Trans-differentiation of Alveolar Epithelial Type II Cells to Type I Cells Involves Autocrine Signaling by Transforming Growth Factor β1 through the Smad Pathway*

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Type II alveolar epithelial cells (AEC II) proliferate and trans-differentiate into type I alveolar epithelial cells (AEC I) when the normal AEC I population is damaged in the lung alveoli. We hypothesized that signaling by transforming growth factor β1 (TGF-β1), through its downstream Smad proteins, is involved in keeping AEC II quiescent in normal cells and its altered signaling may be involved in the trans-differentiation of AEC II to AEC I. In the normal lung, TGF-β1 and Smad4 were highly expressed in AEC II. Using an in vitro cell culture model, we demonstrated that the trans-differentiation of AEC II into AEC I-like cells began with a proliferative phase, followed by a differentiation phase. The expression of TGF-β1, Smad2, and Smad3 and their phosphorylated protein forms, and cell cycle inhibitors, p15Ink4b and p21Cip1, was lower during the proliferative phase but higher during the differentiation phase. Furthermore, cyclin-dependent kinases 2, 4, and 6 showed an opposite trend of expression. TGF-β1 secretion into the media increased during the differentiation phase, indicating an autocrine regulation. The addition of TGF-β1 neutralizing antibody after the proliferative phase and silencing of Smad4 by RNA interference inhibited the trans-differentiation process. In summary, our results suggest that the trans-differentiation of AEC II to AEC I is modulated by signaling through the Smad-dependent TGF-β1 pathway by altering the expression of proteins that control the G1 to S phase entry in the cell cycle.

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Alveolar epithelial cells (AEC) II have a multifunctional role in the lung including secretory, synthetic, and progenitor capacities. AEC II serve as remodeling reservoirs for lung epithelium (1). They are the progenitors for AEC I. By virtue of their squamous shape, AEC I cover the major surface area of alveoli and thus are the main epithelial component of the thin air-blood barrier. In contrast, AEC II are limited to the corners of the alveoli. The progenitor function of AEC II is activated when the lung epithelium encounters a variety of disease conditions including acute lung injury (2). AEC II cell proliferation and hyperplasia, followed by trans-differentiation into AEC I is a hallmark of alveolar epithelial injury. This helps to restore the normal air-blood barrier. However, the molecular mechanisms involved in the trans-differentiation process are not clear. Questions regarding the causes of AEC II proliferation and trans-differentiation into AEC I during lung injury and prevention of this in normal alveoli largely remain unanswered.

The transforming growth factor β (TGF-β) superfamily consists of cytokines that are implicated in the regulation of a variety of biological responses (3–6). Its members include isoforms of TGF-β, bone morphogenetic proteins, Mullerian inhibiting substance, inhibins, and activins. A member of this family, TGF-β1, regulates cell proliferation, differentiation, and migration in many cell and tissue systems. This cytokine is secreted by various cell types and acts both in autocrine and paracrine manners. The binding of TGF-β1 to its specific type II receptor (TβRII) induces the formation of a heterotetrameric complex with the type I receptor (TβRI). This complex phosphorylates the TβRI. The activated TβRII in turn phosphorylates the receptor-regulated Smads (R-Smads), Smad2 and Smad3. The phosphorylated R-Smads disassociate with the Smad anchor for receptor activation (7) and assemble with the common partner Smad (Co-Smad) named Smad4. The Smad heteromeric complex translocates into the nucleus, binds to DNA directly or indirectly through specific DNA-binding proteins, and regulates the transcription of target genes (8, 9). Also there are inhibitory Smads (I-Smads), Smad6 and Smad7, which are antagonistic to the R-Smads and act by competitively binding to the activated TβRII (10, 11). Once the response is achieved, R-Smads and I-Smads are turned off mainly by ubiquitin-mediated degradation (12).

The mechanism by which TGF-β inhibits cell proliferation varies in different epithelial cells (5). Generally, the action is elicited by altering the levels of cell cycle proteins that are involved in the G1 to S phase entry before the restriction point in the cell cycle. TGF-β increases the expression of two cyclin-dependent kinase inhibitors, p15 (p15Ink4b) (13) and p21 (p21Cip1) (14), which in turn inhibit cyclin-dependent kinases (CDKs) 2, 4, and 6. TGF-β can also inhibit the transcription of genes needed for the cell cycle progression. This includes c-Myc (15–17) and CDK activating phosphatase, cdC25A (18). Additionally, TGF-β can initiate signaling through pathways other than those involving Smads, such as phosphatidylinositol

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The abbreviations used are: AEC, alveolar epithelial cell; R-Smad, receptor-regulated Smad; TGF-β, transforming growth factor β; CDK, cyclin-dependent kinase; RNAi, RNA interference; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; MDSF, modified serum-free medium; PBS, phosphate-buffered saline; BrdUrd, 5-bromo-2′-deoxyuridine; siRNA, small interfering RNA.
3-kinase and various members of the mitogen-activated protein kinase (MAPK) (19–21).

