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Wnt3a Mediates the Inhibitory Effect of Hyperoxia on the Transdifferentiation of AECIIs to AECIs

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Summary
The aim of this study is to investigate the effect of Wnt3a in the transdifferentiation of type II alveolar epithelial cells (AECIIs) to type I alveolar epithelial cells (AECIs) under hyperoxia condition. In the in vivo study, preterm rats were exposed in hyperoxia for 21 days. In the in vitro study, primary rat AECIIs were subjected to a hyperoxia and normoxia exposure alternatively every 24 hr for 7 days. siRNA-mediated knockout of Wnt3a and exogenous Wnt3a were used to investigate the effect of Wnt3a on transdifferentiation of AECIIs to AECIs. Wnt5a-overexpressed AECIIs were also used to investigate whether Wnt3a could counteract the effect of Wnt5a. The results showed that hyperoxia induced alveolar damage in the lung of preterm born rats, as well as an increased expression of Wnt3a and nuclear accumulation of β-catenin. In addition, Wnt3a/β-catenin signaling was activated in isolated AECIIs after hyperoxia exposure. Wnt3a knockout blocked the inhibition of the transdifferentiation induced by hyperoxia, and Wnt3a addition exacerbated this inhibition. Furthermore, Wnt3a addition blocked the transdifferentiation-promoting effect of Wnt5a in hyperoxia-exposed Wnt5a-overexpressed AECIIs. In conclusion, our results demonstrate that the activated Wnt3a/β-catenin signal may be involved in the hyperoxia-induced inhibition of AECIIs' transdifferentiation to AECIs. (J Histochem Cytochem 63:879–891, 2015)

Keywords
Wnt3a, Wnt5a, type II alveolar epithelial cell, hyperoxia, preterm born

Premature newborns often suffer from hypoxemia and acute respiratory failure. Supplemental oxygen is one of the most treatments for preterm respiratory support. It has been reported that prolonged exposure to hyperoxia results in oxidative stress–induced tissue damage in the lung, such as acute lung injury and bronchopulmonary dysplasia (BPD) (Kugelman and Durand 2011). BPD is a clinical syndrome of chronic respiratory, which can lead to hypoxemic respiratory failure and death. Advance in mechanical ventilation increases the percentage of infants surviving delivery earlier in gestation but also results in a high morbidity of BPD (Merritt et al. 2009).

In animal models, retarded lung alveolization and differentiation of alveolar epithelial type II cells (AECIIs), fewer and larger alveoli, and enlarged airspace area were found in hyperoxia-exposed lungs (Dauger et al. 2003; Wang et al. 2005; Woyda et al. 2009). These damages were considered the result of the decreased proliferation of alveolar epithelium. Alveoli are lined by two morphologically and functionally different types of cells, type I alveolar epithelial cells (AECIs) and type II alveolar epithelial cells (AECIIs) (Crapo et al. 1982). AECIs cover 95% to 99% of the alveolar surface area and are responsible for gas, ions,

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and water exchange. AECIIs cover only 1% of the alveolar surface and produce surfactant. AECIs are highly vulnerable to oxidative stress damage. Importantly, they are mainly differentiated from AECIIs. Consequently, repair capacity of the damaged epithelium is mainly dependent on the ability of AECIIs to transdifferentiate to AECIs. However, it has been reported that newborn lungs that recovered from hyperoxia exposure displayed a significant reduction in the number of AECIIs, which suggested that hyperoxia might destroy AECIIs' proliferation and differentiation into AECIs (Yee et al. 2006). Thus, the exploration of the molecular pathogenesis of hyperoxia-induced lung injury would be beneficial in the treatment of this disease.

The Wnt family is composed of cysteine-rich secreted glycoproteins with varying expression patterns (Dale 1998) and exerts their function through at least three known pathways: the Wnt/β-catenin pathway, which is also known as the canonical pathway; the Wnt/Ca^{2+} pathway; and the Wnt/JNK pathway (planar cell polarity pathway) (Chien et al. 2009). The Wnt signaling pathway is essential to the development and diseases of the lung. Since the first evidence of Wnt expression in the lung was published (Gavin et al. 1990), a wide range of Wnts has been found expressed in lung tissue and cell lines, including Wnt 3a, 4, 5a, 7a, 7b, 10b, and 11 (Pongracz and Stockley 2006). Wnt3a expresses in the bronchial and alveolar epithelial cells and increases in fibrotic regions and hypertrophic alveolar epithelium (Aumiller et al. 2013). In addition, increased Wnt3a expression may contribute to the secretion of proinflammatory factors in epithelial cells via the Wnt/β-catenin pathway (Aumiller et al. 2013). Wnt5a locates in both the epithelium and mesenchyme in the lung and acts through the non-canonical pathway (Slusarski et al. 1997). Wnt5a may participate in the differentiation of AECIIs to AECIs and contribute to the alveolarization (Boucherat et al. 2007; Ghosh et al. 2013).

The counteractive effect of Wnt3a and Wnt5a has been reported in human dermal papilla cells (Kwack et al. 2013) and lipopolysaccharide-induced mouse primary microglia (Halleskog and Schulte 2013). However, the role of Wnt3a and Wnt5a in hyperoxia-induced lung diseases has not been studied. In the present study, hyperoxia-exposed preterm rats and primary AECIIs were used to investigate the role of Wnt3a in the differentiation of AECIIs to AECIs.

Materials and Methods

Animals

Sprague-Dawley rats were obtained from the Laboratory Animal Center of China Medical University (Shenyang, China). All animal experiments were performed in accordance with the guidelines of the ethics committee of China Medical University. Rats had free access to water and rodent laboratory chow in a facility with a controlled temperature (22C) on a 12/12-hr light/dark cycle.

Preterm Delivery

On day 21 of gestation, rats were anesthetized with ether and hysterectomized. The uterus horns, which contained the fetuses, were taken out and the pups were removed immediately. Pregnant rats were killed after hysterectomy. Rats that had normally delivered 1 to 3 days before the day of caesarean section were used as surrogate mothers.

Hyperoxia Exposure and Tissue Collection

A total of 80 rat pups were randomly assigned into one of two groups, 40 rats in each: normal control group (normoxia) and hyperoxia exposure group (hyperoxia). On the day of birth, pups in the hyperoxia group were placed in a hyperoxic chamber, in which the oxygen level was maintained at 90% and CO_2 level was maintained at less than 0.5%. Pups in the normoxia group were placed in a normal air chamber. The chambers were opened once a day for 1 hr to replace food and water. Surrogate mothers were rotated between two groups daily to avoid oxygen toxicity. Lungs of pups were collected at 1, 7, 14, and 21 days for Wnt 3a expression detection.

