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SEMA3A protects against hyperoxia-induced lung injury in a bronchopulmonary dysplasia model of newborn rat by inhibiting ERK pathway

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Abstract
Background: Hyperoxia induces lung injury through lung inflammation in premature infants, leading to bronchopulmonary dysplasia (BPD). Semaphorin 3A (SEMA3A) participates in diverse biological processes, including cell migration, angiogenesis, and inflammation. The effect of SEMA3A on hyperoxic lung injury of neonatal rats with BPD was investigated in this study.
Methods: Neonatal rats with BPD were established through hyperoxia treatment. Hematoxylin-eosin staining was used to evaluate histopathological analysis in lung tissues. SEMA3A expression was assessed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot assay. Adeno-associated virus (AAV)-mediated over-expression of SEMA3A (AAV-SEMA3A) was administrated into hyperoxia-induced rats, and apoptosis was evaluated by TUNEL staining. Levels of inflammatory cytokines were investigated by enzyme-linked-immunosorbent serologic assay (ELISA).
Results: Hyperoxia-induced histopathological changes in lung tissue reduced alveolar number and enhanced alveolar interval and alveolar volume. SEMA3A was downregulated in lung tissue of hyperoxia-induced rats. AAV-SEMA3A injection attenuated hyperoxia-induced cell apoptosis in lung tissues by increasing Bcl-2 and decreasing Bax and cleaved caspase-3. Moreover, the enhanced levels of Interleukin (IL)-1β, monocyte chemoattractant protein (MCP)-1, and tumor necrosis factor-α (TNF-α) in hyperoxia-induced rats were restored by AAV-SEMA3A injection by the downregulation of nuclear factor *kappa B* (NF-κB) phosphorylation. AAV-SEMA3A injection also ameliorated histopathological changes in lung tissues of hyperoxia-induced rats by increasing the number of radial alveolar count and decreasing the volume of mean linear intercept. Besides, the protein expression levels of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) phosphorylation were reduced in hyperoxia-induced rats post-AAV-SEMA3A injection.
Conclusion: Ectopical expression of SEMA3A suppressed hyperoxia-induced apoptosis and inflammation in neonatal rats, and ameliorated the histopathological changes through inactivation of ERK/JNK pathway.

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Introduction

Bronchopulmonary dysplasia (BPD), a common lung disease in premature infants, is caused by physiological lung dysplasia.[1] BPD is an important cause of respiratory diseases in preterm newborns, leading to significant morbidity and mortality.[2] The main characteristics of BPD are enlarged alveoli, pulmonary growth arrest, mesenchymal cell hyperplasia, abnormal lung function, and fibrosis.[3] Preterm birth, oxygen toxicity, postnatal infection, inflammation, and prenatal infection are considered to be closely related to the progression of BPD[4] Treatment strategies, such as diuretics and glucocorticoids, demonstrated limited clinical efficiency in the treatment of BPD.[5] Therefore, novel treatments are urgently required for BPD.

Although mechanical oxygen supply is one of the key methods for treating BPD, hyperoxia induces severe adverse effects in lung tissue, such as alveolar vascular cell permeability, alveolar epithelial and endothelial cell death.[6] Preterm infants are susceptible to hyperoxia, as it induces excessive accumulation of reactive oxygen species, and activates pathways to promote the secretion of inflammatory factors in alveolar epithelial cells, thus promoting inflammation and alveolar development deficits in neonatal rats, similar to the features found in infants with BPD.[7] Moreover, hyperoxia-induced apoptosis of lung epithelial cells is another prominent feature of hyperoxia-induced acute lung injury.[8] Therefore, strategies that alleviate hyperoxia-induced inflammation and apoptosis of lung tissue are widely investigated in the prevention of BPD.[9]

Class 3 semaphorins, including seven members (3A-3G), are secreted proteins in vertebrates that regulate lymph angiogenesis, angiogenesis, immune responses, and other physiological and developmental functions.[10] SEMA3A has been found to exert anti-cancer and anti-inflammatory properties. For example, the proliferation of brain tumor stem cells of glioblastoma multiform was suppressed by SEMA3A.[11] Silence of SEMA3A promoted tumor cell migration and invasion through activation of mitogen-activated protein kinase (MAPK) cascade.[12] SEMA3A alleviated osteoarthritis by inhibiting the inflammatory response of chondrocytes to excessive mechanical stress.[13] Radiation-induced osteoporosis was also attenuated by SEMA3A through reduction of inflammation and inhibition of osteoclasts.[14] In respiratory system, loss of SEMA3A altered distal lung structure, and was associated with increased alveolar septal cell death in neonatal mice, suggesting that SEMA3A plays an important role in regulating development of the lung and alveolar separation.[15] Moreover, deficiency of SEMA3-neuropilin pathway thickens alveolar septa, misaligns pulmonary veins, and reduces capillary density and pulmonary surfactant secretion, thus leading to atelectatic and immature regions in the lungs; all this suggests the clinical relevance of SEMA3 in neonatal respiratory disorders.[16] However, the protective effect of SEMA3A against hyperoxia-induced BPD has not been reported yet.