Although several studies have shown that TGF β1 has a role in modulating abnormal lung conditions such as idiopathic pulmonary fibrosis (22–25), whether it controls AEC II proliferation and trans-differentiation into AEC I has not been examined in detail. Studies on the effect of TGF β1 on AEC II so far have been limited to its anti-proliferative effects on culture systems that maintain the AEC II phenotype (26). Here, we report the role of Smad-dependent TGF β1 pathway in the trans-differentiation of AEC II to AEC I using a model that mimics the differentiation process that takes place during lung injury. Through the addition of TGF β1 and its neutralizing antibody, RNA interference (RNAi)-mediated silencing of Smad4 and by showing differential expression of the components of TGF β pathway and cell cycle proteins during the trans-differentiation process, we have demonstrated a possible mechanism of controlling this process in the alveolar epithelium.

**MATERIALS AND METHODS**

Rat IgG, deoxyribonuclease 1, n-propyl gallate, 5-bromo-2’-deoxyuridine, and recombinant human TGF β1 were obtained from Sigma. Elastase was from Worthington Biochemical Corp. Mouse monoclonal anti-Smad4, goat polyclonal anti-p15, anti-p21, anti-CDK2, anti-CDK4, anti-CDK6, goat polyclonal anti-purinergic receptor P2X7 (P2X7), and anti-surface protein C (SP-C) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-Smad2, rabbit polyclonal anti-pSmad2, and anti-pSmad3 antibodies were from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal anti-Smad3 was from Abcam (Cambridge, MA). Monoclonal anti-TGF β1 antibody and mouse/rat/porcine TGF β1 Quantikine ELISA Kit were from R & D Systems (Minneapolis, MN). Mouse anti-LB-180 antibody was from Covance (Berkeley, CA). Alexa 568- and 488-conjugated anti-rabbit and anti-mouse secondary antibodies were from Molecular Probes (Eugene, OR). Cy3-conjugated Affini-Pure goat anti-mouse IgG was from Jackson Immunoresearch Laboratories. The Dc protein assay kit and horseradish peroxidase-conjugated anti-mouse antibodies were from Bio-Rad. The enhanced chemiluminescence detection system was from Amershams Biosciences. Anti-rat and anti-mouse IgG-conjugated magnetic beads were from Dynal Biotech (Lake Success, NY). The TRI Reagent was from Molecular Research Center Inc. (Cincinnati, OH). Moloney murine leukemia virus reverse transcriptase, penicillin, streptomycin, and fetal bovine serum (FBS) were from Invitrogen. DNA polymerase was from New England Biolabs (Beverly, MA). Quantitect SYBR Green PCR kit was from Qiagen (Valencia, CA). Dulbecco’s modified Eagle’s medium/Ham’s nutrient mixture F-12 (DMEM/F-12) in 1:1 ratio was from ATCC (Manassas, VA).

**Isolation and Culture of AEC II—**AEC II were isolated from pathogen-free male Sprague-Dawley rats (200–250 g) by an improved method as previously described (27). In brief, adult rat lungs were perfused, lavaged, and digested with elastase (3 units/ml). The cell mixture was filtered through 160- and 37-μm nylon mesh once and 15-μm nylon mesh twice, and plated on rat IgG-coated plates twice for 45 and 30 min to remove macrophages. The unattached cells were further incubated with anti-leukocyte common antigen (40 μg/ml) and rat IgG (70 μg/ml) antibodies for 30 min at 4 °C. This was followed by incubation for 20 min with anti-rat and anti-mouse IgG-conjugated magnetic beads. A magnetic field was applied to remove the cells attached to the magnetic beads. The purity of AEC II was above 95% as determined by modified Papanicolaou staining and the viability was above 98%.