Cell Culture

Rat AECIIs were isolated and purified as previously described (Zhang et al. 2014). Briefly, neonatal rats were anesthetized with 10% chloral hydrate, and the lung tissue was removed immediately under sterile conditions. After being washed by 0.15 M NaCl, lungs were filled with 0.25% trypsin (Beyotime Institute of Biotechnology, Haimen, China) and 0.25% deoxyribonuclease I from bronchia and digested for 20 min at 37C. An equal quantity of DMEM (Gibco, Grand Island, NY) containing 10% FBS (HyClone, Logan, UT) and 0.25% DNase I was added to terminate digestion. Then, the lung was minced, filtered, and centrifuged (1000 rpm/min) at 4C for 5 min. The cell pellets were resuspended in 3 mL DMEM. The cell suspension was purified by discontinuous Percoll (Phamacia, Stockholm, Sweden) density gradient centrifugation. After being centrifuged at 1000 rpm/min, 4 °C for 20 min, the layer of 10% and the interface of 10% and 30% were collected and washed twice by DMEM. The isolated AECIIs were cultured in DMEM supplemented with 10% FBS, 4 mM glutamine, 1% penicillin/streptomycin, and 0.25 μM amphotericin B and grown at 37C in under 5 % CO_2, 95 % air. Immunofluorescence staining of surfactant protein (SP)–C was used to verify the AECIIs. The purity of AECIIs was nearly 60% to 70%.

Construction of Adenoviral Vectors and Transfection

Predesigned small interference RNA (siRNA) for Wnt3a and a scrambled sequence that does not lead to the specific degradation of any known cellular mRNA were obtained from Genechem (Shanghai, China). Wnt5a (Ad-GFP-Wnt5a) and control (Ad-GFP) adenoviruses containing green fluorescent protein were purchased from Genechem (Shanghai, China). Adenoviruses were applied to AECIIs for Wnt5a overexpression according to the manufacturer's instructions. The efficacy of infection was determined by fluorescence microscope examination and PCR and Western blotting analysis.

Hyperoxia Exposure

Normal AECIIs and Wnt5a-overexpressed AECIIs were randomly divided into two groups: normoxia and hyperoxia. Cells in the normoxia group were cultured in a normal environment, and cells in hyperoxia group were exposed to hyperoxia (95% O_2, 5% CO_2) or (21% O_2) normoxia alternatively every 24 hr at 37C (days 1, 3, 5, and 7: hyperoxia; days 2, 4, and 6: normoxia). The culture medium was replaced every 24 hr.

Lung Morphometric Analysis

Lungs samples were pressure fixed by instilling with 4% paraformaldehyde (PFA) at 18 cmH_2O pressure via a cannula inserted into the trachea (Hsia et al. 2010). PFA-saturated lungs were then postfixed in 4% PFA for 24 hr, embedded in paraffin, and cross-sectioned into 5-µm slices. Sections were stained with hematoxylin and eosin (Solarbio Science & Technology, Beijing, China) for morphometric analysis. Mean alveolar diameter (MAD) and mean alveolar intercept (MLI) were measured to quantify interalveolar distance according to the previous study (Bhaskaran et al. 2012).

Immunohistochemistry

The sections were dewaxed and boiled in 0.1 M sodium citrate buffer for 10 min using a microwave oven to perform the heating antigen retrieval. Then, the sections were immersed in 3% H_2O_2 for 15 min at room temperature to quench the endogenous peroxidase activity. After being blocked with goat serum, the sections were incubated with primary antibodies anti-Wnt3a (1:100, WL0199; Wanleibio, Shenyang, China) or isotype-IgG (1:100, A0716; Beyotime Institute of Biotechnology) at 4C overnight. After being washed in PBS, the biotinylated goat anti–rabbit serum (1:200, A0277; Beyotime Institute of Biotechnology) was applied and incubated at room temperature for 30 min. The staining was visualized by reaction with diaminobenzidine tetrahydrochloride (Beyotime Institute of Biotechnology) and observed under an optic microscope (DP73; Olympus, Tokyo, Japan).

Wnt3a Treatment and Immunofluorescence

To evaluate the effect of exogenous Wnt3a on AECIIs, cells were cultured in medium with or without 500 ng/ml recombinant rat Wnt3a (USCN Life Science, Wuhan, China) and exposed to hyperoxia as described above.

For immunofluorescence analysis, cells cultured on glass coverslips or dewaxed sections were fixed with 4% PFA for 15 min. After being treated with 0.5% Triton X-100 for 10 min, cells were blocked with goat serum (Solarbio Science & Technology, Beijing, China) at room temperature for 15 min and then incubated with primary SP-C antibodies (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) at 4C overnight. After being washed with PBS, cells were incubated in secondary antibody coupled to Cy3 (1:100; Beyotime Institute of Biotechnology) in the dark for 1 hr at room temperature. After being washed, cells were counterstained with 4′,6-diamidino-2-phenylindole (Wanleibio) and examined using a confocal system (FV1000S-SIM/IX81; Olympus).

RNA Isolation and Real-Time PCR

Total RNA was isolated from lung tissues or AECIIs using the RNA simple Total RNA Kit (Tiangen, Beijing, China) according to the manufacturer's protocol. The concentration and purity of RNA were determined by calculating the ratio of the absorbance at 260 and 280 nm. Equal amounts of RNA (1 µg) from each sample were reverse-transcribed with oligonucleotide primer in a volume of 20 µl to synthesize cDNA using super M-MLV (BioTeke, Beijing, China). Quantitative real-time PCR reactions were performed on 1 µl cDNA using the 2× Power Taq PCR Master Mix (BioTeke) and SYBR Green (Solarbio Science & Technology) on an Exicycler 96 real-time quantitative thermal block (Bioneer, Daejeon, Korea). Data were analyzed by $2^{-\Delta\Delta Ct}$, and β-actin was used in parallel as an internal control to normalize samples. The sequences of primer used are shown in Table 1.

Western Blotting Analysis

Total proteins of lung tissues and AECIIs were extracted using NP-40 supplemented with 1% phenylmethanesulfonyl fluoride (Beyotime Institute of Biotechnology). Nuclear and cytosolic proteins were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of Biotechnology) following the manufacturer's instruction. The concentration of protein was determined using a

Table 1. Primer Sequences for Real-Time PCR Analysis.

Gene	Primer sequence	
	Upstream	Downstream
Wnt3a	GAGTTTGCCGATGCCAGGGA	CGGAAGTCAGGCTGCGACCA
SP-C	GTCGTCGTGGTGATTGTAGGG	GAAGGTAGCGATGGTGTCTG
T1α	AGATAACGCAGGCGGTGAAAC	CAGGAAGAGGGATGGGGAACA
β-actin	GGAGATTACTGCCCTGGCTCCTAGC	GGCCGGACTCATCGTACTCCTGCTT

bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instruction. After being separated on 10% SDS-PAGE, target protein was wet-transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). After being blocked with 5% non-fat dry milk (w/v) in Tris-buffered saline with 0.1% Tween-20, the membranes were incubated with anti–SP-C (1:200) or anti-T1α (1:200) primary antibodies (Santa Cruz Biotechnology) at 4C overnight and then incubated with a horseradish peroxidase–conjugated secondary antibody (Beyotime Institute of Biotechnology) at room temperature for 1 hr. The blots were visualized with the ECL plus detection system (Wanleibio). The protein levels were semi-quantified by gray analysis using Gel-Pro-Analyzer (Media Cybernetics, Bethesda, MD). Cytoplasm proteins were standardized by β-actin, and nuclear proteins were standardized by Lamin A. All values were normalized by the control group.

