

LncRNA gadd7 promotes mitochondrial membrane potential decrease and apoptosis of alveolar type II epithelial cells by positively regulating MFN1 in an *in vitro* model of hyperoxia-induced acute lung injury

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ABSTRACT

Hyperoxia-induced acute lung injury (HALI) is a complication of oxygen therapy. LncRNA gadd7 shows associations with HALI. We explored the effects of gadd7 on mitochondrial membrane potential (MMP) and apoptosis of alveolar type II epithelial cells (AEC II) in HALI. RLE-6TN cells were transfected with 50 nM silenced and overexpressed gadd7 and MFN1 plasmids (sh-gadd7, oe-gadd7 and/or oe-MFN1), respectively for 48 h, followed by hyperoxic culture for 24 h. gadd7 and MFN1 levels were assessed by RT-qPCR and Western blot. RLE-6TN cells were stained by JC-1 and MMP changes were observed using a fluorescence microscope. The average fluorescence intensity of red and green fluorescence was evaluated with the help of Image J software, and the ratio of red/green fluorescence intensity represented MMP. Cell apoptosis was assessed by flow cytometry using Annexin V-FITC/PI double staining method. The levels of apoptosis proteins [Bax, Bcl-2, Cleaved caspase-3, Cleaved poly ADP-ribose polymerase (PARP)] were measured by Western blot. LncRNA gadd7 and MFN1 were highly expressed in hyperoxia-induced AEC II. Hyperoxia induced MMP decrease and apoptosis promotion in RLE-6TN cells. LncRNA gadd7 positively regulated MFN1 expression. Knockdown of gadd7 inhibited MMP decrease and apoptosis of hyperoxia-induced RLE-6TN cells. MFN1 overexpression annulled the inhibitory effects of gadd7 silencing on MMP decrease and apoptosis in RLE-6TN cells. Briefly, lncRNA gadd7 promoted MMP decrease and apoptosis of AEC II by positively regulating the expression of MFN1 in the HALI model *in vitro*.

Key words: hyperoxia-induced acute lung injury; LncRNA gadd7; MFN1; alveolar type II epithelial cells; mitochondrial membrane potential; apoptosis.

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Introduction

Mechanical ventilation and oxygen therapy are widely used to manage and treat neonatal emergencies in critically ill newborns.¹ However, prolonged exposure to oxygen with high concentrations can cause bronchopulmonary dysplasia and chronic pulmonary diseases in neonates.² In addition, oxygen therapy is also used in adults with chronic lung disease,^{3,4} which may also produce certain side effects. The main pathological characteristics of the injured lung tissues are disordered or stagnant alveolar development that is manifested as a decrease in alveoli number, an increase in simplification, size of the alveolar structure, and pulmonary interstitial fibrosis.¹ Hyperoxia-induced acute lung injury (HALI) is a classic complication of oxygen therapy, which can cause acute respiratory distress syndrome and seriously affects the quality of life of prematurely born infants.⁵ In HALI, hyperoxia can result in necrosis and apoptosis of alveolar epithelial cells (AEC).⁶ The alveolar surface is covered by two functionally and morphologically distinct cells, namely type I AEC (AEC I) and type II AEC (AEC II), among which AEC II recycles, synthesizes, and secretes lung surfactant and maintains the homeostasis of alveolar fluid.⁷ AEC II cells are the major target of hyperoxic lung injury.⁸ Reducing apoptosis of AEC II is a crucial way to repress HALI.^{6,9,10}

Mitochondrial dysfunction is reported to contribute to the alveolar developmental arrest in hyperoxia-induced mice.¹¹ Hyperoxia induces the apoptosis of AECs by regulating mitochondrial function.¹ Mitochondrial fusion is regulated by different proteins, including mitofusin 1 (MFN1) and mitofusin-2, which is a GTPase protein that regulates mitochondrial outer membrane fusion,¹² and is critical to mitochondrial function.^{13,14} MFN1 regulates the synthesis of phospholipids and cholesterol in AEC II cells.¹⁵ Abnormal MFN1 overexpression promotes mitochondrial fusion, induces cell apoptosis, and MFN1 down-regulation promotes cell growth.¹⁶ miR-20b represses mitochondrial dysfunction-mediated apoptosis to suppress HALI by targeting MFN1 and MFN2.¹⁷ Moreover, overexpression of MFN1 can aggravate lung injury and cell apoptosis.¹⁷ Agmatine can inhibit cell apoptosis by indirectly reducing the expression of MFN1 protein.¹⁶ Further study on the regulatory pathway of AEC II apoptosis and mitochondrial function may provide a new strategy for the prevention and treatment of HALI.

Long non-coding RNAs (lncRNAs) are noncoding RNAs with more than 200 nucleotides, which are vital regulators in various biological processes.¹⁸ lncRNA NEF modulates the lung epithelial cells in HALI through FOXA2.¹⁹ lncRNA CASC2 targets CAV1 by competitively binding to miRNA-194-5p to suppress neonatal lung injury.²⁰ lncRNA growth-arrested DNA damage-inducible gene 7 (lncRNA gadd7) is a 754-nt polyadenylated lncRNA, which is isolated from the Chinese hamster ovary cells.²¹ It plays an imperative regulatory role in cell death induced by lipotoxicity.²² It also participates in the regulation of oxidative stress and inflammatory reaction, and overexpression of gadd7 promotes cell apoptosis.²³ Agmatine protects against HALI by repressing the expression of lncRNA gadd7.¹⁶ However, whether lncRNA gadd7 affects mitochondrial membrane potential (MMP) and apoptosis of AECs in HALI through MFN1 remains elusive. This study aims to investigate the mechanism of lncRNA gadd7 in MMP and apoptosis of AECs in HALI, to provide a strategy for the prevention and treatment of HALI.

