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# FAM134B Attenuates Apoptosis and EMT by Inhibiting M1 Macrophage Polarization Via PI3K/AKT Pathway in Rat Lungs Exposed to Hyperoxia

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## ABSTRACT

Oxygen therapy is widely used in pulmonary disease and critical care resuscitation. Unfortunately, prolonged exposure to high concentrations of oxygen may cause oxygen toxicity, leading to hyperoxia-induced acute lung injury (HALI). The injurious effects of lung following hyperoxia exposure are well established, and the effects include cell apoptosis and epithelial-to-mesenchymal transition (EMT). HALI responds to the inflammatory response induced by M1 macrophage polarization. Suppressing inflammation in macrophages protects against HALI. The PI3K/AKT pathway promotes cell survival in oxidative stress injury. Activation of Akt is a beneficial response protects against hyperoxic stress. FAM134B activates ER-phagy through binding to LC3B, ER fragments are degraded by ER-phagy, and ER homeostasis is maintained. We hypothesized that FAM134B may regulate macrophage polarization and inflammatory responses in hyperoxia-exposed rats via PI3K/AKT signaling pathway, thereby attenuating HALI.

In this study, we clarified the role of FAM134B in lung tissues of hyperoxia-exposed rats and the effect of FAM134B on macrophage polarization. The expression of FAM134B and PI3K/AKT pathway were inhibited in rat lung tissues after hyperoxia exposure. We found that overexpression of FAM134B activated the PI3K/AKT pathway and reduced apoptosis and EMT in rat lung tissues after hyperoxia exposure. The PI3K/AKT pathway inhibitor, LY294002, reversed the protective effect of FAM134B in hyperoxia-exposed rats. Overexpression of FAM134B reduced the release of inflammatory factors (IL-1β, TNF-ɑ, IL-6) and polarization of M1 macrophages after hyperoxia exposure, and LY294002 reversed this effect.

In conclusion, our study showed that FAM134B inhibits M1 macrophage polarization and inflammatory factor release via PI3K/AKT pathway and attenuates apoptosis and EMT in rat lung of hyperoxia-exposed FAM134B is a key target for HALI, which provides new ideas for the treatment of HALI.

## INTRODUCTION

Oxygen therapy is widely used in pulmonary disease and critical care resuscitation. However, high oxygen concentrations for a prolonged period of time may cause oxygen toxicity, resulting in hyperoxia-induced acute lung injury (HALI), and it is associated with the increased mortality in humans Tibboel et al. (2013), Chu et al. (2018). The injurious effects of lung following hyperoxia exposure are well established, and the effects include cell apoptosis Bhandari et al. (2006) and epithelial-to-mesenchymal transition (EMT) Vyas-Read et al. (2014). Despite significant advances in the treatment of HALI over time, its pathogenesis remains incompletely cleared and effective preventive or therapeutic strategies are lacking. Therefore, the discovery of new molecular pathways that cause apoptosis and EMT in HALI may lead to potential new therapeutic approaches.

Fundamental to the pathogenesis is the oxygen-induced lung inflammation, which is defined by the release of pro-inflammatory cytokines like interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α), followed by the influx of inflammatory cells that amplify the response and damage lung tissue Shahzad et al. (2022). Hyperoxia promotes macrophage recruitment and triggers M1-like macrophage polarization and increased interleukin release to exacerbate lung injury Hirani et al. (2022). Therefore, attenuating the hyperoxia-induced inflammatory response in the lungs is a very attractive method of preventing HALI.

Through ER-phagy, a selective autophagy pathway, the endoplasmic reticulum undergoes persistent remodeling Vargas et al. (2023). The reticulon-like protein FAM134B forms the ER membrane Khaminets et al. (2015). The ER-phagy receptor FAM134B mediates the selective

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degradation and fragmentation of ER sheets González et al. (2023). FAM134B activates ER-phagy through binding to LC3B, ER fragments are degraded by ER-phagy, and ER homeostasis is maintained Chen et al. (2022). Deletion of FAM134B is a key cause of several diseases. FAM134B knockdown reverses AGE-induced intracellular ROS buildup, apoptosis, and senescence; FAM134B overexpression attenuates these effects Luo et al. (2021). Moreover, ROS levels and apoptosis of hippocampus neurons were reduced by FAM134B overexpression Xie et al. (2020). What part does ER-phagy play in the formation of HALI, and how might ER-phagy affect the course of HALI? It's up for debate.

