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Original Article

LncRNA-NEF regulated the hyperoxia-induced injury of lung epithelial cells by FOXA2

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Abstract: Introduction: Hyperoxia-induced injury is a common form of damage in lung tissues, which could lead to bronchopulmonary dysplasia (BPD) in newborns. Recent studies have discovered that FOXA2 played a substantial role in protecting lung tissues from various injuries and lncRNA-NEF could activate the expression of FOXA2. However, it is unclear whether lncRNA-NEF could alleviate hyperoxia-caused damage of lung tissues by activating FOXA2. Material and methods: In this study, we used the lentivirus to establish the lncRNA-NEF overexpression RLE-6TN and MLE-12 cells. After that, the lentivirus was also used to knockdown the expression of FOXA2 in the two lncRNA-NEF overexpression cells. ELISA was performed to detect the levels of TNF-α, IL-1β and IL-6. The production of ROS, SOD, MDA and LDH was determined with the commercial kits. The apoptosis rates of these cells were measured with the flow cytometry. Results: The secretion of TNF-α, IL-1β and IL-6 was inhibited in RLE-6TN and MLE-12 cells after the overexpression of lncRNA-NEF. Furthermore, the production of ROS, MDA and LDH was also suppressed after the upregulation of lncRNA-NEF. The promotion of lncRNA-NEF also restricted the hyperoxia-induced apoptosis. However, the knockdown of FOXA2 abolished all the inhibitory effects exerted by lncRNA-NEF. Conclusion: LncRNA-NEF regulated hyperoxia-caused inflammatory response, oxidative damage and apoptosis of RLE-6TN and MLE-12 cells by affecting the expression of FOXA2.

Keywords: Bronchopulmonary dysplasia, Hyperoxia-induced lung injury, LncRNA-NEF, FOXA2, inflammation

Introduction

Bronchopulmonary dysplasia (BPD) is one kind of the pulmonary diseases after ventilation and oxygen administration in premature infants with acute respiratory failure [1]. Mechanical oxygen supply is one of the crucial means to treat acute hypoxic respiratory failure [2]. However, this treatment may result in serious damage to the lungs, the toxic responses to organs and acute injuries, such as acute lung injury [3-5]. The lung injury induced by hyperoxia was considered as a bimodal process, which happened under the oxygen toxicity and deposition of inflammatory mediators in the lungs [6]. Although oxygen played a pivotal role in the process of energy metabolism and ATP synthesis, excess oxygen could also increase the production of ROS. Excessive ROS could bring about the damage to the epithelial cells of alveoli and eventually lead to the disorders of gas exchange in the lungs [7-9]. Under high oxygen, excessive ROS could act as the cytotoxin to activate the signal transduction pathway in alveolar epithelial cells, further promoting the secretion of pro-inflammatory factors [10]. Hyperoxia-induced apoptosis of pulmonary epithelial cells is another prominent feature of acute lung injury caused by hyperoxia [11]. Therefore, relieving the inflammation, oxidative damage and apoptosis of lung epithelial cells is the key to treating hyperoxia-induced lung injury.

FOXA2 belongs to the vertebrate forkhead box A (Foxa) gene family and modulates the process of cell differentiation and cell cycle [12]. There is also study revealing that the FOXA2 was an essential factor for the differentiation and functioned in the development of alveolar epithelial cells [13]. Furthermore, lower expression of FOXA2 in respiratory epithelial cells is

conducive to the emphysema [14]. It is obvious that FOXA2 plays the crucial role in protecting epithelial cells from injury in lung tissue.

Moreover, long noncoding RNA (lncRNA) is one subtype of the noncoding RNAs with dysregulated expression and regulatory roles in diversified physiological processes in the body [15]. There is a study indicating that the expression changes of some lncRNAs is associated with the hyperoxia-induced damage of lung tissues [16]. For instance, lncRNA-NEF could promote the expression of FOXA2, therefore suppressing the metastasis and EMT process of hepatocellular carcinoma cells [17]. However, whether the lncRNA-NEF could enhance the expression of FOXA2 and relieve the hyperoxia-damaged injury of lung tissues is still unclear.

Therefore, we studied the effect of lncRNA-NEF on the pulmonary epithelial cells of rats and alveolar epithelial cells of mice cultured under the hyperoxia condition. Next, we used the lentivirus to establish the lncRNA-NEF overexpression and FOXA2 knockdown epithelial cell lines which were separated from lung tissues. After that, the cell viability, contents of pro-inflammation factors, ROS levels and apoptosis rates were determined with multiple types of experiments. All the results from these assays could reveal the effects of lncRNA-NEF/FOXA2 axis on the hyperoxia-induced injury of the epithelial cells in lung tissues.

Material and methods

Cell culture and transfection

The RLE-6TN cells (obtained from lung epithelial tissues of rats) and MLE-12 cells (obtained from alveolar epithelial tissues of mice) were cultured with the RPMI-1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Gibco, USA). These cells were placed in the 37°C humid conditions with 5% CO_2. The concentration of the oxygen was regulated with the incubator. The lncRNA-NEF-overexpressed and FOXA2-silenced lentivirus vectors were obtained from Genechem (Shanghai, China). The polybrene (Genechem, China) was used to promote the transfection efficiency. shRNA-FOXA2 sequence was designed (CACACAAACCAAACCGTCA). lncRNA-NEF overexpression sequence was designed based on the following sequence: ATGAAGAAACNNNNGNNTATNNTGCNNTCNTAAACCAACNNAANNGCNNNNGNNNCNNNCNNNGTTATTTAGGGTGGNGNNNNNNNNTCTNNCAACNAGANAAGGTGGCNNNNGCATGGGGACACNNNAANTGANNAGNTGTTNGGGNAANANNCTGNTTNAGANANTTAAGNCNTCCACNGATGNNAGNGGAATNNNCANNTNCTCANTCCNTCCACTGAGANGTCCNNNNAAAGATGGANAANCCNTAACTGAAACCCNACNTTNNGCACAANGNTCANATTCTNCNNGCATAANGCNAGANATCTNTGTGCCAGATCCATCTTNNANGCANCNCACNCANANGACNNNNTCTGTCNCCTTCCCCNNAACAATANGCNGAAANNACCNTGGTATTTCNTGNNTTCTCACTTAGNAAANNTGAGTTCTGNTCANTGANGNNCCTTCTNCNNTGGNTGGTTTCATGTGACAAAGNGANNTCTAGGNAGNNCTTNTGNNAGNNNAANGNAGATNGTTCCAGCAAGATNAANNCCTNNNNGACCTNGNTGNNCNNGNTGTTTTCTTNCCANGGANNGNAGANGANGNCTGGATTTGNACTNNAGTNTTANGNCTTTGCCNTTGNNGCAACATCCAAAATAAAAATAGCATGCTANCCCNAA. All the operations in this assay followed the instructions.