Materials and Methods

Cell culture and treatment

The standard rat AEC II cell line (RLE-6TN cells) was purchased from BeNa Culture Collection (Beijing, China; BNCC337708) and cultured in the RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Thermo Scientific, Waltham, MA, USA) and 1% 100 U/mL penicillin/100 µg/mL streptomycin (Thermo Scientific) in an incubator containing 5% CO₂ at 37°C. The oxygen concentration was adjusted by the incubator. For the normoxia group, cells were cultured in an environment containing 21% O₂, 74% nitrogen, and 5% CO₂ for 24 h. For the hyperoxia group, cells were cultured in an environment containing 95% O₂ and 5% CO₂ for 24 h. During cell culture, RLE-6TN cells were regularly tested for mycoplasma to avoid pollution of cell growth environment.

Cell transfection and grouping

RLE-6TN cells were allocated into the following 8 groups: Normoxia group, Hyperoxia group, Hyperoxia + sh-gadd7 group (transfected with silencing gadd7 plasmid), Hyperoxia + sh-NC group (transfected with negative control plasmid sh-NC), Hyperoxia + oe-gadd7 group (transfected with overexpression gadd7 plasmid), Hyperoxia + oe-NC group (transfected with negative control plasmid oe-NC), Hyperoxia + sh-gadd7 + oe-MFN1 group (co-transfected with silencing gadd7 plasmid and overexpression MFN1 plasmid), and Hyperoxia + sh-gadd7 + oe-NC group (co-transfected with silencing gadd7 plasmid and negative control plasmid oe-NC). Silencing gadd7 plasmid (sh-gadd7) and its corresponding negative control (sh-NC), overexpression gadd7 plasmid (oe-gadd7) and its corresponding negative control (oe-NC), MFN1 overexpression plasmid (oe-MFN1) and its negative control NC (oe-NC) were purchased from GenePharma (Shanghai, China). The transfection was performed using Lipofectamine 2000 (Invitrogen) in strict accordance with its instructions with 50 nM as the final concentration for each plasmid.²⁴ After 48-h transfection, hyperoxic environment treatment was conducted for 24 h.

RT-qPCR

Total RNA was extracted from RLE-6TN cells using the TRIzol reagent (Invitrogen). The total RNA was reverse transcribed into cDNA using the reverse transcription kit (Roche, Switzerland). The concentration and purity of RNA samples were determined using the spectroscopic method. Complementary DNA was synthesized with specific Taqman® RT primers (Thermo Fisher, Wilmington, USA) and PrimeScript™ II 1st Strand cDNA Synthesis kit (TaKaRa Biotech, Dalian, Liaoning, China). RT-qPCR was performed by TaqMan™ Fast Advanced Master Mix (Thermo Fisher) under the following reaction conditions: pre-denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 15 s, and extension at 60°C for 1 min. With GAPDH as the internal reference gene, the relative expression levels of lncRNA gadd7 and MFN1 standardized by internal parameters were calculated using the 2^{-ΔΔCt} method. The primers were synthesized by Sangon Biotech (Shanghai, China) and their sequences are shown in Table 1.

Table 1. Primer sequence.

Primer	Sequence
lncRNA gadd7	forward: 5'-ACAATGACGCCATCGTTTTCT-3' reverse: 5'-TGTCTCCATCTGGGCATT-3'
MFN1	forward: 5'-GGGTGCTCCTAGGATTATCAGA-3' reverse: 5'-TATCTGGCGTTGCTGGAGT-3'
GAPDH	forward: 5'-GCACCGTCAAGGCTGAGAAC-3' reverse: 5'-ATGGTGGTGAAGACGCCAGT-3'

Western blot

The relative level of the target protein was determined by Western blot. After normoxic and hyperoxic culture, RLE-6TN cells were lysed with radioimmunoprecipitation assay lysate containing protease inhibitor (Beyotime Biotechnology, Shanghai, China) to extract the total protein, and the protein concentration was determined using the bicinchoninic acid kit (Beyotime Biotechnology). Then, 30 μ g protein of the samples in each group was prepared by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto the polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The 5% skim milk was prepared with Tris-buffered saline with Tween-20 (TBST), and the PVDF membranes were placed in the milk, shaken, blocked at room temperature for 1 h to block nonspecific binding, and incubated with primary antibodies anti-MFN1 (ab221661, 1:1000, Abcam, Cambridge, UK), anti-Bax (ab32503, 1:1000, Abcam), anti-Bcl-2 (ab194583, 1:500, Abcam), anti-caspase-3 (ab32351, 1:5000, Abcam), anti-Cleaved caspase-3 (ab32042, 1:500, Abcam), anti-PARP (#3542, 1:1000, Cell Signaling Technology, Beverly, MA, USA), anti-Cleaved PARP (#9185, 1:1000, Cell Signaling Technology), and anti-GAPDH (ab181602, 1:10000, Abcam) overnight at 4°C. Then, the membranes were washed twice with TBST and incubated with horseradish peroxidase-labeled goat anti-rabbit secondary antibody IgG (ab48386, 1:2000, Abcam) at room temperature for 1 h. Subsequently, the samples were developed using the enhanced chemiluminescence working solution (Millipore) and imaged. The protein band density was detected using the Image J software 1.48 (NIH, Bethesda, MD, USA). GAPDH was used as the internal reference. Each experiment was repeated 3 times.

Determination of MMP

The 5,5',6,6',tetrachloro-1,1',3,3';-tetraethyl-imidacarbocyanine iodide (JC-1) was a mitochondrial dye. The changes in MMP were detected using the JC-1 Kit (Solarbio, Beijing, China). The treated cells of each group were incubated with JC-1 staining working solution (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 20 min in the dark. After twice rinses with phosphate buffer saline (PBS), 4',6-diamidino-2-phenylindole staining was performed. Cells were detached with trypsin and centrifuged into cell suspension. The red and green fluorescence intensities were measured by flow cytometry under 485 nm excitation light and 530 and

590 nm emission light, respectively. The ratio of red/green fluorescence intensity represented the mitochondrial MMP, and a decrease in the ratio represented a decrease in the MMP. JC-1 dyeing working solution was prepared according to the manufacturer's instructions. The average fluorescence intensity of red and green fluorescence was evaluated using the Image J software (version 1.52), and 6 random fields were taken under a fluorescence microscope (Olympus, Tokyo, Japan) for observation and image acquisition. The experiment was repeated 3 times.

