Inhibition of PCSK9 improves the development of pulmonary arterial hypertension via down-regulating Notch3 expression

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Research Article

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Abstract

Background: Pulmonary arterial hypertension (PAH) is a fatal disease characterized by continuous constriction and occlusion of small pulmonary arteries (PA), leading to the development of right ventricular failure and death. PCSK9(Proprotein convertase subtilisin/kexin type 9) is a kind of serine protease enzyme that increases low-density lipoprotein cholesterol (LDLC) levels through degrading low-density lipoprotein cholesterol receptors (LDLr). However, whether inhibition of PCSK9 can alleviate PAH has not been reported.

Methods and results: We reported that PCSK9 expression was up-regulated in lung tissues of PAH patients. In addition, we used PCSK9 monoclonal antibody subcutaneously to inhibit PCSK9 expression in mice exposed to chronic hypoxia (10%) in combination with SU5416, a VEGF receptor inhibitor. Hypoxia plus SU5416-induced PAH was attenuated in PCSK9 monoclonal antibody-treated mice compared with wild-type mice. PSCK9 inhibited pulmonary vascular remodeling in mice. Moreover, PCSK9 knockdown significantly altered the proliferation and migration of hypoxia-induced PASMCs. We also found that PCSK9 monoclonal antibody inhibited Notch3 expression in vivo and in vitro experiments.

Conclusion: Our results suggest that the PCSK9-Notch3 signaling pathway is critical for the proliferation and migration of PASMCs and provides a potential drug target for the treatment of PAH.

Introduction

Pulmonary arterial hypertension (PAH) is a fatal disease characterized by continuous constriction and occlusion of small pulmonary arteries (PA)[1], leading to the development of right ventricular failure and death. According to epidemiological studies, the three-year survival rate of newly discovered hereditary pulmonary hypertension is only about 60%[2, 3]. More and more evidence has indicated that excessive proliferation of pulmonary artery smooth muscle cells (PASMC), the inflammatory response of pulmonary vessels, and the infiltration of macrophages are important pathological mechanisms of PAH development[4]. Although multiple drugs interfering with the endothelin, nitric oxide, and prostacyclin pathways have been developed to treat PAH in recent decades, the long-term survival and clinical symptoms of patients with PAH remain uncertain[5, 6]. So, it is urgent to actively search for PAH therapeutic targets.

PCSK9(Proprotein convertase subtilisin/kexin type 9), is a kind of serine protease enzyme and one member of the pro-protein convertase family, subsequently increasing low-density lipoprotein cholesterol (LDLC) levels through degrading low-density lipoprotein cholesterol receptors (LDLr). Accumulating evidence showed that inhibition of PCSK9 has could decrease the development of atherosclerosis and inflammation. The pathophysiological effect of PCSK9 is mainly based on the increase of LDLC, but PCSK9 also is independent of LDLR to induce vascular inflammation and accumulate the severity of cardiovascular disease via affecting chemokine receptor expression, ROS production, initiating mitochondrial DNA damage, activating NLRP3 inflammasome signaling and inducing pyroptosis[7–9].

The enhancing expression of PCSK9 was observed in vascular smooth muscle cells (VSMCs) treated with Pro-inflammatory stimuli TNFα and LPS[10]. Also, it is reported recombinant PCSK9 actives macrophage migration and the release of pro-inflammatory cytokines and enhances the mRNA levels of inflammatory cytokines TNFα and IL-1β. The Inflammatory response is an important factor in inducing VSMC proliferation and the development of PAH. Ferri et al[11] reported that knock out of PCSK9 could improve the neointimal formation through sustaining SMC contractile phenotype and inhibiting SMC proliferation and migration. Inhibiting vascular inflammation, macrophage migration, and SMC proliferation are reported to be important strategies for the treatment of PAH. Now, PCSK9 monoclonal antibody has been widely used to treat atherosclerosis. However, whether inhibition of PCSK9 can alleviate PAH has not been reported.

Notch3, as a member of the Notch family, is involved in the regulation of vascular smooth muscle proliferation and regulation. When the Notch3 receptor is activated, its cytoplasmic domain NICD is cleaved by gamma-secretase, dissociated into the cytoplasm, and transferred into the nucleus[12]. It regulates cell proliferation and differentiation by binding to DNA anchor protein RBPj in the nucleus. Abundant evidence has shown that the Notch3 signaling pathway can regulate the identity, proliferation capacity, and apoptosis of arterial smooth muscle cells[13, 14]. Notch3 plays a crucial role in vascular maturation. Systemic Notch3 knockdown inhibits the formation of hypoxia-induced PAH hypertension[14]. The activation of the Notch3 pathway is recognized pathogenesis of PAH. There is no evidence for an association between PCSK9 and Notch3.

Here, we investigated that PCSK9 expression was significantly increased in the tissue suffered from the patient's PAH. Then, we found that progression of PAH was alleviated in mice injected subcutaneously with PCSK9 monoclonal antibody. Our present evidence showed that PCSK9 and its downstream molecule Notch3 are Potential therapeutic targets for PAH.

