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Research Article

Keywords: hypoxia-induced pulmonary hypertension, NEDD4L, ACE2, ubiquitination, K788, site-directed mutagenesis

Posted Date: October 21st, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10565674/v1>

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Additional Declarations: No competing interests reported.

Blocking NEDD4L-Mediated ACE2 Ubiquitination at Lysine 788 Alleviates Hypoxia-Induced Pulmonary Hypertension: A Mechanistic Study

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Abstract

Background: We previously showed that neural precursor cell-expressed developmentally down-regulated gene 4-like (NEDD4L), an E3 ubiquitin ligase, drives pulmonary hypertension by promoting the ubiquitination and subsequent degradation of angiotensin-converting enzyme 2 (ACE2). Whether this regulation occurs at a specific residue, and whether targeting that residue is therapeutically useful, had not been determined.

Methods: Based on bioinformatic prediction combined with previously validated ubiquitin-acceptor lysines in the intracellular C-terminal tail of ACE2, we substituted lysine 788 with arginine to generate the K788R ACE2 mutant. Co-immunoprecipitation (co-IP) in pulmonary artery smooth muscle cells (PASMCs) was used to examine the interaction of NEDD4L with wild-type or K788R ACE2. For in vivo experiments, ACE2-knockout rats were given adeno-associated virus (AAV) carrying either wild-type or K788R ACE2, followed by 4 weeks of chronic hypoxia (10% O₂). Echocardiography, blood-gas analysis, histology, TUNEL staining, quantitative PCR, and Western blotting were performed to evaluate pulmonary artery pressure, oxygenation, pulmonary vascular remodeling, apoptosis, and gene and protein expression.

Results: Under both normoxic and hypoxic conditions, the K788R mutation greatly weakened the NEDD4L–ACE2 association in PASMCs and in rat lung tissue, although wild-type and mutant ACE2 were expressed at comparable levels. Relative to ACE2-knockout rats reconstituted with wild-type ACE2, those reconstituted with K788R ACE2 showed markedly lower pulmonary artery pressure, less pulmonary arteriolar muscularization and medial wall thickening, a higher oxygenation index, and reduced hypoxia-induced apoptosis in lung tissue.

Conclusion: These findings establish K788, a validated ubiquitin-acceptor lysine in the intracellular tail of ACE2, as an essential residue for the NEDD4L–ACE2 interaction, and they show that selectively blocking ACE2 ubiquitination at this single site alleviates hypoxia-induced pulmonary hypertension in rats. Intervention at the NEDD4L–ACE2 axis through K788 may thus offer a precision therapeutic approach that stabilizes ACE2 while sparing global NEDD4L function.

Keywords: hypoxia-induced pulmonary hypertension; NEDD4L; ACE2; ubiquitination; K788; site-directed mutagenesis

Introduction

Pulmonary hypertension (PH) is a progressive and ultimately fatal cardiopulmonary disease in which pulmonary vascular resistance rises persistently, progressing to right ventricular failure and death. Among its clinical subgroups, pulmonary arterial hypertension (PAH) is hemodynamically defined by a precapillary pattern, whereas chronic hypoxia gives rise to hypoxia-induced pulmonary hypertension (HPH), a separate entity frequently complicating chronic lung disease and high-altitude exposure[1]. Although considerable progress has been made in elucidating the pathogenesis of these disorders, curative therapies are still unavailable, and novel treatment strategies are urgently required.

The renin-angiotensin system (RAS) is closely linked to the development of PH. Its classical arm, the angiotensin-converting enzyme (ACE)-angiotensin II (Ang II)-AT1R axis, drives vasoconstriction, cellular proliferation, and inflammation, while the ACE2-Ang-(1-7)-Mas axis acts as the endogenous counter-regulatory arm, conferring vasoprotection through vasodilatory, anti-proliferative, and anti-inflammatory actions [2]. Acting as a negative regulator of the RAS, ACE2 converts Ang II into Ang-(1-7) and thereby preserves cardiovascular homeostasis. Impaired ACE2 function is increasingly recognized as a key contributor to the pathogenesis of PAH [3, 4]. Nevertheless, how ACE2 is downregulated in PAH at the molecular level remains only partially understood.

Ubiquitination represents a central post-translational modification governing protein stability and function. Within the ubiquitin-proteasome system (UPS), ubiquitinated substrates are targeted for degradation, and E3 ubiquitin ligases, the enzymes that determine substrate specificity, have been implicated in a wide range of cardiovascular diseases[5-7], including PAH [8]. Among them, neural precursor cell-expressed developmentally down-regulated gene 4-like (NEDD4L) has drawn particular interest. As a member of the NEDD4 family of HECT-type E3 ligases, NEDD4L recognizes its substrates via WW domains that engage proline-rich PPxY/PY-type motifs and then catalyzes the transfer of ubiquitin to acceptor lysines on the bound substrate. NEDD4L targets several proteins relevant to cardiovascular pathophysiology for ubiquitination and degradation, among them the epithelial sodium channel (ENaC)[9], the cardiac sodium channel Nav1.5 [10], and ACE2 [11, 12].

In our 2024 study published in *Respiratory Research*, we provided the first evidence that NEDD4L ubiquitinates ACE2 and promotes its degradation in PH, thereby aggravating disease progression[13]. That work defined the NEDD4L–ACE2 regulatory axis but did not map the residues involved. Supporting evidence from hypertensive disease models has also accumulated: in Ang II-induced hypertensive mice, NEDD4L was markedly upregulated, and NEDD4L and ACE2 protein levels were inversely correlated in the brain, heart, and kidneys [11]. Loss-of-function mutation of NEDD4L or knockdown with NEDD4L siRNA restored the ACE2 expression suppressed by Ang II [12]. In addition, Zhu et al. documented elevated NEDD4L expression in the lungs of monocrotaline-induced PAH rats[14], which lends further support to a pathogenic role of NEDD4L in PH.

Two key questions thus remain open. First, ACE2 is a type I transmembrane glycoprotein, and its short intracellular C-terminal tail (amino acids 763–805) harbors five lysine residues (K769, K770, K771, K773, and K788) that can act as ubiquitin acceptor sites, as well as an NPxY motif (amino acids 779–781) recognizable by WW domain-containing proteins[12, 15]. In models of hypertension, NEDD4L has been shown to ubiquitinate ACE2 at these C-terminal lysines[15]. Within the pulmonary vasculature, ubiquitination of ACE2 at K788 by MDM2 promotes experimental PH, whereas the deubiquitination-mimetic ACE2-K788R mutant confers protection[16]. Whether NEDD4L targets the same residue in the pulmonary circulation, and whether blocking ACE2 ubiquitination specifically at K788 can ameliorate HPH, is not yet known. Second, whether selective disruption of this ubiquitination event constitutes a feasible therapeutic strategy remains to be tested. Resolving these questions is a prerequisite for precision therapies that stabilize ACE2 while avoiding global NEDD4L inhibition and the attendant off-target effects on its other substrates.

Materials and Methods

1. Cell Culture

All animal procedures were approved by the Animal Ethics Committee of Xuzhou Medical University (approval number: 2022105068). Male C57BL/6 wild-type mice aged 6–8 weeks were obtained from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. (animal license No. SCXK (Beijing) 2021-0011). Following pentobarbital sodium anesthesia and euthanasia, PSMCs were isolated by an improved tissue-piece inoculation method combined with trypsin digestion [17]. Cell morphology was examined by inverted phase-contrast microscopy, and a cell growth curve was generated by counting the cells. Immunofluorescence (IF) staining of α -smooth muscle actin (α -SMA, anti- α -SMA, ab7817, Abcam, UK) was performed to verify the identity and purity of PSMCs (shown in green, **Supplementary Fig. 1**). PSMCs were cultured in Dulbecco's modified Eagle's medium (DMEM, iCell-0001, iCell Bioscience, China) supplemented with 15% fetal bovine serum,

100 U/mL penicillin, and 100 U/mL streptomycin, and maintained in an incubator at 37°C, saturated humidity, and 5% CO₂. All experiments used cells at passages 3–6, which retained α -SMA positivity consistent with a contractile phenotype. For hypoxic treatment, PSMCs were in a hypoxic chamber (MIC-101, Billups-Roche, USA) with an O₂ concentration of 4% for 24 h.

