The American Journal of Pathology, Vol. 171, No. 4, October 2007

DOI: 10.2353/ajpath.2007.070226

Cardiovascular, Pulmonary and Renal Pathology

Inhibitor of Differentiation 1 Promotes Endothelial Survival in a Bleomycin Model of Lung Injury in Mice

Huimin Zhang,* William E. Lawson,† Vasiliy V. Polosukhin,† Ambra Pozzi,‡ Timothy S. Blackwell,† Ying Litingtung,* and Chin Chiang*

From the Departments of Cell and Developmental Biology and Medicine,‡ and the Department of Medicine,† Division of Allergy, Pulmonary, and Critical Care Medicine, Vanderbilt University Medical Center, Nashville, Tennessee*

The Id family of genes encodes negative regulators of basic helix-loop-helix transcription factors and has been implicated in diverse cellular processes such as proliferation, apoptosis, differentiation, and migration. However, the specific role of Id1 in lung injury has not been investigated. Bleomycin has been widely used to generate animal models of acute lung injury and fibrogenesis. In this study we found that, on bleomycin challenge, Id1 expression was significantly up-regulated in the lungs, predominantly in endothelial cells, as revealed by double immunolabeling and quantitative flow cytometric analysis. Mice with Id1 loss-of-function (*Id1*$^{-/-}$) displayed increased vascular permeability and endothelial apoptosis in the lungs after bleomycin-induced injury. Cultured *Id1*$^{-/-}$ lung microvascular endothelial cells also showed decreased survival when exposed to bleomycin. We detected a decrease in the level of Bcl-2, a primary anti-apoptotic protein, in *Id1*$^{-/-}$ endothelial cells, suggesting that down-regulated Bcl-2 may promote endothelial apoptosis in the lung. Therefore, we propose that Id1 plays a crucial role in promoting endothelial survival in the adult lung on injury. In addition, bleomycin-exposed *Id1*$^{-/-}$ mice showed increased lung collagen accumulation and fibrogenesis, suggesting that Id1 up-regulation in the lung may play a critical role in lung homeostasis. ***(Am J Pathol 2007, 171:1113–1126; DOI: 10.2353/ajpath.2007.070226)***

The Id family of genes consists of four members (Id1 to Id4) encoding helix-loop-helix transcriptional regulators that lack the basic domain. They function primarily by binding to and inhibiting the transcriptional activities of basic helix-loop-helix or Ets transcription factors.[1] One function of Id proteins is to inhibit myofibroblast differentiation by disrupting transcription complex formation involving basic helix-loop-helix proteins.[2] Another important role of Id proteins is their active engagement in cell-cycle regulation as well as promoting cell survival and delaying onset of cellular senescence.[3] Id proteins have been implicated in embryonic development and cancer by playing key roles in cellular processes such as proliferation, apoptosis, differentiation, or migration of various cell types, notably endothelial and neural precursor cells.[1,4–7]

Several lines of evidence have suggested that Id proteins play a key role in endothelial function and homeostasis. For example, Id proteins can regulate angiogenesis and promote endothelial survival during embryonic development and in tumor progression.[4] Mice lacking Id1 and Id3 functions displayed brain hemorrhage during embryonic development and defects in tumor-promoted angiogenesis.[6] Deregulated Id1 expression in endothelial cells substantially affected angiogenesis and tumor growth in various tumor models.[8–11] *In vitro* studies also demonstrated that overexpression of Id1 reduced human endothelial cell apoptosis rate.[12] Id1 has also been shown to delay endothelial senescence by suppressing CDK inhibitor expressions and may be an important component of the cellular stress response pathway.[13–15] Transplantation of Id1-overexpressing human umbilical vein endothelial cells into mice increased capillary density and limb salvage rate, indicating involvement of Id1 in endothelial repair.[12] Up-regulation of Id1 expression in endothelial cells was also detected during hypoxic vascular remodeling in pulmonary hypertension, suggesting a contribution of Id1 in maintaining endothelial homeostasis.[16]

Id1 is highly expressed in the lung mesenchyme during embryogenesis,[17] but its expression was detected at

Supported by the American Heart Association (to C.C.) and the National Institutes of Health (grant KO8HL85406 to W.E.L).

Accepted for publication July 3, 2007.

Address reprint requests to Chin Chiang, Ph.D., Associate Professor of Cell and Developmental Biology, the Department of Cell and Developmental Biology, Vanderbilt University Medical Center, 4114 MRB III, Nashville, TN 37232. E-mail: chin.chiang@vanderbilt.edu.

basal level in the adult murine lung (this work),[18] suggesting that Id1 might play a role in regulating adult lung homeostasis. Bleomycin has been widely used in animal models of acute lung injury and fibrosis, and mice treated with a single dose of bleomycin intratracheally displayed massive epithelial and endothelial cell damage followed by fibrogenesis.[19–25] Up-regulation of Id1 in bleomycin-treated rat lungs has been reported although its detailed tissue distribution and specific functions were not investigated.[18] In this study, we found that on bleomycin-induced injury, Id1 is up-regulated predominantly in endothelial cells, suggesting a potential role of Id1 in these cell types. Loss of Id1 function in the lung endothelium resulted in increased vascular permeability and endothelial cell death after bleomycin instillation. In agreement, we found that bleomycin-treated *Id1*$^{-/-}$ lung microvascular endothelial cells showed decreased survival in culture with significant reduction in the level of anti-apoptotic protein Bcl-2. Lung fibrosis is characterized by an initial accumulation of inflammatory cells, epithelial and endothelial injury and apoptosis, fibroblast proliferation, myofibroblast accumulation, and increased deposition of extracellular matrix proteins resulting in irreversible distortion of lung architecture.[26–31] Loss of Id1 function also resulted in increased susceptibility to fibrogenesis possibly because of increased endothelial damage. Collectively, our studies reveal a new function of Id1 in the lung endothelium in promoting the survival of pulmonary endothelial cells on bleomycin-induced lung injury.

