Original Paper

Angiotensin-Converting Enzyme 2 Inhibits Apoptosis of Pulmonary Endothelial Cells During Acute Lung Injury Through Suppressing SMAD2 Phosphorylation

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Key Words

Acute lung injury (ALI) • Angiotensin converting enzyme 2 (ACE2) • Hydrolyzing angiotensin II (AngII) • Nitric oxide (NO) • SMAD2 • SMAD7

Abstract

Background/Aims: Angiotensin converting enzyme 2 (ACE2) has an established role in suppressing the severity of acute lung injury (ALI), especially when it was applied together with transplantation of human umbilical cord mesenchymal stem cells (uMSCs). Although the effects of ACE2 in ALI are believed to mainly result from its role in hydrolyzing angiotensin II (AngII), which subsequently reduces the vascular tension and subsequent pulmonary accumulation of inflammatory cells, we and others have recently reported a possible role of ACE2 in suppressing the ALI-induced apoptosis of pulmonary endothelial cells. However, the underlying mechanisms remain undetermined. **Methods:** Here, we analyzed the alteration in lung injury severity in ALI after ACE2, by histology and inflammatory cytokine levels. We analyzed apoptosis-associated proteins in lung after ALI, as well as in cultured endothelial cells treated with nitric oxide (NO). We overexpressed SMAD7 to inhibit SMAD2 signaling in cultured endothelial cells and examined its effects on NO-induced cell apoptosis. **Results:** ACE2 alleviated severity of lung injury after ALI. ACE2 significantly decreased the ALI-induced apoptosis of pulmonary cells *in vivo*, and ACE2 protected endothelial cells against NO-induced apoptosis *in vitro*. NO induced phosphorylation of a key factor of transforming growth factor β (TGF β) receptor signaling, SMAD2, which could be dose-dependently inhibited by ACE2. Inhibition of SMAD2 phosphorylation through expression of its inhibitor SMAD7 significantly inhibited NO-induced cell apoptosis, without need for ACE2. **Conclusion:** Our data suggest that ACE2-mediated AngII degradation may inhibit AngII-mediated SMAD2-phosphorylation, possibly through a TGF β -independent manner, which subsequently suppresses the ALI-induced cell death. Our results thus reveal a novel molecular pathway that controls the pathogenesis of ALI.

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Introduction

Recently, the Berlin definition brought an update over the previous definitions for acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) [1-3]. Data over the first 24 hours are now applied for reclassification of the severity of the disease [1-3]. Moreover, compliance to stratify each oxygenation category is also used for the definition [1-3]. ALI is a common pulmonary disease with clinical features of expiratory dyspnea, refractory hypoxemia and non-cardiogenic pulmonary edema, and could further deteriorate into highly lethal ARDS [4-7]. The new criteria for definition of ARDS included 3 categories on the basis of hypoxemia: mild ($\text{PaO}_2/\text{FiO}_2 \leq 300$ mm Hg but > 200 mm Hg), moderate ($\text{PaO}_2/\text{FiO}_2 \leq 200$ mm Hg but > 100 mm Hg), or severe ($\text{PaO}_2/\text{FiO}_2 \leq 100$ mm Hg) [1-3]. Four ancillary variables were also considered for the definition: radiographic severity, respiratory system compliance, positive end-expiratory pressure, and corrected expired volume per minute [1-3].

ALI results from an acute inflammation, and cellular characteristics of ALI include loss of alveolar-capillary membrane integrity, excessive transepithelial neutrophil migration and release of pro-inflammatory cytokines, e.g. interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)- α [4-7]. Injuries of both pulmonary endothelial cells (PECs) and alveolar epithelial cells occur after ALI, and are often followed by lung fibrosis and loss of respiratory capacity [4-7].