Flow cytometry

Apoptosis was detected by flow cytometry. After normoxic and hyperoxic culture, RLE-6TN cells (1×10^6) were resuspended in the medium, centrifuged at 4°C at $400 \times g$ for 5 min, and the supernatant was removed. Then, the cells were resuspended in 1 mL cold PBS and centrifuged at 4°C at $400 \times g$ for 5 min, and the supernatant was removed. After that, the cells were resuspended with PBS buffer containing 10 μ L annexin V-FITC and 10 μ L propidium iodide dye (BD Biosciences, San Jose, CA, USA). After mild mixing, the cells were incubated at 4°C in the dark for 30 min and washed thrice with PBS. Cell apoptosis was detected using a CytoFLEX flow cytometer (Beckman Coulter, Brea, USA).

Statistical analysis

SPSS 21.0 (IBM Corp. Armonk, NY, USA) and GraphPad Prism 8.01 (GraphPad Software Inc., San Diego, CA, USA) were employed for statistical analysis and mapping. Cell experiment was repeated 3 times. The data were expressed as mean \pm SD. Independent *t*-test was employed for comparisons between two groups and one-way analysis of variance (ANOVA) was applied for comparisons among multi-groups, followed by Tukey's multiple comparisons test; $p < 0.05$ was indicative of statistical significance.

Results

LncRNA gadd7 and MFN1 were highly expressed in hyperoxia-induced AEC II cells

Agmatine regulates MFN1 expression by suppressing lncRNA gadd7 expression, thus protecting against HALI.¹⁶ To investigate the

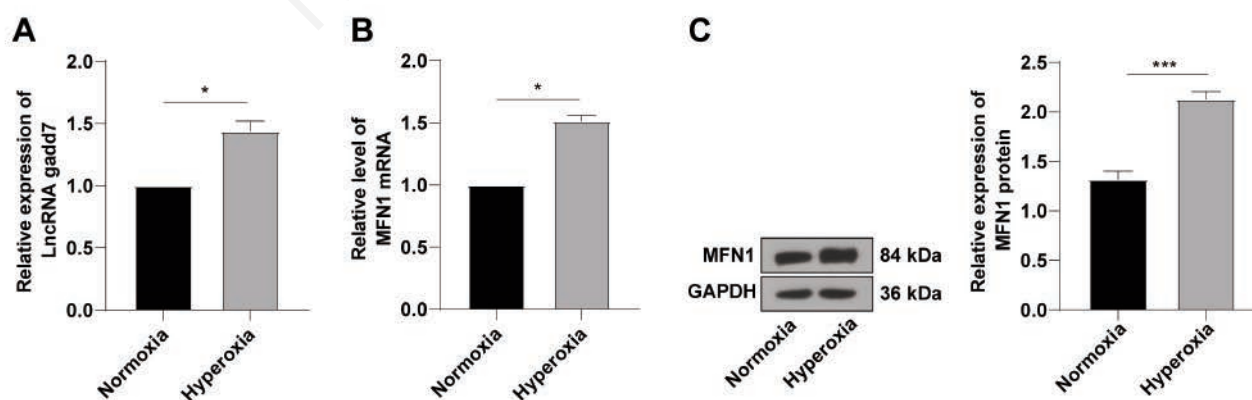


Figure 1. LncRNA gadd7 and MFN1 were highly expressed in hyperoxia-induced alveolar type II epithelial cells. RLE-6TN cells were cultured under normoxic or hyperoxic condition for 24 h. A,B) The lncRNA gadd7 and MFN1 mRNA levels were determined by RT-qPCR. C) The protein level of MFN1 was assessed by Western blot. Cell experiment was conducted 3 times. Data were expressed as mean \pm SD. Independent *t*-test was employed for comparisons between groups; * $p < 0.05$, ** $p < 0.01$.

expression levels of lncRNA gadd7 and MFN1 in AEC II cells induced by hyperoxia, RLE-6TN cells were cultured for 24 h under normoxic or hyperoxic conditions. Firstly, the expression of gadd7 was determined by RT-qPCR. Compared with the normoxia group, gadd7 expression in the hyperoxia group was elevated ($p < 0.05$) (Figure 1A). Subsequently, MFN1 levels were determined by RT-qPCR and Western blot. Compared with the normoxia group, the MFN1 levels in the hyperoxia group were raised (all $p < 0.01$) (Figure 1B,C). These results suggested that lncRNA gadd7 and MFN1 were highly expressed in AEC II cells induced by hyperoxia.

Effects of hyperoxia induction on MMP and apoptosis in AEC II

MMP is an important parameter reflecting the functional state of mitochondria in cells and the decrease of MMP is related to apoptosis.²⁵ Therefore, the effects of hyperoxia on MMP and apoptosis in RLE-6TN cells were further investigated. Firstly, the

changes of MMP of cells were detected. Compared with the normoxia group, the MMP of RLE-6TN cells was significantly reduced by hyperoxia induction ($p < 0.01$) (Figure 2A,B). Next, the apoptosis level was detected by flow cytometry. Compared with the normoxia group, the level of apoptosis in the hyperoxia group was increased ($p < 0.01$) (Figure 2C). In addition, the levels of pro-apoptotic protein Bax, Cleaved caspase-3 and Cleaved PARP, and anti-apoptotic protein Bcl-2 in cells of each group were determined by Western blot. Compared with the normoxia group, the levels of Bax/Bcl-2, Cleaved caspase-3/caspase-3, and Cleaved PARP/PARP were raised in the hyperoxia group (all $p < 0.05$) (Figure 2D). The results suggested that hyperoxia induced the decrease of MMP in RLE-6TN cells and promoted apoptosis.

lncRNA gadd7 positively regulated MFN1 expression

To further explore the regulatory relationship between lncRNA gadd7 and MFN1, RLE-6TN cells were transfected with sh-gadd7