In this study, the expression of SEMA3A in lung tissue of hyperoxia-induced neonatal rats was evaluated, and the effects of SEMA3A on cell apoptosis and inflammation of lungs were explored. The results might provide a novel strategy for preterm infants with hyperoxia-induced BPD.

Materials and Methods

Animals

This study was approved by the Medical Ethics Committee of Guangdong Second Provincial General Hospital (Approval No. 2021-KZ-028-02) in accordance with the Guidance for the Care and Use of Laboratory Animals. A total of 24 neonatal Wistar rats were purchased from the Animal Center of the Chinese Academy of Science (Shanghai, China), and maintained with breastfeeding. Rats were divided into two groups: control (N = 6) and BPD (N = 18). For establishing BPD model, the neonatal rats were exposed to atmosphere with continuous 90% O_2 for 7 days. The control group rats were maintained in normal atmosphere. Rats in the BPD group were further divided into 3 subgroups: BPD (N = 6), BPD+AAV-Scramble (N = 6), and BPD+AAV-SEMA3A (N = 6). AAV-Scramble and AAV-SEMA3A were purchased from Genepharma (Shanghai, China). The BPD rats were injected with AAV-Scramble or AAV-SEMA3A virus (8×10^{11} VG/rat) via tail vein on 7th day of post-hyperoxia induction. After 14 days of hyperoxia induction, rats were anesthetized with 80 mg/kg body weight sodium pentobarbital injection. Lung tissues were harvested for functional assays, and the animals were sacrificed by cervical dislocation.

Histopathology of lung tissues

Lung tissues were isolated from each rat, and fixed in 4% paraformaldehyde. The paraffin-embedded tissues were cut into 6-μm sections. The sections were stained with hematoxylin-eosin (H&E) staining (Solarbio Science & Technology, Beijing, China) and measured under light microscopy (Nikon, Tokyo, Japan). Line was drawn from the center of the peripheral bronchiole to the nearest connective tissue septum, and the number of alveoli along the line was counted; this was viewed as radial alveolar count (RAC). Five lines in each field were drawn, and the mean linear intercept (MLI) was calculated as length of each line divided by the number of alveolar intercepts.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and enzyme-linked-immunosorbent serologic assay (ELISA)

The sections isolated from lung tissue were de-paraffinized and rehydrated in an ethanol series. Sections were incubated with proteinase K (Sigma-Aldrich, Stockholm, Sweden), and treated with TUNEL reaction mixture (Roche Applied Science, Pennsburg, Germany) to determine TUNEL-positive cells under light microscopy (Nikon). Lung tissues were lysed in radioimmunoprecipitation assay lysis buffer (Beyotime, Shanghai, China), and supernatants were collected for ELISA analysis of Interleukin (IL)-1β, monocyte chemoattractant protein (MCP)-1, and tumor necrosis factor (TNF)-α using corresponding commercial kits (Dakewe, Shenzhen, China).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RNAs were isolated from lung tissue by Trizol (Invitrogen, Carlsbad, CA, USA). RNAs were transcribed into complementary DNA (cDNAs), and RT-qPCR analysis of SEMA3A was performed by SYBR Premix Ex Taq II (TaKaRa, Tokyo, Japan). *GAPDH* was used as an endogenous control, and the expression level of SEMA3A was calculated using $2^{-\Delta\Delta Cq}$. The primers used are as follows: SEMA3A (forward primer: 5'-ACCCAACTATCAATGGGTGCCTTA-3' and reverse primer: 5'-AACACTGGATTGTACATGGCTGGA-3') and *GAPDH* (forward primer: 5'-CCATCTTCCAGGAGCGAGAT-3' and reverse primer: 5'-TGCTGATGATCTTGAGGCTG-3').