Cell survival is promoted by the PI3K/AKT pathway in oxidative stress injury. Activated Akt protects the lung from damage and delays death in mice Lu et al. (2001) The finding that constitutive activation of Akt protects against hyperoxia suggests that it is a beneficial response by maintaining mitochondrial integrity Ahmad et al. (2006). PI3K/AKT pathway has a regulatory role in the inflammatory response, Lung injury and inflammation were caused by PI3K/AKT inhibition in rats following hyperoxia exposure Reddy et al. (2015). Interestingly, FAM134B plays an important role in hepatocellular carcinoma through its regulatory effect on the Akt signaling pathwa Zhang et al. (2019).

We hypothesized that FAM134B may regulate macrophage polarization and inflammatory responses in hyperoxia-exposed rats through the PI3K/AKT signaling pathway, thereby attenuating HALI. In this study, we clarified the role of FAM134B in lung tissues of hyperoxia-exposed rats and the effect of FAM134B on macrophage polarization.

## MATERIALS AND METHODS

### Animals

About three-week-old male Sprague–Dawley (SD) rats were purchased from Lanzhou University (Lanzhou, China). Lanzhou University's First Hospital Animal Ethics Committee approved all experiments (LDYYLL2022-216). Rats were treated with humane care at all times during the experiment. They were provided with food and water freely, provided with 12-hour light/dark cycles, were kept at constant temperatures (22 °C) and were kept at a constant humidity level (45-55%). We euthanize rats with isoflurane. All methods are reported in accordance with ARRIVE guidelines.

### A statement to confirm that all experimental protocols were approved by a named institutional and/or licensing committee.

This study and all experimental protocols were approved by the the Animal Ethics Committee of the First Hospital of Lanzhou University (LDYYLL2022-216). Our research activities were carried out in accordance with the requirements of the project application and in line with the ethical requirements of the study.

### Hyperoxia-induced lung injury model

Cages (6 rats per cage) were placed in a hyperoxia exposure chamber for 7 days. Sodium absorbent was used to line the bottom of the chamber to collect CO2. Continuous delivery of sufficient oxygen to the chamber, with the oxygen concentration regulated to 90% and monitored with oxygen monitors.

### Animal treatment

The adeno-associated virus (AAV)-6 vector-mediated FAM134B overexpression was designed by Genechem (Shanghai, China) and provided in a concentration of 1012 v.g/mL, of which 150 μL was endotracheally administered to the rats after one week of adaptive feeding. Three weeks after administration of AAV-FAM134B, rats were exposed to hyperoxia to induce HALI. Rats were divided into four groups (n=6): (1) NC group, (2) HALI group, (3) HALI + AAV-FAM134B group, (4) HALI + AAV-FAM134B + LY294002 group. Rats in the fourth group were injected intraperitoneally with LY294002 (5 mg/kg), a potent Pi3k inhibitor.

### Bronchoalveolar Lavage Analysis

Alveolar lavage of rat lungs with saline was performed twice at the end of the experiment. Bronchoalveolar lavage fluid (BALF) was centrifuged at 200 × g for 10 min.

### Histopathology of lung tissue

A paraffin embedding procedure was performed at the end of the experiment to fix the left lung in 4% paraformaldehyde for 24 hours at room temperature. The lung tissue was cut into 4-micron sections and stained with hematoxylin-eosin (H&E). A scale ranging from 0 to 3 was used to measure the degree of lung injury: In the first grade, there is normal appearance of the lungs; in the second grade, there is perivascular edema, partial leukocyte infiltration, and moderate neutrophil leukocyte infiltration; there is a massive neutrophilic infiltration and structural destruction of the lungs in the third grade.