Since neutrophils are the primary perpetrators of inflammation, neutrophil migration across the vascular epithelia appears to be an important pathological feature of ALI [4-7]. Excessive and prolonged activation of neutrophils leads to basement membrane destruction and increased permeability of the alveolar-capillary barrier [4-7]. Migrating neutrophils further enhance the mechanical enlargement of the neutrophil migratory paths [4-7]. Meanwhile, neutrophils also release pro-inflammatory and pro-apoptotic cytokines to damage adjacent cells to create ulcerating lesions [4-7]. Although neutrophil depletion can be protective, acute lung injury does develop in the absence of circulating neutrophils, suggesting the possible presence of neutrophil-independent pathways that also cause lung injury [4-7].

Angiotensin-converting enzyme 2 (ACE2) is a member from the renin-angiotensin system [8]. In this system, renin induces the production of angiotensin I (Ang I), which is converted to Ang II by Ang I-converting enzyme (ACE) [8]. Ang II is an important vasoconstrictive peptide. ACE2 is the homolog of ACE but counterbalances the ACE activity through induction of degradation of Ang II into the vasodilator peptide Ang 1-7, which counteracts the effects of Ang II after binding to Mas receptor [8]. Recently, we and others have shown that ACE2 has therapeutic effects on ALI, especially when ACE2 is applied together with transplantation of human umbilical cord mesenchymal stem cells (uMSCs) [9-15]. However, the underlying mechanisms remain largely unknown.

Here, we first confirmed that ACE2 significantly decreased the ALI-induced apoptosis of pulmonary cells *in vivo*, and further showed that ACE2 protected PECs against nitric oxide (NO)-induced apoptosis *in vitro*. Moreover, we found that NO [16-18] induced phosphorylation of a key factor of transforming growth factor β (TGF β) receptor signaling, SMAD2 [19-21], which could be dose-dependently inhibited by ACE2. Further, inhibition of SMAD2 phosphorylation through expression of its inhibitor SMAD7 significantly inhibited NO-induced cell apoptosis, without need for ACE2.

Materials and Methods

Animal manipulations

All mouse experiment protocols were approved by the Animal Research and Care Committee at First people's Hospital Affiliated to Shanghai Jiaotong University. All experiments were performed in accordance with the guidelines from the Animal Research and Care Committee at First people's Hospital Affiliated to Shanghai Jiaotong University.

Specific pathogen free (SPF) Balb/c mice (aged 10 weeks, weight 18–22g) were supplied by Laboratory Animal Center of Shanghai Academy of Sciences, Chinese Academy of Sciences, China. After acclimatization for 1 week, totally 10 mice were used as the no-injury group and received no treatments. The lung injury model was induced in another 20 mice, by intraperitoneal administration of the bleomycin solution (10mg/mL, Sigma-Aldrich, St Louis, MO, USA) at 20 mg/kg body weight, as has been applied and described before [9–15]. This ALI model is stable and easy to be built. No death was observed in the current study. After successful induction of ALI, the 20 mice were randomly divided into 2 groups of 10 each. One group did not receive further intervention (termed ALI), while the other group received single tail vein injection of ACE2 (R&D systems, Los Angeles, CA, China) at a dose of 1mg/kg body weight (termed ALI+ACE2).

Cell lines and reagents

Human lung endothelial cell line HULEC-5a was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA), and has been described before [22]. HULEC-5a cells were maintained in MCDB131 media (without L-Glutamine, ATCC) supplemented with 10ng/ml Epidermal Growth Factor (EGF, Sigma-Aldrich), 1μg/ml Hydrocortisone (Sigma-Aldrich), 10mmol/l Glutamine (Invitrogen, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS, Invitrogen). DETA NONOate (a NO releasing compound, Sigma-Aldrich) is used at a dose of 500μmol/l. For administration of low (l), medium (m) and high (h) doses of ACE2 to cultured cells, 100ng/ml, 200ng/ml and 400ng/ml were used, respectively.