2. Vector Construction and Transfection

The UbiBrowser database was used to predict candidate NEDD4L ubiquitination sites on ACE2, and the predictions were cross-checked against experimentally validated ubiquitin-acceptor lysines within the intracellular C-terminal tail of ACE2. Accordingly, site-directed mutagenesis was performed to generate an ACE2 expression vector carrying a lysine-to-arginine substitution at lysine 788 (K788R; designated mutACE2-3xHA). This substitution was selected because it prevents ubiquitin conjugation yet retains the positive charge and overall architecture of the side chain, thereby minimizing perturbation of secondary structure. Expression vectors for wild-type ACE2 (ACE2-3xHA) and Flag-tagged NEDD4L were constructed in parallel. All constructs were assembled in the pCMV3-SV40-Puro backbone and confirmed by Sanger sequencing. PSMCs were transfected with the vectors using Lipofectamine 3000 reagent (L3000001, Invitrogen) and harvested 48 h later for downstream experiments.

3. Experimental Animals and Model Establishment

Male Sprague-Dawley rats (160–180 g) were obtained from Changzhou Cavens Laboratory Animal Co., Ltd. for the animal experiments. All rat procedures were approved by the Animal Ethics Committee of Xuzhou Medical University (approval number: 2022105068). ACE2-knockout rats were generated on the Sprague-Dawley background by CRISPR/Cas9 technology; the genotype of each animal was confirmed by qPCR, and the absence of ACE2 protein in lung tissue was verified by western blotting before the experiments.

The rats were randomly assigned to three groups: (1) the control group (NC group), in which wild-type rats received control AAV; (2) the AAV-WT group, in which ACE2-knockout rats received AAV encoding wild-type ACE2; and (3) the AAV-MUT group, in which ACE2-knockout rats received AAV encoding site-mutated ACE2. Recombinant adeno-associated virus serotype 6 (AAV6) vectors carrying wild-type ACE2 (AAV-WT), K788R-mutated ACE2 (AAV-MUT), or an empty control vector were produced commercially, and the viral titers were determined by quantitative PCR in 293T cells (1.46×10^{13} VG/mL). All AAV vectors were administered by intratracheal instillation at a dose of 1×10^{12} VG per rat under pentobarbital sodium anesthesia. Following AAV delivery, the rats were placed in a normobaric hypoxic chamber (10% O₂) for 4 weeks to induce the HPH model; normoxic control animals were kept under ambient oxygen conditions.

The HPH rat model was established by exposure to sustained hypoxia (10% O₂) for 4 weeks. Successful modeling was verified by ultrasonographic measurement of pulmonary artery pressure, blood gas analysis, and histopathological examination. Throughout the modeling period, food and water were replenished at fixed times each day, and the chamber was opened once daily for 1 h of ventilation; the hypoxia-group rats were then returned to the chamber, and nitrogen was reintroduced.

The surgical procedures in rats followed the protocols previously established by our group[18] and are briefly outlined here: under sodium pentobarbital anesthesia (50 mg/kg, intraperitoneally), the rats were intubated through the trachea, and a catheter was advanced via the right external jugular vein into the right ventricle to record right ventricular systolic pressure. A median sternotomy was then performed, the tracheal tube was disconnected from the ventilator, and bronchoalveolar lavage was carried out through the tube. After excision of the left and right atrial appendages, the heart was perfused through the right atrial appendage, with the perfusate draining from the left atrial appendage; once the effluent ran clear, the heart and lungs were harvested.

Pulmonary arteriolar muscularization and medial wall thickness were evaluated by HE staining(HE; C0105, 0.5% hematoxylin for 5 min followed by 0.5% eosin for 1 min, both at 25°C). Observations were made under a 400x light microscope (magnification, ×400; model, Leica DMI8; Leica Microsystems GmbH, Germany), and images were analyzed with Image-Pro Plus software (version 6.0; Media Cybernetics). From each group, 20–40 pulmonary arterioles approximately 50 µm in diameter were selected and categorized morphologically as non-muscularized, partially muscularized, or fully muscularized; the degree of muscularization was expressed as the percentage of muscularized arterioles among all pulmonary arterioles examined. In addition, 25 pulmonary arterioles of approximately 50 µm in diameter were selected per group, and the medial thickness and external diameter were measured to compute the medial wall thickness ratio (medial thickness/external diameter)[19].

4. Western Blot Analysis

Total protein was extracted from cultured cells or rat lung tissue and quantified with the BCA assay. Equal amounts of protein were separated by SDS-PAGE, transferred onto membranes, and blocked. The membranes were probed with primary antibodies (against HA, Flag, ACE2, NEDD4L, β-actin, and GAPDH) overnight at 4°C, washed, and then incubated with the corresponding secondary antibodies for 1 h at room temperature. Signals were developed by ECL chemiluminescence, and band intensities were quantified with ImageJ software. Anti-ACE2 (21115-1-AP, Proteintech, USA), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 60004-1-Ig, Proteintech, USA), anti-NEDD4L (ab46521, Abcam, UK), anti-DDDDK tag(ab205606, Abcam, UK), anti-HA tag(ab9110, Abcam,

UK) , horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:3,000; 7074, Cell Signaling, USA) ,and goat anti-rabbit IgG (1:3,000; 7074, Cell Signaling, USA).

5. Co-Immunoprecipitation (CO-IP) Assay

Protein extracts prepared from cells or lung tissue were incubated overnight at 4°C with anti-Flag antibody or control IgG, followed by a 2-h incubation with Protein A/G magnetic beads at 4°C. The beads were then washed, resuspended in loading buffer, and boiled, and the immunoprecipitates were analyzed by Western blotting to detect HA-tagged proteins.

6. Assessment of the Pulmonary Hypertension Phenotype in Rats

At the end of the 4-week hypoxia period, pulmonary artery pressure was determined with a Vevo 2100 small-animal ultrasound system. Arterial blood samples were collected, and the arterial partial pressure of oxygen was measured with a blood gas analyzer to derive the oxygenation index. Pulmonary arteriolar morphology was examined on HE-stained lung sections, and the medial wall thickness ratio was calculated.

7. Quantitative PCR (qPCR)

For qPCR, RNA was extracted from rat lung tissue with TRIzol reagent (T9424, Sigma–Aldrich, Germany). qPCR amplification was carried out with ChamQ SYBR color qPCR master mix (Q411, Nanjing Novozan Biotechnology, China) and HiScript® II 1st strand cDNA synthesis kit (R211, Nanjing Novozan Biotechnology, China), using thioredoxin-binding protein (TBP) as an internal control. Relative ACE2 and NEDD4L mRNA levels were computed with the $2^{-\Delta\Delta C_t}$ method. The sequences of primers used for qPCR are listed in **Supplemental Table S1**.

8. Immunohistochemistry

Paraffin-embedded rat lung sections were deparaffinized, subjected to antigen retrieval, and blocked. The sections were probed with an ACE2 primary antibody overnight at 4°C and then with coraLite488-conjugated goat anti-rabbit IgG (H+L) (P0176-1, Beyotime Biotechnology, China) for 1 h at room temperature. Chromogenic development was performed with DAB (cat. no. P0203; Beyotime Institute of Biotechnology) at 25°C for 30 min, after which the sections were counterstained with hematoxylin, dehydrated, cleared, and mounted. ACE2 expression in the pulmonary artery wall was examined microscopically.

9. CCK-8 Assay

PASMCs were plated in 96-well plates (5×10^3 cells per well) and transfected with ACE2-3xHA, mutACE2-3xHA, or control vector as described above. At 24, 48, 72, and 96 h post-transfection, 10 μ L of Cell Counting Kit-8 (CCK-8) reagent was added per well, and the plates were incubated at 37°C for 2 h before the absorbance at 450 nm was read on a microplate reader. Each experiment was repeated independently at least three times.

10. Cell Cycle Analysis

Forty-eight hours after transfection, PASMCs were collected, rinsed with phosphate-buffered saline (PBS), and fixed overnight at 4°C in 70% ice-cold ethanol. The fixed cells were washed with PBS and stained for 30 min at 37°C in the dark with propidium iodide (PI) solution containing RNase A. DNA content was then measured by flow cytometry to determine the percentages of cells in the G1, S, and G2 phases.

11. Statistical Analysis

All statistical analyses were conducted with GraphPad Prism 8.0.2 software. All experiments were independently repeated at least three times, yielding similar results. The Shapiro–Wilk test ($P < 0.05$) and the F test ($P > 0.05$) were first applied to assess normality and homogeneity of variance, respectively. Comparisons between two groups of normally distributed data were made with two-tailed Student's t tests, whereas multiple groups were compared by one-way ANOVA followed by the Bonferroni post-hoc correction. When data were not normally distributed or the sample size was small ($n < 5$), two-group and multiple-group comparisons were performed with the Mann–Whitney U test and the Kruskal–Wallis test, respectively. Data are presented as the mean $\pm$ standard error of the mean (SEM), and differences were considered statistically significant at $P < 0.05$.