Materials And Methods

PASMC isolation and culture

Primary PASMC was isolated from small pulmonary arteries with a diameter of 500-1500μm from healthy donors according to previous reports. Briefly, the pulmonary arterioles were separated from the healthy donors and the surrounding connective tissue was removed. The arterioles were cut along the longitudinal axis of the blood vessels, and the intima and outer membrane tissues were gently scraped with a blade. The fragments were cut into 1-3mm^2, and the inner side was attached to the cell culture dish at 37°C and 5% CO2. 1.5 hours later, smooth muscle cell culture medium (SMCM) (ScienCell, Rockville, MD, USA) contained fetal bovine serum (3%) and 100 U/mL penicillin/streptomycin was added, and when the cells covered the whole dish, then the cells were digested and transferred to a new culture dish. These cells were detected by α-SMA staining. The cells were used from 5 to 8 passages. Cells were treated with PCSK9 monoclonal antibody or 3% oxygen concentration

Experimental PAH modeling

This research protocol was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University and was conducted by the Guidelines for The Care and Use of Laboratory Animals (National Research Council). Mice (8-10 weeks, 20-25g, male) were given a single intraperitoneal injection of SU5416 (VEGF receptor inhibitor, 20mg/kg, weekly, subcutaneously) and then exposed to a hypoxic environment (10% O_2 and 90% N_2) on 4 weeks to establish a hypoxic PAH model. The cabin is open once a week for cleaning and changing food and water. Mice were exposed to normoxic conditions (21% oxygen) with the same 12-hour light / 12-hour dark cycle as hypoxic mice. The PAH mice were subcutaneously injected with PCSK9 monoclonal antibody (alirocumab, 20mg/Kg, once two days, Sanofi, France).

Western blotting

Western blotting follows the previous study. In simple terms, cells and tissues are cleaved in lysis buffers (RIPA buffers contained protease inhibitor complexes and phosphatase inhibitors (Beyotime, China)). After determination of protein concentration, these proteins were separated by SDS-PAGE and transferred to the PVDF membrane. PVDF membranes containing proteins were incubated with primary antibodies overnight, followed by secondary antibodies for 2 hours. Finally, the PVDF membranes were visualized using chemiluminescence and Syngene bioimaging equipment (Syngene, Cambridge, UK), and the immune response band density was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

Immunofluorescence assay

Frozen lung tissue sections and cells were washed with frozen PBS three times. Then, they were immobilized with 0.5% Triton X-100 at room temperature with 4% permeability for 15 min. The non-specific sites were sealed with PBS containing 5% bovine serum albumin (BSA) for 30 min at room temperature and then incubated with PCSK9 primary antibody (Santa Cruz, 1:200), Notch3(Abcam, 1:50), and α-SMA antibody (1:200) at 4℃. After incubation overnight, the slices were washed and incubated at room temperature for 2 hours with their respective secondary antibodies. The nucleus is restained with DAPI (blue). Photographs were taken with a fluorescence microscope (original magnification 400×, Zeiss, Germany)

Histological analysis

Animal lung tissue was fixed in 4% paraformaldehyde solution for 48 hours and embedded in paraffin. Paraffin-embedded lung tissue was cut into 5 μm thick sections and stained with hematoxylin and eosin (H&E). Photographs were taken with a light microscope (original magnification 200 ×, Nikon, Tokyo, Japan)

PASMC proliferation and migration assay

PASMC migration was assessed by wound healing test and PASMC proliferation (Haimen Beyotime, China) was assessed by 5-ethynyl-2 '-deoxyuridine (EdU) test, according to manufacturer's protocol.

Echocardiogram

After successful modeling, mice were anesthetized with isoflurane. To evaluate cardiac function, the Vevo 2100 system (Fujifilvisualsonics, Inc., Toronto, Canada) and high frequency (30mhz) ms-400 transducer were used in the animal center laboratory of Nanjing medical university. Right ventricular diastolic diameter (RVID, D), right ventricular anterior wall (RVAW), and pulmonary artery velocity time integral (PAVTI) were measured.

Right ventricular systolic pressure and right ventricular hypertrophy index measurement

Right ventricular systolic pressure (RVSP) was measured by right heart catheterization as described earlier. Mice were anesthetized by inhalation of 1% isoflurane. Under ventilator anesthesia, mice with left anterior cardiac region ribs were cut to expose the heart. The probe is then inserted into the right ventricle to record the waveform. Right ventricular Hypertrophy Index (RVHI) is defined as the ratio of Right ventricular weight to left ventricular and interventricular septum weight.

Statistical analysis

All continuous variables were expressed as mean ± standard deviation (SD). Student paired or unpaired T-tests were used to assess the statistical significance of differences between the two groups. Univariate analysis of variance (ANOVA) was used to compare differences between groups. Graphpad Prism 8.0 software was used for statistical analysis. $P < 0.05$ was considered statistically significant.

Results

PCSK9 expression in the lungs from patients with PAH is increased.

To explore the potential of PCSK9 as a target for PAH treatment, tissue proteins were extracted from healthy lung tissues and lung tissues of patients with PAH for Western Blot detection. The results (**Figure 1A-B)** showed that the expression of PCSK9 in lung tissues of patients with PAH was significantly increased. Further, immunofluorescence was used to detect the expression of PCSK9 in lung tissues. PCSK9 expression was significantly increased in pulmonary arterioles in the PAH group (**Figure1C**), confirming that the high expression of PCSK9 was highly correlated with the formation of pulmonary hypertension.

PCSK9 monoclonal antibody inhibits the progression of Su/Hypo-induced PAH in mice.