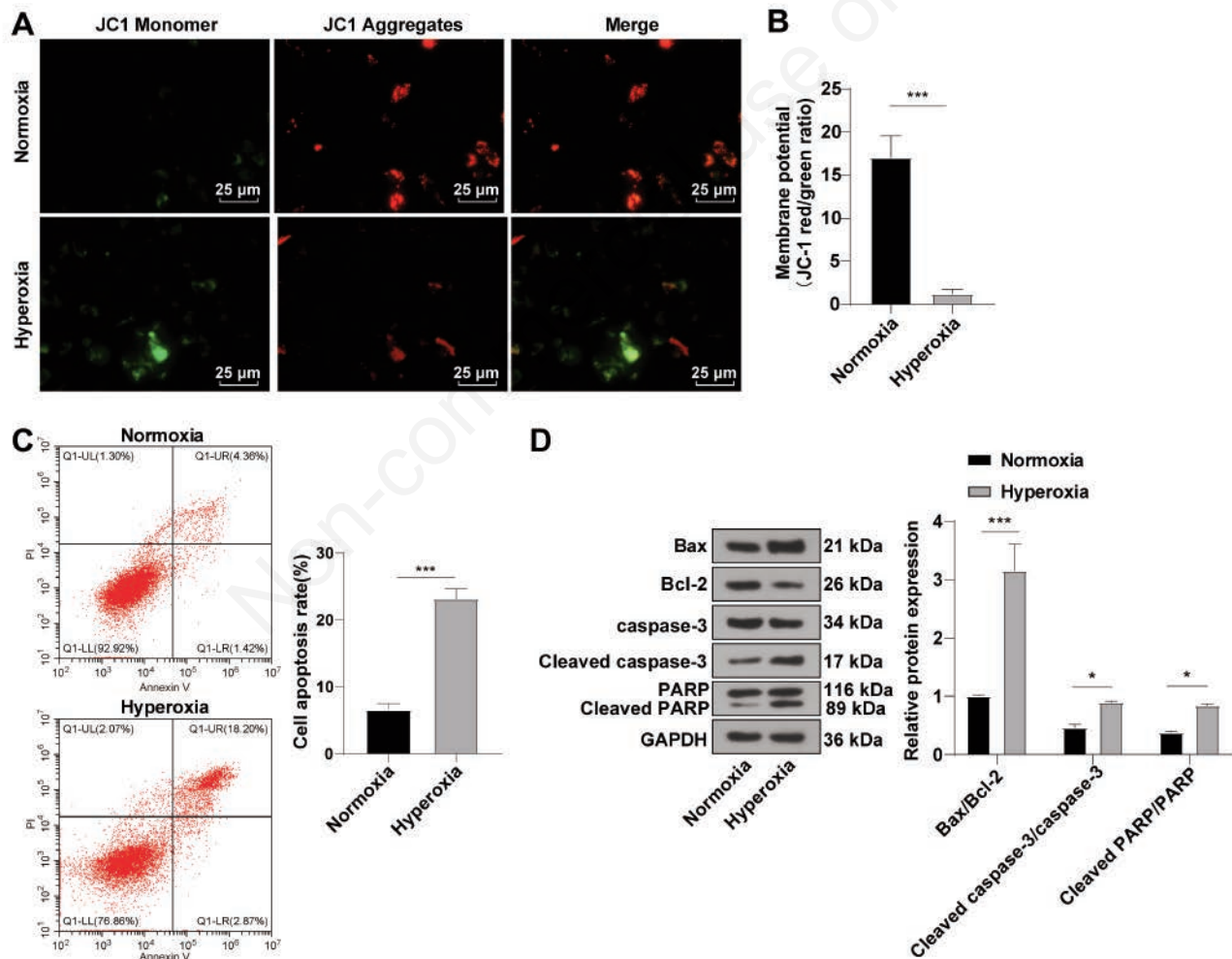


Figure 2. Effects of hyperoxia induction on MMP and apoptosis in alveolar type II epithelial cells. A) JC-1 staining was performed in RLE-6TN cells, and the MMP was observed using a fluorescence microscope; JC-1 monomers emitted green fluorescence, and JC-1 aggregates emitted red fluorescence. B) Ratio of red/green fluorescence intensity. C) The level of apoptosis was detected by flow cytometry. D) The protein levels of Bax, Bcl-2, Cleaved caspase-3 and Cleaved PARP were assessed by Western blot. Cell experiment was conducted 3 times. Data were expressed as mean \pm SD. Independent t-test was employed for comparisons between groups; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

or oe-gadd7, respectively, and then cultured in a hyperoxia environment for 24 h. Firstly, the expression of gadd7 was detected by RT-qPCR. Compared with the Hyperoxia + sh-NC group, gadd7 expression in the Hyperoxia + sh-gadd7 group was lowered, while compared with the Hyperoxia + oe-NC group, gadd7 in the Hyperoxia + oe-gadd7 group was facilitated (all $p < 0.05$) (Figure 3A), indicating that the cells were successfully transfected. Subsequently, the protein level of MFN1 was determined by Western blot. Compared with the Hyperoxia + sh-NC group, MFN1 level in the Hyperoxia + sh-gadd7 group was diminished, while compared with the Hyperoxia + oe-NC group, MFN1 level in the Hyperoxia + oe-gadd7 group was stimulated (all $p < 0.05$) (Figure 3B). Briefly, lncRNA gadd7 positively regulated MFN1 levels.

Knockdown of gadd7 expression inhibited MMP decrease and apoptosis of RLE-6TN cells induced by hyperoxia

To investigate the effects of gadd7 on MMP and apoptosis of RLE-6TN cells induced by hyperoxia, RLE-6TN cells were transfected with sh-gadd7 and cultured in hyperoxia for 24 h. The MMP and apoptosis were detected. Compared with the Hyperoxia + sh-NC group, the MMP was increased and apoptosis was reduced in the Hyperoxia + sh-gadd7 group (all $p < 0.05$) (Figure 4 A-C). In addition, compared with the Hyperoxia + sh-NC group, the level of Bax/Bcl-2, Cleaved caspase-3/caspase-3, and Cleaved PARP/PARP were reduced in the Hyperoxia + sh-gadd7 group (all $p < 0.05$) (Figure 4D). Overall, knockdown of gadd7 inhibited MMP decrease and apoptosis of RLE-6TN cells induced by hyperoxia.