Results

1. Construction and Validation of the ACE2-K788R Mutant Vector and Its Effects on PASMC Proliferation and Cell Cycle

By integrating bioinformatic prediction with published site-mapping data, lysine 788 (K788) in the intracellular C-terminal tail of ACE2 was nominated as the candidate residue responsible for NEDD4L-dependent ubiquitination. The K788R mutant vector (mutACE2-3xHA) and the wild-type ACE2-3xHA vector were therefore constructed (Supplementary Fig. 2). Following transfection into PASMCs, Western blotting detected no HA-ACE2 in untransfected control cells, whereas robust and statistically indistinguishable HA-ACE2 expression was observed in the ACE2-OE and mutACE2-OE groups, confirming efficient and comparable transfection (Figure 1A). We then assessed how ACE2

overexpression affects PASMCM proliferation and cell cycle progression under basal culture conditions. Flow cytometric analysis revealed that, relative to PASMCMs-NC cells, ACE2-OE cells accumulated in G1 phase (approximately 58% vs. 49%, $P < 0.01$), accompanied by reductions in S phase (approximately 16% vs. 25%, $P < 0.05$) and G2 phase (approximately 20% vs. 25%, $P < 0.05$), a pattern indicative of G1-phase arrest (Figure 1B). The mutACE2-OE group displayed an intermediate profile (approximately 54%, 20%, and 23% for G1, S, and G2, respectively) and showed a partial yet statistically significant attenuation of the G1-phase arrest induced by wild-type ACE2 (approximately 54% vs. 58%, $P < 0.05$ vs. ACE2-OE), while the S- and G2-phase fractions did not differ significantly between these two groups. By contrast, CCK-8 assays detected no significant differences in viability among the three groups at 24, 48, 72, or 96 h (Figure 1C), demonstrating that neither ACE2 overexpression nor the K788R mutation is cytotoxic or alters the overall proliferative capacity of PASMCMs under basal conditions.

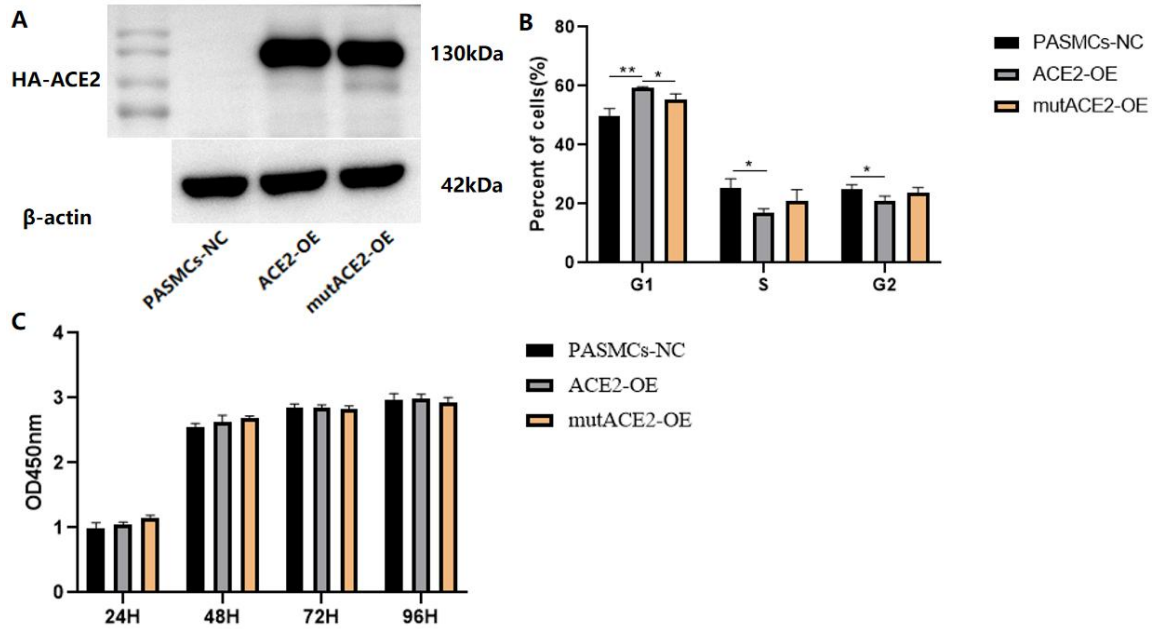


Figure 1. Validation of ACE2-3xHA vector transfection and its impact on PASMCM proliferation and cell cycle distribution. (A) PASMCMs were transfected with wild-type ACE2-3xHA (ACE2-OE) or K788R-mutated ACE2-3xHA (mutACE2-OE), with untransfected cells as controls (PASMCMs-NC); HA-tagged ACE2 (~130 kDa) was detected by Western blotting, using β -actin (42 kDa) as the loading control. (B) Cell cycle distribution (G1, S, and G2 phases) determined by propidium iodide staining and flow cytometry at 48 h post-transfection. (C) Cell viability assessed by the CCK-8 assay at 24, 48, 72, and 96 h post-transfection. Data are expressed as the mean $\pm$ SEM ($n = 3$). * $P < 0.05$, ** $P < 0.01$ vs. PASMCMs-NC; in the G1 phase, the mutACE2-OE group also differed significantly from the

ACE2-OE group ($P < 0.05$). ACE2, angiotensin-converting enzyme 2; CCK-8, Cell Counting Kit-8; PSMCs, pulmonary artery smooth muscle cells.

2. K788 Is Required for the Association between NEDD4L and ACE2

PASMCs were co-transfected with the NEDD4L-Flag vector together with either ACE2-3xHA or mutACE2-3xHA (K788R), and co-IP was subsequently performed. HA-ACE2 was readily detectable in Flag immunoprecipitates from cells expressing wild-type ACE2, whereas it was barely detectable in those from K788R-expressing cells. Given that co-IP reflects protein–protein association, these findings demonstrate that K788 is indispensable for the physical interaction of NEDD4L with ACE2 and support the notion that K788 acts as a key ubiquitin-acceptor lysine for NEDD4L on ACE2 (Figure 2).

