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Hyperoxia stimulates the transdifferentiation of type II alveolar epithelial cells in newborn rats

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Hou A, Fu J, Yang H, Zhu Y, Pan Y, Xu S, Xue X. Hyperoxia stimulates the transdifferentiation of type II alveolar epithelial cells in newborn rats. Am J Physiol Lung Cell Mol Physiol 308: L861–L872, 2015. First published February 13, 2015; doi:10.1152/ajplung.00099.2014.—Supplemental oxygen treatment in preterm infants may cause bronchopulmonary dysplasia (BPD), which is characterized by alveolar simplification and vascular disorganization. Despite type II alveolar epithelial cell (AEC II) damage being reported previously, we found no decrease in the AEC II-specific marker, surfactant protein C (SP-C), in the BPD model in our previous study. We thus speculated that AEC II injury is not a unique mechanism of BPD-related pulmonary epithelial repair dysfunction and that abnormal transdifferentiation can exist. Newborn rats were randomly assigned to model (85% oxygen inhalation) and control groups (room air inhalation). Expressions of AEC I (aquaporin 5, T1α) and AEC II markers (SP-C, SP-B) were detected at three levels: 1) in intact lung tissue, 2) in AEC II isolated from rats in the two groups, and 3) in AEC II isolated from newborn rats, which were further cultured under either hyperoxic or normoxic conditions. In the model group, increased AEC I was observed at both the tissue and cell level, and markedly increased transdifferentiation was observed by immunofluorescent double staining. Transmission electron microscopy revealed morphological changes in alveolar epithelium such as damaged AECs, a fused air-blood barrier structure, and opened tight junctions in the model group. These findings indicate that transdifferentiation of AECs is not suppressed but rather is increased under hyperoxic treatment by compensation; however, such repair during injury cannot offset pulmonary epithelial air exchange and barrier dysfunction caused by structural damage to AECs.

transdifferentiation; alveolar epithelial cells; hyperoxia; bronchopulmonary dysplasia

SUPPLEMENTAL OXYGEN TREATMENT, which is often necessary for preterm infants with respiratory failure, has become a major risk factor for bronchopulmonary dysplasia (BPD), one of the most common, serious complications in preterm infants (6, 34). BPD is characterized by high morbidity in infants with very low birth weight (BW < 1,500 g) soon after birth and may induce multiple complications in respiratory and nervous systems during adolescence (4, 6). Despite several decades of research, the underlying pathophysiological foundation of BPD has not been completely clarified.

Lung tissue consists of many different cell types, and a developmental disorder of alveolar epithelial cells (AEC) is likely a major cause of BPD (40). The alveolar area accounts for ≥99% of the internal surface area of the lungs (49). Mammals have two types of AECs that maintain normal air exchange function by mutual coordination. Type I AEC (AEC I) cover nearly 96% of alveolar spaces and closely adhere to adjacent capillaries. Such cells are the main epithelial constituents of the air-blood barrier (18, 53) and have air exchange functions. Recent studies have revealed that AEC I can also secrete transport proteins to maintain intrapulmonary fluid and electrolyte balance (9, 19, 22). Type II AEC (AEC II) are located at the corners of alveoli and are more abundant than AEC I but only cover 4% of alveolar spaces (18). AEC II are pulmonary epithelial progenitor cells and have many functions, such as surfactant synthesis and secretion (57), intrapulmonary fluid and ion transport (22), and immune support (29). AEC II can proliferate into new AEC II and transdifferentiate into AEC I (1, 24). Following lung injury, AEC II-to-AEC I transdifferentiation is an important mechanism in injury repair (16, 24, 39). Tight junctions between epithelial cells regulate diffusion of solutes through the paracellular pathway (47, 61).

AEC II injury is considered the key mechanism in BPD-related pulmonary epithelial injury (46, 54). Many studies have found necrosis and apoptosis, DNA injury, and oxidative stress injury of AEC II in BPD (15, 41, 42). However, our preliminary studies revealed that levels of the AEC II-specific marker surfactant protein C (SP-C) increased rather than decreased (58) in BPD, indicating no functional harm to AEC II, similar to the results reported by Yee et al. (60). This suggests that AEC II injury is not a unique mechanism of BPD-related pulmonary epithelial repair dysfunction. Regarding the possibility of abnormal transdifferentiation of AEC, previous in vitro studies of cell culture models of BPD have shown increased AEC transdifferentiation in a hyperoxic environment (13, 39), which indicated a possible mechanism of injury repair. However, the cell response to injury does not completely reflect changes in intact lungs (40). Studies in clinical patients with BPD and animal models have demonstrated abnormal expression of some cytokines, such as insulin-like growth factor-I (IGF-I) (30, 38), transforming growth factor (TGF)-β1 (3, 37, 52), keratinocyte growth factor (KGF) (20, 25), and bone morphogenetic protein (BMP) (3), which play regulatory roles in the AEC transdifferentiation process (7, 10, 26, 44, 63). These data support our hypothesis that abnormal
transdifferentiation may be involved in BPD pulmonary epithelial injury.  
Therefore, the primary objectives of this study were to determine whether abnormal AEC transdifferentiation is present in pulmonary epithelial pathological changes with hyperoxic treatment, whether changes in vivo are consistent with changes seen in vitro, and in what ways such changed transdifferentiation influences pulmonary epithelial function.