Western blot assay

Proteins (40 μg) from lysates of the lung tissue were separated by SDS-PAGE, and transferred to PVDF membranes (Millipore, Shanghai, China). The membranes were blocked in PBST with 5% nonfat milk, and incubated with primary and secondary antibodies, including anti-cleaved caspase-3, anti-c-Jun N-terminal kinase (JNK) and anti-p-JNK (1:2000; Abcam, Cambridge, UK), anti-SEMA3A and anti-β-actin (1:2500; Abcam), anti-Bax and anti-Bcl2 (1:3000; Abcam), anti-NF-κB and anti-p-NF-κB (1:3500; Cell Signaling, Boston, MA, USA), and anti-extracellular signal-regulated kinase 1/2 (ERK1/2) and anti-p-ERK1/2 (1:4000; Abcam). ECL reagents (Roche, Shanghai, China) were used to detect protein signals.

Statistical analysis

All the experimental data were depicted as mean ± standard deviation (SD). Differences between groups were analyzed by Student's *t*-test or One-Way Analysis of Variance using SPSS 11.0 software. Independent sample *t*-test was used for comparison between the two groups if assumptions of normality and homogeneity of variance were satisfied, otherwise the nonparametric Mann–Whitney test was used for analysis; $P < 0.05$ was considered as statistically significant.

Results

SEMA3A was downregulated in hyperoxia-induced BPD rats

The neonatal rats were administrated with hyperoxia to establish rat models of BPD. Histopathological changes in lung tissue caused by hyperoxia were examined by H&E staining (Figure 1A). As depicted in Figure 1A, hyperoxia induced severe lung injuries, including inflammatory cell infiltration, reduced alveolar number, and enhanced alveolar interval and alveolar volume in BPD rats. Besides, SEMA3A was downregulated in the lung tissue isolated from hyperoxia-induced rats compared to the control (Figures 1B and 1C).

SEMA3A suppressed hyperoxia-induced cell apoptosis in BPD rats

To investigate the role of SEMA3A in the progression of BPD, hyperoxia-induced rats were injected with AAV-SEMA3A for over-expression of SEMA3A. Rats in the BPD group demonstrated lower body weight, lung weight, and decreased lung-to-body weight ratio than that in the control group (Table 1). However, AAV-SEMA3A injection given to rats of the BPD group restored decreased body weight, lung weight, and lung-to-body weight ratio (Table 1). AAV-SEMA3A injection upregulated the protein expression of SEMA3A (Figure 2A). Over-expression of SEMA3A also attenuated hyperoxia-induced cell apoptosis in BPD rats (Figure 2B), evidenced by the reduced TUNEL-positive cells (Figure 2C). Moreover, the reduced expression level of Bcl-2, enhanced expression levels of Bax, and cleaved caspase-3 in BPD rats were restored by the over-expression of SEMA3A (Figure 2D), suggesting the anti-apoptotic role of SEMA3A in BPD rats.

SEMA3A suppressed hyperoxia-induced inflammation in BPD rats

The levels of IL-1β, MCP-1, and TNF-α were increased in hyperoxia-induced BPD rats (Figure 3A), while AAV-SEMA3A injection decreased the levels of IL-1β, MCP-1, and TNF-α (Figure 3A). The protein expression of NF-κB phosphorylation (p-NF-κB) was enhanced in hyperoxia-induced BPD rats (Figure 3B), suggesting that hyperoxia promoted the activation of NF-κB to induce inflammation. However, over-expression of SEMA3A reduced the level of p-NF-κB in hyperoxia-induced rats (Figure 3B), revealing the anti-inflammatory role of SEMA3A against progression of BPD.

SEMA3A ameliorated hyperoxia-induced histopathological changes in BPD rats

BPD+AAV-SEMA3A group established decreased inflammatory cell infiltration, alveolar interval, and alveolar volume as well as enhanced alveolar number in the lung tissue (Figure 4), thus demonstrating that SEMA3A ameliorated hyperoxia-induced alveolar injury, simplification, and inflammation, which reduced lung injury. Moreover, hyperoxia-induced delayed alveolar development, reduced alveolar diameter (MLI), and enhanced volume of alveolar intercept (RAC) were ameliorated by the over-expression of SEMA3A (Figure 4).

SEMA3A suppressed the activation of ERK/JNK pathway in BPD rats

The protein expressions of ERK and JNK were not significantly affected by hyperoxia treatment or AAV-SEMA3A injection (Figure 5). However, the expression levels of ERK and JNK phosphorylation were upregulated by hyperoxia treatment (Figure 5). Moreover, AAV-SEMA3A injection downregulated the expression levels of ERK and JNK phosphorylation (Figure 5), indicating that SEMA3A might suppress the activation of ERK/JNK pathway to attenuate progression of BPD.