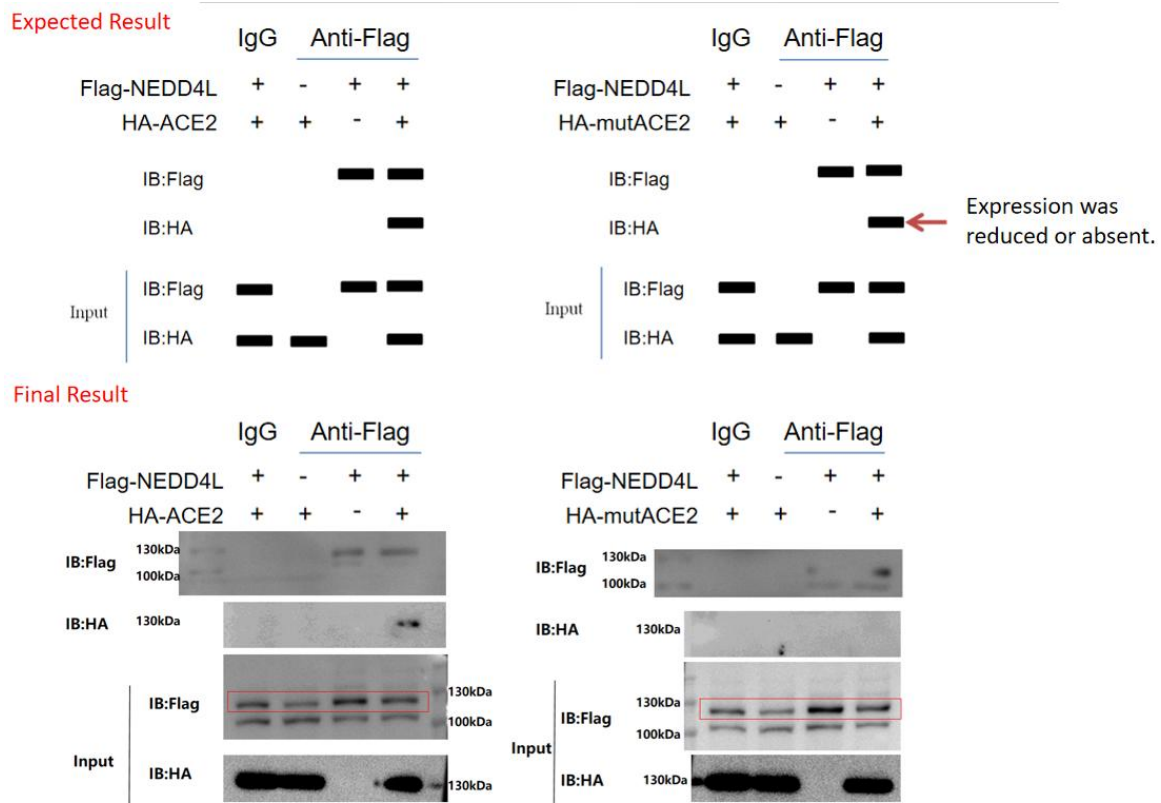


Figure 2. K788 is required for the NEDD4L–ACE2 interaction in PSMCs. PSMCs were co-transfected with Flag-tagged NEDD4L together with either wild-type ACE2-3xHA or K788R-mutated ACE2-3xHA, and cell lysates were immunoprecipitated with an anti-Flag antibody (IgG as the control), followed by immunoblotting for HA-ACE2 (~130 kDa) and Flag-NEDD4L (~120 kDa). NEDD4L, neural precursor cell-expressed developmentally down-regulated gene 4-like; ACE2, angiotensin-converting enzyme 2.

3. Validation of the ACE2-AAV Rat Model

ACE2-knockout rats received AAV encoding either wild-type ACE2 (AAV-WT group) or K788R-mutated ACE2 (AAV-MUT group), and untreated wild-type rats served as the control group (NC group). Western blotting of lung tissue revealed significantly higher ACE2 expression in both AAV groups than in the NC group, with no significant difference between the two AAV groups; NEDD4L expression was comparable across all three groups. These data confirm successful AAV transduction and equivalent expression of wild-type and K788R ACE2 in rat lung tissue (Figure 3).

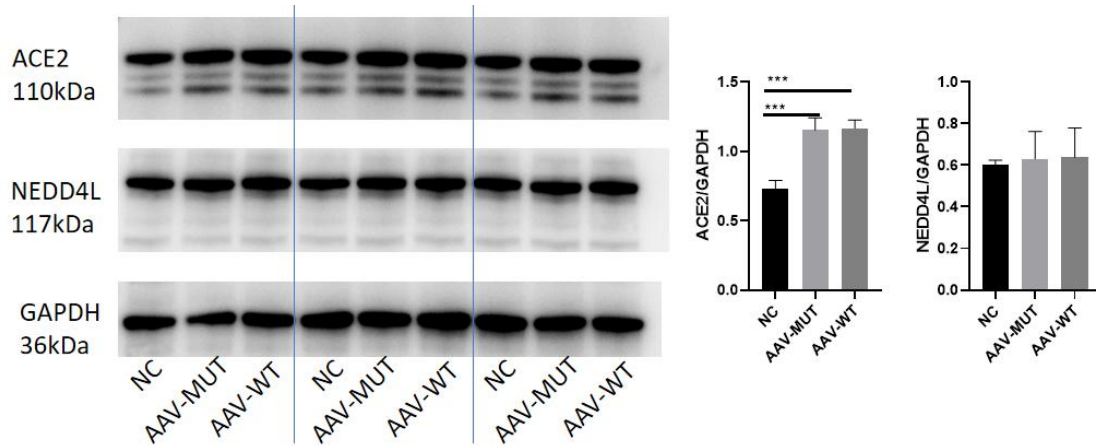


Figure 3. Validation of the ACE2-AAV reconstitution model. ACE2-knockout rats were given intratracheal instillations of AAV6 encoding wild-type ACE2 (AAV-WT) or K788R-mutated ACE2 (AAV-MUT), with wild-type rats as controls (NC). ACE2 and NEDD4L protein levels in lung tissue were measured by Western blotting, using GAPDH as the loading control. $n = 6$ per group; *** $P < 0.001$. NEDD4L, neural precursor cell-expressed developmentally down-regulated gene 4-like; ACE2, angiotensin-converting enzyme 2.

4. Binding of NEDD4L and ACE2 under Normoxic Conditions

In lung tissue harvested under normoxic conditions, co-IP revealed abundant NEDD4L–ACE2 complexes in the AAV-WT group, whereas complex formation was markedly diminished in the AAV-MUT group, demonstrating that K788 is required for the *in vivo* association of the two proteins under physiological conditions (Figure 4).

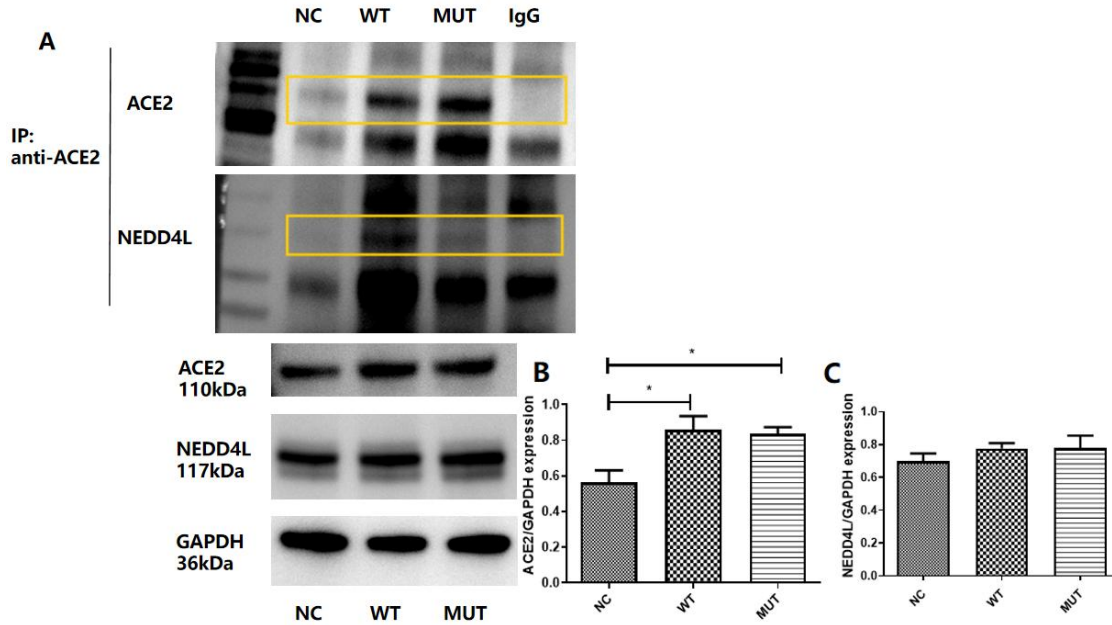


Figure 4. NEDD4L–ACE2 association and protein expression in AAV-reconstituted rats under normoxia. (A) Co-IP and Western blotting of lung lysates from NC, AAV-WT, and AAV-MUT rats; (B, C) quantification of ACE2 (110 kDa) and NEDD4L (117 kDa), using GAPDH (36 kDa) as the loading control. $n = 6$ per group; $*P < 0.05$. NEDD4L, neural precursor cell-expressed developmentally down-regulated gene 4-like; ACE2, angiotensin-converting enzyme 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

5. K788R Mutation Alleviates the Hypoxia-Induced Pulmonary Hypertension Phenotype

Following 4 weeks of hypoxia (10% O₂), echocardiography revealed significantly lower estimated pulmonary artery pressure in the AAV-MUT group than in the AAV-WT group. Blood-gas analysis further showed a significantly higher oxygenation index in the AAV-MUT group. On HE staining, pulmonary arteriolar muscularization and the medial wall thickness ratio were both significantly lower in the AAV-MUT group than in the AAV-WT group (Figure 5). Together, these data demonstrate that disrupting the NEDD4L–ACE2 interaction at K788 markedly attenuates the HPH phenotype.