CCK-8

These cells were plated in the 96-well plates (2×10^3 cells in per well). After the adhesion and hyperoxia treatment (12 h, 24 h and 48 h), the CCK-8 (Dojindo, Japan) was diluted and added into the 96-well plates. Then these cells were incubated in the incubator for 1.5 h. At last, the absorbance of these cells was measured with the spectrophotometer (Thermo Fisher Scientific, USA).

Western blotting

Total proteins of cells were collected with the RIPA (Beyotime, China). The concentration of these samples was determined with BCA (Beyotime, China) methods. After that, these samples were separated with the 10% SDS-PAGE gel (Beyotime, China). Next, these proteins were transferred to the PVDF membranes (Millipore, USA). Subsequently, these membranes were incubated with the primary antibody at 4°C overnight following block with the 5% skim milk powder (Becton, Dickinson and Company, USA). The primary antibodies used in this research were FOXA2 (Abcam, ab108422), Bcl-2 (Abcam, ab185002), Bax (Abcam, ab32503), Cleaved caspase3 (Abcam, ab2302), Cleaved caspase9 (Abcam, ab2324) and GAPDH (Abcam, ab181602). On the second day, the PBST was used to wash these membranes, which then were incubated with the second

antibodies for 1.5 h at room temperature. At last, these membranes were washed with the PBST again and the signals were monitored by Pierce Western Blotting Substrate (Thermo Fisher Scientific, USA). The expression of targeted proteins was normalized to that of GAPDH.

RT-PCR

Total RNA was collected with the Trizol (Thermo Fisher Scientific, USA) method. The reverse transcription kit (Roche, Switzerland) was used to convert RNA to cDNA. SYBR green (Thermo Fisher Scientific, USA) was added in the PCR reaction system. Next, the cDNA was amplified with the Applied Biosystems 7500 Real-Time PCR system (Thermo Fisher Scientific, USA). The expression of targeted genes was normalized to the expression of GAPDH and calculated with the $2^{-\Delta\Delta CT}$ method. The primers used in this research were: lncRNA-NEF forward: 5'-CTGCCGTCTTAAACCAACCC-3', reverse: 5'-GCCCAAACAGCTCCTCAATT-3'; FOXA2 forward: 5'-CCCCTGAGTTGGCGGTGGT-3', reverse: 5'-TTGCTCACGGAAGAGTAG-3'; GAPDH forward: 5'-ACAACTTTGGTATCGTGGAAGG-3', reverse: 5'-GCCATCACGCCACAGTTTC-3'.

ELISA assays

The supernatant of cells were collected and centrifuged with the centrifugal machine. After that, the supernatant was transferred to the sterilized tube. Next, mouse TNF-α ELISA kit (ab208348), rat TNF-α ELISA kit (ab236712), mouse IL-1β (ab197742), rat IL-1β (ab255730), mouse IL-6 (ab100713) and rat IL-6 (ab234570) were used to determine the levels of TNF-α, IL-1β and IL-6 in the supernatant. All the operations during this process were in good accordance with the instructions.

Apoptosis assays

These cells were made into the single-cell suspension by trypsin (Beyotime, China). Then, cells were washed with the cold PBS for three times. After that, the AnnexinV and PI (Beyotime, China) were incubated with cells at 37°C for 0.5 h. The cell suspension was shaken every 5 minutes to ensure that the probe fully bound to these cells. Next, cells were washed utilizing PBS for three times again. At last, the apoptosis rates were detected with the flow cytometry.

Detection of ROS, SOD, MDA and LDH

For the detection of ROS levels, these cells were prepared into the cell suspension and washed with the cold PBS to remove the serum. After that, these cells were incubated with the DCFH-DA (Beyotime, China) for 0.5 h followed by wash with PBS for three times. Finally, the ROS was observed with the confocal laser scanning microscopy (Olympus, Japan). The levels of MDA, LDH and SOD were determined with the commercial kits (Jiancheng Institute of Bioengineering, Nanjing, China). All the operations in these assays followed the guidebook.

Statistical analysis

All the data was analyzed with the Graphpad Prism 7.0. All the experiments in this study were repeated for three times. The data in this research was presented as mean ± SD. One-way analysis of variance was performed to compare the data among different groups, following with Turkey's test. The value of *P* less than 0.05 was considered that the difference between these groups was prominent.

Results

Hyperoxia suppressed the expression of lncRNA-NEF and FOXA2 in RLE-6TN and MLE-12 cells

The RLE-6TN and MLE-12 cells were cultured under the hyperoxia condition for 12 h, 24 h and 48 h. After that, the cell viability was determined with the CCK-8 assays. As shown in the **Figure 1A**, the cell viability of RLE-6TN and MLE-12 cells was inhibited after the stimulation of hyperoxia. Next, the expression of lncRNA-NEF and FOXA2 was determined with the RT-PCR and western blotting. The results (**Figure 1B**) showed that the levels of lncRNA-NEF and FOXA2 were gradually inhibited in these cells with the extension of the hyperoxia stimulation time.