Luciferase Reporter Assay

The activity of β-catenin was assessed using the firefly luciferase reporter assay. AECIIs grown for 24 hr in 12-well plates were transiently transfected with 1 μg/well of pTOP-Luc (β-catenin/Tcf4 responsive elements reporter plasmid) with 1 μg/well of *Renilla* luciferase control plasmid PRL-TK as an internal control reporter using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Five hours after transfection, transfection medium was replaced by normal medium and cultured in a normal or hyperoxia condition as described above. At indicated time points, cells were lysed and subjected to luciferase activity assays according to the protocol of the kit (Promega, Madison, WI). Luciferase activity was normalized with total protein concentrations of the cells.

Statistical Analysis

SPSS 19 software (SPSS, Inc., Chicago, IL) was used for statistical analysis, and data were presented as the mean ± standard deviation (SD). Data that changed with time were analyzed using repeated-measures analysis of variance (ANOVA). Differences in data at each time point between groups were determined by two-way ANOVA followed by Fisher's least significant difference tests.

Results

Hyperoxia Exposure Induces Alveolar Damage in Preterm Rats

Enlarged alveoli and decreased alveolar septa formation were observed in hyperoxia-exposed preterm born rats since day 7 (Fig. 1A), and the damage became more serious in the following days. The alveolar septa were thickened and the formation of secondary crests was reduced in hyperoxia-exposed lungs. The alveolar size was quantified by measuring MAD and MLI. No significant change was observed at days 1 and 7. At days 14 and 21, an almost 30% to 40% increase in MAD and 50% to 60% increase in MLI were found in the hyperoxia-exposed lungs compared with the control lungs, respectively (Fig. 1B,C).

Hyperoxia Exposure Increases Wnt3a Expression in the Lung of Preterm Born Rats

To determine whether expression of Wnt3a would change with hyperoxia exposure, real-time PCR, Western blotting, and immunohistochemistry (IHC) were used to detect the expression of Wnt3a in lung tissues of preterm born rats at 1, 7, 14, and 21 days of hyperoxia exposure (Fig. 2). Relative mRNA level of Wnt3a in the lung was significantly upregulated 1 day after hyperoxia exposure (Fig. 1A, $p<0.01$ compared with normoxia group) and followed by a higher expression in the following days. Results of Western blotting showed similar observations with PCR analysis (Fig. 2B). By IHC staining, Wnt3a immunoreactivity was found in lungs and increased with time in the hyperoxia group. Lungs of rats in the normoxia group were less stained, and no change was observed with time (Fig. 2D). In agreement with the results in Wnt3a, nuclear accumulation of β-catenin was significantly increased in the lung of hyperoxia exposure rats compared with normoxia exposure rats ($p<0.01$ at days 1, 7, 14, and 21; Fig. 2C).

Hyperoxia Exposure Inhibits the Differentiation of AECIIs to AECIs

To evaluate the effect of hyperoxia exposure on the differentiation of AECIIs to AECIs, the immunoactivities of SP-C

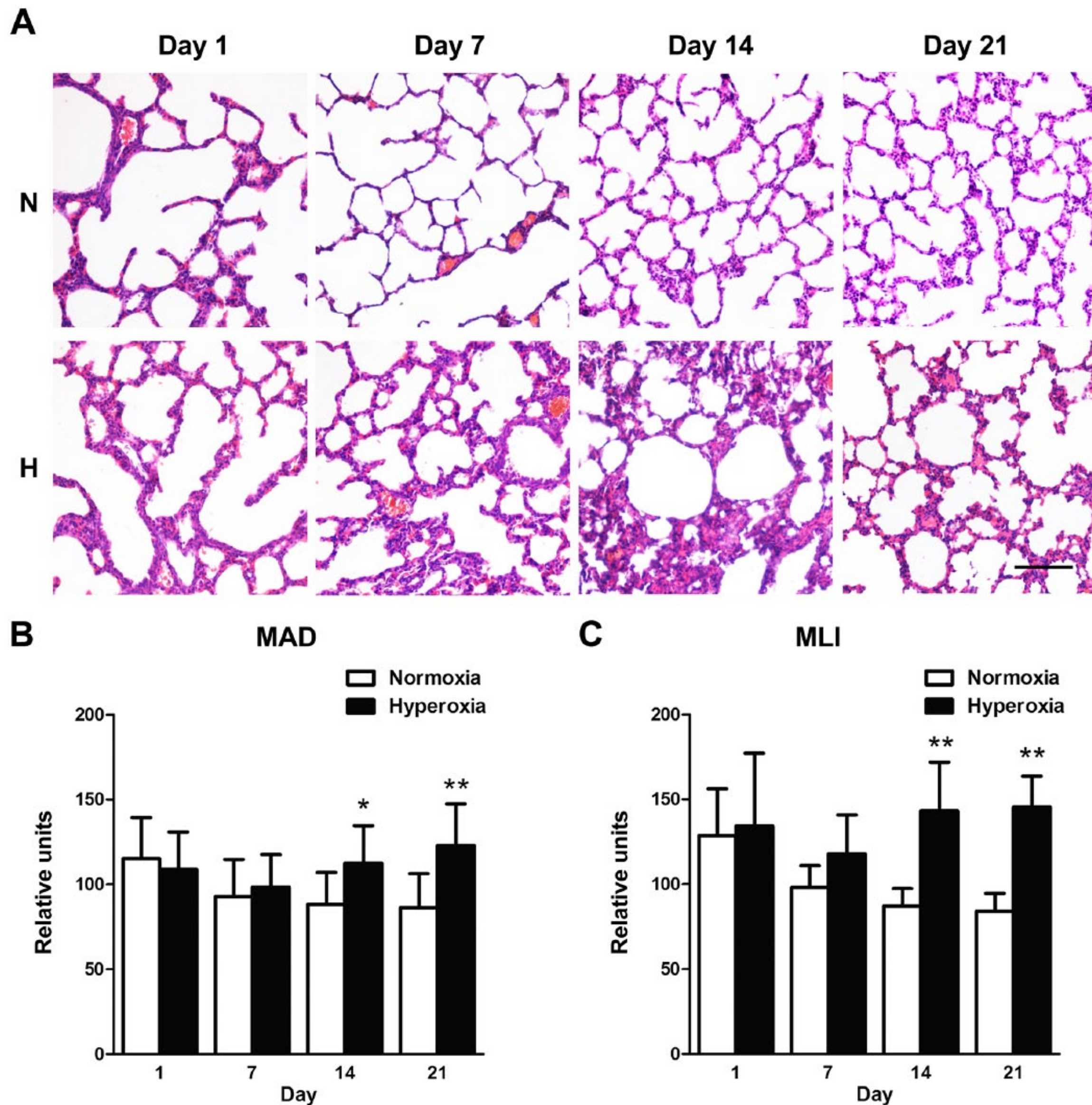

Figure 1. Hyperoxia exposure induces alveolar damage in preterm born rats. (A) Hematoxylin and eosin staining of lungs in preterm born rats exposed to hyperoxia or normoxia for 1, 7, 14, or 21 days. (B) Mean alveolar diameter (MAD). (C) Mean linear intercept (MLI). Data are expressed as the mean ± SD (n=5). *p<0.05 compared with normoxia group at the same day, **p<0.01 compared with normoxia group at the same day. Scale bar: 100 μm.

and T1α were detected by double immunofluorescence staining. As shown in Fig. 3, in normal lungs, SP-C expression was not obviously changed, but T1α expression was significantly upregulated, which indicated the transdifferentiation of AECIIs to AECIs. In the lungs of hyperoxia-exposed rats, SP-C expression was upregulated at days 14 and 21 compared with that in normal rats (p<0.05). In addition, expression of T1α was increased at day 1 (p<0.05), which is in agreement with a previous study (Hou et al. 2015), but was markedly diminished at days 7 and 14 compared with normal rats (p<0.01), which indicated that transdifferentiation was inhibited in the lungs of hyperoxia-exposed rats.