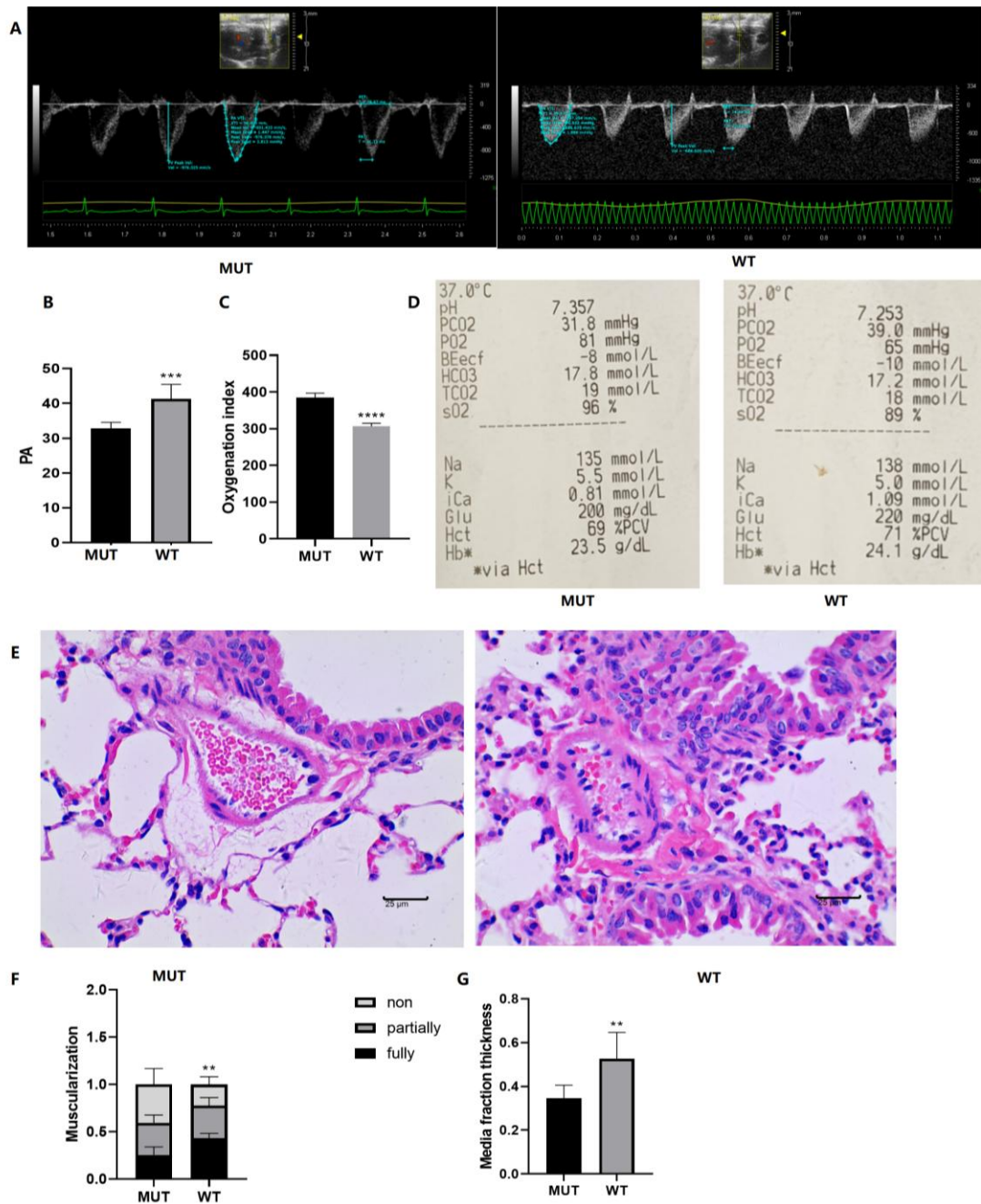


Figure 5. K788R ACE2 attenuates hypoxia-induced pulmonary hypertension. ACE2-knockout rats reconstituted with AAV encoding wild-type (WT) or K788R-mutated (MUT) ACE2, together with wild-type controls (NC), were maintained at 10% O₂ for 4 weeks. (A) Representative pulmonary artery Doppler tracings and (B) quantification of estimated pulmonary artery pressure; (C) oxygenation index derived from arterial blood-gas analysis, with (D) representative readouts; (E) representative HE-stained pulmonary arterioles, with quantification of (F) muscularization and (G) the medial wall thickness ratio (medial thickness/external diameter). n = 6 per group; ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. PA, pulmonary artery.

6. K788R Mutation Reduces Hypoxia-Induced Lung Tissue Apoptosis

TUNEL staining revealed that hypoxic exposure significantly increased apoptosis in the lung tissue of the AAV-WT group relative to the NC group, whereas apoptosis in the AAV-MUT group was significantly reduced compared with the AAV-WT group, demonstrating that disrupting the NEDD4L–ACE2 interaction at K788 suppresses hypoxia-induced apoptosis in lung tissue (Figure 6).

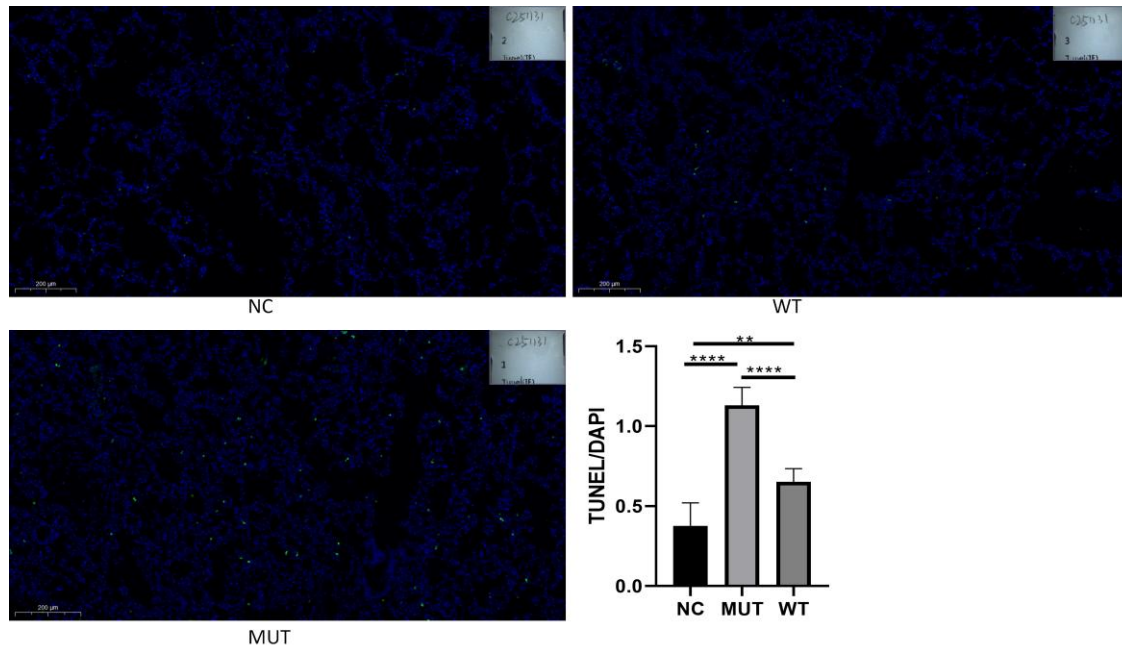


Figure 6. K788R ACE2 reduces hypoxia-induced apoptosis in rat lung. Following 4 weeks of hypoxia, apoptosis in lung sections from NC, AAV-WT, and AAV-MUT rats was evaluated by TUNEL staining, and the fluorescence intensity was quantified. $n = 6$ per group; $**P < 0.01$, $****P < 0.0001$.

7. Effects of K788R Mutation on ACE2 and NEDD4L mRNA Expression

qRT-PCR analysis showed that, after hypoxic exposure, lung ACE2 mRNA levels were comparable between the AAV-WT and AAV-MUT groups, consistent with a post-translational mode of action. By contrast, NEDD4L mRNA expression was significantly higher in the AAV-MUT group than in the AAV-WT group (Figure 7), which raises the possibility of a compensatory transcriptional response upon disruption of the NEDD4L–ACE2 interaction.