**MATERIALS AND METHODS**

**Animals and hyperoxic exposure protocol.** All animal procedures were reviewed and approved by the Laboratory Animal Ethics Committee of China Medical University. Pregnant Wistar rats (200–250 g) were purchased from the Animal Lab, Experimental Research Center, Shengjing Hospital, China Medical University, and the BPD model for the present study has been previously described (51, 61). In brief, term pups of pregnant Wistar rats were randomly marked and assigned to two groups: model group rats were exposed to 85% oxygen in a sealed Plexiglas chamber for 1–21 days beginning on the day of birth, and the control group rats were exposed to room air. The oxygen concentration was continuously monitored using a strip-chart recorder (model 572; Servomex, Norwood, MA).

**Lung sample collection and lung histology.** Pups were killed, and lungs harvested at the end of 1, 3, 7, 14, and 21 days of exposure as described previously (58, 61). Briefly, lung samples from the right middle lobe were inflated at 18 cmH₂O pressure with PBS and immersed in 4% paraformaldehyde (PFA) at 4°C, overnight. Lung tissue was dehydrated with graded alcohol and then embedded in paraffin. Sections (4 μm) were cut for hematoxylin and eosin staining and also for immunohistochemistry staining. By drawing a perpendicular line from the center of the most peripheral bronchiole to the pleura or the nearest interlobular septum, the number of alveoli transected by this line was defined as the radial alveolar count (RAC), an important index used to evaluate the stage of lung development (32).

**Immunohistochemistry and double immunofluorescence staining.** Paraffin-embedded tissue was used in immunohistochemistry staining for α-SMA, using mouse anti-α-SMA (1:100; Abcam, New Territories, Hong Kong), as previously described (62). A subsequent morphometric study was performed to measure the optical density of the α-SMA stain in 10 random fields using an image analyzer (NIS-Elements BR 3.0 software; Nikon Corp, Tokyo, Japan). Lung tissue double immunofluorescence staining was performed on frozen sections for immunofluorescent double staining, PFA-fixed tissues were immersed in 30% sucrose for 12 h at 4°C and then frozen at −80°C. The right upper lung lobes were excised and weighed to determine the wet lung weight. After being dried in an oven at 80°C for 48 h, lung tissues were weighed again to yield a final dry weight. Finally, the pulmonary wet/dry (W/D) ratio was calculated to determine the presence of pulmonary edema (61).

**Immunoblotting and double immunofluorescence staining.** Western blot analysis. Immunoblotting was performed as described previously (27, 61). Briefly, equal amounts of protein from extracts were mixed with sample loading buffer. After being heated, proteins were electrophoresed on 12% precast SDS-polyacrylamide gels (70 V for 90 min) and transferred to polyvinylidene difluoride (PVDF) membranes (70 V for 90 min). Membranes were blocked with 5% (wt/vol) nonfat milk in TBS-T [100 mM Tris base (pH 7.5), 0.9% (wt/vol) NaCl, 0.1% (vol/vol) Tween 20] for 1 h at room temperature. PVDF membranes were then incubated overnight at 4°C with primary antibodies: goat anti-AQP5 (1:500, Santa Cruz Biotechnology), rabbit anti-Podoplanin (T10; 1:1,000, Abcam), rabbit anti-SP-C (1:500, Santa Cruz Biotechnology) and donkey anti-goat IgG (H&L) Affinity Pure donkey anti-rabbit IgG (H+L) (1:100; EarthOx, San Francisco, CA), polyclonal rabbit anti-SP-C (1:50; Santa Cruz Biotechnology, Santa Cruz, CA), donkey anti-goat IgG (H+L) Affinity Pure, DyLight594 Conjugate (1:100; ImmunoReagents, Raleigh, NC), and polyclonal goat anti-aquaporin 5 (AQP5; 1:100, Santa Cruz Biotechnology). Negative controls included the substitution of primary antibodies with PBS. DAPI (Sigma-Aldrich, St. Louis, MO) was used for counterstaining nuclei. Double immunofluorescence imaging was observed using a confocal laser-scanning microscope (C1, Nikon). Ten randomly selected microscopic fields of lung tissue in each group were used to calculate the percentage of AQP5-positive cells/total cells, SP-C-positive cells/total cells, and AQP5-SP-C double-stained cells/SP-C-positive cells.