### Immunohistochemistry and immunofluorescence

The lung tissue slices underwent a series of procedures including xylene deparaffinization, rehydration in graded alcohol, boiling in 0.01 M sodium citrate buffer (pH 6.0), room temperature cooling, and three PBS washes. Sections were blocked for 30 minutes at room temperature using 1% goat serum and 3% hydrogen peroxide for endogenous peroxidase. After that, sections were incubated for an entire night at 4 °C with the

matching primary antibodies:anti-FAM134B antibody (21537-1-AP, Proteintech), anti-LC3B antibody (T55992, Abmart), anti-caspase3 antibody (19677-1-AP, Proteintech), anti-Bax antibody (T40051, Abmart), anti-Bcl-2 antibody (T40056, Abmart), anti-E-cadherin antibody (TA0131, Abmart), anti-N-cadherin antibody (T55015, Abmart), anti-vimentin antibody (T55134, Abmart), anti-PI3K antibody (20584-1-AP, Proteintech), anti-P-PI3K antibody (T40065, Abmart), and anti-P-AKT antibody (80455-1-RR. Proteintech). After that, sections were cleaned and treated for 30 minutes with rat-specific horseradish peroxidase polymer anti-rabbit antibody. Next, horseradish peroxidase substrate was added and incubated for an additional three minutes. Hematoxylin was then used to stain the lung slices. After washing, enzyme-labeled secondary antibodies were added and the mixture was incubated for 50 minutes at room temperature to achieve immunofluorescence. After that, 4',6-diamidino-2-phenylindole (DAPI) was used to stain the sections. Images were examined with fluorescence microscope, and Image J software was used to compute the average OD values and fluorescence intensities.

### Elisa

IL-1β, IL-6 and TNF-α were assayed based on kit instruction (RuixinBiotech, China) and the plate was then transferred to a microplate reader (synergy H1, BioTek Instruments, USA) and measured at 450 nm optical density.

### Western blotting

The electrophoretic PVDF membrane was combined with the anti-P-PI3K antibody (T40065, ABMART), anti-AKT antibody (60203-2-Ig, Proteintech), anti-P-AKT antibody (80455-1-RR. Proteintech) overnight at 4°C. Membranes were washed and then treated with secondary antibodies for 1 hour.

### Statistical analysis

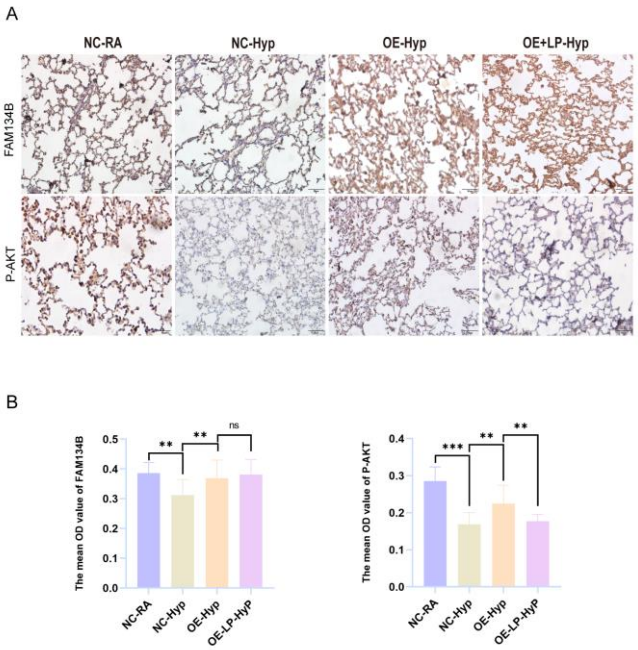
ImageJ was used to analyze the images. GraphPad Prism 9 was used to evaluate the experimental data. The mean ± standard deviation (SD) is used to express normally distributed data, and one-way analysis of variance (ANOVA) was used to compare the groups. $P < 0.05$ is considered statistically significant.

## RESULTS

### Hyperoxia exposure inhibits ER-phagy and PI3K/AKT pathway and activated by FAM134B overexpression

First, FAM134B and P-AKT was significantly inhibited following hyperoxia exposure in rat lungs, Overexpression of FAM134B increased FAM134B expression that was inhibited by hyperoxia, LY294002 inhibited the AKT Pathway activated by FAM134B following hyperoxia exposure (Fig1, A-B). Immunofluorescence showed that hyperoxia inhibited ER-phagy as evidenced by diminished fluorescence of FAM134B and LC3B, and activation of ER-phagy was promoted by overexpression of FAM134B(Fig2, A-B).

**Figure 1:** Hyperoxia exposure inhibits ER-phagy and PI3K/AKT pathway and activated by FAM134B overexpression.




(A) Immunohistochemical staining of FAM134B and P-AKT in lung tissues of different treatment groups (Bar = 100 μm, magnification 200 ×, ±SD, n=6).