Overexpression of lncRNA-NEF promoted the expression of FOXA2 in RLE-6TN and MLE-12 cells

According to the results of CCK-8 assays, the 48 h of hyperoxia stimulation was applied for the next experiments. The lentivirus was utilized to overexpress lncRNA-NEF in RLE-6TN

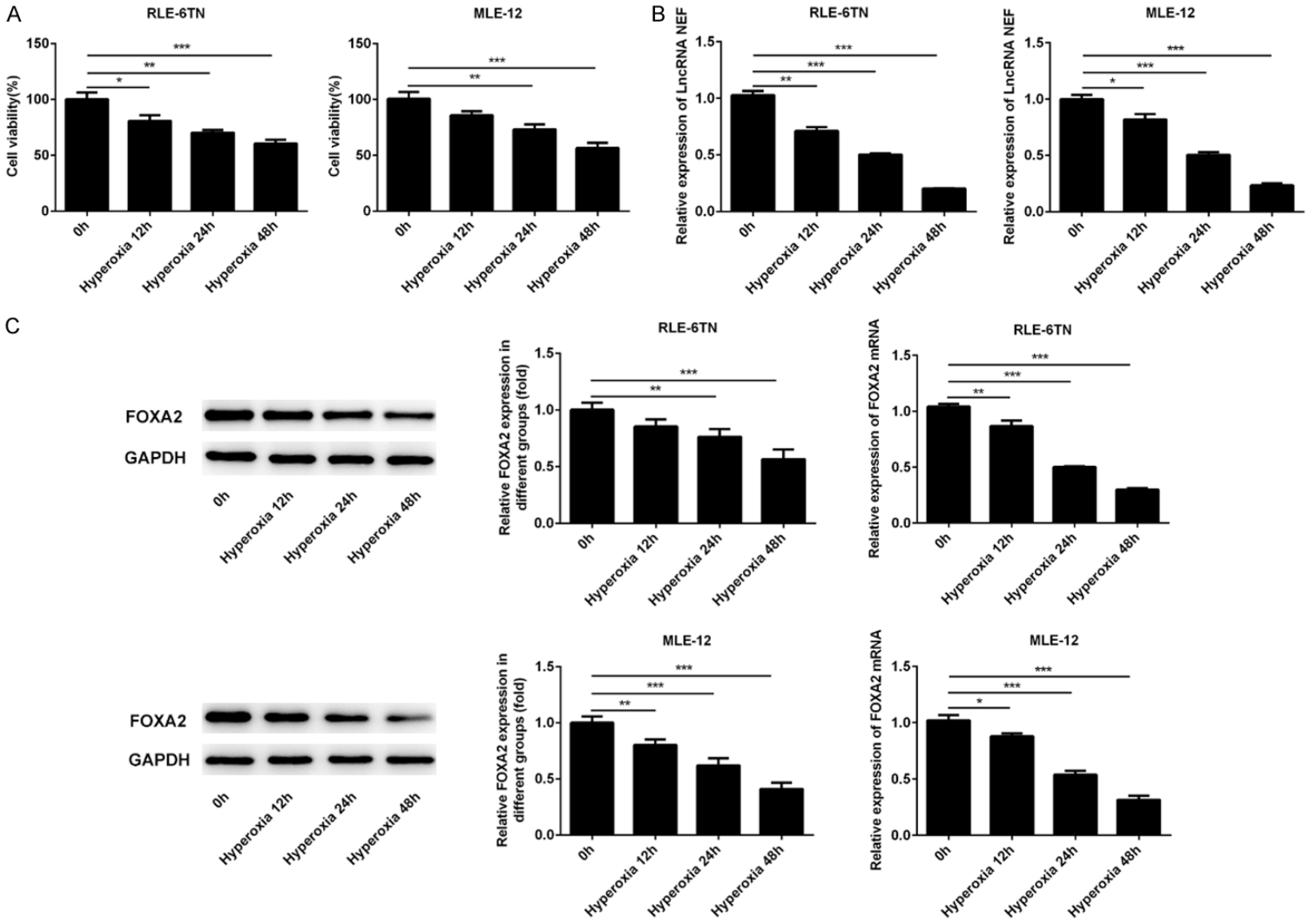

Figure 1. Hyperoxia inhibited the expression of lncRNA-NEF and FOXA2 in RLE-6TN and MLE-12 cells. A. Cell viability of RLE-6TN and MLE-12 cells was detected with the CCK-8 assays. B. The levels of lncRNA-NEF in RLE-6TN and MLE-12 cells were determined with the RT-PCR. C. Protein and mRNA levels of FOXA2 in RLE-6TN and MLE-12 cells were detected with the western blotting and RT-PCR, respectively. *P<0.05, **P<0.01, ***P<0.001.