**AEC II isolation and culture.** AEC II were isolated from newborn rats within 24 h after birth by a modified method as previously described (10, 33, 36). In brief, newborn rat lungs were perfused, rinsed, minced, digested with 0.25% Trypsin-EDTA (Gibco Life Technologies, Grand Island, NY), filtered through a 200-μm mesh cell strainer, and digested with 0.1% collagenase I (Gibco Life Technologies). After centrifugation, the cells were resuspended and purified by differential adherence steps: cells were plated 3 × 50 min to remove fibroblasts and unattached cells and transferred to rat IgG-coated plates to remove macrophages. Cells were then seeded onto tissue culture-treated polycarbonate filter inserts at a density of 2–3 × 10⁶/ml in minimum essential medium with 10% FBS. The purity of AEC II was above 90% as assessed by immunofluorescence staining of SP-C, and the viability was above 95% as determined by Trypan blue staining. The medium was changed after the first 24 h, and cells were randomly divided into two groups, the model and control groups, followed by culturing for 48 h in an 85% O₂–5% CO₂ incubator (CB150; BINDER, Tuttlingen, Germany) or 21% O₂–5% CO₂ incubator (3111; Thermo Fisher Scientific, Marietta, OH).

To isolate AEC II from rats of the model and control groups at different time points, animals exposed to normoxia or hyperoxia for 7, 14, and 21 days were killed, and AEC II were isolated as described above.

**Western blot analysis.** Immunoblotting was performed as described previously (27, 61). Briefly, equal amounts of protein from extracts were mixed with sample loading buffer. After being heated, proteins were electrophoresed on 12% precast SDS-polyacrylamide gels (70 V for 90 min) and transferred to polyvinylidene difluoride (PVDF) membranes (70 V for 90 min). Membranes were blocked with 5% (wt/vol) nonfat milk in TBS-T [100 mM Tris base (pH 7.5), 0.9% (wt/vol) NaCl, 0.1% (vol/vol) Tween 20] for 1 h at room temperature. PVDF membranes were then incubated overnight at 4°C with primary antibodies: goat anti-AQP5 (1:500, Santa Cruz Biotechnology), rabbit anti-Podoplanin (T10; 1:1,000, Abcam), rabbit anti-SP-C (1:500, Santa Cruz Biotechnology), mouse anti-PCNA (1:1,000, Abcam), rabbit anti-cleaved caspase 3 (1:1,000; Cell Signaling, Beverly, MA), mouse anti-α-SMA (1:1,000, Abcam), mouse anti-N-cadherin (N-cad; 1:1,000, Abcam), or rabbit anti-β-actin (1:1,000, Sigma-Aldrich). After being washed three times in TBS-T, membranes were incubated at room temperature for 90 min with horseradish peroxidase-conjugated secondary antibody (Cell Signaling, Beverly, MA). After being washed, membranes were treated with a chemiluminescence ECL kit (Santa Cruz Biotechnology) and analyzed with an Alpha Ease RFC Imaging System (Alpha Innotech, San Leandro, CA).

**Quantitative real-time PCR.** Quantitative changes in mRNA expression of genes encoding AEC markers were assessed by quantitative real-time PCR as described previously (28). Total RNA was extracted from lungs and cells using Trizol reagents (Invitrogen, Camarillo, CA), and cDNA was reverse-transcribed from 1 μg of each sample (SuperScript III, Invitrogen). Real-time quantitative PCR was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Gene-specific primers for real-time PCR were designed and synthesized by TaKaRa (Shiga, Japan) Primers used were as follows: AQP5, forward: 5'-CTC CGA GCT GTC TTC TAC GTG-3', reverse: 5'-CAG GCG TTG TGT TGT TTG TC-3'; T1α, forward: 5'-GCG AGG TGC TAG AAA CCA AGA AC-3', reverse: 5'-GCC GGT GAC GAA CCA AGA AC-3'; SP-C, forward: 5'-GTT AGC AAA GAG GTA CTG ATG G-3', reverse: 5'-CAC CAC GAC GAC AAG GAC TA-3'; SP-B, forward: 5'-AAT GAC GTC TGC
CAAGAGTGTG-3', reverse: 5'-AGG ACC AGC TTG TAC AGC AGA G-3'; α-SMA, forward: 5'-CTT GCT AAC GGA GGC G-3', reverse: 5'-TCC AGA GTC CAG CAC AAT A-3'; N-cad, forward: 5'-GAC CCA GAA GAT GAT GTA AG-3', reverse: 5'-CTC AGC GTG GAT AGG C-3'. β-actin was used as an internal control, forward: 5'-ACC GTG AAA GAT GAT GTA AG-3', reverse: 5'-CAG TGG TAC GAC CAG AGG CAT A-3'. Dissociation curve analysis was performed for each gene to ensure the specificity of PCR products. Relative changes in target gene/β-actin mRNA ratios were determined by the power of 2$^\Delta\Delta C_T$.

Transmission electron microscopy. To compare morphological changes occurring with time in lungs of the model and control groups, representative tissues were examined by transmission electron microscopy (TEM) as previously described (61). Specimens were fixed in 2.5% glutaraldehyde overnight at 4°C, washed once in PBS, and postfixed in 1% osmium tetroxide in phosphate buffer (pH 7.4). Specimens were rinsed, dehydrated in graded ethanols, treated in propylene oxide, and embedded in epoxy-resin-embedding media. Ultrathin (silver-gray) sections were cut with a diamond knife in an ultramicrotome (MT-2B; Sorvall, Norwalk, CT) and viewed by transmission electron microscope (JEM-1200EX; Hitachi Electronic, Tokyo, Japan). Images were documented using Kodak SO163 EM film.