Cell transfection

The SMAD7-expressing plasmid pCMV5-Smad7-HA [23] was purchased from Addgene (Cambridge, MA, USA). For cell transfection experiments, PECs were grown to 70 to 80 % confluence in 6-well plates. Transfection was performed using the Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Cells that were transfected with the same plasmid vector carrying a scrambled sequence rather than SMAD7 were used as controls.

Western blot

The protein from the mouse lung or cultured cells was extracted using RIPA lysis buffer (1% NP40, 0.1% Sodium dodecyl sulfate (SDS), 100μg/ml phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, in PBS) on ice. The supernatants were collected after centrifugation at 12000×g at 4°C for 20min. Protein concentration was determined using a BCA protein assay kit (Bio-rad, China), and whole lysates were mixed with 4×SDS loading buffer (125mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100mmol/l Dithiothreitol (DTT), and 0.2% bromophenol blue) at a ratio of 1:3. Samples were heated at 100 °C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies were anti-p53, anti-Bad, anti-Bid, anti-Bak, anti-Bax, anti-Cytochrome C (CytoC), anti-caspase9 (casp9), anti-Bcl-2, anti-Bcl-xl, anti-SMAD2, anti-phosphorylated SMAD2 (pSMAD2) and α-tubulin (all purchased from Cell Signaling, St Louis, MO, USA). α-tubulin was used as a protein loading control. Secondary antibody is HRP-conjugated anti-rabbit (Jackson Labs). Images shown in the Figure were representative from 5 repeats. Densitometry of Western blots was quantified with NIH ImageJ software. The protein levels were first normalized to loading controls, and then normalized to experimental controls.

Tunel assay

Tunel staining was performed on the cultured cells with In Situ Cell Death Detection Kit (Roche, San Francisco, CA USA). DAPI was used to stain nuclei.

Statistical analysis

All statistical analyses were carried out using the SPSS 18.0 statistical software package. All values are depicted as mean ± standard deviation from 5 individuals and are considered significant if $p < 0.05$. All data were statistically analyzed using one-way ANOVA with a Bonferoni correction, followed by a Student-Newman-Keuls test or Fisher's exact test, as necessary.

Fig. 1. ACE2 significantly reduced the severity of lung injury by bleomycin. (A) Representative HE staining of lung tissue. (B-C) We isolated protein from the lung of the mice treated with bleomycin and ACE2 (ALI+ACE2) and then analyzed the inflammatory cytokine levels by Western blot, compared to the mice without treatment (UT) and to the mice treated with bleomycin only (ALI). The data were shown by quantification (B), and by representative images (C). * $p < 0.05$ (ALI+ACE2 vs ALI). NS: non-significant. $N = 5$. Statistics: one-way ANOVA, followed by a Student-Newman-Keuls test. Scale bar is $50\mu\text{m}$.

Results

ACE2 reduced ALI-induced cell apoptosis in lung

The mouse bleomycin-ALI model has been performed as has been described before [9-15]. HE staining of lung tissue showed significant pneumonitis, inflammatory exudates, fibroblastic foci and distortion of the normal architecture of the lung after ALI, while ACE2 reduced the severity of these features (Fig. 1A). We isolated protein from the lung of the mice treated with bleomycin and ACE2 (ALI+ACE2) and then analyzed the inflammatory cytokine levels by Western blot, compared to the mice without treatment (UT) and to the mice treated with bleomycin only (ALI). We found that ALI induced significantly increases in TNFα, IL6 and CXCL1 levels, by quantification (Fig. 1B), and by representative images (Fig. 1C). These data suggest that ACE2 significantly reduced the severity of lung injury by bleomycin.