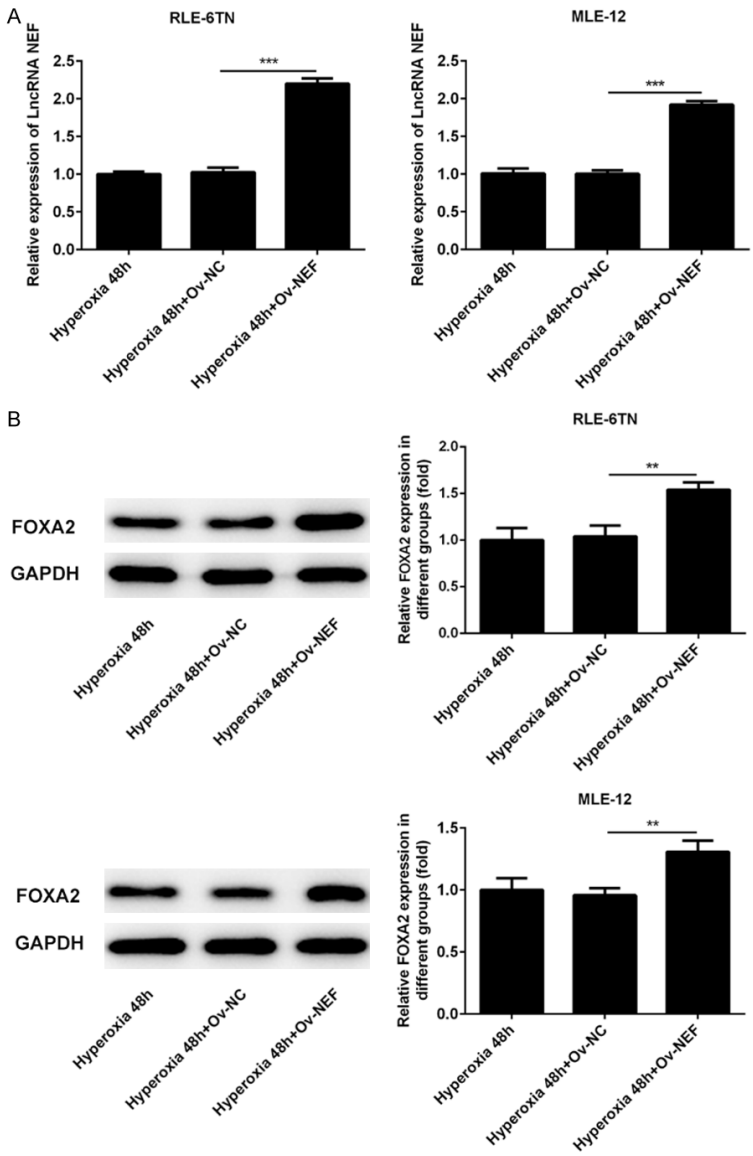

Figure 2. The expression of FOXA2 was enhanced after the overexpression of lncRNA-NEF in RLE-6TN and MLE-12 cells. A. The overexpression of lncRNA-NEF was confirmed with the RT-PCR in RLE-6TN and MLE-12 cells. B. The protein and mRNA levels of FOXA2 were determined with the western blotting and RT-PCR, respectively. **P<0.01, ***P<0.001. Ov-NEF: overexpressing lncRNA-NEF. Ov-NC: empty plasmids.

and MLE-12 cell lines. After 48 h of culture under the hyperoxia condition, RT-PCR was performed to validate the overexpression of lncRNA-NEF. The levels of lncRNA-NEF were significantly increased in the overexpression group (**Figure 2A**). Next, the expression of FOXA2 in these cells was determined with the western blotting and RT-PCR. The results (**Figure 2B**) showed that both the protein and mRNA levels of FOXA2 were enhanced after the overexpression of lncRNA-NEF.

Knockdown of FOXA2 restricted the inhibitory effect of lncRNA-NEF on the secretion of proinflammation factors in RLE-6TN and MLE-12 cells

In this part, we used the lentivirus to knockdown FOXA2 in the lncRNA-NEF-overexpressed RLE-6TN and MLE-12 cells. The mRNA level of FOXA2 and FOXA2 expression were respectively validated with the RT-PCR and Western blot. As shown in **Figure 3A**, the levels of FOXA2 and FOXA2 mRNA declined in the knockdown group. Given that the hyperoxia could lead to the inflammatory damage of lung tissue [18], we determined the levels of proinflammation factors (TNF-α, IL-1β and IL-6) in the supernatant of RLE-6TN and MLE-12 cells by the ELISA assays. The results uncovered that the levels of TNF-α, IL-1β and IL-6 were decreased after the overexpression of lncRNA-NEF. However, the secretion of TNF-α, IL-1β and IL-6 was restored after the knockdown of FOXA2 in these two cell lines (**Figure 3B**).

lncRNA-NEF regulated the expression of oxidative stress-related markers by FOXA2 in RLE-6TN and MLE-12 cells

The oxidative damage, another kind of injury, happens when tissues are chronically exposed under the hyperoxia condition. Therefore, the probe method was used for the observation of ROS. As exhibited in **Figure 4A**, the levels of ROS decreased after lncRNA-NEF was overexpressed. However, the production of ROS was recovered after the knockdown of FOXA2 in lncRNA-NEF-upregulated RLE-6TN and MLE-12 cells. Next, the levels of SOD, MDA and LDH were determined with specific commercial kits. The results (**Figure 4B**) displayed that the levels of MDA and LDH were suppressed in RLE-6TN and MLE-12 cells with