Further, wild-type mice were selected and subcutaneously injected with PCSK9 monoclonal antibodies to inhibit the expression of PCSK9. Meanwhile, SU5416 combined with 10% hypoxia (Su/Hypo) was to construct the PAH model. Four weeks later, echocardiography (**Figure 2A-E**) showed that the acceleration

time of pulmonary artery blood flow (PAAT) of Su/Hypo mice was significantly shorter than that of wild-type mice. Also, Su/Hypo-induced PAH resulted in the increased right ventricle internal diameter (RVID) and the right ventricle anterior wall (RVAW). Right ventricular systolic pressure (RVSP) was higher in Su/Hypo mice than in wild-type mice (**Figure 2A-E**). These changes were significantly alleviated in mice given PCSK9 monoclonal antibody and it was statistically significant. These results confirmed that inhibition of PCSK9 alleviates PAH in mice.

Next, we used morphological and histological assessments to demonstrate that lowering PCSK9 improves hypoxic combined with SU5416-induced PAH. By H&E staining (**Figure 3**), we found that PCSK9 monoclonal antibody-treated mice had a significantly lower percentage of vessel wall thickness and partial and full muscularization of distal pulmonary arteries than Su/Hypo mice. These results suggested that down-regulation of PCSK9 in PAH mice can reduce hypoxia-induced pulmonary vascular remodeling. Also, the right ventricular hypertrophy index (RVHI) was calculated by weighing the heart weight of mice. It was found that the RVHI of PAH mice treated with PCSK9 monoclonal antibody was significantly lower than that of PAH mice. Taken together, these data supported inhibition of PCSK9 had could significantly alleviate the progression of PAH.

PCSK9 monoclonal antibody inhibits hypoxia-induced proliferation and migration of PASMC.

To further confirm the role of inhibition of PCSK9 in PAH, PASMC was cultured in vitro and treated with PCSK9 monoclonal antibody under hypoxia. It was confirmed by wound-healing assay and Edu fluorescence staining (**Figure 4**) that inhibition of PCSK9 could significantly inhibit the migration and proliferation of hypoxia cells.

PCSK9 monoclonal antibody mitigates PAH progression by inhibiting Notch3 signaling in vivo and vitro.

It has been previously confirmed that overactivation of the Notch3 signaling pathway plays an important role in the formation of PAH, while whether inhibition of PCSK9 occurs through the Notch3 signaling pathway is unknown. We extracted lung tissue proteins from the above experimental mice and performed Wb detection. The results (**Figure 5A-B**) showed that Notch3 expression was significantly increased in lung tissues of PAH mice, whereas it was inhibited in lung tissues of PAH mice treated with PCSK9 monoclonal antibody. Immunofluorescence staining (**Figure 5C**) showed the same results. In vitro, we treated PASMC with PCSK9 monoclonal antibody to inhibit PCSK9 expression. Wb and immunofluorescence staining (**Figure 6**) confirmed that hypoxia-induced the expression of Notch3, and inhibition of PCSK9 also inhibited the expression of Notch3. These results confirmed that down-regulation of PCSK9 alleviated PAH by inhibiting Notch3 signaling.

Discussion

In the present study, we demonstrated for the first time the role of PCSK9/Notch3 signaling in the pathogenesis of PAH. First, we confirmed that PCSK9 was significantly increased in pulmonary hypertension. In animal experiments, subcutaneous injection of PCSK9 monoclonal antibody in mice

significantly alleviated SU5416/Hypo-induced PAH, and down-regulation of PCSK9 was also confirmed to inhibit PASMC migration and proliferation in vitro. Mechanistically, the suppression of PCSK9 alleviated PAH by inhibiting Notch3 expression.

The important pathogenesis of pulmonary hypertension includes overproliferation of PASMCs and overactivation of the inflammatory response, whose pathological basis mainly involves pulmonary vascular remodeling and excessive contraction of pulmonary arteriole[1, 6, 12]. Excess body fat has also been linked to several lung diseases, including chronic obstructive pulmonary disease, obstructive sleep apnea syndrome, pulmonary embolism, and asthma, according to previous studies[15]. One clinical study confirmed that 44% of obese patients had varying degrees of pulmonary artery media thickening, which is associated with inflammatory activation induced by LDL[16, 17]. The expression of PCSK9 promotes LDL receptor degradation and leads to increased LDL expression[18]. It has been reported that PCSK9 activation can also induce the increased expression of inflammatory factors such as TNF-α, IL-1β, and IL-6[8–10]. However, whether the expression of PCSK9 is related to the formation of PAH has not been reported. In our study, we observed that the expression of PCSK9 increased in the patients with PAH, and the treatment of PCSK9 monoclonal antibody attenuated PAH, which is associated with the inhibition of inflammation.

PCSK9 monoclonal antibody has been widely used in the treatment of hypercholesterolemia and has been shown to reduce the incidence of a variety of cardiovascular adverse events[19–21]. In addition to the function of targeting LDL receptor degradation, PCSK9 can also promote inflammatory response, autophagy, cell proliferation, and apoptosis[10]. A study in hepatoma cells showed that recombinant PCSK9 treatment affected the expression of several genes involved in the cell cycle and cell growth independent of its cholesterol uptake[22], and PCSK9-deficient mice showed impaired hepatocyte proliferation and enhanced apoptosis after partial hepatectomy[23]. In gastrointestinal and liver cancers, a higher level of PCSK9 is associated with the increased ability of tumor development and metastasis and decreased overall survival, and PCSK9 monoclonal antibody suppresses tumor growth[24]. Also, PCSK9 knockout mice can alleviate neointimal formation by inhibiting the proliferation of vascular smooth muscle cells[11]. Pathological stimulation can promote PCSK9 binding to LOX1, increase mitochondrial ROS production, and ultimately activate the NF-κB signaling pathway[25]. So, in a variety of pathological models, PCSK9 activation is an important factor leading to cell proliferation. In our study, PCSK9 monoclonal antibody could also alleviate PAH by inhibiting PASMC proliferation.