Overexpression of MFN1 partially annulled the inhibitory effects of gadd7 silencing on MMP decrease and apoptosis in RLE-6TN cells

To further explore the role of MFN1 in the decrease of MMP and apoptosis of RLE-6TN in HALI, MFN1 was overexpressed in sh-gadd7-transfected RLE-6TN, and then the cells were cultured in a hyperoxia environment for 24 h. Western blot manifested that compared with the Hyperoxia + sh-gadd7 + oe-NC group, the MFN1 level in the Hyperoxia + sh-gadd7 + oe-MFN1 group was facilitated ($p < 0.05$) (Figure 5A). Subsequently, the MMP and apoptosis of RLE-6TN cells were detected. Compared with the

Hyperoxia + sh-gadd7 + oe-NC group, the MMP was lowered and the apoptosis level was increased in the Hyperoxia + sh-gadd7 + oe-MFN1 group (all $p < 0.05$) (Figure 5B-D). In addition, the level of Bax/Bcl-2, Cleaved caspase-3/caspase-3 and Cleaved PARP/PARP were all augmented in the Hyperoxia + sh-gadd7 + oe-MFN1 group (all $P < 0.05$) (Figure 5E). Collectively, overexpression of MFN1 partially abrogated the inhibitory effects of gadd7 silencing on MMP decrease and apoptosis of RLE-6TN cells.

Discussion

Hyperoxia promotes inflammation and results in infiltration of neutrophils, macrophages, and cytokines, and causes edema, alveolar damage, and even death.²⁶ HALI is a kind of iatrogenic pulmonary dysfunction resulting from prolonged exposure to oxygen with high concentrations, which is prevalently seen in the treatment of refractory hypoxemia.¹⁶ It can cause acute respiratory distress syndrome and bronchopulmonary dysplasia.^{6,27} Evidence has shown that lncRNA plays crucial roles in HALI.²⁸ This study found that lncRNA gadd7 was highly expressed in hyperoxia-induced AEC II and promoted MMP decrease and apoptosis of AEC II by positively regulating the expression of MFN1 in HALI.

LncRNAs, including lncRNA NEF, lncRNA CASC2, and lncRNA H19, are reported to have prominent roles in HALI.¹⁸⁻²⁰ Our results manifested that the lncRNA gadd7 expression and MFN1 levels were augmented after hyperoxia treatment, consistent with the already reported results.^{16,17} Hyperoxia induces cell apoptosis and mitochondrial dysfunction in HALI.²⁹ Mitochondria are the main organelle in cells, which are involved in apoptosis and regulate apoptotic pathways, and the decrease of MMP is related to apoptosis.²⁵ In the early stage of hyperoxia exposure, unfolded protein response is activated in rat lung tissue and starts the apoptosis process in AECII.³⁰ Hyperoxia exposure is positively correlated with the apoptosis of alveolar epithelial cells.³¹ Hyperoxic conditions decreased Bcl-2 level and increased Bax level, which induced proliferation restriction and apoptosis of AECIIs.² Our results revealed that MMP was decreased and apoptosis was promoted in hyperoxia-induced RLE-6TN cells. In brief, hyperoxia induction decreased MMP and stimulated apoptosis in RLE-6TN cells.

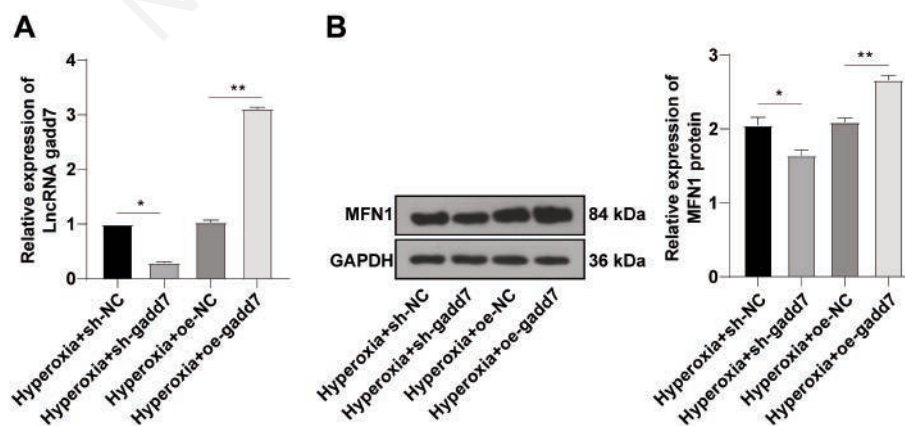


Figure 3. LncRNA gadd7 positively regulated MFN1 expression. A) The level of lncRNA gadd7 was assessed by RT-qPCR. B) The protein level of MFN1 was determined by Western blot. Cell experiment was conducted 3 times. One-way ANOVA was applied for comparisons among groups, followed by Tukey's multiple comparisons test; * $p < 0.05$, ** $p < 0.01$.

Furthermore, we knocked down *gadd7* and overexpressed *gadd7* in RLE-6TN cells and treated RLE-6TN cells with hyperoxia for 24 h, and discovered that MFN1 expression was blocked in hyperoxia-treated RLE-6TN cells with silenced *gadd7* expression, while MFN1 expression was facilitated in hyperoxia-treated RLE-6TN cells with overexpressed *gadd7* expression. Consistently, transfection of lncRNA *gadd7* promotes the MFN1 protein level.¹⁶ In summary, lncRNA *gadd7* positively regulated the expression of MFN1. MFN1 levels were accumulated, along with mitochondria elongation under hypoxic conditions.³² Overexpression of MFN1 promoted cell death in these cells, indicating that fine-tuning of MFN1 levels is necessary for cell survival.³³ Furthermore, we knocked down *gadd7* expression or overexpressed MFN1 levels in RLE-6TN cells and treated RLE-6TN cells by hyperoxia for 24 h. As a result, MMP was augmented, apoptosis was repressed.

Knockdown of lncRNA *gadd7* effectively inhibits the apoptosis of spermatocytes.²² MFN1 overexpression attenuated the protective effects of miR-20b on ACE II cell apoptosis, mitochondrial function, and lung injury.¹⁷ However, there is no report on the effect of lncRNA *gadd7* silencing on the MMP and apoptosis of HALI cells. This study highlighted that knockdown of *gadd7* inhibited hyperoxia-induced MMP decrease and apoptosis for the first time. To conclude, overexpression of MFN1 partially abrogated the effects of *gadd7* silencing on inhibiting MMP decrease and apoptosis of RLE-6TN cells.