AEC II were seeded onto 35-mm tissue culture-treated plastic dishes at a density of 1.3 × 10⁶ cells/dish in minimum essential medium with 10% FBS and cultured for 1 to 7 days. The media were changed after the first 24 h and thereafter on alternate days. In this culture system, the trans-differentiation of AEC II to AEC I-like cells was evident from day 3 onwards and was nearly complete by day 5. For the sandwich ELISA experiment to quantitate TGF β1 secreted into media, modified serum-free medium (MDSF) was used. The MDSF contained a 1:1 ratio of Dulbecco’s modified Eagle’s medium/F-12 media, bovine serum albumin (1.5 mg/ml), 10 mM HEPES, 0.1 mM non-essential amino acids, 2 mM glutamine, 1000 units/ml penicillin G, and 100 μg/ml streptomycin.

**Immunohistochemistry and Immunocytochemistry—**Paraffin-embedded tissue sections of perfused rat lungs were de-waxed using xylene and rehydrated using descending grades of ethanol. They were then washed in phosphate-buffered saline (PBS, pH 7.4). Antigen retrieval was done by boiling the slides for 15 min in 20 mM citrate buffer (pH 6.0). Cytospinned cells or cells cultured in 35-mm plastic dishes were fixed in 4% paraformaldehyde. The tissue sections or cells were permeabilized with 0.4% Triton X-100 for 20 min and blocked for 1 h in 10% FBS. The slides were then incubated with mouse anti-Smad4 (1:50), mouse anti-TGF β1 (1:10), LB-180 (1:200), SP-C (1:100), or P2X7 (1:50) antibodies at 4 °C overnight. Slides were washed and incubated with Alexa 568-conjugated anti-rabbit/anti-goat and Alexa 488-conjugated anti-mouse/anti-rabbit secondary antibodies or Cy3-conjugated Affini-Pure anti-mouse IgG (1:250 dilution). Finally, slides were then washed and mounted on an anti-fade medium (5% n-propyl gallate and 80% glycerol in PBS) and viewed through a Nikon Eclipse E600 fluorescence microscope or Nikon Eclipse TE 2000 U inverted fluorescence microscope.

**Real Time PCR—**Total RNA (1 μg) was isolated using TRI Reagent and reverse-transcribed into cDNA using 200 units of Moloney murine leukemia virus reverse transcriptase and a mixture of random and oligo(dT) primers. Gene sequence information was obtained using nucleotide data bases. All primer sequences (Table 1) were designed using primer express 2.0 software and confirmed for specificity using the non-redundant basic local alignment search. The reverse transcribed cDNA was further diluted 5 times and quantitative real time PCR was done using an ABI prism 7700 system (PE Applied Biosystems, Foster City, CA). The reactions were carried out on 96-well plates. 18 S rRNA was amplified on the same plates and used to normalize the data. The reaction volume was 10 μl containing 5 μl of the SYBR master mix, 0.4 μl of 25 mM MgCl₂, 0.3 μl each of 5 μM forward and reverse primers, 2 μl of RNase-free water, and 2 μl of the cDNA samples. Each sample was prepared in duplicate and at least 3 different sets of cell prepa-
TABLE 1

| GenBank ID | Gene name | Forward | Reverse |
|------------|-----------|---------|---------|
| BC076380   | TGF β1    | AGTCTGCGCTTGACCTTGTTCAT   | TGGTACCTTGGGATGGTTCATCA   |
| AB010147   | Smad2     | AATGGTTGCTCCGCCCTGCTGCTG  | CGGGTGTACCGCTGGTCCTCTCT   |
| BC064437   | Smad3     | TCACCACGCTTGCTGGGAAATCA   | TGCTGTGACACGTTCGGAGAATT   |
| AF056002   | Smad4     | CCATTGATGACCATGGCTGCAAT   | TGGCTGACACGTTCGGAGAATT   |
| NM_005842  | Smad6     | AGGCCACCAACTCCCTCATC      | AGGCCACCAACTCCCTCATC      |
| BC086959   | p15       | ATGCTGTGCACATGGCTGCAAT    | ATGCTGTGCACATGGCTGCAAT    |
| NM_080782  | p21       | GCTTCTCATGGCGGATGCA       | GCTTCTCATGGCGGATGCA       |
| M11188     | I8S       | TCCGGAAGGCTGCTGCAAT       | TCCGGAAGGCTGCTGCAAT       |
|            |           | CAGGGGTCCGCTCTAAAACTCAT   | CAGGGGTCCGCTCTAAAACTCAT   |

rations were used. The thermal cycling conditions used were: 95 °C for 15 min followed by 40 cycles at 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s. The data acquisition temperature varied from 76 to 78 °C depending on the melting temperature of the individual PCR products. Dissociation curve analysis was performed for each gene to ensure the specificity of PCR products. The relative abundance of each gene was calculated by subtracting the Ct value of each sample of an individual gene from the corresponding value for the 18 S gene (βCt). Freshly isolated AEC II (day 0) were used as the reference point. βCt were obtained by subtracting Ct of the reference point. These values were raised to the power of 2 (2βCt) to give expression levels relative to day 0 expression.