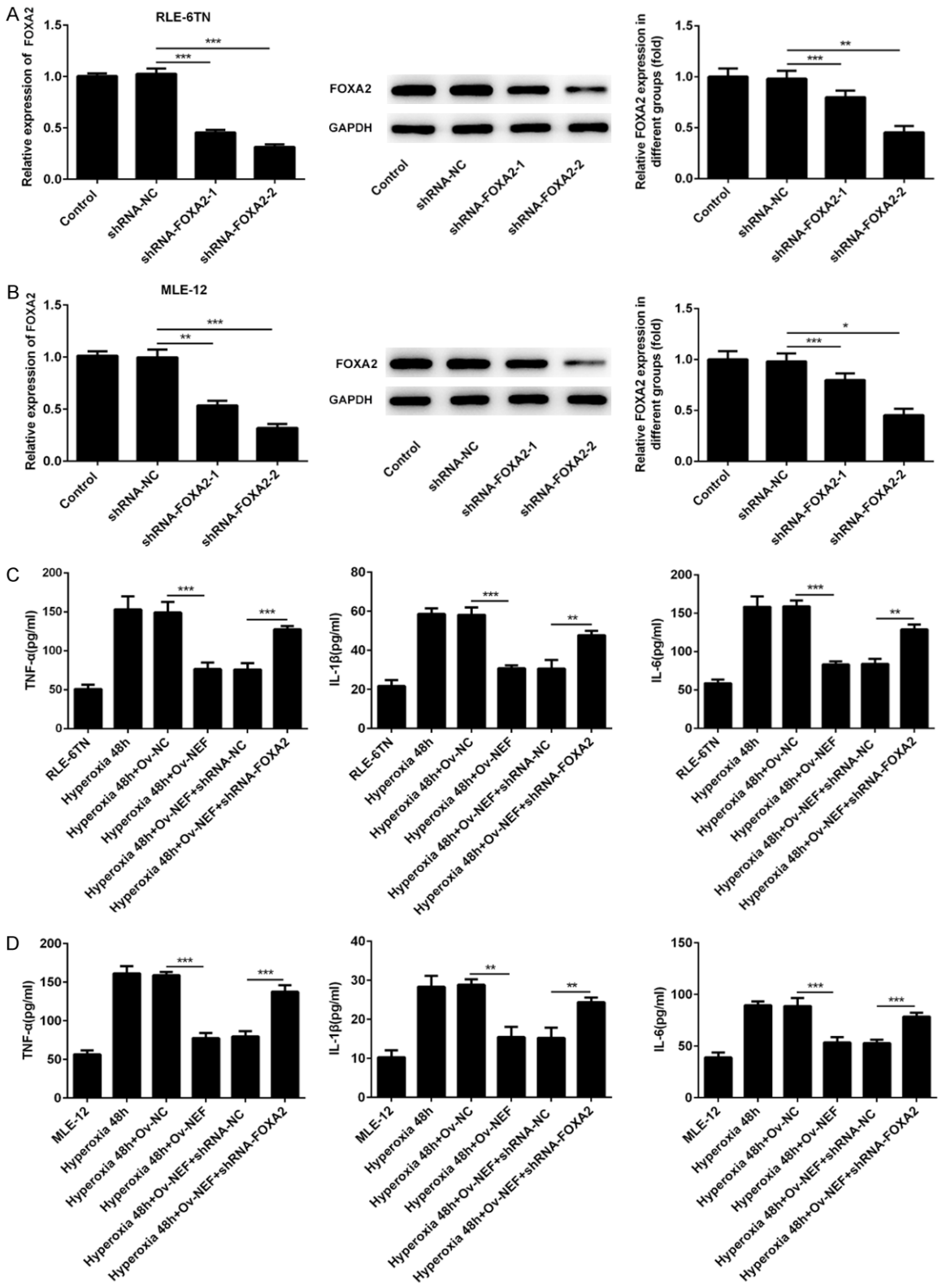

Figure 3. The suppression effect of lncRNA-NEF on proinflammation factors (TNF-α, IL-1β and IL-18) was restricted after the knockdown of FOXA2. A, B. The transfection efficacy of shRNA-FOXA2-1 or shRNA-FOXA2-2 was determined in RLE-6TN and MLE-12 cells by the RT-PCR and Western blot. C, D. The levels of pro-inflammatory markers determined with ELISA, TNF-α, IL-1β and IL-18, were significantly reversed through downregulating FOXA2 levels after overexpressing lncRNA-NEF. **P<0.01, ***P<0.001. Ov-NEF: overexpressing lncRNA-NEF. Ov-NC: empty plasmids. ShRNA-FOXA2: silencing FOXA2. ShRNA-NC: Scrambled RNA, the negative control of ShRNA-FOXA2.