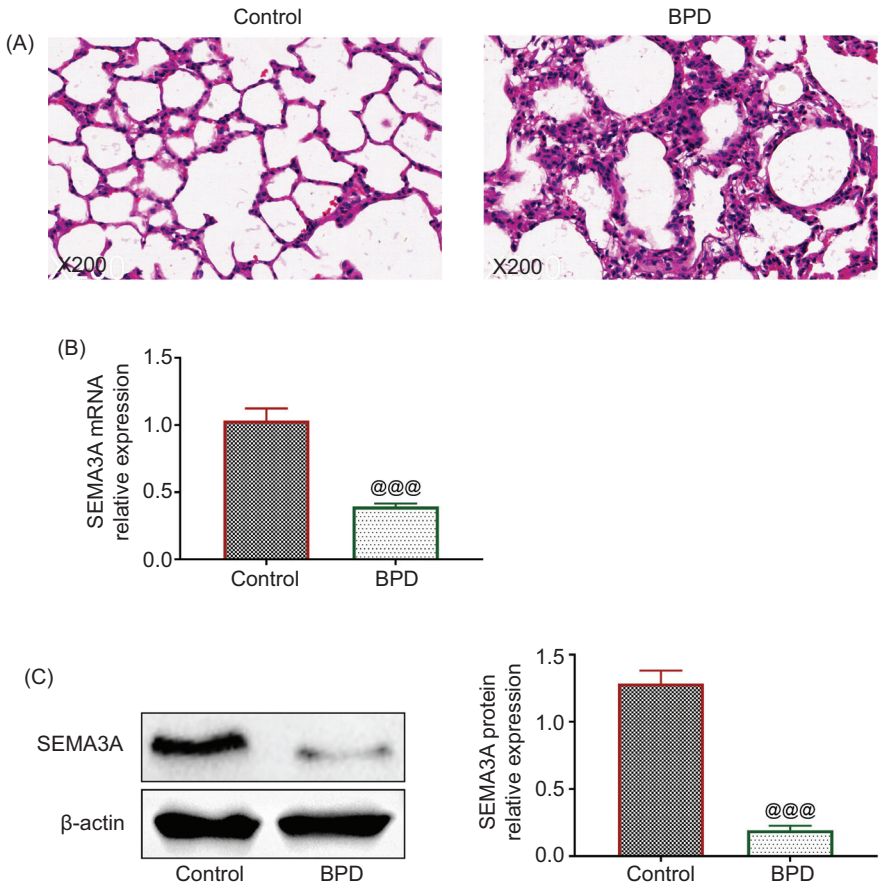

Figure 1 SEMA3A was downregulated in hyperoxia-induced BPD rats. **(A)** Hematoxylin-eosin staining established that hyperoxia induced severe lung injuries, including inflammatory cell infiltration, reduced alveolar number, and enhanced alveolar interval and alveolar volume in BPD rats. (B) The mRNA expression of SEMA3A was reduced in lung tissue isolated from hyperoxia-induced rats compared to the control group. (C) The protein expression of SEMA3A was reduced in lung tissue isolated from hyperoxia-induced rats compared to the control group. @@@ vs. control, P < 0.001. BPD: bronchopulmonary dysplasia; SEMA3A: semaphorin 3A.

Table 1 Basic characteristics of rats in each group.

Group	n	Body weight (g)	Lung weight (g)	Lung-to-body weight ratio (%)
Control	6	13.06 ± 1.14	0.29 ± 0.04	2.25 ± 0.24
BPD	6	10.42 ± 0.64**	0.17 ± 0.03**	1.59 ± 0.30*
BPD+AAV-Scramble	6	10.29 ± 0.54	0.16 ± 0.03	1.50 ± 0.22
BPD+AAV-SEMA3A	6	11.94 ± 0.93†	0.24 ± 0.03††	2.06 ± 0.33†

Values are presented as mean ± standard deviation (SD).
*P < 0.01 **P < 0.001 compared with the Control.
†P < 0.05 ††P < 0.01 compared with the BPD+AAV-Scramble.

Discussion

SEMA3 binds to neuropilin receptors and recruits plexin family to participate in the process of pulmonary vascular development, and the disruption of pulmonary vascular morphogenesis is implicated in the pathogenesis of pulmonary diseases in infancy, including BPD.[17] SEMA3C was reportedly reduced in the lungs of O_2-induced newborn rats, and treatment with SEMA3C attenuated hyperoxia-induced decreased viability of alveolar epithelial cells, promoted wound healing, and preserved the lung and alveolar vascular growth, thus ameliorating progression of BPD.[18] Considering the association between SEMA3A and alveolar septal cell death in neonatal mice,[15] the protective effect of SEMA3A against hyperoxia-induced BPD was investigated in this study.