We then analyzed the apoptosis-associated proteins by Western blot. We found that the levels of pro-apoptotic proteins p53, Bad, Bid, Bak and Bax all significantly increased in ALI group, compared to UT group. However, these increases in pro-apoptotic proteins significantly reduced in ALI+ACE2 group (Fig. 2A-B). On the other hand, although the levels of anti-apoptotic proteins Bcl-2 and Bcl-xl both significantly increased in ALI group, compared to UT group, the levels of these anti-apoptotic proteins further significantly increased in ALI+ACE2 group (Fig. 2A-B). The levels of apoptotic feature proteins Cytochrome C (CytoC) and caspase 9 (casp9) both significantly increased in ALI group, compared to UT group. However, these increases in CytoC and casp9 proteins significantly reduced in ALI+ACE2 group (Fig. 2A-B). These data are consistent with some previous findings, suggesting that ALI induces apoptosis of pulmonary cells and ACE2 seems to protect the cells from apoptosis, possibly through decreasing expression of pro-apoptotic proteins and through increasing expression of anti-apoptotic proteins.

Fig. 2. ACE2 reduced ALI-induced pulmonary cell apoptosis through SMAD2. We isolated protein from the lung of the mice treated with bleomycin and ACE2 (ALI+ACE2) and analyzed by Western blot, compared to the mice without treatment (UT) and to the mice treated with bleomycin only (ALI). (A-B) Quantification (A) and representative images (B) of proteins in lung. Cytochrome C: CytoC; caspase 9: casp9. (C-D) Quantification (C) and representative images (D) of SMAD2 proteins in lung. Phosphorylated SMAD2: pSMAD2. UT: untreated mice. * $p < 0.05$ (ALI+ACE2 vs ALI). $N = 5$. Statistics: one-way ANOVA, followed by a Student-Newman-Keuls test.

ACE2 reduced SMAD2 phosphorylation in lung

Since it has been reported that SMAD2 phosphorylation could be controlled by downstream signaling of Ang II in a TGF β receptor signaling independent manner [24], and phosphorylated SMAD2 (pSMAD2) is well known to regulate cell proliferation and apoptosis [19-21], we thus examined the phosphorylation of SMAD2 in these samples. We found that ALI significantly increase the phosphorylation of SMAD2, which was significantly attenuated by ACE2 (Fig. 2C-D). These data suggest a possible role of SMAD2 signaling in the regulation of ALI/ACE2-associated apoptosis.

NO-induced PEC apoptosis was dose-dependently inhibited by ACE2

NO has an established role in the pathogenesis of ALI. In lung, PECs are the only cells that harbor receptors for Ang II, which is a unique target of ACE2 [16-18]. Thus, we hypothesize that the direct anti-apoptosis effect of ACE2 after ALI is conducted on PECs. To prove it, we used DETA NONOate (a NO releasing compound) to induce ALI-like injury to the cultured human lung endothelial cell line HULEC-5a (simplified as PEC). For administration of low (l), medium (m) and high (h) doses of ACE2 to the cultured cells, 100ng/ml, 200ng/ml and 400ng/ml were used, respectively.

Fig. 3. NO-induced PEC apoptosis was dose-dependently inhibited by ACE2 through SMAD2. We used DETA NONOate (a NO releasing compound) to induce ALI-like injury to the cultured human lung endothelial cell line HULEC-5a (simplified as PEC). For administration of low (l), medium (m) and high (h) doses of ACE2 to the cultured cells, 100ng/ml, 200ng/ml and 400ng/ml were used, respectively. SMAD7-overexpressing HULEC-5a cells were termed PEC-SMAD7. (A-B) Quantification (A) and representative images (B) of proteins in PECs. Cytochrome C: CytoC; caspase 9: casp9. Phosphorylated SMAD2: pSMAD2. CTL: untreated controls. * $p < 0.05$. NS: non-significant. N=5. Statistics: one-way ANOVA, followed by a Student-Newman-Keuls test.