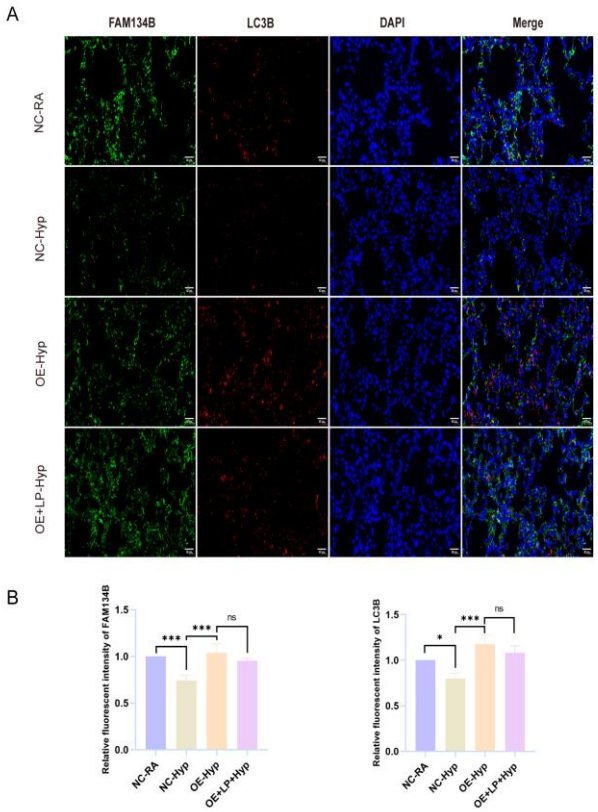
(B) Quantitative immunohistochemical analysis of FAM134B and P-AKT (Bar = 20 μm, magnification 400 ×, ±SD, n=6).

### Overexpression of FAM134B in Sprague–Dawley rat lung tissues attenuates cell death, whereas LY294002 reverses these effects.

P-PI3K, P-AKT expression was decreased in rat lung tissues following hyperoxia exposure, and overexpression of FAM134B activated the PI3K/AKT pathway, which was inhibited by LY294002 (Fig3A). Hyperoxia-exposed rats showed significant weight loss compared to controls, and overexpression of FAM134B increased the body weight of hyperoxia-exposed rats, whereas the favorable effect of FAM134B overexpression was reversed by LY294002(Fig3B). Immunohistochemistry showed that apoptosis was markedly elevated in the lung tissues of

hyperoxia-exposed rats, as evidenced by an increase in the expression of caspase3 and Bax and a decrease in the expression of Bcl-2, whereas overexpression of FAM134B attenuated apoptosis in lung tissues of hyperoxia-exposed rats, and the favorable effect of FAM134B was reversed by LY294002(Fig3, C-D).

**Figure 2:** Hyperoxia exposure inhibits ER-phagy.



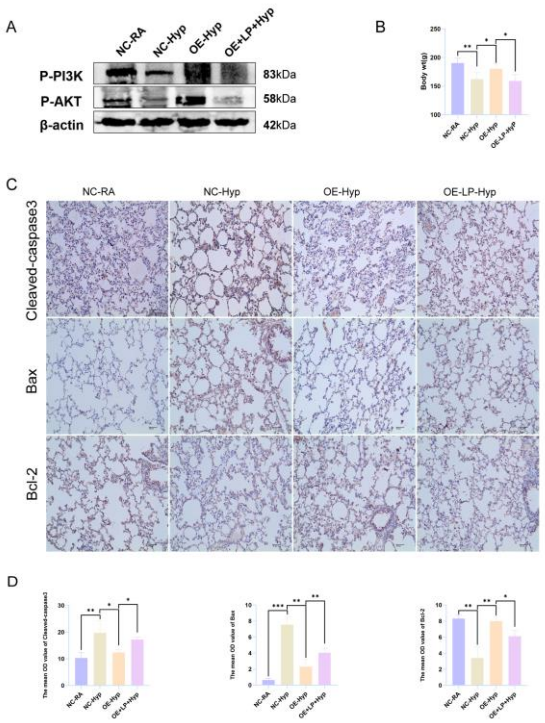

(A) Immunofluorescence analysis of FAM134B and LC3B. (Bar = 50 μm, magnification 400 ×, ±SD, n=6). (B) Quantitative analysis of immunofluorescence.