Hyperoxia Exposure Activates the Wnt3a/β-Catenin Pathway

SP-C is expressed solely by AECIIs and is used as an AECII marker (Wert et al. 1993; Mason et al. 2000). As shown in Supplemental Fig. S2, SP-C expression was detected in primary cultured AECIIs, which indicated successful cell isolation.

Because Wnt3a is known to act through the β-catenin pathway, we examined whether hyperoxia exposure activated this signaling pathway in primary rat AECIIs. Consistent with the result of the in vivo study, hyperoxia exposure induced a significant upregulation of Wnt3a

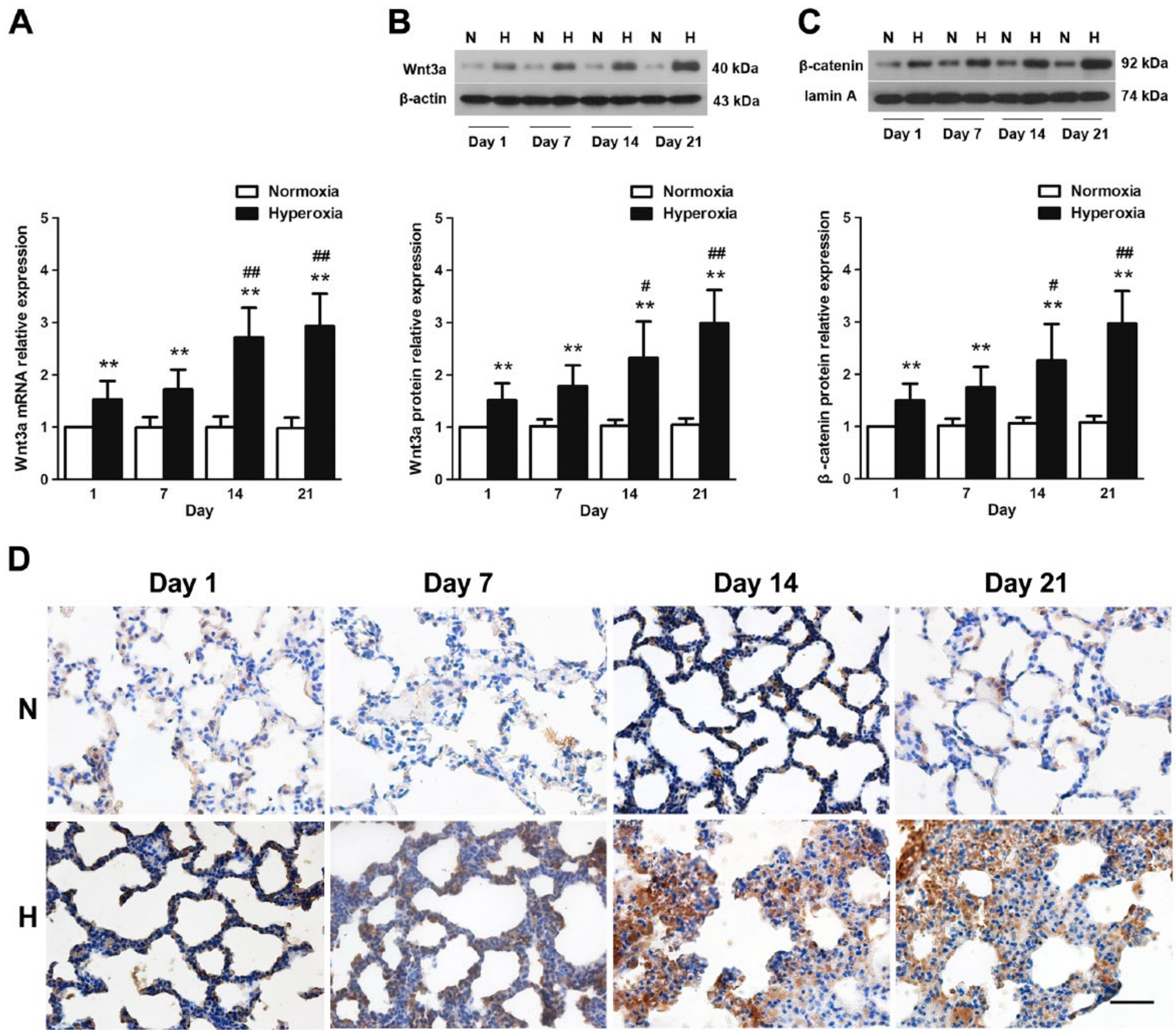

Figure 2. Hyperoxia upregulated Wnt3a expression in the lung of preterm rats. (A) Wnt3a mRNA expression in lung, (B) Wnt3a protein expression in lung, (C) β-catenin protein expression in the nucleoli of the lung, and (D) Wnt5a immunohistochemistry stain of lung sections. Data are expressed as the mean ± SD (n=5). $^{**}p<0.01$ compared with the normoxia group on the same day, $^{\#}p<0.05$ compared with the hyperoxia group at day 1, $^{\#\#}p<0.01$ compared with the hyperoxia group at day 1. Scale bar: 40 μm. N, normoxia-exposed rats; H, hyperoxia-exposed rats.

protein expression in primary AECIIs at day 1 compared with the control group, followed by a more significant increase over time (Fig. 4A). As expected, increased nuclear accumulation and decreased cytoplasmic expression of β-catenin were found in the hyperoxia group (Fig. 4B,C). Immunofluorescence staining showed that hyperoxia induced the nuclear translocation of β-catenin (Fig. 4E). In addition, the β-catenin/Tcf4 reporter assay results indicate hyperoxia exposure increased the reporter activity over time as well (Fig. 4D). These results indicate hyperoxia exposure activates the β-catenin pathway through upregulating Wnt3a expression.

Hyperoxia Exposure Inhibits the Transdifferentiation of AECIIs to AECIs

Expressions of AECII marker SP-C and AECI marker T1α were examined using Western blot analysis to evaluate the effect of hyperoxia on the transdifferentiation of AECIIs to AECIs in vitro. In the normoxia-exposed cells, expression of SP-C was decreased and T1α was increased over time after being isolated (Fig. 5), which indicated the transdifferentiation of AECIIs to AECIs. However, in the hyperoxia-exposed cells, expressions of SP-C and T1α remained unchanged during the experimental period,