We found that the levels of pro-apoptotic proteins p53, Bad, Bid, Bak and Bax all significantly increased in NO group, compared to control (CTL) group. However, these increases in pro-apoptotic proteins significantly and dose-dependently reduced in NO+ACE2 group (Fig. 3A-B). On the other hand, although the levels of anti-apoptotic proteins Bcl-2 and Bcl-xl both significantly increased in NO group, compared to CTL group, the levels of these anti-apoptotic proteins further significantly and dose-dependently increased in NO+ACE2 group (Fig. 3A-B). The levels of apoptotic feature proteins CytoC and casp9 both significantly increased in NO group, compared to CTL group. However, these increases in CytoC and casp9 proteins significantly and dose-dependently reduced in NO+ACE2 group (Fig. 3A-B). These data are consistent with our findings in mouse ALI model, suggesting that ALI induces

Fig. 4. Effects of ACE2 on apoptosis-related proteins resulted in alteration in PEC apoptosis. We performed TUNEL assay on these experimental conditions. Our data confirm that effects of ACE2 on apoptosis-related proteins result in alteration in PEC apoptosis, shown by representative images (A), and by quantification (B). SMAD7-overexpressing HULEC-5a cells were termed PEC-SMAD7. CTL: untreated controls. * $p < 0.05$. NS: non-significant. $N = 5$. Statistics: one-way ANOVA, followed by a Student-Newman-Keuls test.

apoptosis of PECs and ACE2 seems to protect the PECs from apoptosis, possibly through decreasing expression of pro-apoptotic proteins and increasing expression of anti-apoptotic proteins.

Protection of PECs from apoptosis by ACE2 is mediated by regulation of SMAD2 phosphorylation

In order to find out whether the protection of PECs from apoptosis by ACE2 may be mediated by control of SMAD2 phosphorylation, we transfected PECs with a SMAD7-expressing plasmid. SMAD7 is an inhibitor for SMAD2 phosphorylation and downstream signaling [19-21]. We found that overexpression of SMAD7 in PEC, without the need for ACE2, was sufficient to decrease NO-induced increases in pro-apoptotic proteins, decreases in anti-apoptotic proteins and increases in apoptosis-related proteins (Fig. 3A-B). These data suggest that protection of PECs from apoptosis by ACE2 is mediated by regulation of SMAD2 phosphorylation. Since the TGF β 1 levels were not altered by ACE2 in the injured lung (Fig. 1C), these data also imply that the anti-apoptotic effects of ACE2 may be also mediated by regulation of SMAD2 phosphorylation in lung after ALI.

Fig. 5. Schematic of the model

Effects of ACE2 on apoptosis-related proteins resulted in alteration in PEC apoptosis

In order to confirm that the effects of ACE2 on apoptosis-related proteins indeed result in alteration in PEC apoptosis, we performed TUNEL assay on these experimental conditions. Our data confirm that effects of ACE2 on apoptosis-related proteins result in alteration in PEC apoptosis (Fig. 4A-B). Thus, this model was summarized in a schematic (Fig. 5).

Discussion

ALI is characterized with refractory hypoxemia and respiratory distress. As a common postoperative complication, ALI-induced ARDS has high incidence and mortality and appears to be one of the leading causes for the death of patients in intense care unit. The current ALI therapy mainly applies supportive treatments, such as protective ventilation. However, these supportive treatments are only effective to less severe subjects, and their effects on severe cases are quite limited [4-7].

In the past decades, the molecular pathogenesis of ALI has been extensively studied. To date, it is known that ALI is characterized with loss of alveolar-capillary membrane integrity, excessive transepithelial neutrophil migration and release of pro-inflammatory cytokines [4-7]. ALI damages both PECs and alveolar epithelial cells to lead to lung fibrosis and loss of respiratory capacity [4-7]. Moreover, the damages to PECs appear to occur ahead of the damages to epithelial cells.