**Overexpression of FAM134B in Sprague–Dawley rat lung tissues attenuates EMT, whereas LY294002 reverses these effects**

HE showed that lung injury was evident in hyperoxia-exposed rats, whereas overexpression of FAM134B attenuated lung injury in hyperoxia-exposed rats, and LY294002 reversed the favorable effect of FAM134B(Fig4, A-B).

Immunohistochemistry showed that EMT was markedly elevated in the lung tissues of hyperoxia-exposed rats, as evidenced by an increase in the expression of N-cadherin and Vimentin and a decrease in the expression of E-cadherin, whereas overexpression of FAM134B attenuated EMT in lung tissues of hyperoxia-exposed rats, and the favorable effect of FAM134B was reversed by LY294002(Fig4, C-D).

**Figure 3:** Overexpression of FAM134B attenuates cell death, whereas LY294002 reverses these effects.



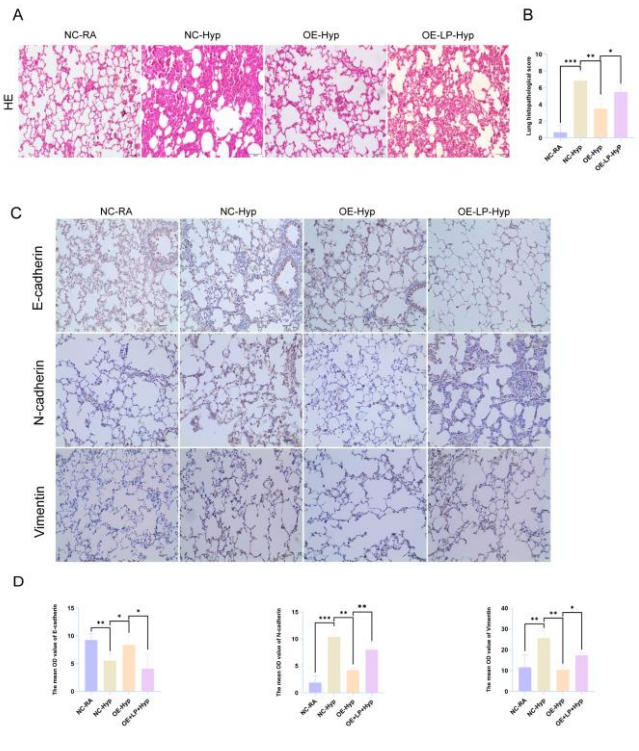

(A) Western blot analysis of P-PI3K, P-AKT in rat lung. (B) Body weight of rats in each group(±SD, n=6) The grouping of gels/blots cropped from different parts of the same gel. (C) Immunohistochemical staining of Cleaved-caspase3,Bax and Bcl-2 in lung tissues of different treatment groups, (Bar = 40 μm, magnification 200 ×,±SD, n=6).(D) Immunohistochemical quantification of Cleaved-caspase3, Bax, Bcl-2.

**Overexpression of FAM134B in Sprague–Dawley rat lung tissues attenuates M1 macrophage polarization, whereas LY294002 reverses these effects.**

Hyperoxia increased the expression of inflammatory factors in alveolar lavage fluid of rats, with increased expression of IL-1, IL-1β, and TNF-a, whereas overexpression of FAM134B significantly reduced the expression of inflammatory factors, and this favorable effect was reversed by LY294002 (Fig5A). It suggests that FAM134B attenuates the expression of inflammatory factors after hyperoxia exposure through the PI3K/AKT pathway. M1 polarization of macrophages promotes the expression of inflammatory factors. Immunofluorescence results showed that hyperoxia promoted macrophage and M1 macrophage polarization, overexpression of FAM134B decreased macrophage and M1 macrophage polarization, whereas LY294002 increased macrophage and M1 macrophage polarization decreased by overexpression of FAM134B following