Hyperoxia has been demonstrated to induce reduction in dynamic lung compliance and enhancement in airway resistance, thus impairing lung function.[5] Therefore, hyperoxia-induced oxygen toxicity was widely used to establish BPD model in newborn rats.[5] In this study, prevention of alveolarization, and simpler and wider alveoli formation, was observed in the lungs of hyperoxia-induced neonatal rats, thus demonstrating hyperoxia-induced lung injury in newborn rats. SEMA3A was identified to be downregulated in the lung tissue of hyperoxia-induced neonatal rats, suggesting that SEMA3A could be involved in the development of BPD. AAV-mediated over-expression of SEMA3A ameliorated hyperoxia-induced histopathological changes in rats

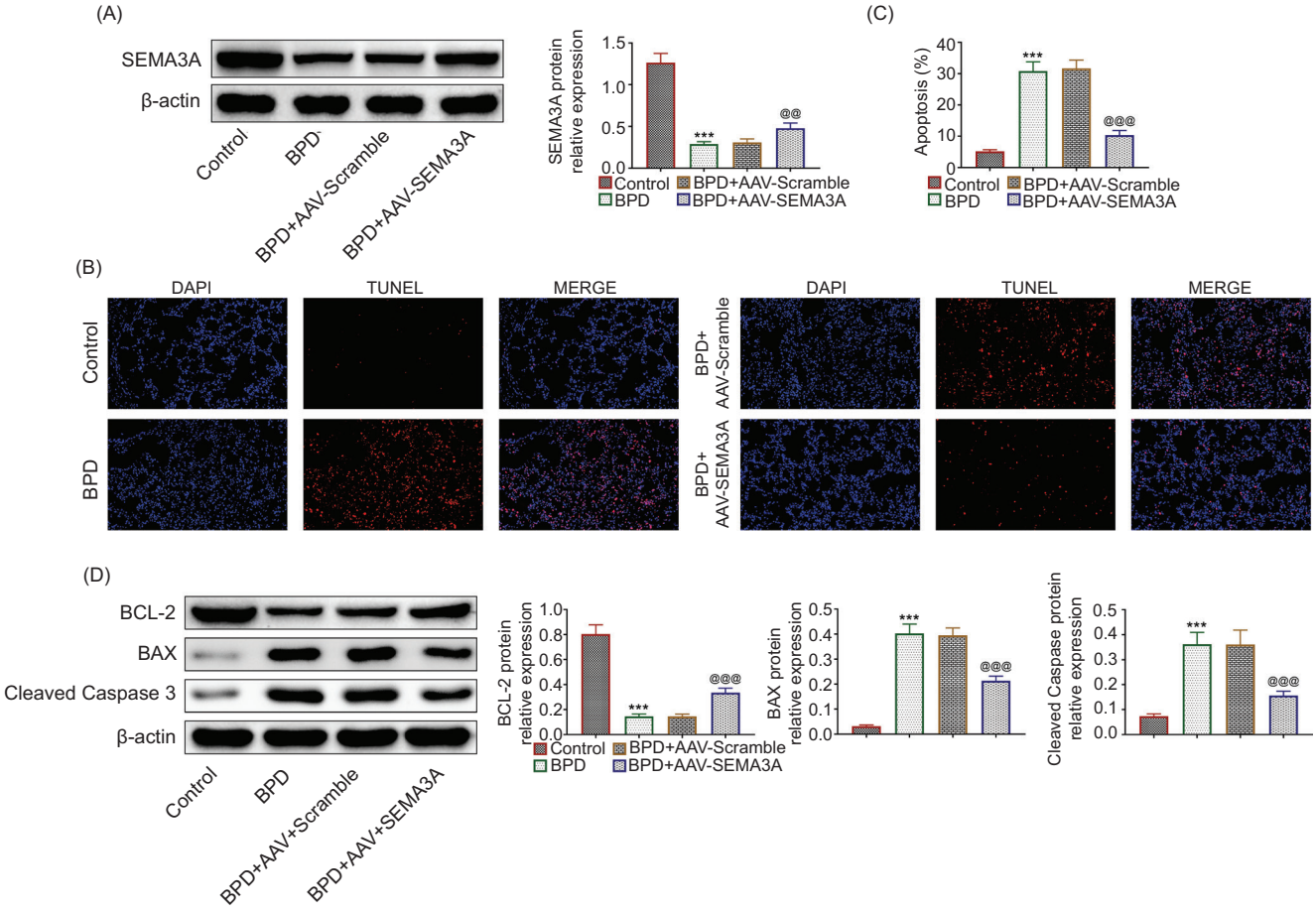

Figure 2 SEMA3A suppressed hyperoxia-induced cell apoptosis in BPD rats. (A) AAV-SEMA3A injection promoted the protein expression of SEMA3A. (B) AAV-SEMA3A injection attenuated hyperoxia-induced cell apoptosis in BPD rats. (C) AAV-SEMA3A injection attenuated hyperoxia-induced increased number of TUNEL-positive cells in BPD rats. (D) AAV-SEMA3A injection attenuated hyperoxia-induced reduced expression of Bcl-2, and enhanced expression levels of Bax and cleaved caspase-3 in BPD rats. *** vs. control, $P < 0.001$. @@, @@@ vs. BPD+AAV-Scramble, $P < 0.01$, $P < 0.001$. BPD: bronchopulmonary dysplasia; SEMA3A: semaphorin 3A.

by enhancing the count of RAC and reducing the volume of MLI, thereby confirming the protective effect of SEMA3A against BPD.

Apoptosis is a common manifestation of hyperoxia-induced lung injury.[19] Suppression of cell apoptosis is regarded as a potential therapeutic strategy for the treatment of hyperoxia-induced lung injury in BPD.[19] SEMA3A induced pro-apoptotic program in ischemia-reperfusion-induced acute kidney injury,[20] and repression of SEMA3A protected retinal ganglion cell against the optic nerve crush-induced apoptosis.