Previous studies have reported that Notch3 is highly expressed in lung tissues of patients with PAH and that Notch3 knockout can significantly improve PAH progression[14]. Therefore, we explored whether PCSK9 could affect the Notch3 signaling pathway in terms of mechanism. In our experiment, it was confirmed that inhibition of PCSK9 could lead to the decrease of Notch3 expression in vitro and in vivo. It has been reported that Ox-LDL can promote the high expression of Notch3[26], while the monoclonal antibody of PSCK9 can promote the decomposition of Ox-LDL, thus inhibiting the expression of Notch3. In this study, PCSK9 monoclonal antibody inhibited the expression of Notch3, possibly through promoting Ox-LDL degradation.

In conclusion, PSCK9 is highly expressed in lung tissues of patients with PAH, and PCSK9 monoclonal antibody can reduce the expression of Notch3, inhibit the proliferation of PASMC, and ultimately alleviate the development of PAH. This study also provides new targets and therapeutic strategies for the treatment of PAH.

Declarations

Data Availability Statement

All relevant data are within the paper.

Compliance with ethical guidelines

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YP performed the immunoblot analyses, analyzed the echo data, and drafted the manuscript. JXM performed the mouse work, including feeding and coordinating the echography and harvest dates and times. YP and JXM added in harvesting the mice and analyzing the data. ZJ and QWC designed and oversaw the study, and edited the manuscript. All authors read and approved the final manuscript.

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Figures

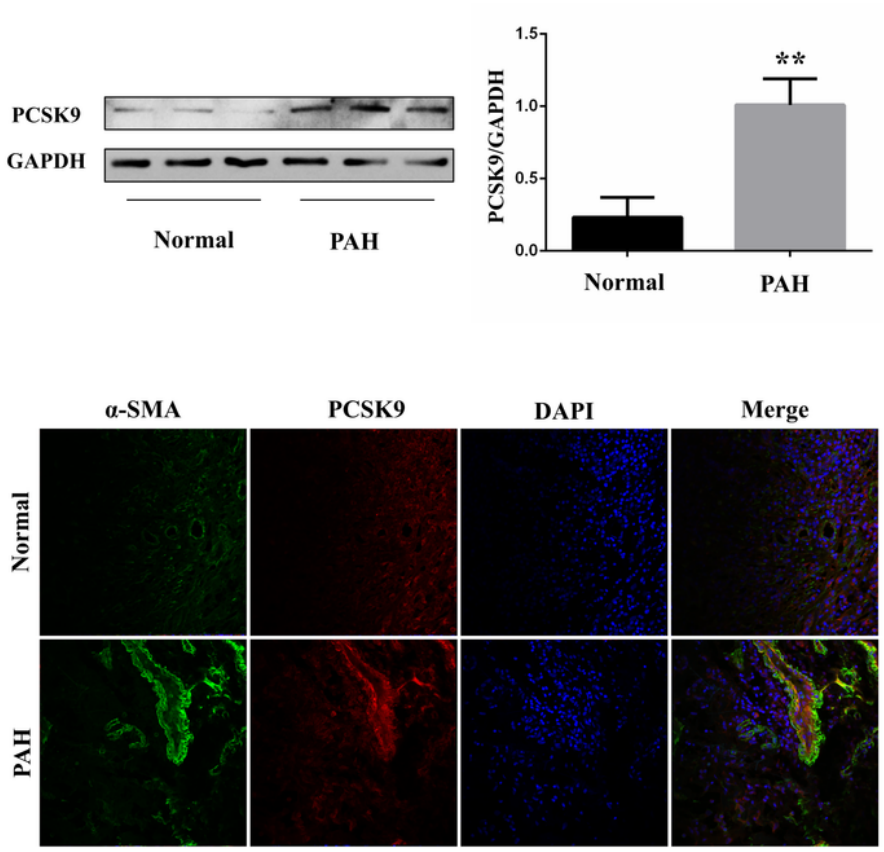

Figure 1

PCSK9 expression in the lungs are upregulated in patients with PAH. (A) Representative Western blot images of PCSK9 in the lungs from normal donors and PAH patients; (B)Quantification of PCSK9 in the A. (C) Representative confocal immunofluorescence images of lung tissue from normal donors and PAH patients stained with antibodies against α-SMA (green), PCSK9 (red) and DAPI (blue) in the lung. The results are expressed as the mean ± SD. n = 3 per group. **, $p < 0.05$ versus normal.