Western Blot—Cells were lysis in lysis buffer (10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The protein concentration was determined by the DC protein assay kit.

20 μg of protein was separated on 12% SDS-polyacrylamide gels, transferred to a nitrocellulose membrane using the semi-dry transfer apparatus (Bio-Rad) at 17 mA for 60 min. The membrane was stained with Ponceau S to ensure proper transfer. After being washed in TBS-T 3 times, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse, -goat, or -rabbit IgGs (dilution 1:100 dilutions overnight at 4 °C. After being washed in TBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-polyclonal antibody specific to TGF β1 antibody was precoated on a microplate. The latent TGF β1 in the media was assayed using an ELISA kit. Briefly, monoclonal anti-TGF β1 antibody was precoated on a microplate. The latent TGF β1 in the media was activated by the addition of 1 N HCl, followed by neutralization using 1.2 N NaOH, 0.5 M HEPES. Serially diluted standards, controls, and activated samples were incubated on the microplate for 2 h at room temperature on an orbital shaker. This was followed by thorough washing and the addition of horseradish peroxidase-conjugated polyclonal antibody specific to TGF β1. After 2 h of incubation and subsequent washing, the substrate solution was added to each well. The plate was protected from light and incubated for 30 min at room temperature so that color developed in proportion to the amount of TGF β1 bound in the initial step. The reaction was terminated by adding the stop solution. The optical density was measured within 30 min using a microplate reader set to 450 nm with a wavelength correction set at 540 nm.

Construction of Adenovirus Vectors—A new pK4 short hairpin RNA vector, developed in our laboratory, was used to silence Smad4. The single vector expresses four short hairpin RNA driven by four different promoters after transfecting into cells. The details for the construction of the new vector will be published elsewhere. The four small interfering RNA (siRNA) sequences for Smad4 were: 5'-GGTTGGAGAGTTGAGGACATT-3' (374–394), 5'-GGCGTCTGTGTCGGAACCAT-ATC-3' (374–394), 5'-GGAGATCGAGTCTCTGGATTA-3' (418–428), and 5'-GGAGTGCAGTTGGAGTGAATGAAT-3' (1156–1176). A vector containing four non-relevant siRNA sequences was used as a control. The sequences were 5'-ACGTGACGTCGTTGGACATT-3', 5'-GATCGTGATGAACTCAGCTGTC-3', 5'-ATCGGTACCGGCACGCTAAGC-3', and 5'-GGCTACTGACGACGCTGTGAAT-3'. The expression cassettes were ligated into pENTR vectors (Invitrogen) using the Invitrogen Gateway® technology. They were switched to the adenoviral vectors by LR recombinations. The viral vectors were transfected into 293A cells to produce the first generation of virus. After amplification, the second generation of viruses was used to infect target cells. The virus with Smad4 silencing sequence was named AdSmad4S and the control with the four non-relevant sequences AdCon.

Statistical Analysis—Data were expressed as mean ± S.E. Statistical analysis was performed by using one-way analysis of variance using GraphPad Prism software, followed by Dunnnett’s Multiple Comparison Test. The significance was assigned at p < 0.05.

RESULTS

TGF β1 and Smad4 Were Highly Expressed in AEC II—To identify cellular localization of TGF β1 and Smad4, double immunolabeling was performed on perfused normal rat lung
tissue sections by using monoclonal antibodies for TGF β1 or Smad4, and goat polyclonal antibody for SP-C (an AEC II marker). The results revealed a strong co-localization of both proteins with SP-C in AEC II (Fig. 1). The negative controls with only secondary antibodies did not show any signals. TGF β1 and Smad4 were also present to a lesser extent in AEC I. A high expression of TGF β1 was also seen in alveolar macrophages and bronchial epithelial cells (data not shown).