[21] However, in this study, over-expression of SEMA3A decreased cell apoptosis through upregulation of Bcl-2 and downregulation of Bax and cleaved caspase-3 in hyperoxia-induced neonatal rats, indicating the anti-apoptotic role of SEMA3A in BPD. The pathway involved in SEMA3A-mediated cell apoptosis in hyperoxia-induced BPD must be investigated in the future research. Moreover, alveolar simplification and reduced pulmonary vessel density were considered as typical histological patterns of BPD.[18] A previous study has indicated that promotion of alveolar epithelial cell proliferation by SEMA3C attenuated hyperoxia-induced lung injury.[18] Therefore, the effect of SEMA3A on alveolar epithelial cell proliferation could be helpful to confirm its protective effect against BPD. Functions of airway epithelial cells are important for lung homeostasis, and differential functional activities of airway epithelial cells are implicated in different diseases, such as asthma[22] and BPD.[23] Environmental factors regulating epigenetic modifications are implicated in the pathogenesis of asthma.[24] Epigenetic modification of SEMA3A was involved in the progression of breast cancer.[25] The epigenetic modification of SEMA3A involved in the development of BPD must be investigated in the future research.

Inflammation is involved in the pathogenesis of BPD, and the anti-inflammatory effect of omega-3 fatty acids prevented hyperoxic lung injury in newborn rats.[26] Previous studies have demonstrated that SEMA3A reduced inflammation in mice with bronchial asthma[27] and autoimmune arthritis.[28] The present results have demonstrated that over-expression of SEMA3A attenuated hyperoxia-induced increased levels of IL-1β, MCP-1, and TNF-α in the lung tissue of BPD rats. Moreover, NF-κB pathway was reported to be involved in SEMA3A-mediated immune dysfunction during l*ipopolysaccharide* (LPS)-induced sepsis.[29] Enhanced