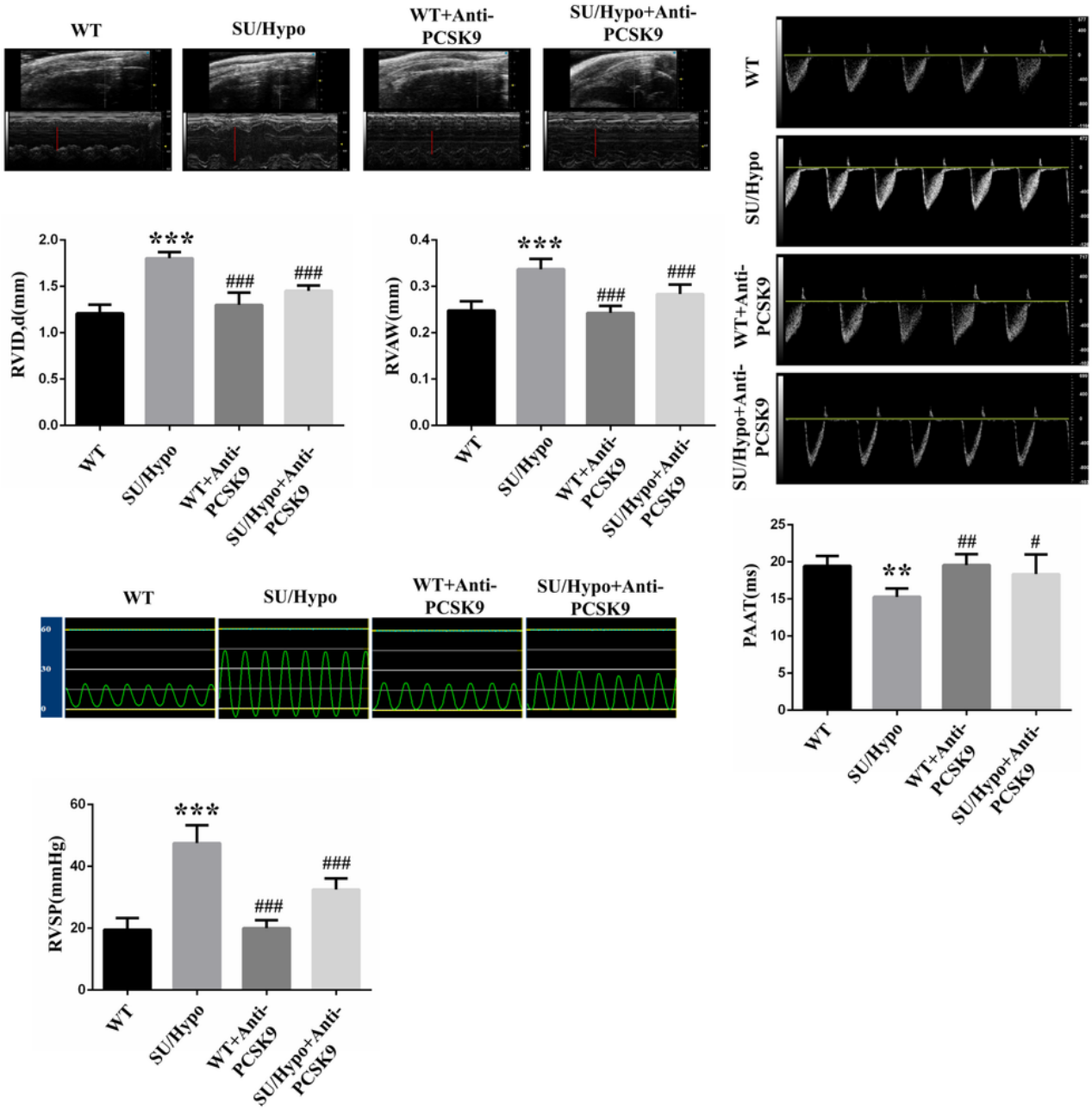

Figure 2

PCSK9 monoclonal antibody prevents SU/Hypo-induced pulmonary arterial hypertension in mice. C56B6/L mice (8-10weeks, male) were exposed to normoxia or hypoxia (10% O2) for 4 weeks after SU5416 (20mg/kg, once a week) subcutaneous injection. During the 4 weeks, the mice continuously were subcutaneously injected with PCSK9 monoclonal antibody (Anti-PCSK9,60mg/kg, once a week). After treatment, Cardiac ultrasound was performed on mice under anesthesia(A-E). (A, D) Representative

ultrasound images. (B) RVID(d)/mm, (C) RVAW/mm, and PAAT /ms (E) were assessed in the WT group, SU/Hypo group, WT+Anti-PCSK9 group, and SU/Hypo+Anti-PCSK9 group. (F) Representative pressure curve of the right ventricular systolic pressure (RVSP). (G) Quantitative images of F. The results are expressed as the mean ± SD; n = 6–8 per group. **, p < 0.01and ***, p < 0.001 versus WT. #, p < 0.05, ##, p < 0.01 and ###, p < 0.001 versus SU/Hypo.