Cell cycle analysis. Cells (500,000) were seeded into 6-cm tissue culture dishes. Twelve hours later, cells were transfected with the indicated amounts of siRNA. At the indicated time points, cells were harvested, fixed in 1% paraformaldehyde, washed with chilled PBS, and stained in 5 mg/ml propidium iodide (PI) in PBS supplemented with RNase A (Roche, Indianapolis, IN) for 30 min at room temperature. Incubation in the dark for 30 min, cells were analyzed by FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Cell apoptosis detection. Cell apoptosis was detected by annexin V/PI double staining. Briefly, 48 h after transfection, cells were harvested with 0.25% trypsin, washed twice with chilled PBS, and resuspended in 250 μl of binding buffer. Staining solution containing annexin V/FITC and PI was added to the cell suspension. After incubation in the dark for 30 min, cells were analyzed by FACS Calibur flow cytometer (Becton Dickinson).

Statistical analysis. Statistics were performed using SPSS 18.0 software. Data were presented as means ± SD, and all experiments were performed at least three times with similar results. An unpaired Student’s t-test or χ² test was used to determine any significant difference between two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Morphological changes in lung tissue. Figure 1A shows increasing severity of lung injury over time during hyperoxic exposure, with alveolation interruption as a major manifestation, a pathological criterion of BPD. At 1 day, lung tissue in the control group exhibited an original, irregular morphology and a low number of alveoli, as well as thick alveolar septa; there was no significant difference between the control and model groups. At 3 days, the terminal alveolar spaces of lung tissue were reduced, and the number of alveoli was increased in the control group; the model group also developed increased alveoli, but no significant difference between two groups was observed. At 7 days, alveoli in the control group showed a regular morphology and a consistent size, whereas a decreased number of alveoli with an increased size and obvious interstitial edema were observed in the model group. At 14 days, alveoli were distributed uniformly, alveolar septa were thinner, and the number of both secondary alveolar septa and alveoli was significantly increased in the control group, whereas a decreased number of both secondary alveolar septa and alveoli and thickened alveolar septa were observed in the model group. At 21 days, for the control group, alveolation was more complete, secondary alveolar septa were significantly increased, and an increased number of alveoli with a consistent size and regular morphology were noted. In the model group, an arrested number of enlarged alveoli, blunted secondary alveolar septa, and significantly thickened alveolar septa were observed, giving the appearance of aborted development.

Using quantitative RAC (Fig. 1B), an important index to evaluate the stage of lung development, the number of alveoli in the model group was significantly lower than the control group starting at 7 days after hyperoxic exposure ($P < 0.05$), suggesting the interruption of alveolation. We performed morphometry to quantify alveolar wall thickness (Fig. 1C) and
found alveolar septa were significantly thickened from 14 days after hyperoxic exposure in the model group \( (P < 0.05) \). A comparison of W/D ratios of lung weights (Fig. 1D) yielded a significantly higher ratio for the model group starting 7 days after hyperoxic exposure \( (P < 0.01) \) and reflected that edema had occurred in lung tissue of the model group.

Alveoli form by the division of terminal air sacs with secondary septa during development (12, 50). The localization of myofibroblasts at the tips of secondary septa is critical for alveolar development and thus is often used as a symbol of secondary septa development and alveolar development (11, 50). Immunohistochemistry staining for the myofibroblast marker \( (\alpha\)-SMA) demonstrated that secondary septation decreased in the model group compared with the control group (Fig. 2A). The difference in \( \alpha\)-SMA-positive cells was seen only in the septal tips, and the percentage of \( \alpha\)-SMA-positive septa decreased significantly in the model group compared with the control group \( (Fig. 2B; P < 0.05) \). These data demonstrate that hyperoxia disrupted alveolar development via interference of secondary septa development. On the other hand, according to the total expression of \( \alpha\)-SMA in the alveolar epithelium, we saw a significant increase, instead of a decrease, in total \( \alpha\)-SMA expression in the model group \( (Fig. 2C; P < 0.05) \), which mostly located at the mesenchyme of alveolar epithelium. In fact, we previously had found increased total \( \alpha\)-SMA expression in the alveolar epithelium in the hyperoxia-treated group compared with the control group \( (59) \), which indicated the occurrence of epithelial-mesenchymal transition (EMT).