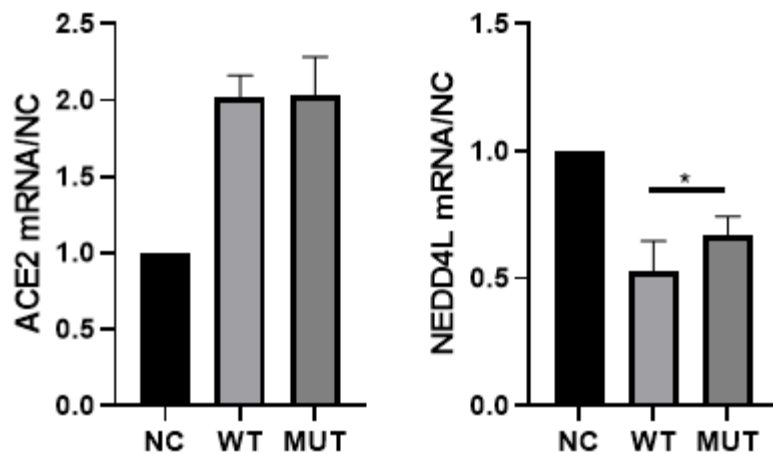


Figure 7. ACE2 and NEDD4L mRNA expression after hypoxia. Lung ACE2 and NEDD4L mRNA levels in NC, AAV-WT, and AAV-MUT rats were quantified by qPCR, with TBP as the internal reference. $n = 6$ per group; $*P < 0.05$. NEDD4L, neural precursor cell-expressed developmentally down-regulated gene 4-like; ACE2, angiotensin-converting enzyme 2.

8. NEDD4L–ACE2 Association and Protein Expression under Hypoxia

Under hypoxic conditions, co-IP again revealed abundant NEDD4L–ACE2 complexes in the lung tissue of the AAV-WT group, with markedly lower complex levels in the AAV-MUT group. Western blotting detected no significant difference in ACE2 protein expression between the AAV-WT and AAV-MUT groups, excluding differential transgene expression as an explanation for the phenotypic differences. In contrast, NEDD4L protein expression was significantly lower in the AAV-WT group than in the AAV-MUT and NC groups (Figure 8).

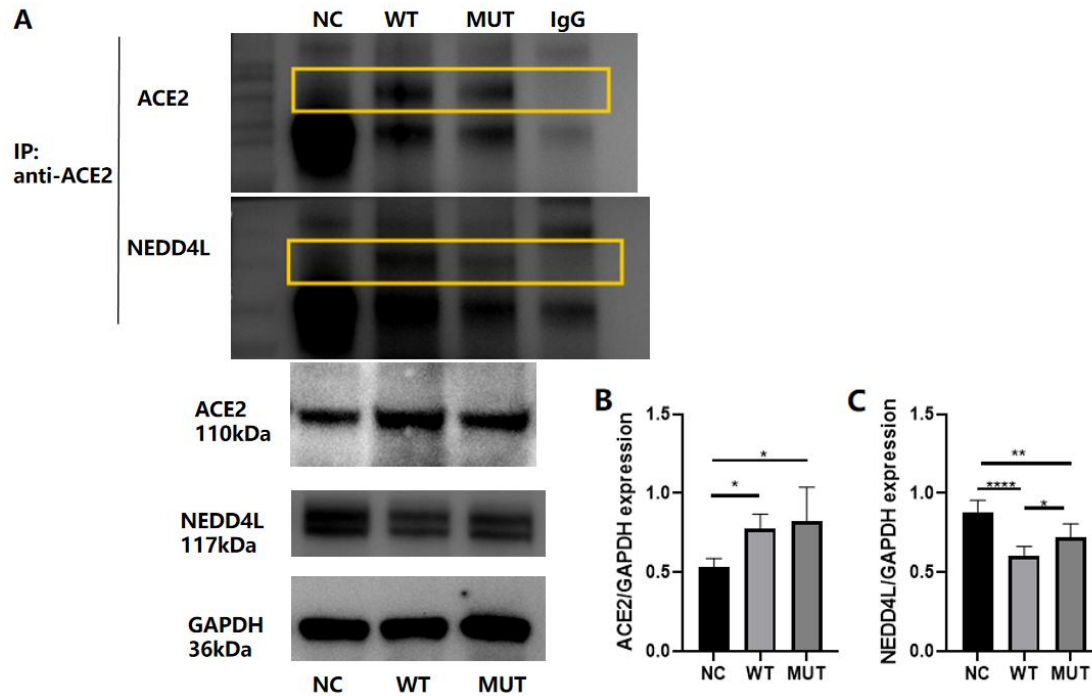


Figure 8. NEDD4L–ACE2 association and protein expression under hypoxia. (A) Co-IP and Western blotting of lung lysates from hypoxia-exposed NC, AAV-WT, and AAV-MUT rats; (B, C) quantification of ACE2 (110 kDa) and NEDD4L (117 kDa), using GAPDH (36 kDa) as the loading control. $n = 6$ per group; $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$. NEDD4L, neural precursor cell-expressed developmentally down-regulated gene 4-like; ACE2, angiotensin-converting enzyme 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Immunohistochemical analysis revealed no significant difference in pulmonary artery wall ACE2 expression between the AAV-WT and AAV-MUT groups, although both were higher than that in the NC group (Figure 9), which further confirms the comparable reconstitution efficiency of the two AAV constructs.

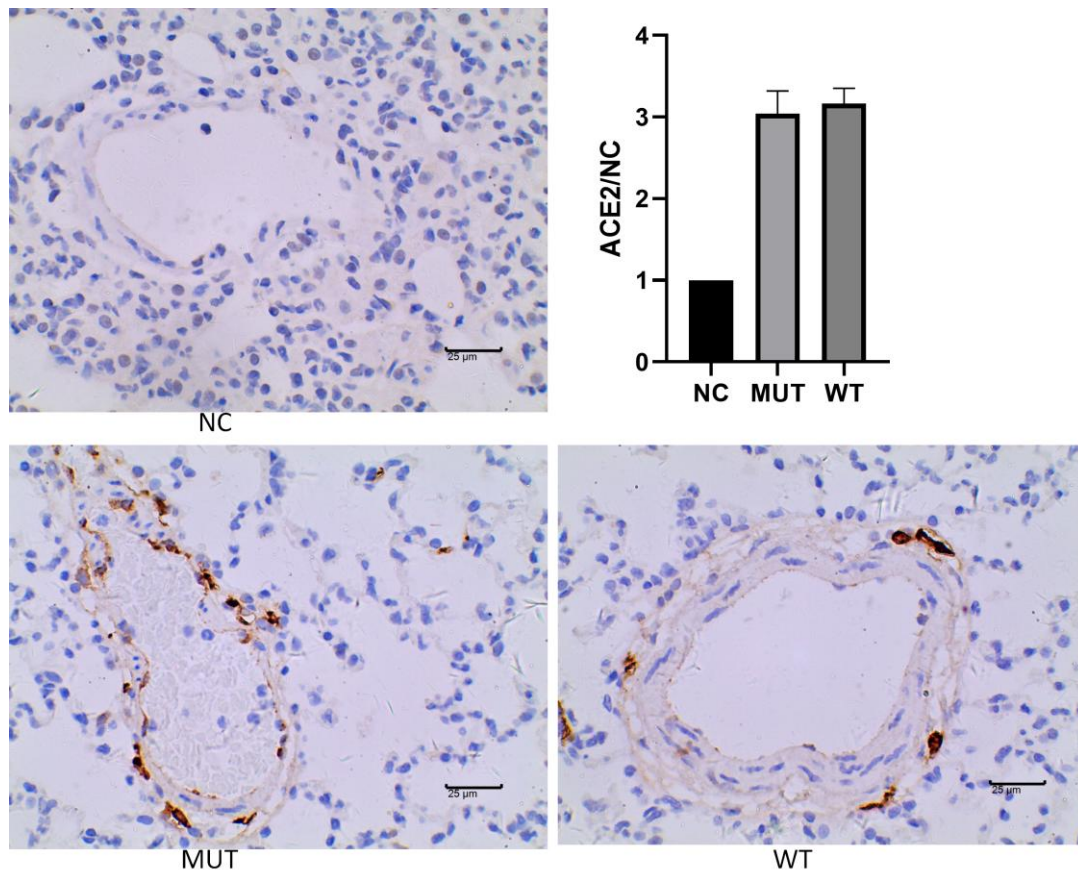


Figure 9. ACE2 expression in the pulmonary artery wall. After 4 weeks of hypoxia, ACE2 in the pulmonary artery walls of NC, AAV-WT, and AAV-MUT rats was assessed by immunohistochemistry and quantified. n = 6 per group.

Discussion

Building on our previous finding that NEDD4L mediates the ubiquitination and degradation of ACE2 in pulmonary hypertension, the present study identified lysine 788 (K788), situated in the intracellular C-terminal tail of ACE2, as a critical residue for the NEDD4L–ACE2 interaction, and demonstrated that AAV-mediated delivery of K788R-mutated ACE2 significantly attenuated the HPH phenotype in ACE2-knockout rats. These findings offer a residue-level molecular target for site-specific therapeutic intervention in HPH.