In summary, this study supported that lncRNA *gadd7* was highly expressed in hyperoxia-induced AEC II and promoted MMP decrease and apoptosis of RLE-6TN cells by positively regulating MFN1 levels in HALI, which provided an important theoretical basis for the prevention and treatment of HALI. However,

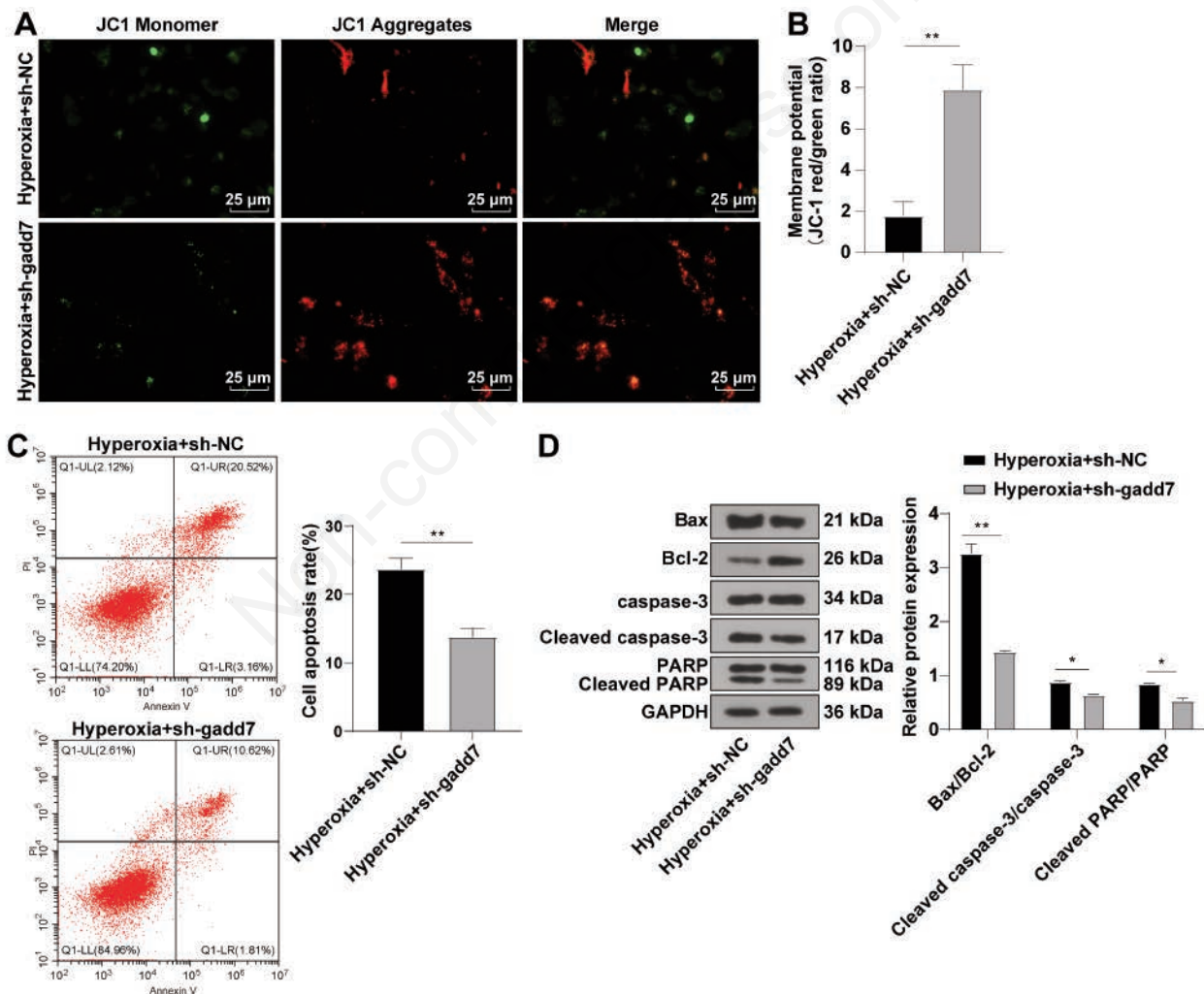


Figure 4. Knockdown of *gadd7* expression inhibited the decrease of MMP and apoptosis of RLE-6TN cells induced by hyperoxia. A) The changes in MMP were observed using a fluorescence microscope. B) Ratio of red/green fluorescence intensity. C) The level of apoptosis was assessed by flow cytometry. D) The protein levels of Bax, Bcl-2, Cleaved caspase-3, and Cleaved PARP were assessed by Western blot. Cell experiment was conducted 3 times. Data were expressed as mean \pm SD. Independent t-test was employed for comparisons between groups; * $p < 0.05$, ** $p < 0.01$.

