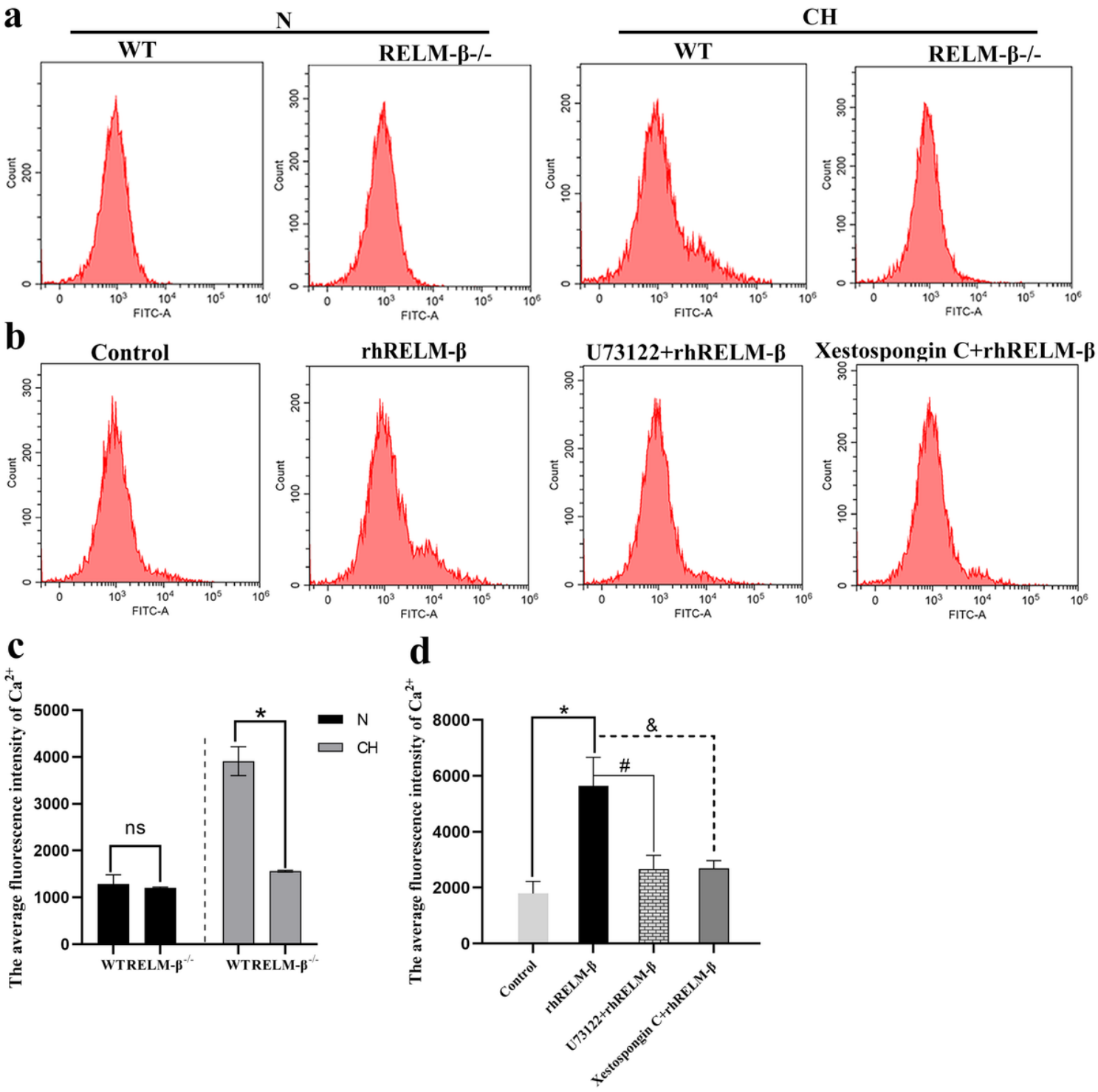

Figure 4

RELM-β Modulates $[Ca^{2+}]_i$ through the PLC-IP_3R pathway. PASMCs were maintained in a Ca^{2+} -free buffer. the average fluorescence intensity of Ca^{2+} in PASMCs were detected by FCM. **a, c** The average fluorescence intensity of Ca^{2+} of the N+WT, N+RELM-$\beta^{-/-}$, CH+WT, and CH+RELM-$\beta^{-/-}$ groups. (n=3, *P<0.05, nsP>0.05). **b, d** the average fluorescence intensity of Ca^{2+} of the control, rhRELM-β (100ng/ml), U73122(20umol/l) + rhRELM-β, Xestospongin C (1umol/l, An inhibitor of IP_3R) + rhRELM-β groups.