AEC II Proliferated and Then Differentiated to AEC I-like Cells in the in Vitro Trans-differentiation Model—It is well known that AEC II gradually trans-differentiate into AEC I-like cells when cultured on plastic dishes (28, 29). However, the kinetics of cell proliferation and differentiation during this process has not been examined. The trans-differentiation of AEC II to AEC I-like cells was confirmed by immunostaining with AEC II and AEC I markers, LB-180 for AEC II, and P2X7 for AEC I (30, 31). On day 1, LB-180 expression was strong with no expression of P2X7, but it disappeared on days 3 and 5 along with the concurrent appearance of P2X7 (Fig. 2A). This indicates that the trans-differentiation occurs between days 3 and 5. Some day 3 cells showed the expression of both AEC II and AEC I markers, demonstrating the possibility of an intermediate cell type. The BrdUrd staining experiments showed that AEC II proliferation increased from day 1 to day 2 and decreased thereafter (Fig. 2B). This suggests that there is a proliferation phase (day 0 to day 2) that precedes the differentiation phase (day 3 to day 5) in the in vitro model.

mRNA and Protein Expression Levels of TGF β1 and Smads Were Altered during the Trans-differentiation from AEC II to AEC I-like Cells—Because there is a proliferation phase immediately following AEC II isolation and culture, we hypothesized that the expression of TGF β1 and its downstream components, R-Smads, would be lower during the proliferation phase, but increase during the differentiation phase so as to facilitate the cell cycle exit. We therefore examined the mRNA and protein expression levels of TGF β1 and Smads in the in vitro trans-differentiation model by real time PCR and Western blotting. The TGF β1 mRNA expression level was low during proliferation, but increased significantly during the later stage of differentiation (Fig. 3A). The same trend was observed at the protein level (Fig. 3B).

TGF β1 activates its signal transduction pathway by binding to the specific TβRII-TβRI receptor complex on the extracellular side of the cell membrane. We therefore investigated whether TGF β1 was secreted from the cultured cells as they trans-differentiated. AEC II were cultured in MDSF media, the media collected on days 2, 3, 4, and 5, and the TGF β1 concentration determined by sandwich ELISA. TGF β1 secretion into the surrounding media increased significantly during trans-differentiation and corresponded with the increase in TGF β1 production within the cells (Fig. 3C). The same experiment, when repeated in minimum essential medium + 10% FBS, yielded similar results (data not shown).

The mRNA expression of Smad2 and Smad3 remained lower during the proliferation phase and increased on day 3 as differentiation became evident (Fig. 3, D and E). This almost corresponded to their protein levels (Fig. 3F). Because the presence of their phosphorylated form would be a better indicator of the activation of the Smad-dependent TGF β pathway, we further determined the phosphorylation of Smad2 and Smad3 using

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**FIGURE 1.** Immunohistochemistry of rat lung tissue sections for TGF β1 and Smad4. Double labeling of rat lung tissue sections was done using anti-TGF β1 and anti-SP-C antibodies (panels a–d) or anti-Smad4 and anti-SP-C antibodies (panels e–h). This was followed by incubation with the corresponding Alexa 488- or 568-conjugated secondary antibodies. Panel d shows the overlay between TGF β1 and SP-C signals, whereas panel h shows the overlay between Smad4 and SP-C. The negative control was without primary antibodies (panels i–l). Scale bars, 5 μm.

**FIGURE 2.** Expression of AEC I and AEC II markers and BrdUrd labeling during the trans-differentiation. Freshly isolated AEC II were seeded on plastic dishes in minimum essential medium containing 10% FBS and cultured for 1–5 days. A, double labeling of the cultured cells on days 1, 3, and 5 was done using anti-LB-180 (panels b, e, and h) and anti-P2X7 antibodies (panels c, f, and i). The protein expression was visualized using corresponding Alexa 488- or 568-conjugated secondary antibodies. B, BrdUrd labeling: BrdUrd was added at a concentration of 10 μM to the culture media 12–14 h before fixing cells. Immunostaining was done on the cells cultured for 1–5 days using anti-BrdUrd antibody and Alexa 488-conjugated secondary antibody. Scale bar, 5 μm.
The Expression of Cell Cycle-related Proteins, p15, p21, CDK2, CDK4, and CDK6, Were Altered during the Trans-differentiation—TGF \( \beta \) mediates its action by altering the levels of cell cycle proteins that are involved in the G1 to S phase transition before the restriction point. TGF \( \beta \) increases the expression of two cyclin-dependent kinase inhibitors, p15 (13) and p21 (14). The latter, in turn, inhibit the activity of CDK2, CDK4, and CDK6, which are required for the progression of the cell cycle. We examined the relative mRNA expression of p15 and p21 by real time PCR. Their expression remained low from day 0 to day 3 and increased significantly as the differentiation progressed (Fig. 4, A and B). These results were verified at the protein level by Western blot (Fig. 4C). The protein levels of CDK2, CDK4, and CDK6 showed an opposite trend of expression (Fig. 4C). This low expression of cell cycle inhibitors during the proliferation and their increase during differentiation with an opposite expression trend of CDKs further strengthen our view that these two phases of cell events may be mediated by the endogenous TGF \( \beta \) released from cultured cells in this model.