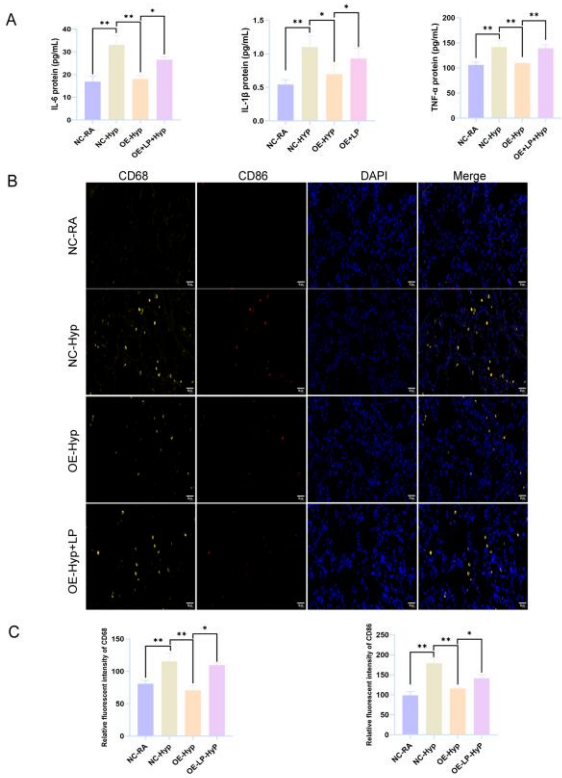
hyperoxia exposure in rat lungs (Fig5,B-C).

**Figure 4:** Overexpression of FAM134B attenuates EMT, whereas LY294002 reverses these effects.




(A) HE staining in lung tissues of different treatment groups (Bar = 100 μm, magnification 200 ×, ±SD, n=6).(B) HE staining score of different treatment groups. (C) Immunohistochemical staining of E-cadherin, N-cadherin, and Vimentin in lung tissues of different treatment groups, (Bar = 40 μm, magnification 200 ×,±SD, n=6). (D) Immunohistochemical quantification of E-cadherin, N-cadherin, and Vimentin.

**Figure 5:** Overexpression of FAM134B attenuates M1 macrophage polarization, whereas LY294002 reverses these effects.




(A) IL-1β, IL-6 and TNF-αexpression in alveolar lavage fluid. (B) Immunofluorescence analysis of CD68 and CD86. Macrophages were labeled using CD68 and MI-type macrophages were labeled using CD86 (Bar = 50 μm, magnification 400 ×, ±SD, n=6). (C) Quantitative immunofluorescence analysis of CD68 and CD86.

## DISCUSSION

An imbalance between pro-oxidant trigger factors and related counter-regulatory mechanisms is known as oxidative stress. Excessive accumulation of ROS is a key factor in hyperoxia-induced lung injury. Autophagy is a vital survival mechanism adopted by cells subjected to stressed or starving situations Bravo-San et al. (2017), Das et al. (2012). In this study, we found that hyperoxia aggravated lung tissue damage and reduced rate of body weight gain in rats. We showed that in rat lung tissue, oxygen tension (hyperoxia) was lower than ambient air, and this decreased the expression of FAM134B and LC3B. Autophagy is critical for embryonic development Tsukamoto et al. (2008), and inhibition of autophagy may result in accumulation of toxic abnormal proteins, which regulates an inflammatory response and ultimately causes cell apoptosis Zhang et al. (2013). Autophagy inhibits cancer development by regulating inflammation and immunity Zhong et al. (2016). Enhancing Autophagy can prevent HALI by reducing Apoptosis in Neonatal Mice Sureshbabu et al. (2016). ER-phagy is part of autophagy, and its absence in hyperoxia exacerbates HALI in the present study. Rats exposed to hyperoxia lost weight and had significant lung injury, overexpression of FAM134B increased body weight and attenuated lung injury after hyperoxia exposure, and LY294002 reversed the favorable effect of FAM134B. FAM134B attenuates lung injury in rats following hyperoxia exposure via P13K/AKT.

PI3K/AKT signal pathways were a recognized survival route that affected lung epithelial cells. Lung epithelial cells subjected to hyperoxia exhibit a progressive loss of Akt activation, although AKT activity makes up for this loss and delays the cells' eventual death Truong et al. (2004). Exposure to hyperoxia causes cells to gradually lose both total

Akt protein and Akt activation. However, AKT activity makes up for this loss of survival effects by postponing the death of hyperoxia-exposed lung epithelial cells Jin et al. (2005). In our study, hyperoxia inhibited AKT expression, while overexpression of FAM134B promoted AKT expression. This suggests that FAM13B regulates the PI3K/AKT pathway in hyperoxia-exposed rat lung tissues.