**Increased transdifferentiation of AEC in intact lungs in the BPD model.** AEC II, as pulmonary epithelial progenitor cells, can transdifferentiate into AEC I when lung injury occurs in an important mechanism of injury repair. AEC II can secrete surfactants, lowering surface tension at the air-liquid interface of the alveoli. In immunofluorescent double staining of lung sections from BPD model rats \( (Fig. 3A) \), green fluorescence-labeled SP-C represented AEC II, red fluorescence-labeled AEC I marker AQP5 represented AEC I, and double-stained cells appeared orange, indicating AEC II-to-AEC I transdifferentiation. SP-C expression was sparse and mainly located at the corners of alveoli in the control group, whereas, in the model group, SP-C expression progressively increased starting 7 days after hyperoxic exposure. SP-C expression sites were aggregated and disordered at 14 and 21 days. In the control group, AQP5 was continuously and uniformly expressed along the alveolar edge, whereas, in the model group, AQP5 expression was increased. Markedly enlarged AEC I protruding inward into alveolar spaces were observed 7 days after hyperoxic exposure. At 21 days, AQP5 expression was significantly increased, disordered, and nonuniform; double-stained cells in the model group were more evident than in the control group. Quantitative results, \( Fig. 3 \), B and C, demonstrate a significantly increased percentage of AQP5- or SP-C-positive cells/total cells, respectively, in the model group \( (P < 0.05) \). Figure 3D indicates that the percentage of double-stained cells/SP-C-positive cells within rat lung tissue was significantly higher in the model group compared with the control group starting 7

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**Fig. 2.** Immunohistochemical analysis of secondary septa development of lung tissue in model and control groups. A: \( \alpha\)-smooth muscle actin \( (\alpha\)-SMA)-positive immunostaining of secondary alveolar septal tips (scale bar = 50 \( \mu \)m). In the control group, \( \alpha\)-SMA-positive cells were concentrated at alveolar septal tips (indicated by black arrows), highlighting normal secondary septa development in lung tissue. While in the model group, many alveolar septal tips were \( \alpha\)-SMA-negative instead of \( \alpha\)-SMA-positive (indicated by red arrows). Instead, \( \alpha\)-SMA-positive cells were mostly seen in the mesenchyme (indicated by red triangles). B: analyses of the percentage of \( \alpha\)-SMA-positive secondary alveolar septal tips was found in the model group compared with the control group. These results highlighted the delay in secondary septa development of lung tissue in the model group. C: analyses of mean OD of \( \alpha\)-SMA-positive cells. An increase of total \( \alpha\)-SMA expression was found in the model group compared with the control group. *\( P < 0.05 \).
days after hyperoxic exposure ($P < 0.001$), suggesting an increase in AEC transdifferentiation.

Protein levels of AEC markers in lung tissue were quantified using Western blot analysis. Figure 4A shows increased AEC I marker (AQP5 and T1α) expression in the model group after 1 day of hyperoxic exposure, followed by a more significant increase over time, compared with the control group. For AEC II markers, SP-C expression in the model group was slightly decreased at 1 and 3 days but increased starting at 7 days compared with the control group, whereas SP-B expression was similar within the two groups at 1 and 3 days and increased in the model group compared with the control group starting at 7 days. Marker mRNA expression levels in lung tissue were also detected using real-time PCR (Fig. 4B). Results revealed that AQP5 and T1α mRNA expression gradually increased in both groups and was significantly higher in the model group than the control group starting at 1 day after hyperoxic exposure ($P < 0.05$). SP-C and SP-B mRNA expression increased slowly in the control group and increased sharply in the model group. SP-C and SP-B expression in the model group was slightly decreased or very close to the control group 1 and 3 days after hyperoxic exposure and increased starting at 7 days compared with the control group; the inter-group difference became statistically significant beginning at 7 and 14 days, for SP-C and SP-B, respectively ($P < 0.05$).

**Injured AEC structure, air-blood barrier, and opened tight junctions between AECs in the BPD model.** Respiratory dysfunction and difficulty in oxygen withdrawal are observed in clinical neonates with BPD and also in animal models. As to why decreased respiratory function follows increased AEC transdifferentiation, we hypothesized that, despite an increased number of AEC I, cellular function may be damaged at the time of injury repair. We observed the ultrastructure of the pulmonary epithelium in the BPD model by TEM. As shown in Fig. 5, AECs with a regular morphology and complete organelles, close tight junctions between cells, and a clear, continuous three-layer structure in the air-blood barrier were found in rat lung tissue from the control group. In the model group, transdifferentiation from AEC II to AEC I was observed 7 days after hyperoxic exposure, manifested by the discharge and vacuolization of lamellar bodies, a reduction in microvilli, and a flattening of cells and nuclei. Tight junctions between cells were irregularly wide, and the three-layer structure of the air-blood barrier became partially fused and blurred. At 14 days, AEC I became round and isolated and morphologically protruded inward into alveolar spaces. Cell nuclei displayed notches and heterochromatin margination. AEC II appeared more seriously damaged, with ruptured cell membranes, cell nuclei protruding toward the alveolar surface, a large number of discharged lamellar bodies, and with a localized, irregular air-blood barrier displaying a fused, three-layer structure. At 21 days, tight junctions between AEC I and between AEC II were open, and the air-blood barrier became more locally blurred. These findings indicate, not only AEC transdifferentiation, but also that structural damage to these cells occurred and that injury to the air-blood barrier and tight junctions between cells existed in the BPD model, which may be a cause of BPD-related pulmonary epithelial dysfunction.
AEC marker expression in cells isolated from rat lungs in the model and control groups. To eliminate the effects of other cell constituents in lung tissues, AEC II were isolated from rat lungs at different time points (7, 14, and 21 days) after normoxic or hyperoxic exposure, and proteins and mRNA were extracted directly from cell samples. Western blot (Fig. 6A) and real-time PCR (Fig. 6B) results indicated that AQP5 and T1α expression increased in the model group after 1 day of hyperoxic exposure, followed by a more significant increase over time compared with the control group. SP-C expression in the model group was slightly decreased at 1 and 3 days but increased starting at 7 days compared with the control group, whereas SP-B was expressed similarly within 2 groups at 1 and 3 days and increased in the model group compared with the control group starting at 7 days. B: relative mRNA expression levels of AQP5, T1α, SP-C, and SP-B in lung tissue detected by real-time PCR. AQP5 and T1α mRNA expression gradually increased in both groups and was significantly higher in the model compared with the control group starting 1 day after hyperoxic exposure. SP-C and SP-B mRNA expression increased slowly in the control group and increased sharply in the model group. SP-C and SP-B expression in the model group was slightly decreased or very close to the control group, 1 and 3 days after hyperoxic exposure and increased starting at 7 days compared with the control group; the inter-group difference became statistically significant beginning at 7 and 14 days for SP-C and SP-B, respectively. *P < 0.05, **P < 0.01.