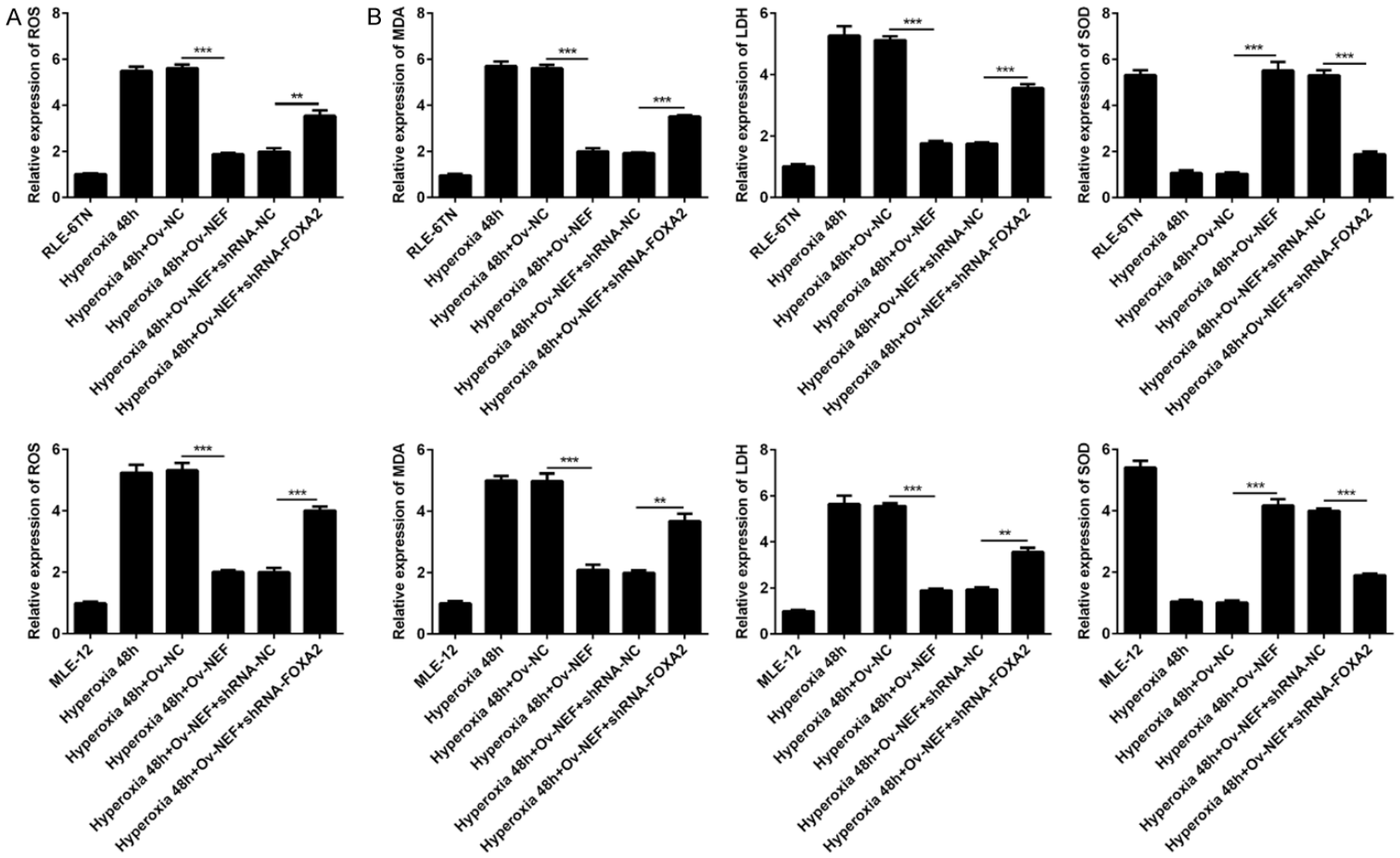

Figure 4. Overexpression of lncRNA-NEF relieved the hyperoxia-induced oxidative damage of RLE-6TN and MLE-12 cells by upregulating the FOXA2. A. The levels of ROS were detected with the DCFH-DA probe. B. The levels of MDA, SOD and LDH were determined with the related kits. Ov-NEF: overexpressing lncRNA-NEF. Ov-NC: empty plasmids. ShRNA-FOXA2: silencing FOXA2. ShRNA-NC: Scrambled RNA, the negative control of ShRNA-FOXA2. **P<0.01, ***P<0.001.

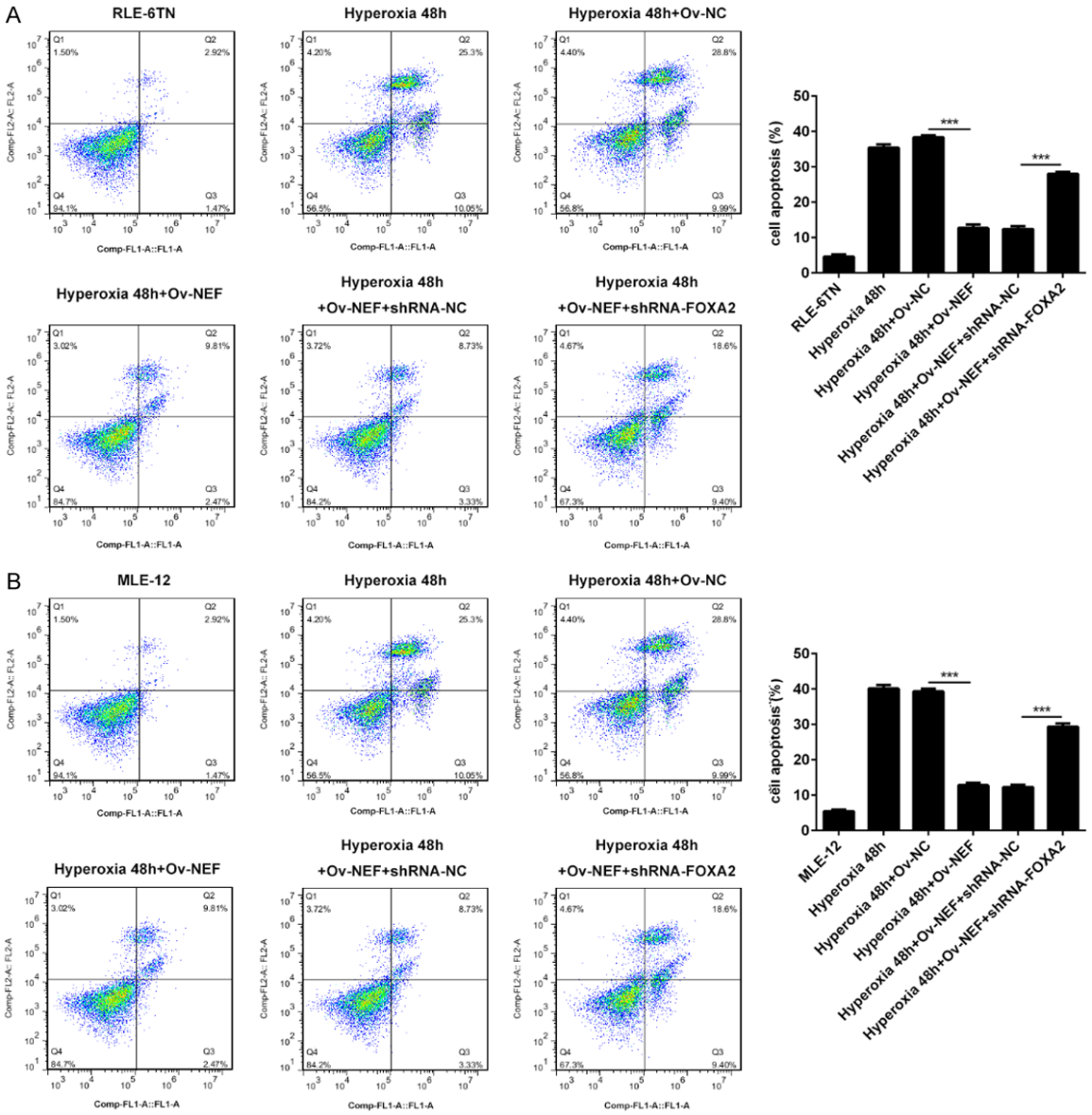

Figure 5. Overexpression of lncRNA-NEF alleviated the apoptosis of RLE-6TN and MLE-12 cells induced by hyperoxia by upregulating the FOXA2. A, B. The apoptosis rates of RLE-6TN and MLE-12 cells in diverse groups were determined with flow cytometry. Ov-NEF: overexpressing lncRNA-NEF. Ov-NC: empty plasmids. ShRNA-FOXA2: silencing FOXA2. ShRNA-NC: Scrambled RNA, the negative control of ShRNA-FOXA2. ***P<0.001.

lncRNA-NEF elevation. On the other side, the production of SOD was increased after the overexpression of lncRNA-NEF. Moreover, the inhibitory effects of lncRNA-NEF on the MDA and LDH and its inducing effect on SOD were abolished by the knockdown of FOXA2.