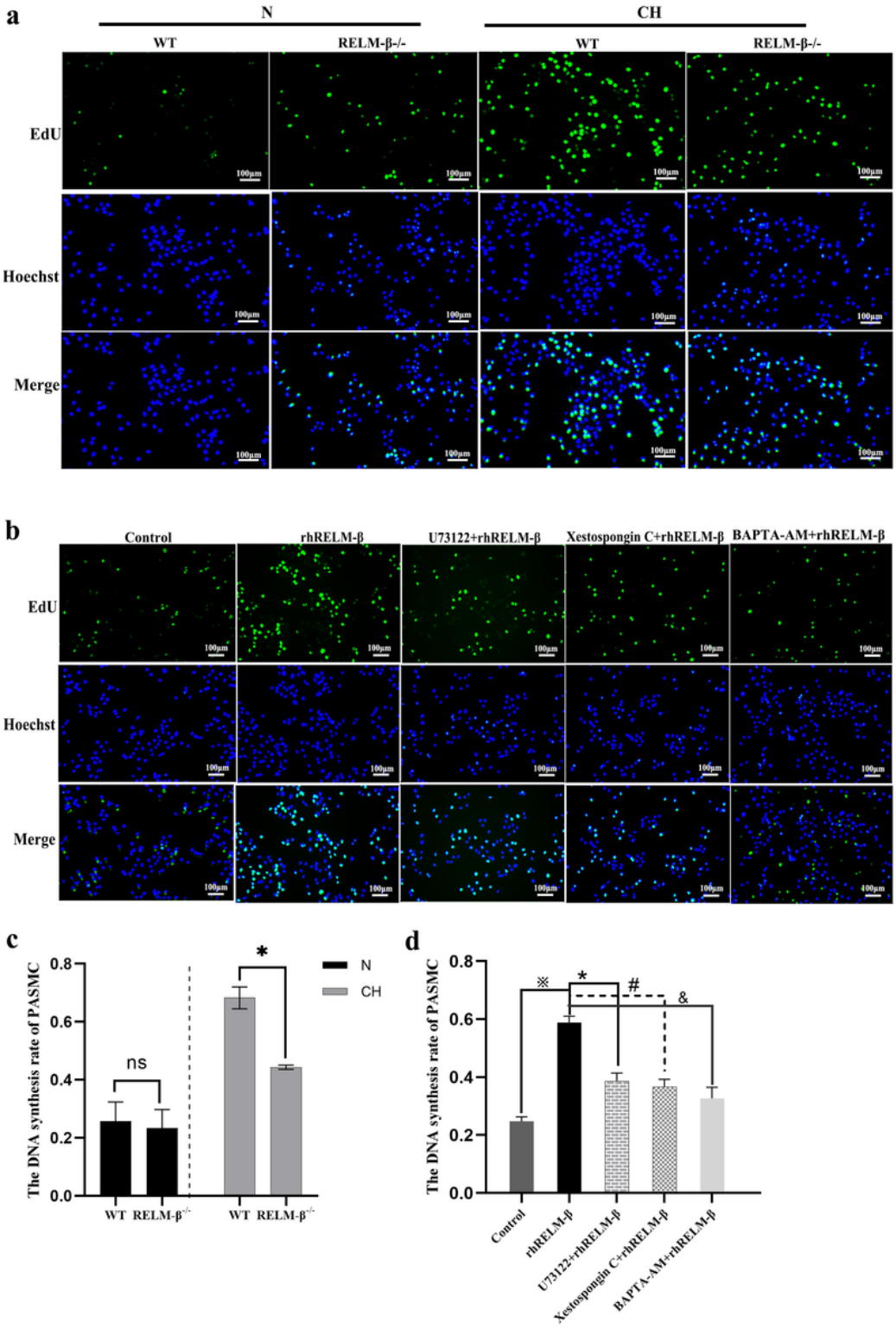

Figure 5

RELM-β regulated $[Ca^{2+}]_i$ through the PLC-IP_3R signaling pathway to regulate PASMCs proliferation. The DNA synthesis rate of PASMCs (ratio of the number of green fluorescent to blue fluorescent cells) was detected by the EdU method. PASMCs were cultured in a calcium-free buffer. **a, c** Fluorescent staining images of PASMCs and corresponding DNA synthesis rates were shown for the N+WT, N+RELM-$β^{-/-}$,

CH+WT, and CH+RELM-β$^{-/-}$ groups. (n=3, *P<0.05, nsP>0.05). **b, d** Fluorescent staining images of PASMCs and corresponding DNA synthesis rates were shown for the rhRELM-β (100ng/ml), U73122(20umol/l) + rhRELM-β, Xestospongin C (1umol/l) + rhRELM-β, BAPTA-AM (10μM, an intracellular Ca^{2+} antagonist) + rhRELM-β, and Control groups. (n=3, ※P 0.05 *P 0.05, #P 0.05 &P 0.05).