Transdifferentiation of AEC II isolated from newborn rats was regulated by environmental oxygen concentrations. To further study the influence of the growth environment on AEC transdifferentiation, we isolated AEC II from newborn rat (within 24 h after birth) lung tissue for 72 h culture in vitro. After wall adherence and medium replacement within 24 h, AEC II were cultured in either a normoxic (21% oxygen, control group) or hyperoxic (85% oxygen, model group) incubator for 48 h. As observed by inverted phase-contrast microscopy (Fig. 7A), AEC II grew in cubic shapes and displayed many protruding pseudopodia 24 h after isolation. However, after isolation and standardizing levels relative to β-actin, SP-C and SP-B expressed by transdifferentiated cells on a per-unit basis were decreased.
was reduced in the model group compared with the control group although fine cellular detail could not be observed. Double-staining immunofluorescence (Fig. 7B) demonstrated that SP-C expression was dominant, whereas AQP5 expression was rare, 24 h after isolation. At 72 h, coexpression of SP-C and AQP5 was observed in both the model and control groups, indicating that transdifferentiation occurred in the two groups. Compared with the control group, more cells displayed coexpression, increased AQP5 expression, and decreased SP-C expression in the model group, which suggested a higher degree of transdifferentiation. Marker protein and mRNA expression levels for AEC I and for AEC II were detected using Western blot analysis and real-time PCR, respectively. Significantly increased AQP5 and T1/H9251 expression and decreased SP-C and SP-B expression in the model group (Fig. 7, C and D; P < 0.001) were noted, which also indicated a higher degree of transdifferentiation.

Changes in proliferation, apoptosis, and EMT of cultured AECs. To explore alternative cell fates, including proliferation, apoptosis, and EMT, we evaluated markers for these cell outcomes using cultured cells under hyperoxic or normoxic conditions. For AEC II proliferation and cell cycle progression, we studied PCNA protein expression levels in the control and model groups using Western blot analysis and found decreased PCNA expression in the model group (Fig. 8A). Meanwhile, in cell cycle analysis, we found that the percentage of cells in S phase was significantly decreased in the model group compared with the control group (Fig. 8C; P < 0.05). Taken together, including changes in SP-C and SP-B expression, these data indicate that hyperoxia inhibited the proliferation of AEC II significantly. For AEC II apoptosis, we examined cleaved caspase 3 protein expression levels using Western blot and annexin V/PI analyses. Increased cleaved caspase 3 protein expression (Fig. 8A) and a significantly increased percentage of apoptosis in cells from the model group were noted compared with the control group (Fig. 8D; P < 0.001), indicating that hyperoxia stimulated the apoptosis of AEC II. Regarding EMT, immunoblotting and real-time PCR were performed to analyze the expression of EMT-related proteins (α-SMA and N-cad) as shown in Fig. 8, A and B, respectively. A significant increase...
in expression of both protein ($P < 0.01$) indicated that hyperoxia also stimulated EMT in cultured AEC II, in accordance with our previous in vivo study concerning EMT in the BPD model (59).

**DISCUSSION**

Although the occurrence of severe BPD has been reduced by gentler ventilation techniques, antenatal glucocorticoid therapy, and surfactant treatments, there still exists a disturbingly high incidence of BPD in very low birth weight, especially in infants with a birth weight $<1,200$ g (5, 23). In this study, we used a well-established animal model for BPD (8, 21, 45) to study the pathological characteristics and pathogenesis of BPD. We found alveolar secondary septation interruption, fewer, larger, and simplified alveoli within the lungs, a significantly lower RAC, thicker alveolar walls, an increased W/D weight ratio, and delayed secondary septa development in the model group compared with the control group. This indicates lagged alveolar development, which matches the pathological characteristics of a new BPD displaying a pulmonary developmental disorder. Meanwhile, we must realize that there are pitfalls of the morphometric analysis using paraffin-embedded tissue because tissue-processing procedures may cause nearly 40% shrinkage of the lung tissue (48).