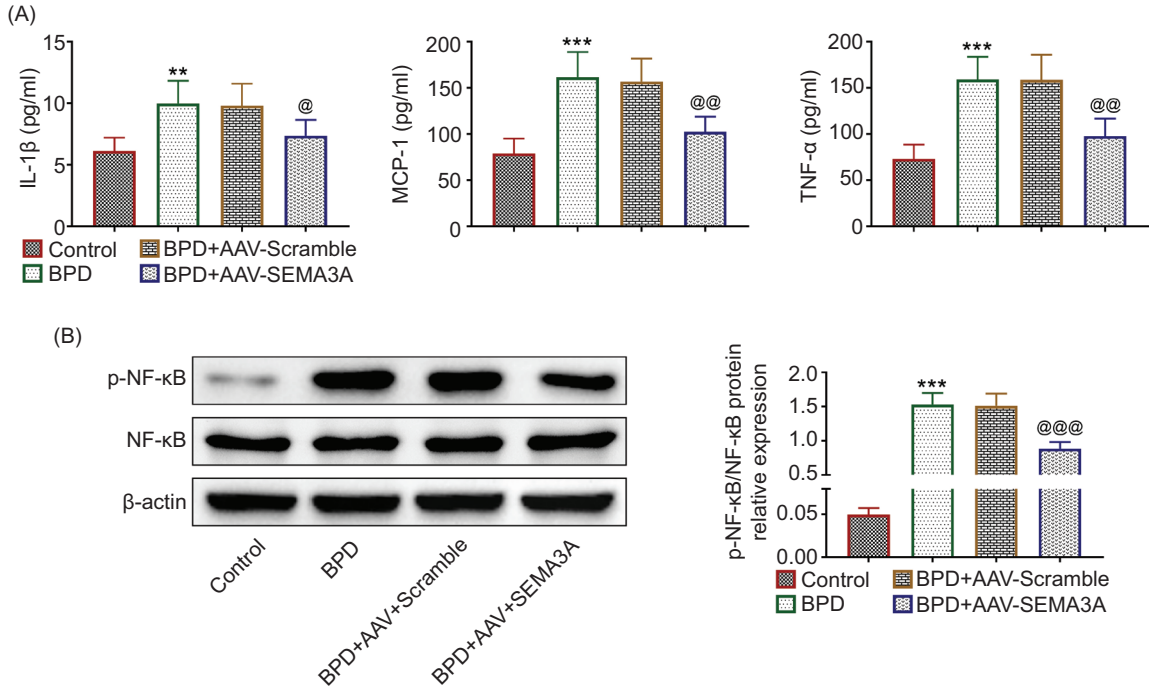

Figure 3 SEMA3A suppressed hyperoxia-induced inflammation in BPD rats. **(A)** AAV-SEMA3A injection attenuated hyperoxia-induced increased levels of IL-1β, MCP-1, and TNF-α. (B) AAV-SEMA3A injection attenuated hyperoxia-induced increased expression of NF-κB phosphorylation. ** vs. control, P < 0.001. @, @@, @@@ vs. BPD+AAV-Scramble, P < 0.05, P < 0.01, P < 0.001. IL-1β: interleukin-1β; MCP-1: monocyte chemoattractant protein-1; TNF-α: tumor necrosis factor alpha; SEMA3A: semaphorin 3A.

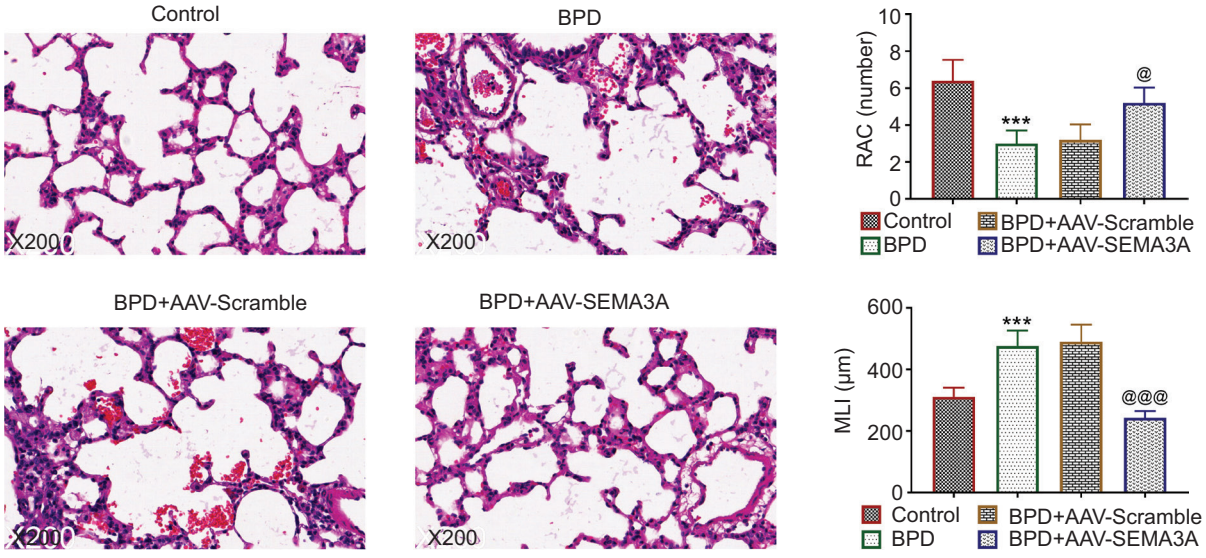

Figure 4 SEMA3A ameliorated hyperoxia-induced histopathological changes in BPD rats. AAV-SEMA3A injection ameliorated hyperoxia-induced alveolar injury, simplification, and inflammation, and attenuated hyperoxia-induced decreased number of RAC and increased volume of MLI. **, *** vs. control, P < 0.01, P < 0.001. @, @@@ vs. BPD+AAV-Scramble, P < 0.05, P < 0.001. RAC: radial alveolar count; MLI: mean linear intercept; SEMA3A: semaphorin 3A.

protein expression of NF-κB phosphorylation in hyperoxia-induced rats was reduced by the over-expression of SEMA3A, suggesting that SEMA3A suppressed hyperoxia-induced inflammation in BPD rats through inactivation of NF-κB pathway.