K788 lies within the 43-amino-acid intracellular C-terminal tail of ACE2 (amino acids 763–805), the sole region of the protein accessible to cytosolic E3 ligases. This tail harbors five ubiquitin-acceptor lysines (K769, K770, K771, K773, and K788), which were systematically validated as NEDD4L target residues in hypertension by Mohammed et al[15], together with an NPxY motif (amino acids 779–781) immediately adjacent to K788 that serves as a canonical recognition element for the WW

domains of NEDD4-family ligases and additionally functions as an endocytic sorting signal[12]. K788 thus sits at the convergence of substrate recognition and ubiquitin conjugation, offering a plausible structural explanation for why the K788R mutation abolished the NEDD4L–ACE2 association in both PSMCs and rat lung. Of note, K788 is also the residue ubiquitinated by MDM2 in pulmonary endothelial cells, where the deubiquitination-mimetic K788R mutant attenuated experimental PH[16]; our data broaden this principle by implicating NEDD4L, rather than MDM2, at the same hotspot residue in the hypoxic pulmonary vasculature. Since K788 is spatially remote from the extracellular residues mediating SARS-CoV-2 spike binding, intervention at this intracellular site may permit modulation of ACE2 protein stability without disturbing its enzymatic activity or receptor function.

The cell-based experiments reported here add mechanistic context to these *in vivo* observations. Under basal culture conditions, overexpression of ACE2 triggered G1-phase arrest in PSMCs, lowering the proportions of cells in S and G2 phases while leaving overall viability unchanged, as evidenced by the CCK-8 assay. This behavior agrees with the established anti-proliferative function of the ACE2–Ang-(1-7)–Mas axis in pulmonary vascular cells, whereby ACE2 restrains PSMC cell cycle progression instead of merely supporting cell survival. Importantly, the K788R mutant significantly blunted the ACE2-induced G1-phase arrest ($P < 0.05$ vs. ACE2-OE) without fully restoring the control distribution, implying that K788-dependent post-translational regulation contributes substantially to, but does not entirely explain, ACE2-mediated cell cycle control under normoxic conditions. Together with the comparable HA-ACE2 levels in the ACE2-OE and mutACE2-OE groups, these results argue that the superior therapeutic efficacy of K788R-ACE2 in hypoxic rats stems not from cytotoxicity or nonspecific transgene effects but from stabilization of the ACE2 protein under hypoxic stress, when NEDD4L-mediated ubiquitination is active.

Site-specific ubiquitination blockade at K788 attenuates HPH. In our animal experiments, ACE2-knockout rats treated with AAV carrying K788R-mutated ACE2 displayed significantly lower pulmonary artery pressure, less pulmonary arteriolar muscularization, a higher oxygenation index, and reduced lung tissue apoptosis than rats treated with wild-type ACE2. These observations align with the well-established protective role of ACE2 in PH: earlier work showed that ACE2 overexpression mitigates pulmonary hypertension in diverse animal models via the ACE2–Ang-(1-7)–Mas axis, which counteracts the vasoconstrictive and pro-proliferative actions of the ACE–Ang II–AT1R axis[2-4]. Our group previously reported that ACE2 alleviates PAH by inhibiting focal adhesion kinase expression[18]. Extending these findings, the present study demonstrates that preventing NEDD4L-mediated degradation of ACE2 at a defined residue—rather than simply overexpressing exogenous ACE2—is by itself sufficient to achieve therapeutic benefit, an approach that leaves the endogenous mechanisms governing ACE2 expression intact. The reduced apoptosis in the AAV-MUT group is also consistent with the anti-apoptotic actions of the ACE2–Ang-(1-7)–Mas axis described in

cardiovascular tissues[20]. Moreover, the improved oxygenation index hints that stabilized ACE2 may enhance alveolar fluid clearance, potentially via interactions with ENaC, since hACE2 supplementation has been reported to activate ENaC and restore endothelial barrier function in vitro[20].

NEDD4L expression patterns in the context of ACE2 ubiquitination blockade. A notable observation was that, under hypoxic conditions, NEDD4L protein expression was significantly lower in the AAV-WT group than in the AAV-MUT group, whereas NEDD4L mRNA showed the opposite trend, being higher in the AAV-MUT group. The diminished NEDD4L protein in the AAV-WT group may reflect auto-ubiquitination and ensuing degradation of NEDD4L during active ubiquitination of ACE2, a regulatory behavior documented for other E3 ubiquitin ligases[8]. The elevated NEDD4L mRNA in the AAV-MUT group points to a possible compensatory transcriptional response: once NEDD4L-mediated ACE2 ubiquitination is blocked, cells may increase NEDD4L transcription in an effort to re-establish ubiquitination balance. Comparable feedback mechanisms have been described for other components of the ubiquitin–proteasome system[21, 22]. This observation is also consistent with reports of elevated NEDD4L expression in the lungs of monocrotaline-induced PAH rats[14] and in the lung tissue of COVID-19 patients [23], implying that NEDD4L upregulation may represent a common reaction to pathological stimuli. Finally, the unchanged ACE2 mRNA levels between the AAV-WT and AAV-MUT groups confirm that the K788R mutation acts at the post-translational level, in line with the role of ubiquitination as a determinant of protein stability rather than of transcription.

The advantage of site-specific targeting over global NEDD4L inhibition. The pleiotropic nature of this E3 ligase is a critical consideration when designing NEDD4L-targeted therapies. Beyond ACE2, NEDD4L ubiquitinates multiple substrates, including ENaC[9] and Nav1.5[10], and indiscriminate inhibition of NEDD4L would disturb the regulation of these substrates and might provoke adverse effects. In mouse lung epithelial cells, for instance, NEDD4L knockout causes excessive ENaC activation and pulmonary inflammation, and ENaC knockout likewise produces severe pulmonary inflammation, underscoring the necessity of ENaC homeostasis[24]. This consideration is especially pertinent to PH, in which hypoxia compromises ENaC activity and alveolar fluid clearance, thereby promoting pulmonary vasoconstriction and edema[25, 26]. By selectively disrupting the NEDD4L–ACE2 interaction at K788, our strategy circumvents such off-target effects, because the capacity of NEDD4L to regulate ENaC and its other substrates is preserved. This notion is bolstered by work on targeted protein degradation technologies—including molecular glues and heterobifunctional degraders—which achieve substrate-specific modulation of E3 ligase activity[21, 22]. K788 is an attractive target for such strategies, as it lies within the intracellular tail of ACE2, immediately next to the NPxY motif implicated in NEDD4-family substrate recognition[12, 15]. Small molecules or

peptides engineered to obstruct the NEDD4L–K788 interaction could therefore stabilize ACE2 selectively, without global NEDD4L inhibition.

Several limitations of this study should be acknowledged. First, co-immunoprecipitation reveals protein association rather than direct ubiquitination; although K788 has been independently validated as a ubiquitin-acceptor residue for NEDD4-family ligases and MDM2[15, 16], direct ubiquitination assays, ubiquitin-linkage profiling, and protein half-life measurements will be needed to verify that the K788R mutation acts by preventing ACE2 degradation in this model. It is also noteworthy that ACE2 protein levels were comparable between the AAV-WT and AAV-MUT groups; while this excludes confounding by transgene dosage, it further implies that the protective mechanism may involve altered ACE2 localization, enzymatic activity, or downstream signaling rather than protein abundance alone, an issue meriting further study. Second, although chronic hypoxia recapitulates key features of HPH, it does not capture the full spectrum of human PAH, and confirmation in additional models, such as monocrotaline-induced PH, is warranted. Third, pulmonary artery pressure was evaluated by echocardiography; more comprehensive invasive hemodynamic measurements would strengthen future work. Fourth, only male rats were studied, and sex-specific effects cannot be ruled out. Finally, a NEDD4L-knockout model would furnish complementary genetic evidence; however, the high mortality of NEDD4L-knockout rats in preliminary experiments precluded the establishment of a stable PH model, so ACE2-knockout rats were chosen as the alternative.

In summary, this study establishes K788 as a critical residue for the NEDD4L–ACE2 interaction and shows that site-specific blockade of ACE2 ubiquitination at K788 effectively alleviates the progression of hypoxia-induced pulmonary hypertension in rats, offering a potential precision-targeted intervention strategy for HPH.