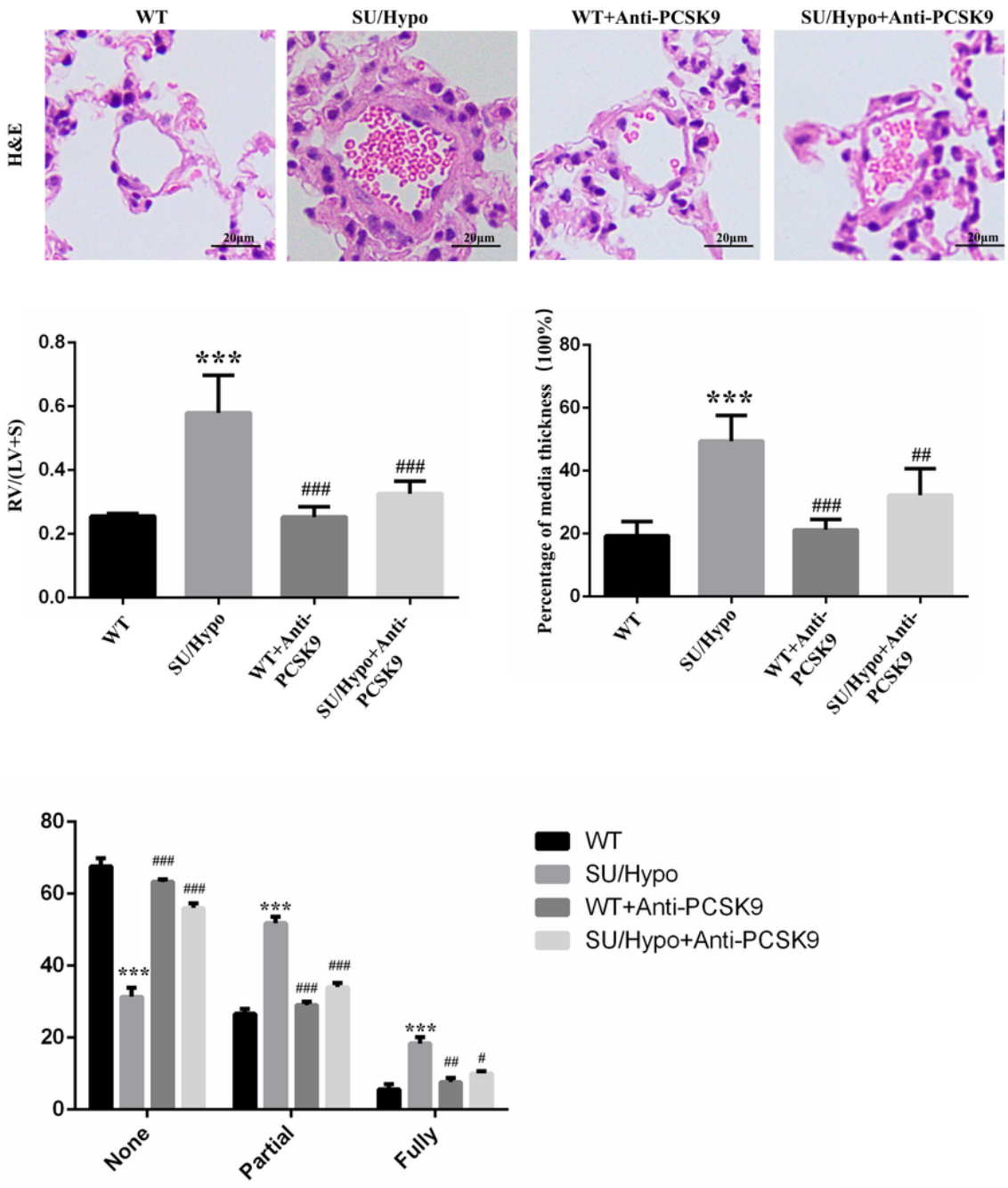

Figure 3

PCSK9 monoclonal antibody prevents SU/Hypo-induced pulmonary arterial hypertension in mice. C56B6/L mice (8-10weeks, male) were exposed to normoxia or hypoxia (10% O2) for 4 weeks after SU5416 (20mg/kg, once a week) subcutaneous injection. During the 4 weeks, the mice continuously were subcutaneously injected with PCSK9 monoclonal antibody (Anti-PCSK9,60mg/kg, once a week) (A) Representative H&E staining of pulmonary arteries. (B) Changes in right ventricular hypertrophy RV/(LV + S). (C–D) Changes in the percentage of medial wall thickness (C) and muscularization in pulmonary arteries. The results are expressed as the mean ± SD; n = 6–8 per group. ***, $p < 0.001$ versus WT. ##, $p < 0.01$ and ###, $p < 0.001$ versus SU/Hypo.