The protective roles of ACE2 in acute and chronic lung diseases have been demonstrated, including anti-inflammation and anti-oxidation [9-15]. Of note, we recently reported a possible role of ACE2 in inhibiting cell apoptosis after ALI [15]. However, the underlying mechanisms remain undetermined. Here we confirmed that ACE2 significantly decreased the lung cell apoptosis after ALI. Since the direct target of ACE2 in lung is Ang II, the receptor of which is uniquely expressed in PECs, we hypothesize the direct effects of ACE2 should be Ang II-related, and most likely to be conducted on PECs. To prove it, we exposed PECs to NO as an injury inducer *in vitro* [16-18], and found that the NO-mediated apoptosis of PECs were indeed similarly affected by ACE2, as *in vivo*. These data suggest that the protective effects of ACE2 on ALI-induced PEC apoptosis at least partially contribute to the protective effects of ACE2 on the injured lung after ALI.

Next, we found that NO induced phosphorylation of a key factor SMAD2 of TGF β receptor signaling [19-21], which could be dose-dependently inhibited by ACE2. Further, inhibition of SMAD2 phosphorylation through expression of its inhibitor SMAD7 significantly inhibited NO-induced cell apoptosis, without need for ACE2. This set of gain-of-function experiments perfectly support that the regulation of SMAD2 phosphorylation is responsible for the ACE2-mediated anti-apoptotic effects on PECs after ALI. Based on the knowledge on this pathway, it appears that after ALI, Ang II may activate SMAD2 by inducing its phosphorylation. pSMAD2

then induces cell apoptosis through increasing pro-apoptotic proteins and decreasing anti-apoptotic proteins, which leads to increases in apoptosis-related proteins, and subsequently reduction in cell apoptosis. We have also checked SMAD3 but found little expression in the endothelial cells. Thus, SMAD2 signaling plays a predominant role in the current model.

In future, it may be interesting to examine whether similar mechanisms also underlie the therapeutic effects of ACE2-expressing uMSCs. In line with these results, an independent protective role of uMSCs as well as a role as an ACE2-carrier can be distinguished and compared. Moreover, further experiments may be applied to examine the protective roles of ACE2 besides its inhibition on apoptosis. These approaches may greatly improve our understandings of the molecular basis of ACE2-mediated protection against ALI, and may shed light on development of novel therapies.

Disclosure Statement

The authors have declared that no conflict of interest exists.