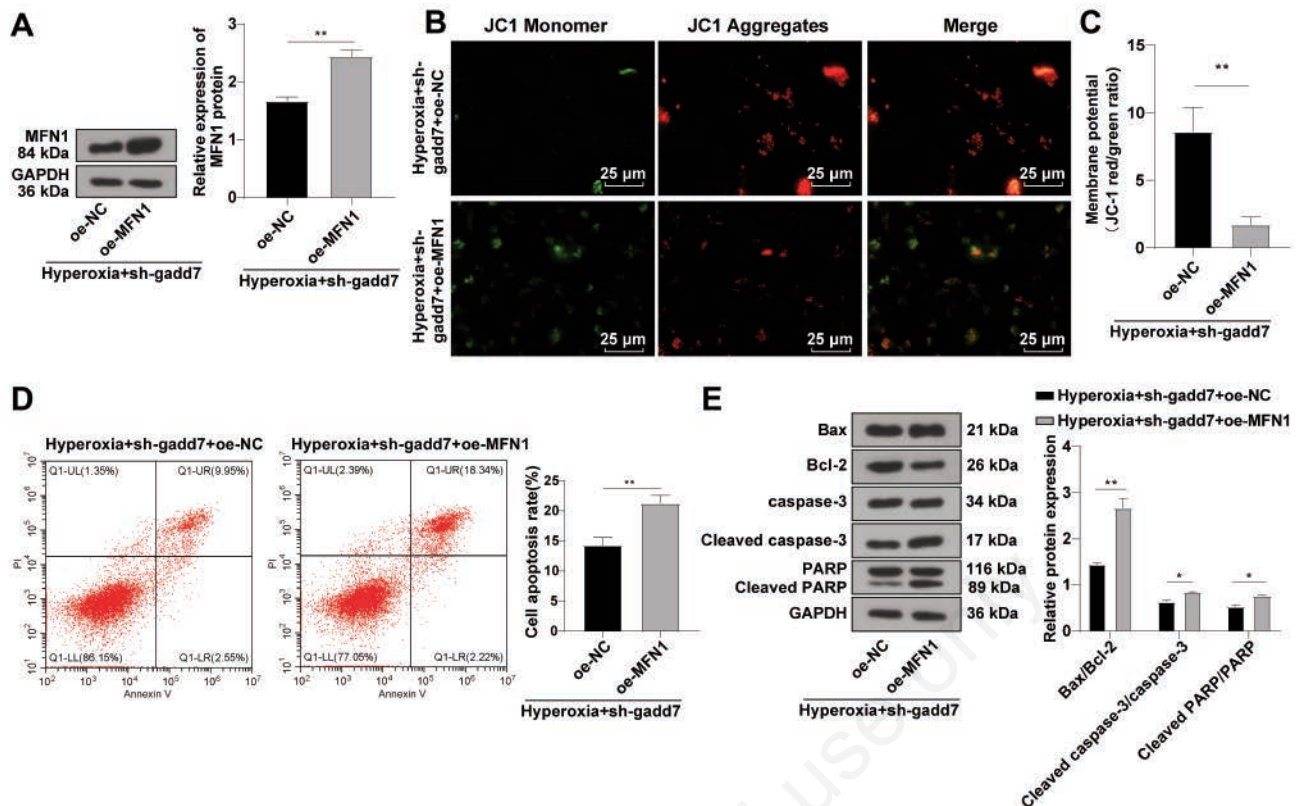


Figure 5. Overexpression of MFN1 partially averted the inhibitory effects of gadd7 silencing on MMP and apoptosis in RLE-6TN cells. A) MFN1 level was assessed by Western blot. B) The changes of MMP were observed using a fluorescence microscope. C) Ratio of red/green fluorescence intensity. D) The level of apoptosis was assessed by flow cytometry. E) The protein levels of Bax, Bcl-2, Cleaved caspase-3 and Cleaved PARP were measured by Western blot. Cells experiment was conducted 3 times. Data were expressed as mean \pm SD. Independent t-test was employed for comparisons between groups; * p <0.05, ** p <0.01.

this study only preliminarily discussed these results, and how lncRNA gadd7 regulated apoptosis of AEC II and its transcription mechanism still needed to be further studied. The possible mechanism of how lncRNA gadd7 regulated MFN1 was not explored. As a competitive endogenous RNA, lncRNA gadd7 might regulate the biological function of miRNAs, thus affecting the expression of downstream target gene MFN1. In addition, this study did not examine the mechanism from many aspects to explore the mitochondrial function of AEC II and the internal relationship between mitochondrial dysfunction and apoptosis. In the future, we will further explore the specific mechanism of lncRNA gadd7 in regulating the MMP and apoptosis of AEC II in HALI at the molecular level, to further study the signal pathways related to apoptosis, and to further explore the possible mechanism of lncRNA gadd7 regulating MFN1 and find the key miRNA connecting lncRNA gadd7 and MFN1, and study the molecular mechanism.

References

- Jiang J, Wang J, Li C, Mo L, Huang D. Hyperoxia induces alveolar epithelial cell apoptosis by regulating mitochondrial function through small mothers against decapentaplegic 3 (SMAD3) and extracellular signal-regulated kinase 1/2 (ERK1/2). *Bioengineered* 2022;13:242-52.
- Wu D, Liang M, Dang H, Fang F, Xu F, Liu C. Hydrogen protects against hyperoxia-induced apoptosis in type II alveolar

epithelial cells via activation of PI3K/Akt/Foxo3a signaling pathway. *Biochem Biophys Res Commun* 2018;495:1620-7.

- Jacobs SS, Krishnan JA, Lederer DJ, Ghazipura M, Hossain T, Tan AM, et al. Home oxygen therapy for adults with chronic lung disease. An Official American Thoracic Society Clinical Practice Guideline. *Am J Respir Crit Care Med* 2020;202:e121-41.
- Wenger HC, Cifu AS, Lee CT. Home oxygen therapy for adults with chronic obstructive pulmonary disease or interstitial lung disease. *JAMA* 2021;326:1738-9.
- Zhu X, Lei X, Wang J, Dong W. Protective effects of resveratrol on hyperoxia-induced lung injury in neonatal rats by alleviating apoptosis and ROS production. *J Matern Fetal Neonatal Med* 2020;33:4150-8.
- Qin S, Wang H, Liu G, Mei H, Chen M. miR215p ameliorates hyperoxic acute lung injury and decreases apoptosis of AEC II cells via PTEN/AKT signaling in rats. *Mol Med Rep* 2019;20:4953-62.
- Ao X, Fang F, Xu F. Vasoactive intestinal peptide protects alveolar epithelial cells against hyperoxia via promoting the activation of STAT3. *Regul Pept* 2011;168:1-4.
- Lee HS, Kim C K. Effect of recombinant IL-10 on cultured fetal rat alveolar type II cells exposed to 65%-hyperoxia. *Respir Res* 2011;12:68.
- Li XG, Song X, Wang JY, Sun CH, Li ZQ, Meng LL, et al. Fibroblast growth factor 18 alleviates hyperoxia-induced lung injury in mice by adjusting oxidative stress and inflammation.