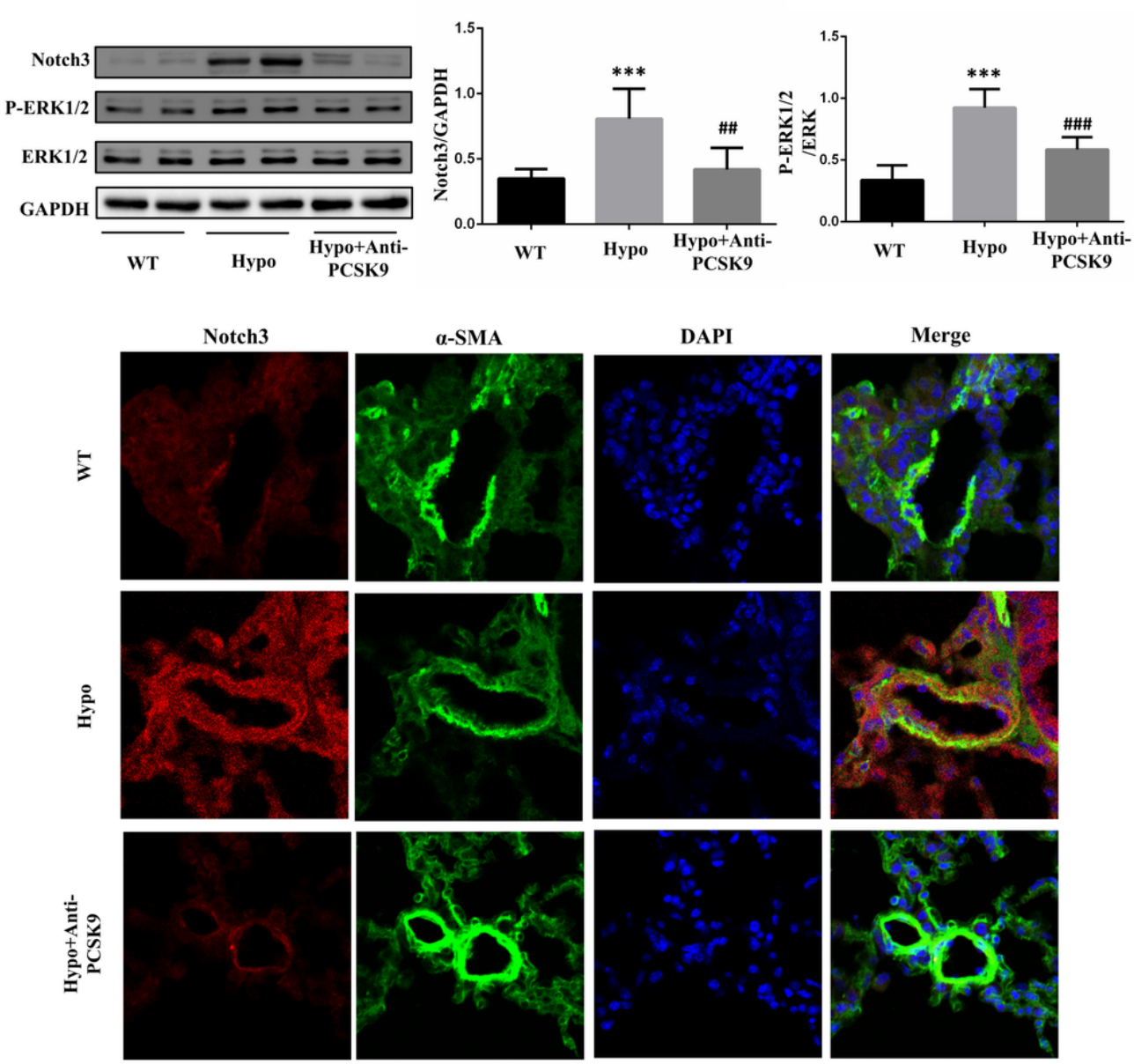

Figure 4

PCSK9 monoclonal antibody inhibits the expression of Notch3 in mice. C56B6/L mice (8-10weeks, male) were exposed to normoxia or hypoxia (10% O2) for 4 weeks after SU5416 (20mg/kg, once a week) subcutaneous injection. During the 4 weeks, the mice continuously were subcutaneously injected with PCSK9 monoclonal antibody (Anti-PCSK9,60mg/kg, once a week). (A) Representative Western blot images in mice with Anti-PCSK9 or not, exposed to normoxia or hypoxia. (B-C) Western blot analysis of

Notch3, p-ERK1/2, and ERK1/2 expression. (C) Representative confocal immunofluorescence images of PASMCs stained with antibodies against α-SMA (green), PCSK9 (red), and DAPI (blue) in the lung. The results are expressed as the mean ± SD. n = 6-8 per group. ***, p < 0.001 versus WT. ##, p < 0.01 and ###, p < 0.001 versus SU/Hypo.