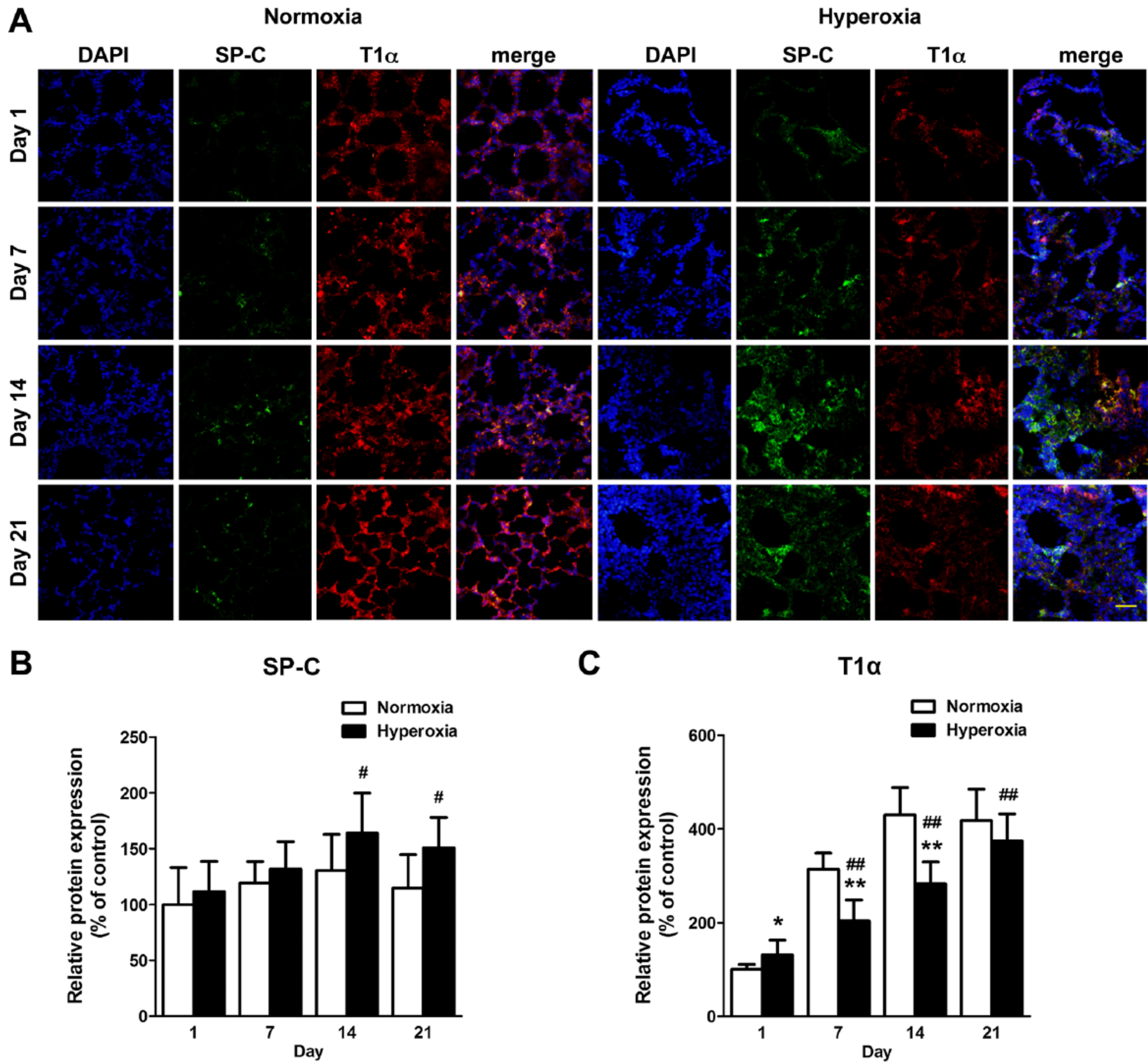

Figure 3. Hyperoxia inhibits transdifferentiation of type II alveolar epithelial cells (AECIIs) to type I alveolar epithelial cells (AECIs) in vivo. Expression of surfactant protein (SP)–C and T1α was detected using an immunofluorescence assay (A). Hyperoxia increased SP-C expression (B) at days 14 and 21, increased T1α expression at day 1, and decreased T1α expression at days 7 and 14. Data are expressed as the mean ± SD (*n*=5). $^{*}p<0.05$ compared with the normoxia group on the same day, $^{**}p<0.01$ compared with the normoxia group on the same day, $^{\#}p<0.05$ compared with the hyperoxia group at day 1, and $^{\#\#}p<0.01$ compared with the hyperoxia group at day 1. Scale bar: 40 µm.

which suggested that transdifferentiation was inhibited by hyperoxia exposure.

Wnt3a Gene Knockout Blocks the Hyperoxia-Induced Inhibition of the Transdifferentiation of AECIIs to AECIs

To investigate the role of Wnt3a in the transdifferentiation of AECIIs to AECIs, siRNA-mediated Wnt3a gene knockout AECIIs were established and the ability of transdifferentiation under hyperoxia was evaluated. As shown in Fig. 6A,B, the Wnt3a mRNA level was obviously diminished in Wnt3a knockout AECIIs compared to vector control AECIIs and parental AECIIs, and a significant decrease in Wnt3a protein expression was found in Wnt3a knockout AECIIs when detected by Western blotting analysis.

To avoid cell death, AECIIs were treated with hyperoxia and normoxia alternatively every 24 hr. At day 7,