- Eur Rev Med Pharmacol Sci 2021;25:1485-94.
10. Lu X, Wang C, Wu D, Zhang C, Xiao C, Xu F. Quantitative proteomics reveals the mechanisms of hydrogen-conferred protection against hyperoxia-induced injury in type II alveolar epithelial cells. *Exp Lung Res* 2018;44:464-75.
 11. Ratner V, Starkov A, Matsukevich D, Polin RA, Ten V S. Mitochondrial dysfunction contributes to alveolar developmental arrest in hyperoxia-exposed mice. *Am J Respir Cell Mol Biol* 2009;40:511-8.
 12. Chen H, Detmer SA, Ewald AJ, Griffin EE, Fraser SE, Chan DC. Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. *J Cell Biol* 2003;160:189-200.
 13. Sidarala V, Zhu J, Levi-D'Ancona E, Pearson GL, Reck EC, Walker EM, et al. Mitofusin 1 and 2 regulation of mitochondrial DNA content is a critical determinant of glucose homeostasis. *Nat Commun* 2022;13:2340.
 14. Rovira-Llopis S, Banuls C, Diaz-Morales N, Hernandez-Mijares A, Rocha M, Victor VM. Mitochondrial dynamics in type 2 diabetes: Pathophysiological implications. *Redox Biol* 2017;11:637-45.
 15. Chung KP, Hsu CL, Fan LC, Huang Z, Bhatia D, Chen YJ, et al. Mitofusins regulate lipid metabolism to mediate the development of lung fibrosis. *Nat Commun* 2019;10:3390.
 16. Liu G, Mei H, Chen M, Qin S, Li K, Zhang W, et al. Protective effect of agmatine against hyperoxia-induced acute lung injury via regulating lncRNA gadd7. *Biochem Biophys Res Commun* 2019;516:68-74.
 17. Mu G, Deng Y, Lu Z, Li X, Chen Y. miR-20b suppresses mitochondrial dysfunction-mediated apoptosis to alleviate hyperoxia-induced acute lung injury by directly targeting MFN1 and MFN2. *Acta Bioch Bioph Sin* 2021;53:220-8.
 18. Zhang L, Wang P, Shen Y, Huang T, Hu X, Yu W. Mechanism of lncRNA H19 in regulating pulmonary injury in hyperoxia-induced bronchopulmonary dysplasia newborn mice. *Am J Perinatol* 2022;39:1089-96.
 19. Mei M, Nie J, Sun H, Wang H, Rong L. LncRNA-NEF regulated the hyperoxia-induced injury of lung epithelial cells by FOXA2. *Am J Transl Res* 2020;12:5563-74.
 20. Ji L, Liu Z, Dong C, Wu D, Yang S, Wu L. LncRNA CASC2 targets CAV1 by competitively binding with microRNA-194-5p to inhibit neonatal lung injury. *Exp Mol Pathol* 2021;118:104575.
 21. Liu X, Li D, Zhang W, Guo M, Zhan Q. Long non-coding RNA gadd7 interacts with TDP-43 and regulates Cdk6 mRNA decay. *EMBO J* 2012;31:4415-27.
 22. Zhao J, Ma W, Zhong Y, Deng H, Zhou B, Wu Y, et al. Transcriptional inhibition of lncRNA gadd7 by CRISPR/dCas9-KRAB protects spermatocyte viability. *Front Mol Biosci* 2021;8:652392.
 23. Zhao J, Li H, Deng H, Zhu L, Zhou B, Yang M, et al. LncRNA gadd7, increased in varicocele patients, suppresses cell proliferation and promotes cell apoptosis. *Oncotarget* 2018;9:5105-10.
 24. Luo Y, Hao T, Zhang J, Zhang M, Sun P, Wu L. MicroRNA-592 suppresses the malignant phenotypes of thyroid cancer by regulating lncRNA NEAT1 and downregulating NOVA1. *Int J Mol Med* 2019;44:1172-82.
 25. Zaib S, Hayyat A, Ali N, Gul A, Naveed M, Khan I. Role of mitochondrial membrane potential and lactate dehydrogenase A in apoptosis. *Anticancer Agents Med Chem* 2022;22:2048-62.
 26. Sidramagowda Patil S, Hernandez-Cuervo H, Fukumoto J, Krishnamurthy S, Lin M, Alleyn M, et al. Alda-1 attenuates hyperoxia-induced acute lung injury in mice. *Front Pharmacol* 2020;11:597942.
 27. Wu Y, Zhang Z, Li J, Zhong H, Yuan R, Deng Z, et al. Mechanism of adipose-derived mesenchymal stem cell-derived extracellular vesicles carrying miR-21-5p in hyperoxia-induced lung injury. *Stem Cell Rev Rep* 2022;18:1007-24.
 28. Zou DM, Zhou SM, Li LH, Zhou JL, Tang ZM, Wang SH. Knockdown of long noncoding RNAs of maternally expressed 3 alleviates hyperoxia-induced lung injury via inhibiting thioredoxin-interacting protein-mediated pyroptosis by binding to miR-18a. *Am J Pathol* 2020;190:994-1005.
 29. Zhu X, Wang F, Lei X, Dong W. Resveratrol alleviates alveolar epithelial cell injury induced by hyperoxia by reducing apoptosis and mitochondrial dysfunction. *Exp Biol Med (Maywood)* 2021;246:596-606.
 30. Zhu Y, Ju H, Lu H, Tang W, Lu J, Wang Q. The function role of ubiquitin proteasome pathway in the ER stress-induced AECII apoptosis during hyperoxia exposure. *BMC Pulm Med* 2021;21:379.
 31. Lu HY, Zhang J, Wang QX, Tang W, Zhang LJ. Activation of the endoplasmic reticulum stress pathway involving CHOP in the lungs of rats with hyperoxia induced bronchopulmonary dysplasia. *Mol Med Rep* 2015;12:4494-500.
 32. Oanh NTK, Park YY, Cho H. Mitochondria elongation is mediated through SIRT1-mediated MFN1 stabilization. *Cell Signal* 2017;38:67-75.
 33. Park YY, Nguyen OT, Kang H, Cho H. MARCH5-mediated quality control on acetylated Mfn1 facilitates mitochondrial homeostasis and cell survival. *Cell Death Dis* 2014;5:e1172.

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