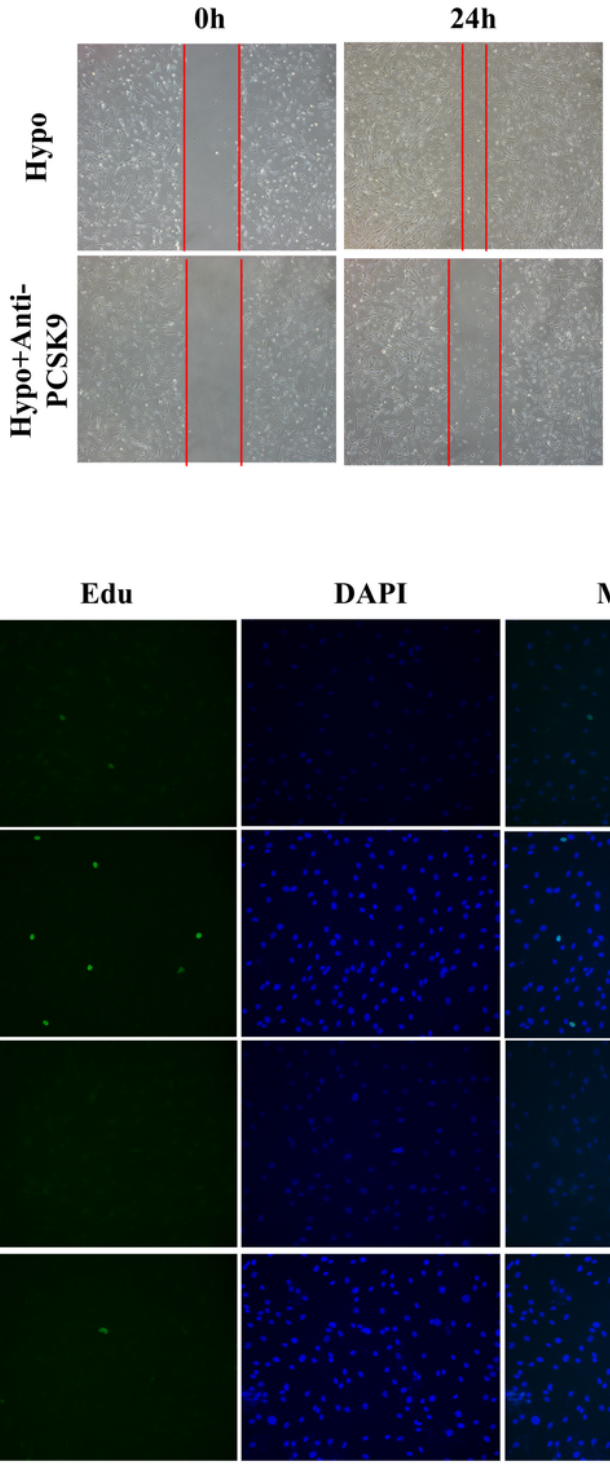

Figure 5

PCSK9 monoclonal antibody inhibits hypoxia-induced proliferation of PASMC. PASMC were treated with PCSK9 monoclonal antibody, before exposed normoxia or hypoxia (3% O_2) for 24h. The proliferative activity of PASMC was assessed via a Wound Healing assay(A) and EdU staining(B). n = 3 in each group.

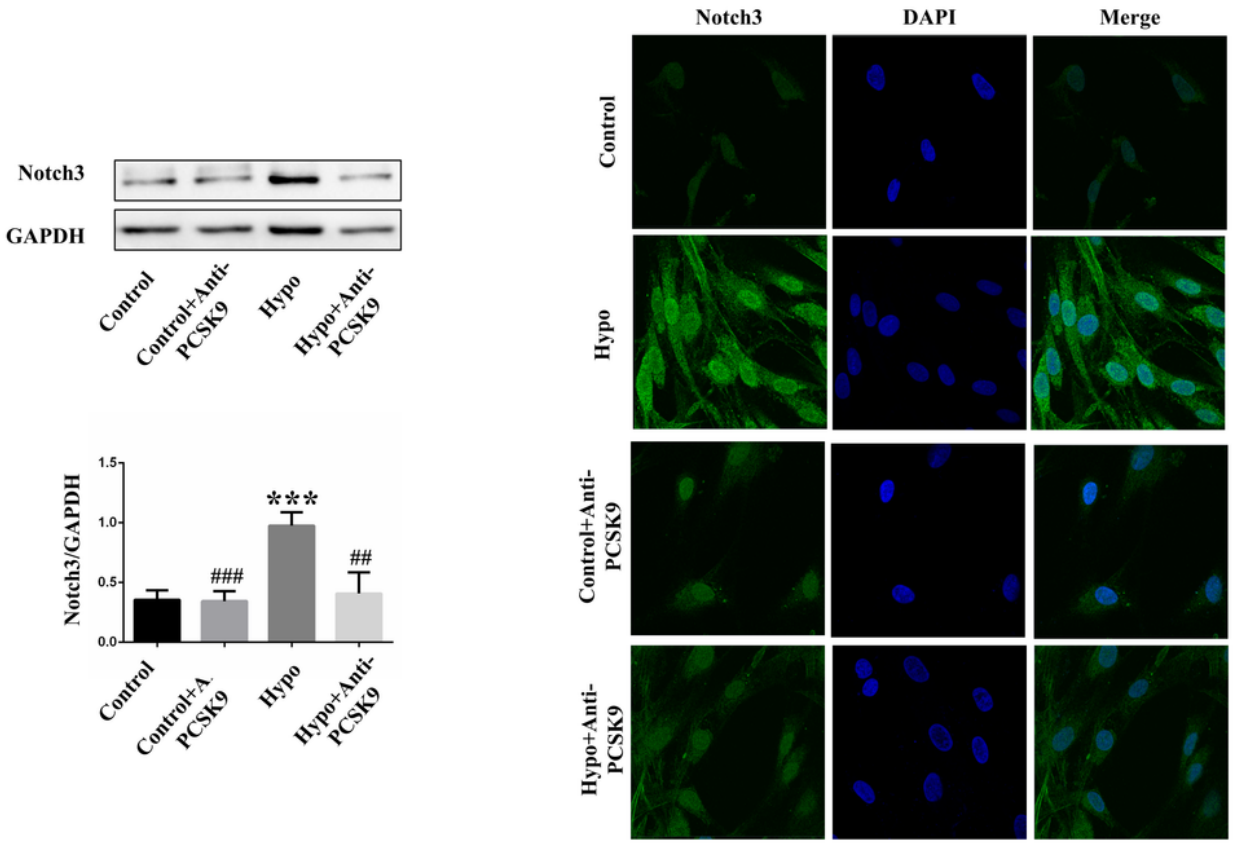

Figure 6

PCSK9 monoclonal antibody inhibits the expression of Notch3 in PASMCs. PASMC were treated with PCSK9 monoclonal antibody, before exposed normoxia or hypoxia (3% O_2) for 24h. (A) Representative Western blot images of Notch3 in PASMCs with Anti-PCSK9 or not, exposed to normoxia or hypoxia. (B) Western blot analysis of Notch3 expression. (C) Representative confocal immunofluorescence images of PASMCs stained with antibodies against Notch3 (green) and DAPI (blue) in the lung. The results are expressed as the mean ± SD. n = 3 per group. ***, $p < 0.001$ versus Control. ##, $p < 0.01$ versus Hypo.