Overexpression of lncRNA-NEF relieved hyperoxia-induced apoptosis of RLE-6TN and MLE-12 cells by enhancing the expression of FOXA2

There is a study reporting that the hyperoxia could induce the apoptosis of cells in lung tissues [19]. Therefore, the proportion of apoptosis cells was determined with the flow cytometry. According to the results (**Figure 5A** and **5B**), the apoptosis rates of RLE-6TN and MLE-12 cells declined after the overexpression of lncRNA-NEF, which were rescued after the silencing of FOXA2. Next, the expression of proteins related to apoptosis was determined with the western blotting. As shown in **Figure 6A** and **6B**, in RLE-6TN and MLE-12 cells with lncRNA-NEF upregulation, the levels of Bax, Cleaved caspase3 and Cleaved caspase9 were inhibited, whereas, the expression of Bcl-2 was

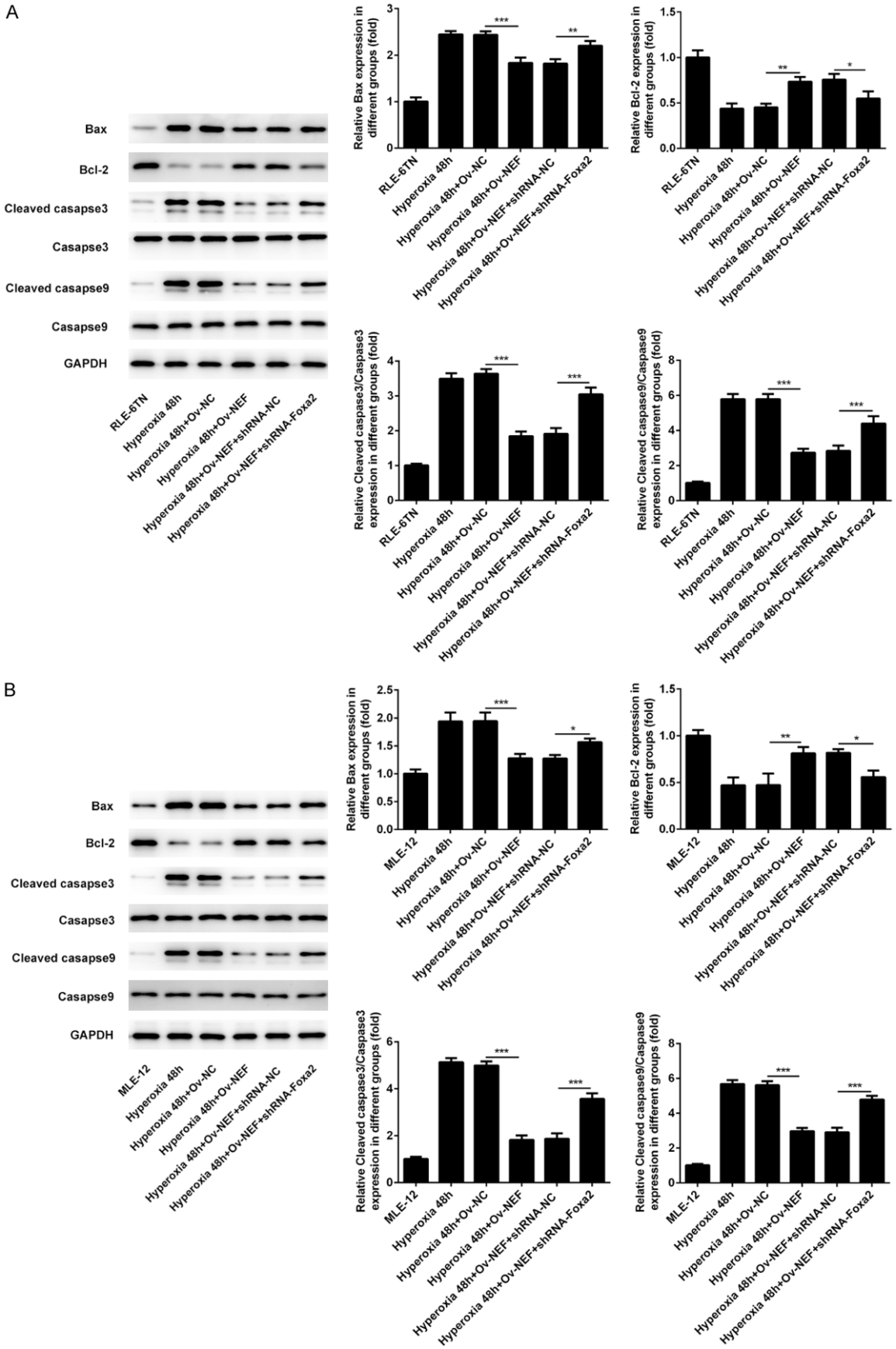
A
Bax
Bcl-2
Cleaved casapse3
Casapse3
Cleaved casapse9
Casapse9
GAPDH
RLE-6TN
Hyperoxia 48h
Hyperoxia 48h+Ov-NC
Hyperoxia 48h+Ov-NEF
Hyperoxia 48h+Ov-NEF+shRNA-NC
Hyperoxia 48h+Ov-NEF+shRNA-Foxa2
Relative Bax expression in different groups (fold)
Relative Bcl-2 expression in different groups (fold)
Relative Cleaved caspase3/Caspase3 expression in different groups (fold)
Relative Cleaved caspase9/Caspase9 expression in different groups (fold)
B
MLE-12
Hyperoxia 48h
Hyperoxia 48h+Ov-NC
Hyperoxia 48h+Ov-NEF
Hyperoxia 48h+Ov-NEF+shRNA-NC
Hyperoxia 48h+Ov-NEF+shRNA-Foxa2
Relative Bax expression in different groups (fold)
Relative Bcl-2 expression in different groups (fold)
Relative Cleaved caspase3/Caspase3 expression in different groups (fold)
Relative Cleaved caspase9/Caspase9 expression in different groups (fold)