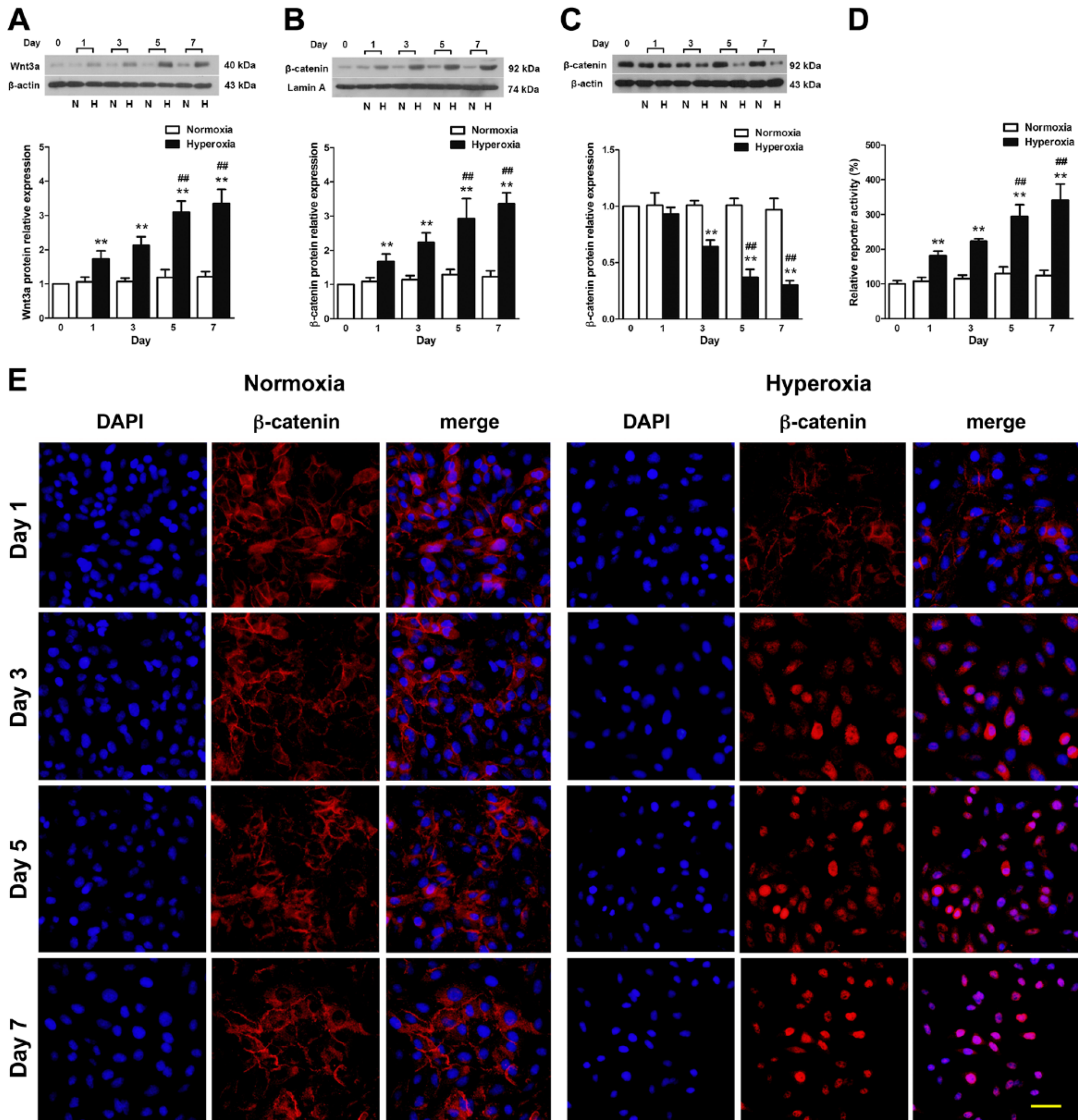

Figure 4. Hyperoxia activates Wnt3a/β-catenin signaling pathway in primary rat type II alveolar epithelial cells (AECIIs). Protein expression of Wnt3a in AECIIs (A) was upregulated after hyperoxia and normoxia alternative exposure every 24 hr for 7 days compared with control cells. β-Catenin expression of AECIIs was upregulated in the nucleolus (C) and downregulated in the cytoplasm (D). The β-catenin/Tcf reporter activity was also increased by hyperoxia exposure. (E) Immunofluorescence staining showed the nuclear translocation of β-catenin. Scale bar: 40 μm. Data are expressed as the mean ± SD (*n*=5). $^{**}p<0.01$ compared with the normoxia group on the same day, $^{\#\#}p<0.01$ compared with the hyperoxia group at day 1.

non-treatment or Ad-GFP control AECIIs showed loss of AECII marker SP-C and gain of AECI marker T1α under hyperoxia exposure, which indicated that transdifferentiation was prevented by hyperoxia. However, when transfected with siRNA-targeted Wnt3a, less SP-C and more T1α expressions were found under both normoxia and hyperoxia exposure (Fig. 6C–F), which indicated that siRNA-mediated Wnt3a knockout promoted the transdifferentiation of AECIIs to

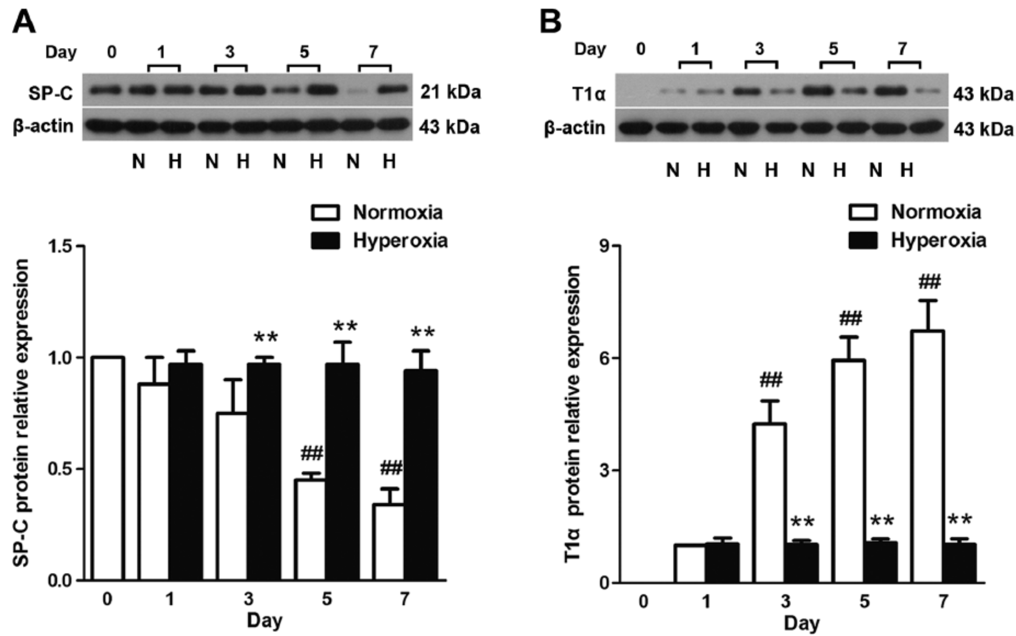

Figure 5. Hyperoxia exposure inhibited transdifferentiation of type II alveolar epithelial cells (AECIIs) to type I alveolar epithelial cells in vitro. Surfactant protein (SP)–C expression (A) was decreased and T1α expression (B) was increased in normal AECIIs after being isolated. Hyperoxia exposure inhibited these changes. Data are expressed as the mean ± SD (n=5). $^{**}p<0.01$ compared with the normoxia group on the same day, $^{\#\#}p<0.01$ compared with the normoxia group at day 1. N, normoxia-exposed rats; H, hyperoxia-exposed rats.

AECIs. This result indicates that the inhibitory effect of hyperoxia exposure on the transdifferentiation of AECIIs to AECIs may be mediated by Wnt3a.

Exogenous Wnt3a Counteracts the Effect of Wnt5a on the Transdifferentiation of AECIIs to AECIs after Hyperoxia Exposure

Wnt5a overexpression was found to promote the transdifferentiation of AECIIs to AECIs in our previous study (data not published). Here, whether Wnt3a counteracts this effect of Wnt5a in hyperoxia-exposed AECIIs was investigated. As shown in Fig. 7A,B, exogenous Wnt3a significantly decreased the Wnt5a protein expression in Wnt5a-overexpressed AECIIs. In addition, Wnt3a addition inhibited the transdifferentiation of AECIIs to AECIs, as represented by increased SP-C and decreased T1α intensity of immunofluorescence (Fig. 7C). In agreement with our previous findings, Wnt5a overexpression promoted the transdifferentiation of AECIIs to AECIs. However, Wnt3a addition inhibited the effect of Wnt5a and blocked the transdifferentiation.

Discussion

In this study, we have demonstrated that 1) hyperoxia exposure induced upregulation of Wnt3a expression and promoted β-catenin nuclear translocation in the lung of preterm rats, and 2) Wnt3a counteracted against Wnt5a and exacerbated the inhibition of the transdifferentiation induced by hyperoxia in AECIIs. To our knowledge, this is the first report to demonstrate that Wnt3a contributes to the inhibitory effect of hyperoxia in the transdifferentiation of AECIIs to AECIs.