Reference

- 1 Kangelaris KN, Calfee CS, May AK, Zhuo H, Matthay MA, Ware LB: Is there still a role for the lung injury score in the era of the berlin definition ards? *Ann Intensive Care* 2014;4:4.
- 2 Khemani RG, Wilson DF, Esteban A, Ferguson ND: Evaluating the berlin definition in pediatric ards. *Intensive Care Med* 2013;39:2213-2216.
- 3 Thompson BT, Matthay MA: The berlin definition of ards versus pathological evidence of diffuse alveolar damage. *Am J Respir Crit Care Med* 2013;187:675-677.
- 4 Ashbaugh DG, Bigelow DB, Petty TL, Levine BE: Acute respiratory distress in adults. *Lancet* 1967;2:319-323.
- 5 Herold S, Gabrielli NM, Vadasz I: Novel concepts of acute lung injury and alveolar-capillary barrier dysfunction. *Am J Physiol Lung Cell Mol Physiol* 2013;305:L665-681.
- 6 Dengler V, Downey GP, Tudor RM, Eltzschig HK, Schmidt EP: Neutrophil intercellular communication in acute lung injury. Emerging roles of microparticles and gap junctions. *Am J Respir Cell Mol Biol* 2013;49:1-5.
- 7 Vlaar AP, Juffermans NP: Transfusion-related acute lung injury: A clinical review. *Lancet* 2013;382:984-994.
- 8 Imai Y, Kuba K, Penninger JM: The discovery of angiotensin-converting enzyme 2 and its role in acute lung injury in mice. *Exp Physiol* 2008;93:543-548.
- 9 Chen D, Jiao G, Ma T, Liu X, Yang C, Liu Z: The mechanism of rapamycin in the intervention of paraquat-induced acute lung injury in rats. *Xenobiotica* 2014;1-9.
- 10 Liu W, Shan LP, Dong XS, Liu Z: Toll-like receptor 4 implicated in acute lung injury induced by paraquat poisoning in mice. *Int J Clin Exp Med* 2014;7:3392-3397.
- 11 Liu F, Gao F, Li Q, Liu Z: The functional study of human umbilical cord mesenchymal stem cells harbouring angiotensin-converting enzyme 2 in rat acute lung ischemia-reperfusion injury model. *Cell Biochem Funct* 2014;32:580-589.
- 12 Gao F, Li Q, Hou L, Li Z, Min F, Liu Z: Mesenchymal stem cell-based angiotensin-converting enzyme 2 in treatment of acute lung injury rat induced by bleomycin. *Exp Lung Res* 2014;40:392-403.
- 13 Liu D, Dong Y, Liu Z, Niu B, Wang Y, Gao X: Impact of trem-2 gene silencing on inflammatory response of endotoxin-induced acute lung injury in mice. *Mol Cell Biochem* 2014;394:155-161.
- 14 Min F, Gao F, Li Q, Liu Z: Therapeutic effect of human umbilical cord mesenchymal stem cells modified by angiotensin-converting enzyme 2 gene on bleomycin-induced lung fibrosis injury. *Mol Med Rep* 2015;11:2387-2396.
- 15 Zhang X, Gao F, Li Q, Dong Z, Sun B, Hou L, Li Z, Liu Z: Mscs with ace ii gene affect apoptosis pathway of acute lung injury induced by bleomycin. *Exp Lung Res* 2015;41:32-43.

- 16 Boshier PR, Hanna GB, Marczin N: Exhaled nitric oxide as biomarker of acute lung injury: An unfulfilled promise? *J Breath Res* 2013;7:017118.
- 17 Mehta S: The effects of nitric oxide in acute lung injury. *Vascul Pharmacol* 2005;43:390-403.
- 18 Royall JA, Kooy NW, Beckman JS: Nitric oxide-related oxidants in acute lung injury. *New Horizons* 1995;3:113-122.
- 19 Stopa M, Anhuf D, Terstegen L, Gatsios P, Gressner AM, Dooley S: Participation of smad2, smad3, and smad4 in transforming growth factor beta (tgf-beta)-induced activation of smad7. The tgf-beta response element of the promoter requires functional smad binding element and e-box sequences for transcriptional regulation. *J Biol Chem* 2000;275:29308-29317.
- 20 Ulloa L, Doody J, Massague J: Inhibition of transforming growth factor-beta/smad signalling by the interferon-gamma/stat pathway. *Nature* 1999;397:710-713.
- 21 Xiao X, Gaffar I, Guo P, Wiersch J, Fischbach S, Peirish L, Song Z, El-Gohary Y, Prasadan K, Shiota C, Gittes GK: M2 macrophages promote beta-cell proliferation by up-regulation of smad7. *Proc Natl Acad Sci U S A* 2014;111:E1211-1220.
- 22 Goebel NA, Babbey CM, Datta-Mannan A, Witcher DR, Wroblewski VJ, Dunn KW: Neonatal fc receptor mediates internalization of fc in transfected human endothelial cells. *Mol Biol Cell* 2008;19:5490-5505.
- 23 Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MA, Jr., Wrana JL, Falb D: The mad-related protein smad7 associates with the tgfbeta receptor and functions as an antagonist of tgfbeta signaling. *Cell* 1997;89:1165-1173.
- 24 Rodriguez-Vita J, Sanchez-Lopez E, Esteban V, Ruperez M, Egido J, Ruiz-Ortega M: Angiotensin ii activates the smad pathway in vascular smooth muscle cells by a transforming growth factor-beta-independent mechanism. *Circulation* 2005;111:2509-2517.