EMT and apoptosis are significant signs of lung damage after exposure to hyperoxia Zhang et al. (2016). In rats exposed to prolonged hyperoxia, there is immediate lung damage and apoptosis. In the lungs subjected to hyperoxia, there was a large rise in IL-1β expression and a considerable increase in Bax expression Husari et al. (2006). Apoptosis may be related to the evolution of the inflammatory process. In present study, hyperoxia exposure promoted apoptosis in rat lung tissues, overexpression of FAM134B reduced apoptosis, and LY294002 reversed the beneficial effect of overexpression of FAM134B. FAM134B attenuates apoptosis in lung tissues of hyperoxia-exposed rats via PI3K/AKT pathway. EMT refers to the process wherein epithelial cells lose their polarity and junctions and take on mesenchymal cell traits. Hyperoxia induces EMT in rat lung following hyperoxia exposure. E-cadherin protein was epithelial phenotypic protein whose expression was reduced following hyperoxia exposure, expression of N-cadherin and Vimentin proteins, which represent mesenchymal cell phenotypes, were increased in hyperoxia. FAM134B overexpression reduces EMT in rat lung tissue following hyperoxia exposure, LY294002 reversed the protective effect of overexpression of FAM34B on EMT. FAM34B exerts a protective effect on EMT via PI3K/AKT pathway.

HALI Responds to Inflammatory Response. suppressing inflammation in macrophages protects against oxidative stress-induced acute lung injury Guo et al. (2021). This was in line with the idea that hyperoxia induces the production of mediators linked to the oxidative stress response. Rats subjected to hyperoxia showed higher levels of inflammatory factors (IL-1β, TNF-α, IL-6) than rats exposed to air Zhang et al. (2021). This is consistent with our findings. Overexpression of FAM134B reduced the release of inflammatory factors, and LY294002 reversed the favorable effect of overexpression of FAM134B. FAM134B regulates the release of inflammatory factors through the PI3K/AKT pathway. Macrophage-derived inflammatory factors were related to HALI, hyperoxia triggered M1-like polarization, IL-6[6], IL-1 Cardenas-Diaz et al. (2023), IL1-β Mian et al. (2019), and TNF-α Lin et al. (2003) inhibiting lung growth and inducing cell death. Our study showed that hyperoxia promoted M1 macrophage polarization, which was reduced by overexpression of FAM134B, and that LY294002 reversed the effect of overexpression of FAM134B in reducing M1 macrophage polarization in rat lung tissue following hyperoxia exposure.

In conclusion, the expression of FAM134B and PI3K/AKT pathway were inhibited in rat lung tissues after hyperoxia exposure. Overexpression of FAM134B activates the P13K/AKT pathway and reduces apoptosis and EMT in rat lung tissue after hyperoxia exposure. The PI3K/AKT pathway inhibitor LY294002 reverses the protective effect of FAM134B in hyperoxia-exposed rats. Overexpression of FAM134B reduced the release of inflammatory factors and M1 macrophage polarization after hyperoxia exposure, and this effect was reversed by LY294002.

## CONCLUSIONS

Our study showed that FAM134B inhibits M1 macrophage polarization and inflammatory factor release through the PI3K/AKT pathway and attenuates apoptosis and EMT in lung tissues of hyperoxia-exposed rats. FAM134B is a key target for HALI, which provides new ideas for the treatment of HALI.

## LIMITATIONS OF THE STUDY

First, this study did not explore whether FAM134B directly regulates the PI3K/AKT signaling pathway; in addition; the issue of the time course of changes in injury endpoints in control or lentiviral protected rats may be very important and is not addressed.

## DECLARATIONS

### Competing interests

The authors confirm that they are not aware of any financial or interpersonal conflicts that might have affected the findings of this study.

### Author Contributions and statements

**Hong Guo:** designed the research and conducted the most of the experiments. **Xin Zhao:** collated data, Data curation. **Ying Yao:** collated data, Data curation. **Kai-Hua Yu:** assisted experiments. **Su-Heng Chen:** assisted experiments. **Yu-Lan Li:** coordinated and directed the project.

### Consent to participate

Informed consent was obtained from all individual participants included in the study.

### Consent to publish

The authors affirm that human research participants provided informed consent for publication of all images.

### Data Availability

The data that support the findings of this study are

available from the corresponding author, upon reasonable request.

**Ethical Statements**

All experiments were approved by the Animal Ethics Committee of the First Hospital of Lanzhou University (LDYYLL2022-216).

**Acknowledgments**

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