Hyperoxia exposure was first found to result in BPD-like changes in newborn guinea pigs (Northway et al. 1969; Han et al. 1996) and was replicated in many species, including rodents. A hundred percent O_2 exposure for 7 days led to an initial phase of acute injury followed by a chronic repair phase in newborn mice (Bonikos et al. 1975). Sixty-eighty percent O_2 exposure caused a decrease in lung cell proliferation and durable impairments in lung structure (Warner et al. 1998; Dauger et al. 2003). AECIIs, which play a key role in lung repair, were also found to be impaired by hyperoxia exposure (Clement et al. 1992; Liu et al. 2013). Wnt family proteins are expressed widely in the lung and participate in pathophysiological processes of the lung. In the present study, Wnt3a, a member of Wnt family, was found to be overexpressed in hyperoxia-exposed lungs of preterm born rats. In addition, the increase of Wnt3a expression was in paralleled with the histological impair of lungs, which indicated the detrimental effects of Wnt3a overexpression in this pathological change.

Wnt3a was reported to participate in numerous pathophysiological processes through β-catenin signaling

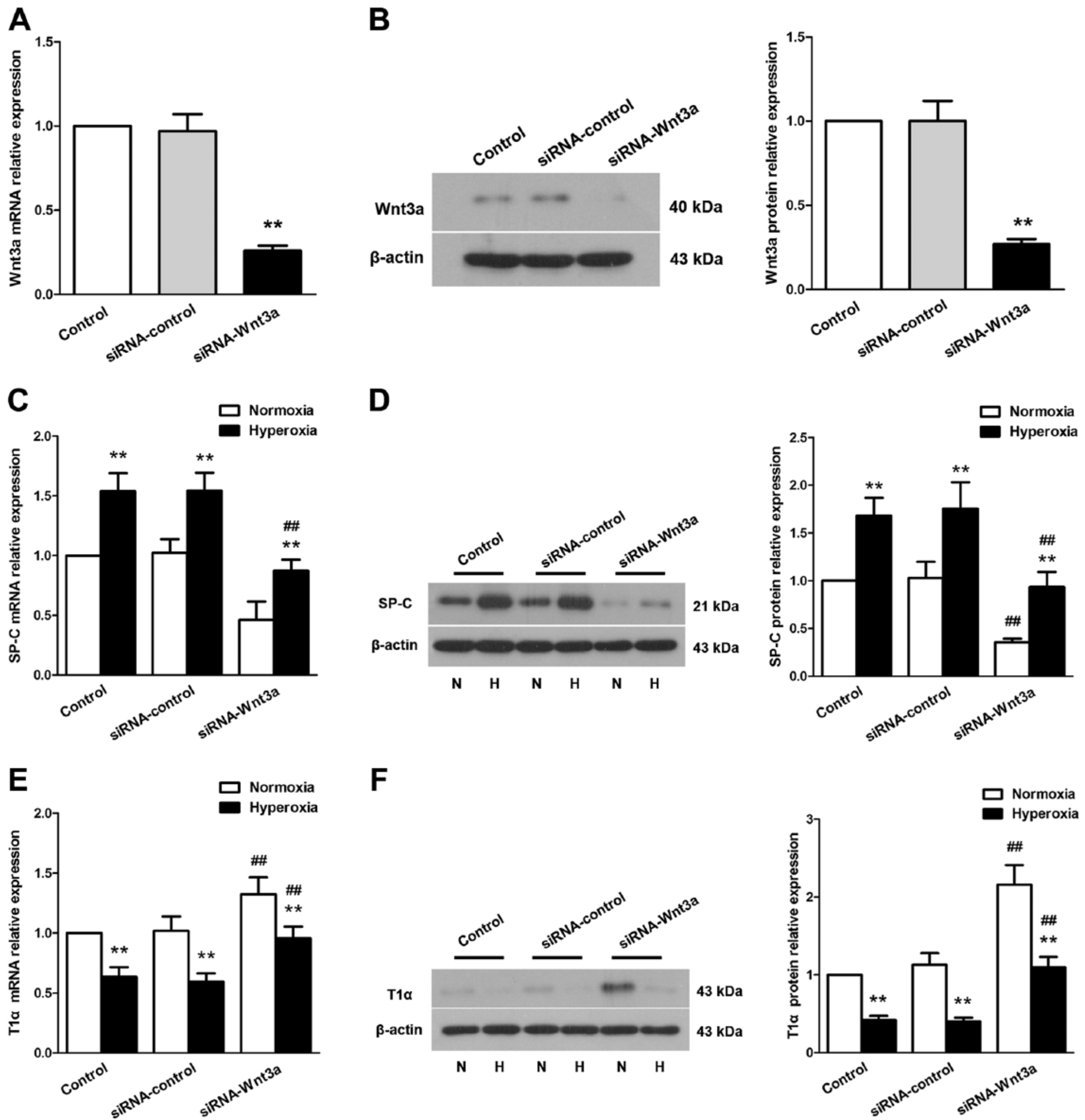

Figure 6. siRNA-mediated Wnt3a gene knockout promotes the transdifferentiation of type II alveolar epithelial cells (AECIIs) to type I alveolar epithelial cells. mRNA (A) and protein (B) levels of Wnt3a were significantly decreased by RNA interference in primary rat AECIIs. Data are expressed as the mean ± SD (*n*=5). ***p*<0.01 compared with siRNA control. Surfactant protein (SP)–C mRNA (C) and protein (D) expression were downregulated and T1α mRNA (E) and protein (F) expression were upregulated under both normoxia and hyperoxia exposure by siRNA-mediated Wnt3a gene knockout. Data are expressed as the mean ± SD (*n*=5). ***p*<0.01 compared with corresponding normoxia group, ##*p*<0.01 compared with siRNA control group. N, normoxia-exposed rats; H, hyperoxia-exposed rats.

pathway (Kaur et al. 2013). In normal state, β-catenin is phosphorylated by glycogen synthase kinase-3β (GSK-3β). When Wnt proteins bind to frizzled cell surface receptors and low-density lipoprotein cell surface coreceptors, GSK-3β is inhibited and β-catenin is dephosphorylated. Hypophosphorylated β-catenin is then translocated to the nucleus, where it binds to members of the LEF/TCF family of transcription factors. Binding of β-catenin activates LEF/TCF factors, thereby switching on cell-specific gene transcription. In the present in vitro study, hyperoxia exposure