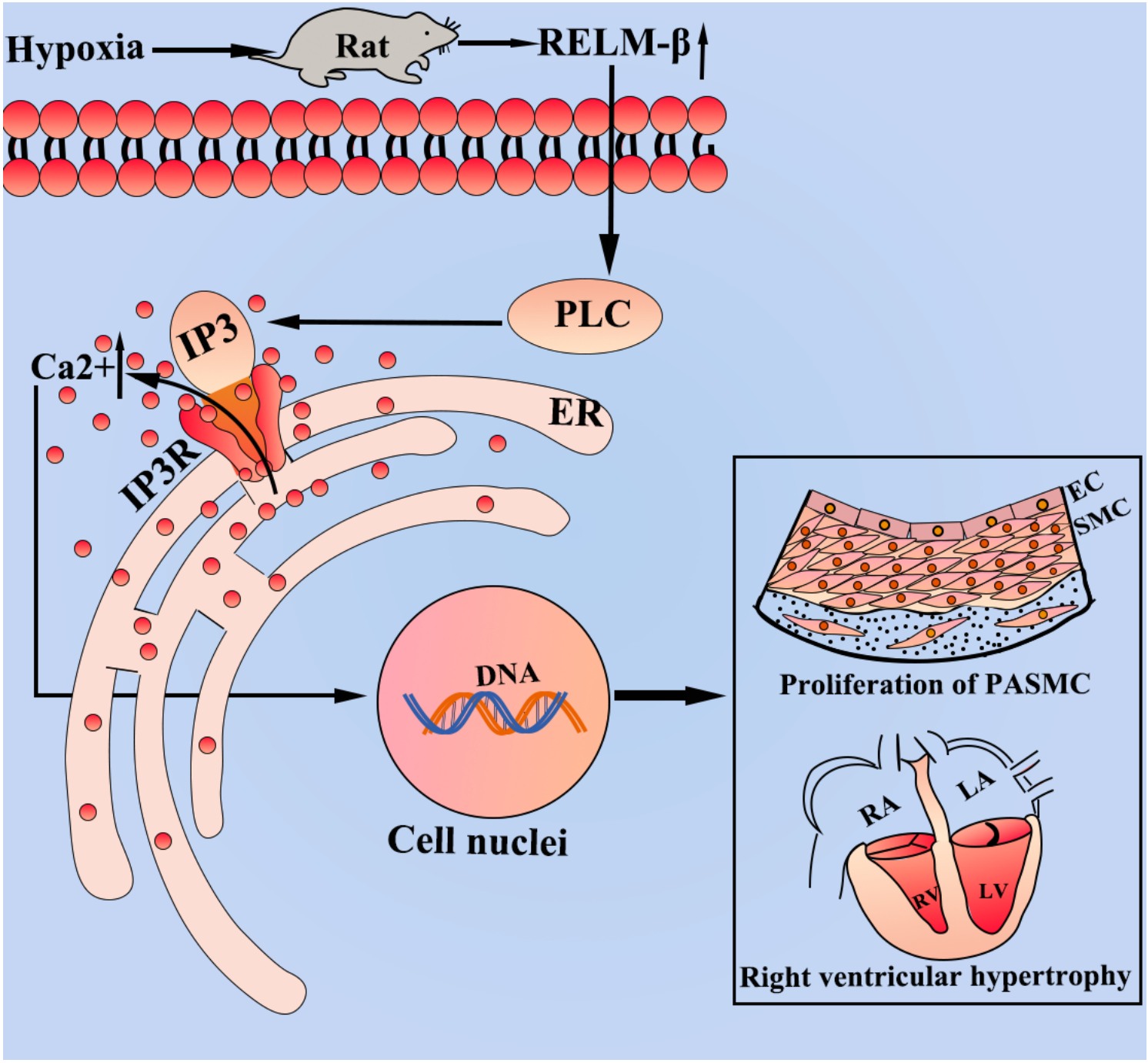

Figure 6

The regulatory effect of RELM-β on the development of HPH through the PLC/IP_3R/Ca^{2+} pathway. Hypoxia upregulates RELM-β, and RELM-β activates and upregulates IP_3R via PLC-IP_3, leading to the

release of Ca^{2+} from the ER into the cytoplasm. The increase of Ca^{2+} concentration in the cytoplasm can promote PASMC proliferation, vascular remodeling and right ventricular hypertrophy.