ERK pathway is important for the growth and survival of epithelial cells in response to hyperoxia.[30] ERK1/2 pathway was implicated in the proliferation and migration of lung fibroblasts in newborn rats,[31] and endothelial ERK2 was identified as a potential target for BPD in infants.[32] Inhibition of ERK1/2 pathway contributed to the prevention of tetrandrine in hyperoxia-induced rats.[5] ERK/JNK was known as a downstream signal pathway of SEMA3A, and SEMA3A suppressed the activation of ERK

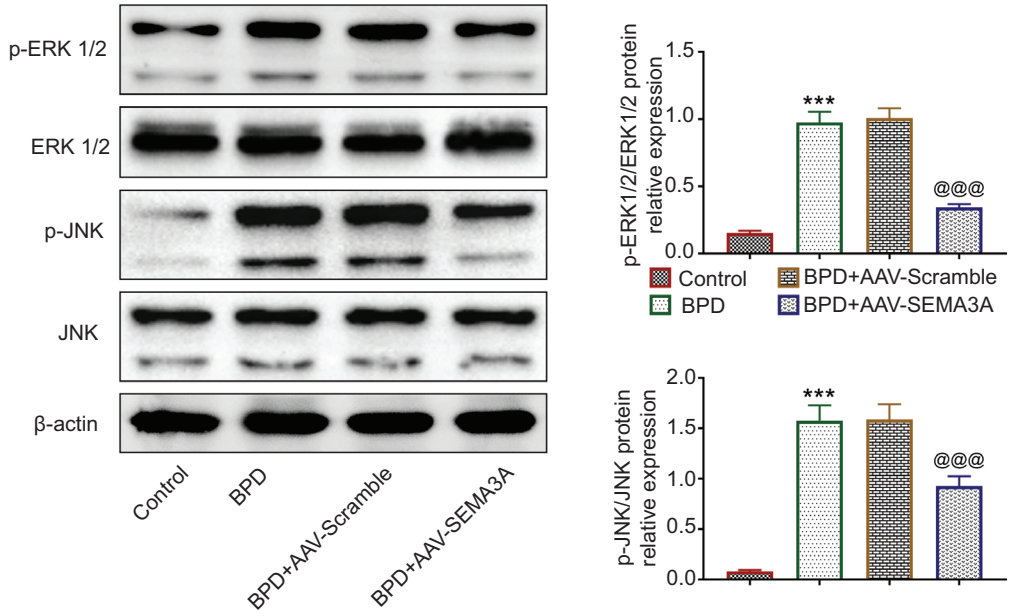

Figure 5 SEMA3A suppressed the activation of ERK/JNK pathway in BPD rats. AAV-SEMA3A injection attenuated hyperoxia-induced increased expression levels of ERK and JNK phosphorylation. *** vs. control, $P < 0.001$. @@@ vs. BPD+AAV-Scramble, $P < 0.001$. SEMA3A: semaphorin 3A.

to inhibit excessive mechanical stress-induced inflammation in chondrocytes.[13] Here, results established that SEMA3A counteracted with the promotive effect of hyperoxia on the phosphorylated protein expressions of ERK1/2 and JNK, indicating that SEMA3A suppressed the activation of ERK/JNK pathway in hyperoxia-induced BPD rats. Oxidative stress caused alveolar epithelial cell injuries and contributed to the development of BPD.[33] SEMA3A was involved in miR-203-3p-mediated oxidative stress of high glucose-induced mice podocytes.[34] Taken together, SEMA3A might exert an anti-oxidant effect in hyperoxia-induced BPD rats.

Conclusion

SEMA3A exerted anti-apoptotic and anti-inflammatory effects against hyperoxia-induced lung injury in neonatal rats through inhibition of NF-κB and ERK1/2 pathways. Our findings provided evidence that SEMA3A might be a potential target for BPD. However, the effect of SEMA3A on alveolar epithelial cell proliferation and oxidative stress, as well as the pathway involved in SEMA3A-mediated cell apoptosis in hyperoxia-induced BPD rats, must be investigated in the future research.

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