Figure 6. Overexpression of lncRNA-NEF regulated the expression of apoptosis-related proteins (Bax, Bcl-2, Cleaved Caspase3, Cleaved Caspase9) in RLE-6TN and MLE-12 cells through FOXA2. A, B. Images of immunoblotting for Bax, Bcl-2, Cleaved caspase3, caspase3, Cleaved caspase9, caspase9 and GAPDH as loading control were shown. Ov-NEF: overexpressing lncRNA-NEF. Ov-NC: empty plasmids. ShRNA-FOXA2: silencing FOXA2. ShRNA-NC: Scrambled RNA, the negative control of ShRNA-FOXA2. *P<0.05, **P<0.01, ***P<0.001.

enhanced. After the knockdown of FOXA2, the inhibition of Bax, Cleaved caspase3 and Cleaved caspase9 expression and the enhancement of Bcl-2 expression were all abolished in RLE-6TN and MLE-12 cells.

Discussion

Recent studies have indicated the great potential of stem cells to repair damaged epithelial cells in lung tissues, but these could not be achieved through transplantation [20-22]. The occurrence and development of BPD were in close association with many factors and signaling pathways [23]. In the present study, we observed that the level of LncRNA-NEF or FOXA2 showed significant decrease in hyperoxia-stimulated RLE-6TN and MLE-12 cells. Further, we confirmed that LncRNA-NEF regulated FOXA2 levels to affect the response of RLE-6TN or MLE-12 cells to hyperoxia, including inflammatory response, oxidative stress and apoptosis.

LncRNAs are the critical molecules in the various life activities of the body. They are associated with the occurrence and development of BPD since it has been proved that lncRNAs are abnormally regulated in the lung tissues of BPD patients compared with normal lung tissues [16]. In addition, there is a study demonstrating that the lncRNA MALAT1 relieves the injury of lung tissues of infants by suppressing the apoptosis of epithelial cells [24]. These researches indicated that the development of BPD is associated with the changes of lncRNA expression. Nonetheless, the exact mechanism whereby lncRNA affects BPD remains unclear. Among lncRNAs, the lncRNA-NEF has been validated to emerge as an activator of FOXA2 and form a feedback loop with FOXA2 [17]. Thus, we had reason to suppose that the lncRNA-NEF might protect the lung epithelial cells against hypoxia-induced injury by modulating the expression of FOXA2. Besides, there possibly existed a feedback loop between lncRNA-NEF and FOXA2 in hypoxia-induced lung injury.

Similarly, FOXA2 is an essential molecule for airway epithelial cell differentiation, and also develops a critical function in the process of alveolar epithelial cells [13]. The inhibition of FOXA2 could induce neutrophil infiltration and mucous formation. Moreover, the expression of FOXA2 is negatively regulated when the inflammation response occurs in the airway [14]. All these conclusions indicated the crucial role that FOXA2 played in the physiological activities of the lungs. In this study, the levels of lncRNA-NEF and FOXA2 declined in the epithelial cells of mice lung tissues which was cultured under the hyperoxia condition. And overexpression of lncRNA-NEF enhanced the expression of FOXA2 in these cells. These data elucidated that the lncRNA-NEF had the potential to relieve hyperoxia-induced damage of lung tissues by promoting the expression of FOXA2.

It is worth noting that the inflammation-caused injury and the abnormal repair after lung tissue injury are the main reasons of the BPD occurrence. During the development of BPD, inflammatory response caused by neutrophilic infiltration is the main characteristic of lung injury induced by hyperoxia [25]. In this current study, the levels of pro-inflammation factors (TNF-α, IL-1β and IL-6) were inhibited when lncRNA-NEF was promoted. However, the decreased levels of TNF-α, IL-1β and IL-6 by promoted lncRNA-NEF were restored after the knockdown of FOXA2. Moreover, the oxidative stress is another nonnegligible factor in lung injury for promoting the occurrence and development of BPD, hence we detected the levels of ROS, SOD, MDA and LDH. It was observed that overexpression of lncRNA-NEF enhanced the production of SOD and inhibited the releasing of ROS, MDA and LDH. However, the impacts of lncRNA-NEF on these molecules were abolished after the knockdown of FOXA2. These results further suggested that lncRNA-NEF could alleviate hyperoxia-stimulated inflammatory damage of lung tissues by promoting the expression of FOXA2.

Furthermore, hyperoxia could induce the apoptosis of alveolar epithelial cells [26]. And hyperoxia could also activate the apoptosis of lung tissues cells from newborn mice [27]. In this work, the proportion of apoptosis cells was

declined after the overexpression of lncRNA-NEF and the phenomenon was promoted after the downregulation of FOXA2, indicating that the activation of lncRNA-NEF/FOXA2 relieved the hyperoxia-produced damage to lung tissues.

Above all, the results in this study suggested that the lncRNA-NEF modulated the hyperoxia-induced injury of lung epithelial cells by affecting the expression of FOXA2. And this research also possibly provides the new target and therapy for the clinic treatment of BPD.

Disclosure of conflict of interest

None.

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