Addition of Exogenous TGF \( \beta \) Prior to Proliferative Phase Blocked Both Proliferation and Trans-differentiation of AEC II, whereas Its Addition after the Proliferative Phase Did Not Affect the Trans-differentiation—The anti-proliferative effect of TGF \( \beta \) on AEC II has been documented previously (26). We investigated the effects of addition of exogenous TGF \( \beta \) prior to and after the proliferation phase in our trans-differentiation culture model. For this purpose, AEC II were cultured for 5 days in the presence of recombinant TGF \( \beta \) at a concentration of 40 ng/ml from day 0 in one group (pre-proliferative addition) and from the end of day 2 in another group (post-proliferative addition). A control group with no additions was also prepared. The phenotypes of the cells on days 3 and 5 were determined by double labeling with AEC II markers, LB-180 and P2X7, respectively (Fig. 5). When compared with the control (Fig. 5, panels a–c and m–o), the group with pre-proliferative addition of TGF \( \beta \) showed reduced proliferation and differentiation (Fig. 5, panels d–f and p–r). The cell counting revealed a 54% reduction in the cell number on day 3. The LB-180 expression in this group persisted even on day 5 showing that they retained the lamellar bodies and AEC II phenotype. Also, the AEC I marker (P2X7) expression was absent in this group indicating a failure to trans-differentiate to AEC I-like cells. A dose experiment (4, 10, 20, and 40 ng/ml) showed that TGF \( \beta \) as low as 4 ng/ml if added at the pre-proliferative phase can still retain the AEC II phenotype although the number of LB-180-positive cells appears to be higher at a higher concentration of TGF \( \beta \) (data not shown). On the other hand, in the cells subjected to
post-proliferative addition of TGF β1, the normal trans-differentiation process was seen as in the untreated control with gradual loss of LB-180 expression and subsequent appearance of P2X7 expression (Fig. 5, panels g–i and s–u). The results indicate that for differentiation to take place there must be a preceding proliferative phase. Maybe that is the reason why TGF β1 and related Smad expression increase after the proliferative phase when cells are ready for differentiation. To further clarify this view, we added an excess of neutralizing antibody to TGF β1 at the end of day 2 (post-proliferative addition). The antibody binds with the endogenous TGF β1 and makes it unavailable to bind with the receptors and to initiate Smad-mediated signaling. The cells retained the AEC II phenotype and failed to trans-differentiate properly when the endogenous TGF β1 was blocked. They retained the LB-180 expression and failed to express P2X7 on days 3 and 5 (Fig. 5, panels j–l and v–x). These experiments demonstrate that altered signaling through TGF β1 is important in the trans-differentiation of AEC II to AEC I and that the initial proliferation phase may be essential for subsequent differentiation process.

Silencing of Smad4 by RNAi Prevented the Trans-differentiation—Because the previous experiment involved exogenous addition of a higher quantity of TGF β1 that may not reflect the normal physiological conditions, we decided to block the Smad pathway within the cell by knocking down Smad4 expression using RNAi. Smad4 was chosen because it is the common partner of Smad that binds with Smad2 and Smad3 and is an essential component of Smad-dependent TGF β signaling. Adenovirus-based RNAi targeted to Smad4 (AdSmad4Si) was used to silence Smad4 in AEC II. The viral control (AdCon) contained non-relevant siRNA sequences. AEC II were treated with the adenoviruses on day 1 and Smad4 protein expres-

FIGURE 4. The expression of cell cycle inhibitors, p15 and p21, and CDK2, CDK4, and CDK6 during trans-differentiation. A and B, mRNA levels of p15 and p21: AEC II were seeded on plastic dishes and cultured for 1–5 days. Total RNA was extracted from the cultured cells collected on days 0, 1, 3, and 5 and reverse transcribed to cDNA. Real-time PCR was done to determine the mRNA abundance of p15 (A) and p21 (B). Data were normalized to 18S RNA and expressed as a ratio to the day 0 value. The error bars represent S.E. Absence of an error bar denotes very low S.E. value. *, p < 0.05 versus day 0; ***, p < 0.001 versus day 0, n = 3 biological preparations, each assay was performed in duplicate. C, p15, p21, CDK2, CDK4, and CDK6 protein levels were detected by Western blot using corresponding antibodies. A representative glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as the loading control, is shown in the lowest panel.