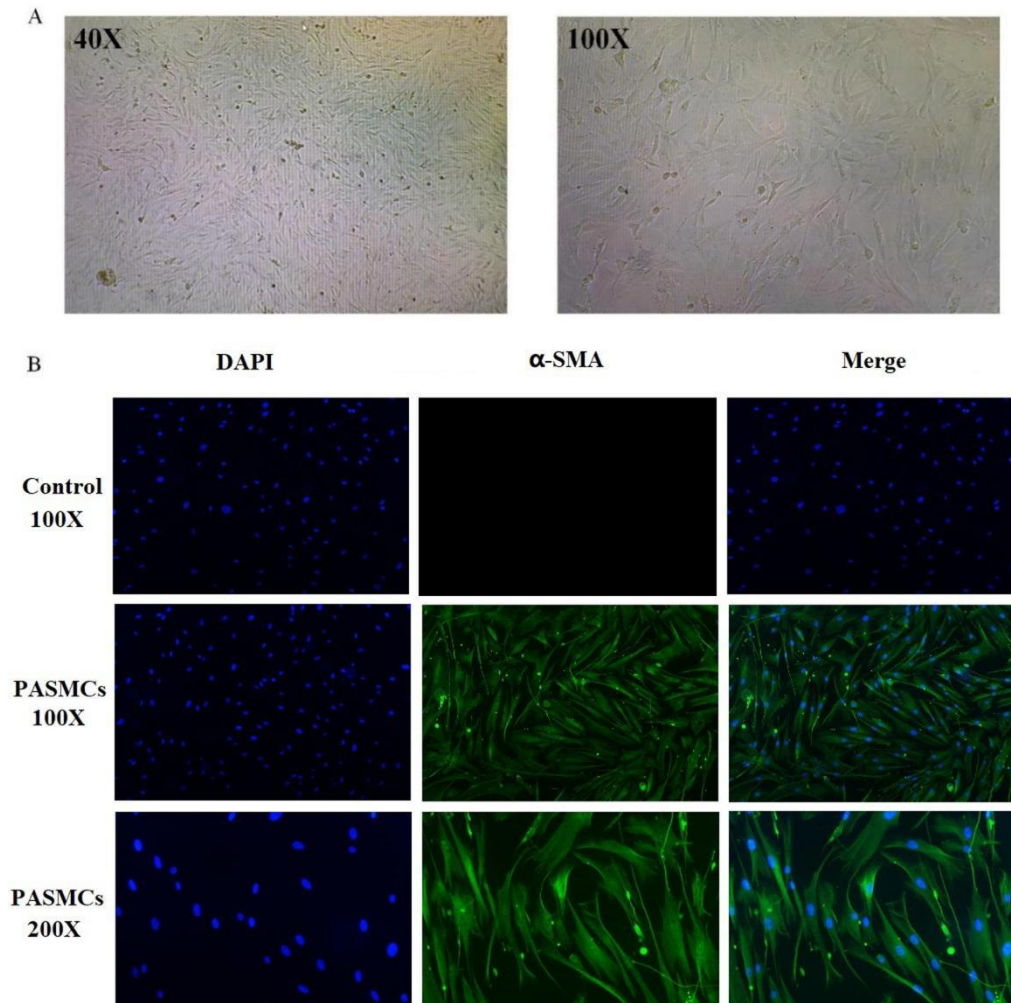
Author contributions Rui Wang¹, Rui Wang², and Zhiping Wang designed the study. Rui Wang¹ and Rui Wang² carried out the formal analysis, and Rui Wang¹, Rui Wang², and Tianya Liu conducted the experiments. Tianya Liu and Qianmin Chen were responsible for sample collection. Zhiping Wang contributed resources and secured funding. Rui Wang¹ drafted the manuscript, and Rui Wang¹ and Rui Wang² revised it. All authors read and approved the final manuscript.

Funding This work was supported by the National Natural Science Foundation of China 82270059, and Project for Clinical Research on New Medical Technologies at the Affiliated Hospital of Xuzhou Medical University GSPJS202523.

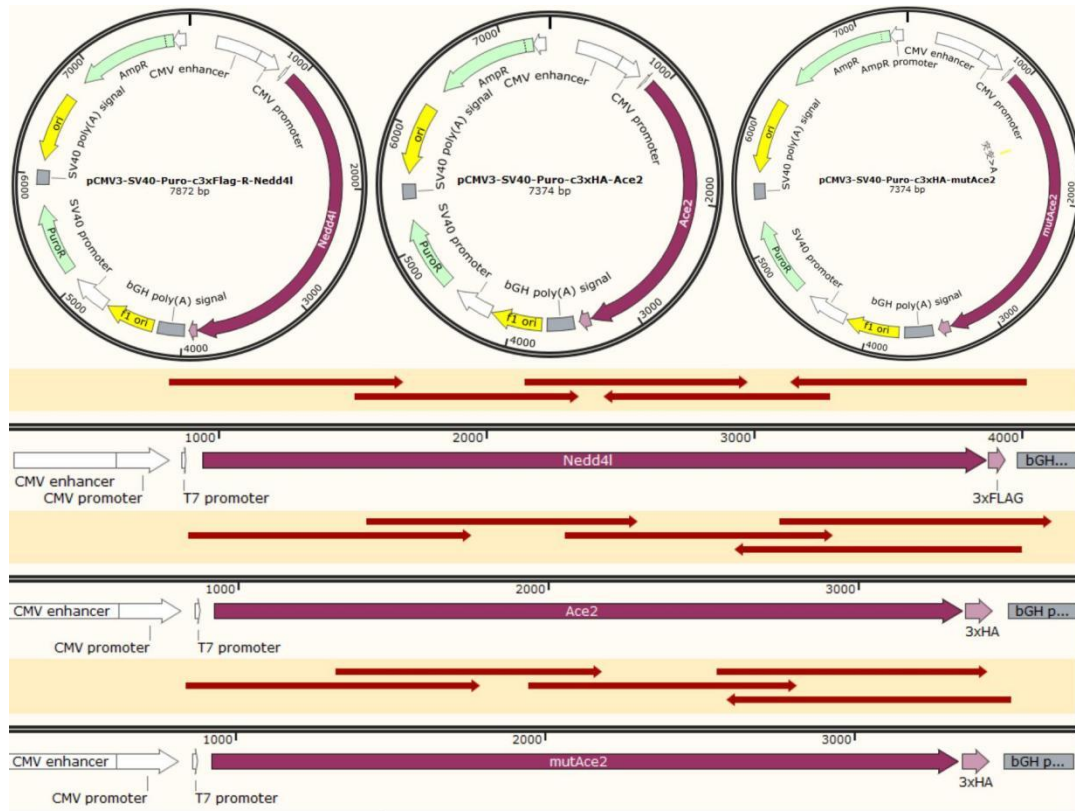
Data availability The datasets generated and analyzed during this study are available from Rui Wang¹ upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.



Supplementary Fig. 1 Results of the isolation and cultivation of PASMCs. Cell morphology was examined by inverted phase-contrast microscopy, and a cell growth curve was plotted by cell counting and observed under $\times 40$ and $\times 100$ mirrors (**A**). Immunofluorescence staining of α -smooth muscle actin (α -SMA) was performed to verify the identity and purity of PASMCs and A549 cells (control, CL0024, Fenghuishengwu, China), which were observed at $\times 100$ and $\times 200$ (**B**).



Supplementary Fig. 2 Vector maps and sequencing results of NEDD4L, ACE2, and K788R-mutated ACE2. The NEDD4L and ACE2 sequences were obtained from the NCBI Gene database, and the ACE2 ubiquitination site (K788) was predicted with the UbiBrowser database and cross-checked against published site-mapping data. The Nedd4l-3xFlag sequence was inserted into the pCMV3-SV40-Puro vector through BamHI and XbaI, while the ACE2-3xHA and mutACE2-3xHA (K788R) sequences were inserted into the pCMV3-SV40-Puro vector through HindIII and XhoI. The vector carries ampicillin resistance in prokaryotes. Correct insertion of the target sequences was confirmed by Sanger sequencing. NEDD4L, neural precursor cell-expressed developmentally down-regulated gene 4-like; ACE2, angiotensin-converting enzyme 2.

Supplementary Table S1. Sequences of Primers Used for qPCR

Target Gene	Forward/Reverse Strand	Primer Sequences
TBP	Forward	5'-CCCACCAGCAGTTCAGTAGC-3'
	Reverse	5'-GAATTCTGGGTTTGATCATTCTG-3'

ACE2	Forward	5'-AAGCCACCTTACGAGCCTCCTG-3'
	Reverse	5'-AACAATGCCAACCCTACCGTTCC-3'
NEDD4L	Forward	5'- CCACCAGGTTGGGAAGAAAAA-3'
	Reverse	5'-TCCAAGTAGTCGTTCTGGAATTG-3'

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