Previous studies of BPD-related pulmonary epithelial injury have focused on AEC II injury, which was regarded as the pathological foundation of BPD. McGrath-Morrow and Stahl (41) observed massive AEC II apoptosis in a BPD model, whereas Roper et al. (46) and O’Reilly et al. (42) reported AEC II DNA injury in in vivo and in vitro BPD models, respec-
tively. According to Chen et al. (15), a large number of reactive oxygen species were produced in AEC II in a hyperoxic environment, which caused cell apoptosis and necrosis. However, our preliminary study (58) and a study by Crapo et al. (18) revealed that surfactant secretion function and the number of AEC II increased rather than decreased in a hyperoxic environment, which suggests that further studies of AEC II in hyperoxic conditions are required. Regarding abnormal transdifferentiation from AEC II to AEC I, we validated AEC transdifferentiation at the tissue and cellular levels with surprisingly interesting results: rather than suppression, both in vivo and in vitro experiments confirmed a compensatory increase in transdifferentiation in the model group.

With the use of immunofluorescent double staining of lung tissue from the model group, more SP-C-positive cells became double-stained with anti-AQP5 as hyperoxic exposure time increased; the ratio of double-stained cells in SP-C-positive cells was also significantly increased, indicating an increase in transdifferentiation. Western blot and real-time PCR quantification demonstrated that SP-C expression was slightly decreased at 1 and 3 days after hyperoxic exposure in the model group but began to increase at 7 days, with a significant difference in SP-B expression observed between groups at 14 days. This is consistent with the results of our preliminary study (58) and the study by Crapo et al. (18) and provides an explanation for decreased SP-C expression in alveolar epithelium 5 days after hyperoxic exposure, as reported by Wikenheiser et al. (54). This suggests that there may be a “suppression-proliferation-repair” process in AEC II after hyperoxic stimulation. Roper et al. (46) pointed out that hyperoxia could cause AEC II DNA injury, but in this case AEC II survived without any morphological abnormalities, which indicates that AEC II may have a good capacity for DNA repair. The present study shows that increased AQP5 and T1α proteins increased, whereas SP-C and SP-B proteins decreased in the model group compared with the control group. β-Actin immunoreactive bands were used to demonstrate equal loading. D: relative mRNA expression levels of AQP5, T1α, SP-C, and SP-B in AEC primary culture detected by real-time PCR. Corresponding marker mRNA expression levels showed increased AQP5 and T1α expression and decreased SP-C and SP-B expression in the model group. ***P < 0.001.

Fig. 7. Hyperoxia promoted transdifferentiation of AEC II in primary culture. A: as shown by inverted phase contrast microscopy (scale bar = 200 μm), AEC II grew in cubic shapes and exhibited many protruding pseudopodia 24 h after isolation. After 72 h, cell bodies became extended and thinner, with a blurred morphological image observed through the Transwell membrane. When comparing the photos of the control and model groups and the blank membrane without cells seeded on it, decreased numbers of cells were obvious in the model group. B: immunofluorescence double staining of cell markers (scale bar = 25 μm). Red fluorescence-labeled AQP5 represents AEC I, green fluorescence-labeled SP-C represents AEC II, and double-stained cells appear orange representing transdifferentiated cells; DAPI-stained nuclei appear blue. At 24 h after isolation, SP-C expression was dominant, whereas AQP5 expression was rare. At 72 h, coexpression of SP-C and AQP5 was observed in both the model and control groups, with more cells showing coexpression, increased AQP5, and decreased SP-C expression in the model group compared with the control group. C: Western blot of marker protein expression levels in AEC primary culture. AQP5 and T1α proteins increased, whereas SP-C and SP-B proteins decreased in the model group compared with the control group. β-Actin immunoreactive bands were used to demonstrate equal loading. D: relative mRNA expression levels of AQP5, T1α, SP-C, and SP-B in AEC primary culture detected by real-time PCR. Corresponding marker mRNA expression levels showed increased AQP5 and T1α expression and decreased SP-C and SP-B expression in the model group. ***P < 0.001.
heterochromatin margination), opening of tight junctions between AEC I and also AEC II, and a fused blurred three-layer structure of the air-blood barrier were observed. Similarly, in a preliminary study (61), we found opened tight junctions in the pulmonary epithelium and a reduction in the tight junction proteins, zonula occludens 1 (ZO-1), and occludin 7 days after hyperoxic exposure. In addition, we also confirmed a continuous decrease in ZO-1 expression in the model group, 14 and 21 days after hyperoxic exposure (data not shown). These add to our proposition that, in the hyperoxia-induced BPD-like model, AECs do not decrease in number but undergo morphological and functional changes, resulting in air-blood barrier dysfunction and open tight junctions in the pulmonary epithelium. Furthermore, air-blood barrier damage may be a type of vascular endothelial cell injury, which has been previously validated (2, 18, 31), and production of increased AEC I attributable to transdifferentiation may be a compensatory effect for the reduction in vascular endothelial cells. However, such compensation is structurally and functionally incomplete. Air-blood barrier damage may lead to weakened air exchange function in lung tissue such that it fails to effectively utilize inhaled oxygen to maintain a normal blood oxygen level. In addition, opened tight junctions in the pulmonary epithelium may be associated with intrapulmonary fluid retention and the development of infection in BPD.