FIGURE 5. Effect of pre-proliferative and post-proliferative addition of exogenous TGF β1 and anti-TGF β1 antibody on AEC II trans-differentiation. AEC II were cultured for 3 or 5 days. Recombinant TGF β1 (40 ng/ml) was added at day 0 (pre-proliferative addition) (panels d–f and p–r) in one group and at the end of day 2 (post-proliferative addition) (panels g–i and s–u) in another. A third group was post-proliferative addition of anti-TGF β1 antibody at a concentration of 40 μg/ml (panels j–l and v–x). The control was without any additions (panels a–c and m–o). Cells were fixed on days 3 and 5 and were double-labeled with anti-LB-180 (AEC II marker) and anti-P2X7 (AEC I marker). Scale bar, 7 μm.
TGF β1 Signaling during Epithelial Cell Trans-differentiation

FIGURE 6. Effect of Smad4 silencing on trans-differentiation of AEC II to AEC I-like cells. A, after 24 h of plating, AEC II were transduced with adenoviruses carrying siRNAs targeted to Smad4 (AdSmad4Si) or non-relevant siRNAs (AdCon). The blank control was without any additions. Multiplicity of infection used was 7 for AdSmad4Si and 36 for AdCon. Total protein was extracted on days 3 and 5 and Western blots for Smad4 were performed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control. B, the effect of Smad4 silencing on trans-differentiation. Double labeling using anti-LB-180 and anti-P2X7 antibodies was done on AEC II treated for 5 days with AdSmad4Si (panels g–i) and AdCon (panels d–f). The untreated blank control is shown in panels a–c. Scale bar, 5 μm.

In the cells possessing the capability to proliferate and differentiate, there is substantial coupling between these processes. The quiescence of AEC II in normal alveoli and their proliferation and trans-differentiation into AEC I in injured lung illustrates how differential signaling through the same TGF β1 pathway might be responsible for controlling both processes.

TGF β1 has been reported to be expressed in alveolar epithelial cells under pathological conditions. For example, TGF β1 has been observed in hypoxic AEC II and in fibrotic areas immediately beneath them (25, 32, 33). However, TGF β1 expression in normal AEC II has not been reported previously. Our immunostaining results showed that TGF β1 and its downstream component, Smad4, were expressed in AEC II of normal lung. These proteins were also detectable in AEC I although to a lesser extent. TGF β1 signaling may play a role in keeping the AEC II quiescent in normal lung.

TGF β1 can be synthesized and secreted by isolated AEC II. The protein level of TGF β1 was increased as AEC II trans-differentiated to AEC I-like cells in vitro as determined by Western blot. The reasons for apparent low expression of TGF β1 in AEC I in the intact lung and its high expression in the trans-differentiated AEC I-like cells in vitro are unclear. However, there are several possibilities: (i) AEC I are very thin and squamous cells that cover ~95% of the alveolar surface, whereas AEC II are cuboidal cells that occupy only ~5% of the surface even though the number of type II cells is twice as many as type I cells in the lung. Therefore, the signal for TGF β1 immunostaining is weak compared with type II cells because of the shape of the cells; (ii) trans-differentiated AEC I in vitro may not reflect all the properties of the normal AEC I in vivo (34). The changes in the cellular environment may alter its expression.

A novel finding in the current study is the identification of a proliferation phase preceding the differentiation phase in the in vitro trans-differentiation cell culture model. The appearance of AEC I-like cells by day 3 and subsequent differentiation was confirmed by differential labeling using established AEC II (LB-180) and AEC I (P2X7) markers. The previous studies have demonstrated the presence of various AEC I markers in these trans-differentiated cells, indicating that they are indeed similar to AEC I (35–38). Although this differentiation pattern has been observed previously, the initial proliferation phase has not been reported. BrdUrd labeling, which is indicative of active DNA synthesis preceding cell division, was found to be the highest on day 2. The expression pattern of the cell cycle inhibitors and CDKs, reflective of the cell cycle stages substantiated this biphasic cell proliferation and differentiation.

AEC I are the most susceptible to injury in alveolar epithelium. After lung injury, AEC I are destroyed and basement membrane denuded and AEC II proliferate and differentiate into AEC I to repair the epithelium (39–42). Therefore, this in vitro trans-differentiation model may closely relate to the process taking place in vivo during lung injury and repair. It should also be noted that isolating AEC II from the lung and culturing them may mimic lung injury and repair. The loss of contact of AEC II with the surrounding cells after isolation may initiate AEC II proliferation and then differentiate into AEC I-like cells.