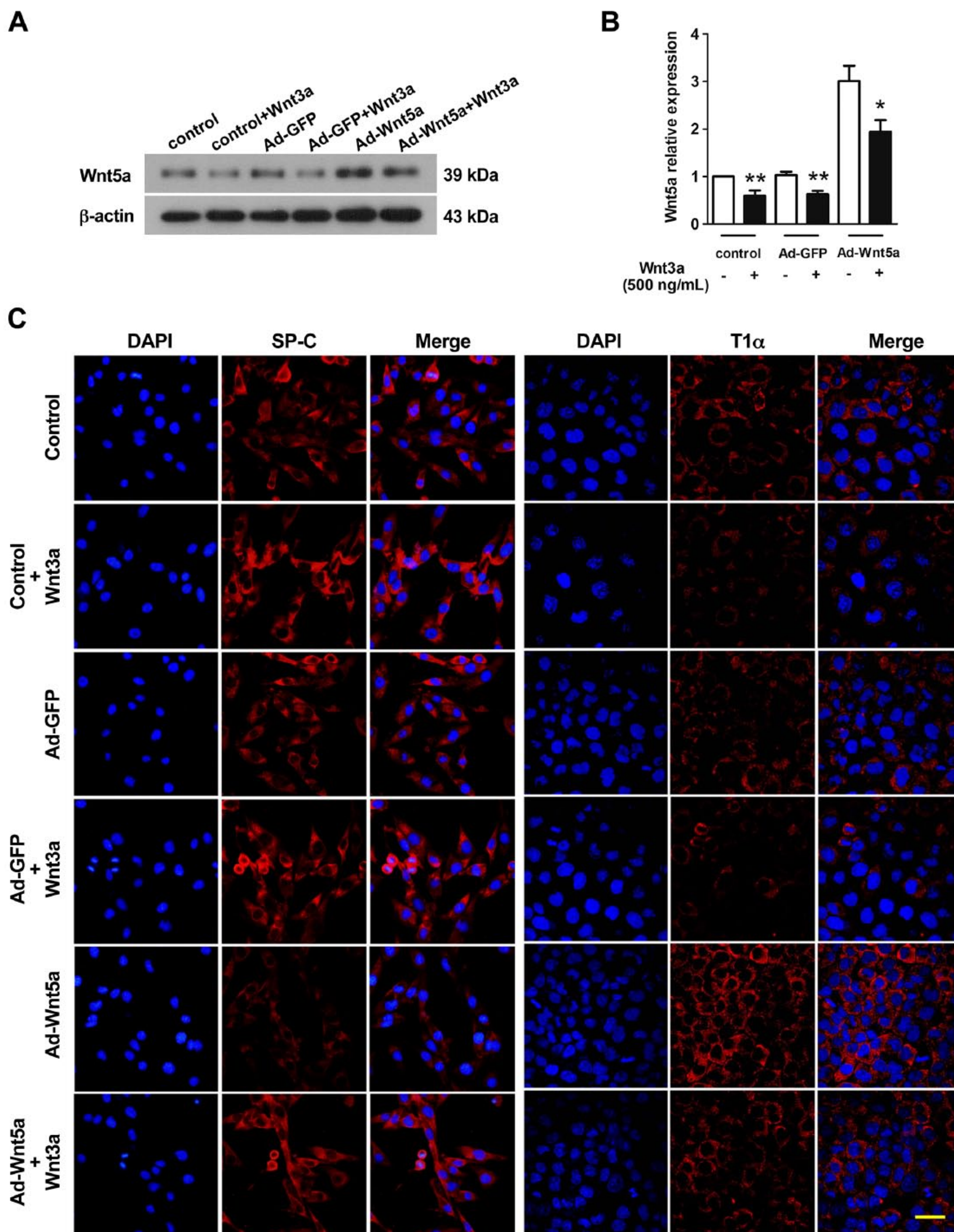

Figure 7. Exogenous Wnt3a counteracts the effect of Wnt5a on transdifferentiation of type II alveolar epithelial cells (AECIIs) to type I alveolar epithelial cells. (A, B) Wnt3a downregulated Wnt5a expression in Wnt5a-overexpressed AECIIs. (C) Immunofluorescence staining showed that Wnt5a overexpression downregulated surfactant protein (SP)–C expression and upregulated T1α expression, but Wnt3a addition blocked this effect. Scale bar: 40 μm. **p*<0.05 compared with corresponding non-wnt5a group,***p*<0.01 compared with corresponding non-wnt5a group.

increased expression of Wnt3a in AECIIs, promoted β-catenin nuclear translocation, and increased β-catenin/Tcf4 reporter activity. The results indicate that hyperoxia activates the Wnt3a/β-catenin signaling pathway in AECIIs, which may contribute to the hyperoxia-induced lung tissue damage.

SP-C is one of the surfactant-associated proteins that are essential for gas exchange and integrity of alveolar structure. It has been considered a biomarker of AECIIs because it is specifically produced by AECIIs (Akella and Deshpande 2013). T1α is the first molecular marker to be cloned and sequenced that is specific for the AECIs (Rishi et al. 1995). Although its molecular function is still unclear, T1α knockout mice die of respiratory failure shortly after birth and show aberrant lung structure (Williams 2003). When AECIIs transdifferentiate to AECIs, they will lose the features of AECIIs and gain characteristics of AECIs, which is represented by downregulation of SP-C and upregulation of T1α. In the present study, we found that hyperoxia exposure led to the inhibition of the transdifferentiation of AECIIs to AECIs both in vivo and in vitro. However, when the Wnt3a gene was knocked out, the inhibitory effect of hyperoxia on the transdifferentiation was prevented in vitro. These results suggest that Wnt3a may contribute to the AECII impairment by inhibiting the transdifferentiation under hyperoxia exposure. In contrast to Wnt3a, Wnt5a acts through the non-canonical pathway, and its expression promotes the transdifferentiation of AECIIs to AECIs (Ghosh et al. 2013). In accordance with our previous study (data not published), Wnt5a-overexpressed AECIIs showed resistance to hyperoxia. However, the addition of Wnt3a broke this resistance and inhibited the transdifferentiation. Although both Wnt3a and Wnt5a belong to the Wnt family, the functions of the two proteins are counteractive. Previous studies reported that Wnt3a and Wnt5a activate different, even opposing, signaling profiles in other cells such as human dermal papilla cells (Kwack et al. 2013), hematopoietic stem cells (Nemeth et al. 2007), microglia (Halleskog and Schulte 2013), and several cell lines (Topol et al. 2003). In agreement with these observations, we found that Wnt3a and Wnt5a counteracted the hyperoxia-induced inhibition of transdifferentiation of AECIIs to AECIs in primary AECIIs. Our finding not only supports the previous theory but also reveals the possible pathological mechanisms of hyperoxia-induced lung injury. It needs to be noted that lung branching morphogenesis is regulated by multiple signaling pathways, including the Wnt signaling pathway (De Langhe and Reynolds 2008). β-Catenin is the central signaling mediator of canonical Wnt signaling, which has been found to proximalize the epithelium by expanding the proximal airway and inhibiting distal airway growth (Mucenski et al. 2003). However, in the present study, Wnt3a/β-catenin activation has been demonstrated to be detrimental to development of the lung in preterm born rats. This discrepancy may be due to the hyperoxia exposure in our study. Namely, this signaling pathway may exert a two-way regulating function in the development of lung, which is dependent on whether the lung tissue has a normal or pathological condition.

It should be noted that the purity of the primary AECII culture in the present study was not 100%, and there was some mesenchymal/fibroblast cells contamination. The Wnt/β-catenin signal should also be activated in these cells under hyperoxia exposure. However, because the β-catenin nuclear translocation was observed in all cells in the visual field, AECIIs should be included. Therefore, although we could not exclude the signal changes from other cells in this study, the hyperoxia-induced β-catenin nuclear translocation in AECIIs was definite.

In conclusion, the present study demonstrates that the inhibitory effect of hyperoxia exposure on the transdifferentiation of AECIIs to AECIs is mediated by Wnt3a. Inhibiting the overexpressed Wnt3a in hyperoxia may be an option for therapy of hyperoxia-induced lung injury.

Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions

WX and CL designed the study. WX and YZ wrote the manuscript. YZ, BZ, BX, YY, and YW performed the experiments and collected the data. YZ and BZ analyzed the data. All authors have read and approved the final manuscript.

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