We further validated the transdifferentiation phenomenon at a cellular level and demonstrated that AQP5 and T1 expression in the model group increased over time during hyperoxic exposure compared with the control group, which is consistent with tissue results. This indicates increased AEC II transdifferentiation in the model group. However, SP-C and SP-B expression in cells was different to that of lung tissue and significantly lower in the model group than the control group starting at 7 days after hyperoxia exposure. Differences between cells and tissue samples may be because, unlike in cells, AEC II in tissues from the model group completed self-repair and transdifferentiation that was dependent on proliferation; this would have significantly increased the number of AEC II, increasing their proportion among various cells in lung tissue to maintain cell number and total surfactant protein expression. This argument is supported by the study of Crapo et al. (18), which calculated the total number of the major classes of cells in the alveolar region of rat lungs and found an increased number of AEC II at 85% oxygen exposure. However, AEC II were functionally injured, as demonstrated by the discharge of lamellar bodies, swelling of mitochondria, and other changes as observed by TEM. The lamellar body is the location of synthesis and a storage pool of alveolar surfactants (43). After discharge of lamellar bodies, AEC II expressed less surfactant protein on average.

After 72 h of primary culture of AEC II isolated from newborn rats, natural transdifferentiation of cells in the control group was observed; however, a higher level of transdifferentiation occurred after 48 h of hyperoxic treatment. After using immunofluorescent double-staining, more cells coexpressing AQP5 and SP-C were observed. Western blot and real-time PCR quantification also suggested increased AQP5 and T1 expression and decreased SP-C and SP-B expression in the...
model group compared with the control group. This validates the results of in vivo experiments at a cellular level and provides a convenient in vitro model for further studies regarding mechanisms of this type of change.

The mechanism regulating AEC II transdifferentiation in BPD is still unknown. Some studies provide evidence indicating that the mechanism may be related to regulation by IGF-I, TGF-β1, KGF, and BMP. Serum IGF-I levels were decreased in patients with BPD (30, 38), but Capoluongo et al. (14) and Chetty et al. (17) found a localized increase in IGF-I in the lungs of patients with BPD that was not consistent with serum levels. IGF-I can promote transdifferentiation from AEC II to AEC I by activating Wnt5a (26). Liu et al. (37) observed a significant increase in TGF-β1 expression in bronchodevalve lavage fluid from patients with BPD. Alejandro-Alcazar et al. (3, 52) noted that TGF-β1 expression was increased in a hyperoxia-induced BPD model and that cellular secreted TGF-β1 could promote self-transdifferentiation (7, 63). Danan et al. (20) found that preterm infants with less KGF in their tracheal aspirates were more susceptible to BPD, and Franco-Montoya et al. (25) observed a preventive and protective effect of KGF on BPD in animal experiments. KGF can inhibit AEC transdifferentiation (10, 44). In BPD models, the BMP signaling pathway is damaged (3), and BMP plays an inhibitory role in AEC transdifferentiation (63). However, these mechanisms need to be confirmed.

Depending on the cellular environment and stimuli, AECs respond to injury by undergoing one of the following pathways: 1) apoptosis/necrosis; 2) proliferation, transdifferentiation, and reepithelialization; or 3) EMT (55). Our study confirmed that hyperoxia stimulated apoptosis and inhibited proliferation of AEC II, consistent with previous reports (41, 42). Alveolar epithelial EMT was observed after TGF-β1 stimulation (35) and in adult idiopathic pulmonary fibrosis (56). Our preliminary study found EMT in lung tissue in a hyperoxia-induced BPD-like animal model (59). The present study has revealed that cultured AEC II also underwent EMT, in addition to transdifferentiation in response to hyperoxia stimulation, which means that AECs travel along several pathways in an hyperoxic environment.

In conclusion, we validated an increase in AEC II-to-AEC I transdifferentiation in a hyperoxia-induced, BPD-like model at both the tissue and cellular levels. This may represent a self-repair process in the body in which functional injuries to AECs, the air-blood barrier, and tight junction structures are also present. Methods of regulating the repair process to avoid or reduce the occurrence of such injuries will require further research.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: A.H., J.F., and X.X. conception and design of research; A.H., J.F., and X.X. performed experiments; A.H. and H.Y. analyzed data; A.H. and H.Y. interpreted results of experiments; A.H. prepared figures, A.H. drafted manuscript; A.H. and J.F. edited and revised manuscript; A.H., J.F., H.Y., and X.X. approved final version of manuscript.

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