TGF β can be a positive or a negative regulator of cell proliferation and differentiation (43). This depends on cell type and the physiological or pathological stage of the cells. The presence of other growth factors and signaling cascades also determines how TGF β acts at a particular stage of growth and differentiation. For example, TGF β inhibits myogenic differentiation (44), but promotes differentiation in mammary epithelium (45). TGF β1 can inhibit the proliferative effects of KGF on cultured AEC II (26). During the recovery of hyperoxia-exposed AEC II, there is a proliferative phase marked by an escape from the negative autocrine regulation by TGF β3 (46). However,
whether TGF β has the pro-differentiation effects on AEC is unknown.

The TGF β1, produced inside AEC, is secreted out as the trans-differentiation takes place and thus regulates this process in an autocrine manner. The secretion of TGF β1 was also observed in alveolar macrophages and fibroblasts from fibrotic lungs (47, 48). An autocrine loop of TGF β1 in pregnant mouse mammary epithelium has been shown to aid the differentiation process (45).

When AEC II trans-differentiated into AEC I-like cells in vitro, we found that the changes in expression of TGF β1 and R-Smads, Smad2 and Smad3, were biphasic: lower in the proliferation phase and higher in the differentiation phase. The increased phosphorylation of Smad2 and Smad3, a hallmark of activation of the TGF β pathway, showed a similar trend. However, an opposite trend of expression of Smad6, an inhibitory Smad was observed. The high expression of Smad6 during the proliferation phase may further inhibit TGF β signaling and ensures that the initial proliferation occurs before the differentiation. Although Smad6 is an inhibitor of BMP signaling through its binding with the activated Smad1 (49), overexpression of Smad6 also blocks TGF β signaling (11). The results suggest that TGF β signaling is depressed during the proliferation phase and activated during the differentiation phase.

TGF β can regulate cell cycle proteins involved in the G1 to S phase entry (50). The activated Smad complexes caused by TGF β result in the transcriptional repression of the c-myc gene (15–17). Concurrent induction of two cell cycle inhibitors, p15 (13) and p21 (14), inhibits the activity of CDK2, CDK4, and CDK6, which are required for the progression of the cell cycle. The expression profile of p15 and p21 during the trans-differentiation of AEC matched that of TGF β1 and R-Smads. However, protein levels of CDKs 2, 4, and 6 showed an opposite trend. These results suggest that the coordinated TGF β signaling through cell cycle points plays a role in the temporal regulation of cell cycle entry and exit by the cells.

TGF β1, when added from day 0, stopped AEC II proliferation and later differentiation. The addition of TGF β1 must have aided in stopping the cell cycle during proliferative phase. This indicates that for differentiation to take place there must be a proliferative phase. This view was substantiated by the fact that the cells were refractory to the above stated effect of TGF β1 when it was added after the proliferative phase where normal differentiation ensued. Also the addition of excess anti-TGF β1 antibody to block the endogenous TGF β1 secreted caused the differentiation to stop, indicating that TGF β1 is essential for the differentiation process.

The effect of exogenous TGF β1 may be quite different from that which is secreted during a particular stage of growth and differentiation at normal physiological levels. So we wanted to block the pathway within the cell. Also because Smad-independent signaling has been reported for TGF β1, we wanted to make sure that signaling is indeed through the Smad pathway. To achieve these aims, the common mediator for Smad-mediated TGF β signaling, Smad4, was silenced using RNAi. The results have clearly demonstrated a halting of the trans-differentiation process in the silenced group. Additionally, these results validated our view that signaling through this pathway is essential for the differentiation of AEC II to AEC I to occur.

Our current studies suggest bifunctional roles of TGF β1 during the trans-differentiation of AEC II to AEC I: inhibit the proliferation of AEC II at the proliferative phase and promote their differentiation into AEC I at the differentiation phase. Once the proliferative phase is finished, endogenous TGF β1 induces the differentiation. This is supported by (i) the production of TGF β1 and the phosphorylation of Smad2/3 are induced at the differentiation phase; and (ii) TGF β1 antibodies if added at the end of the proliferative phase or silencing of Smad4 block the trans-differentiation of AEC II to AEC I.

Because our model closely mimics the proliferation and trans-differentiation process that takes place in the lung during acute injury, understanding the signaling mechanism involved in this process may help to identify the components involved as potential targets for rational therapeutics.

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