Materials and Methods

Mice and Bleomycin Treatment

Id1-null mice (*Id1*$^{-/-}$) (gift of Dr. Robert Benezra, Memorial Sloan-Kettering Cancer Center, New York, NY) and *Tie1-Cre* mice[32] were bred in the C57BL/6J background, and *ShhCre-ZEG* mice[33] were bred in the C57BL/6J;129 background. For the Id1 time-course study, C57BL/6J mice (8 to 10 weeks of age) were purchased from the Jackson Laboratory (Bar Harbor, ME). *Id1*$^{-/-}$ mice were backcrossed for five generations to C57BL/6 background, and pairs of wild-type and *Id1*$^{-/-}$ littermates from *Id1*$^{+/-}$ matings were used for studies. Mice were treated with either saline or bleomycin (0.08 U) by intratracheal injection in a total volume of 50 μl of saline.[34] The experimental protocol was reviewed and approved by the Institutional Animal Care and Utilization Committee at Vanderbilt University.

Immunohistochemistry

ShhCre-GFP embryonic lungs were fixed in 4% paraformaldehyde for 5 hours at 4°C and embedded in OCT cryoprotectant embedding medium. Cryosections at 15 μm were collected and immunostained with PECAM-1 antibody (BD Pharmingen, San Diego, CA) followed by Alexa 568-conjugated secondary antibody (Molecular Probes, Eugene, OR) for signal visualization. *ShhCre-GFP* adult lungs were perfused using phosphate-buffered saline (PBS), then inflated and fixed in 4% paraformaldehyde for 5 hours at 4°C. OCT was subsequently injected intratracheally into fixed lung to preserve the lung architecture. Lungs were embedded in OCT and 15-μm sections were collected and green fluorescent protein (GFP) fluorescence visualized using a BX60F5 microscope (Olympus, Center Valley, PA).

Adult lungs were perfused, inflated, excised, and fixed in 4% paraformaldehyde at 4°C overnight. Subsequently, lungs were embedded in paraffin blocks and 5-μm sections were collected and processed for immunolabeling. Antibodies against Id1 (clone C-20; Santa Cruz Biotechnology, Santa Cruz, CA), smooth muscle α-actin (α-SMA; Sigma Chemical, St. Louis, MO), CD34 (Labvision, Fremont, CA), and β-galactosidase (LacZ) (Sigma) were used for immunostaining. For general immunolabelings, slides were antigen-retrieved using citrate buffer (pH 6.0) and incubated at 4°C overnight with primary antibody. Alexa-conjugated secondary antibodies or horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) and horseradish peroxidase detection kit (Labvision) were used for signal visualization.

For Id1 immunolabeling, lungs were perfused with PBS and fixed in EFA solution (100% ethanol, 37% formaldehyde, and 100% acetic acid at v/v/v ratio of 6:3:1) at 4°C for 5 hours. Lungs were subsequently dehydrated and embedded in paraffin blocks and 5-μm sections were collected and processed for immunolabeling. Slides were incubated at 4°C overnight with primary antibody, Id1, at 1:6000. Detection was performed using polymer-horseradish peroxidase secondary antibodies (Zymed, South San Francisco, CA) diluted at 1:4 and visualized using the TSA Plus Fluorescence System (Perkin-Elmer, Emeryville, CA) diluted at 1:200. Slides were counterstained with TO-PRO-3 (Invitrogen, Carlsbad, CA) to highlight nuclei. For double labelings involving Id1, sequential immunostainings were performed instead of a one-step double labeling. Confocal images were taken using the Zeiss Upright LSM510 confocal microscope (Carl Zeiss, Thornwood, NY) at the Vanderbilt Cell Imaging Core. Regular images were taken using the Olympus BX60F5 microscope.

Western Blotting

Left lungs of bleomycin-treated wild-type and *Id1*$^{-/-}$ mice were harvested and homogenized in radioimmunoprecipitation assay lysis buffer at pH 7.4. Protein lysates of 100 μg each were resolved on sodium dodecyl sulfate-polyacrylamide gels (Bio-Rad, Hercules, CA). Primary antibodies against Id1 or α-SMA were used for detection. Equal loading of protein samples was monitored and normalized to the level of α-tubulin (Calbiochem-EMD Biosciences, La Jolla, CA). Blots were analyzed using QuantityOne software (Bio-Rad). For Western blotting using fluorescence-activated cell sorting (FACS)-sorted cells, cells were immediately frozen in liquid nitrogen after collection. Sorted cells were lysed in radioimmunoprecipitation assay buffer and loaded at 50 μg/lane on

sodium dodecyl sulfate-polyacrylamide gels. Additional antibodies used for blotting were PECAM-1 (BD Pharmingen), TTF-1 (Labvision), Bcl-2, Bcl-xL (Santa Cruz Biotechnology), p-MEK1/2, MEK1/2, p-ERK1/2, and ERK1/2 (Cell Signaling Technology, Beverly, MA). Blots were scanned and quantified as described above.

Pulmonary Vascular Permeability Assay

Vascular permeability was examined using the Evans blue extravasation method as previously described.[35] In brief, Evans blue dye at 20 mg/kg body weight was injected into each animal via the retro-orbital sinus. Three hours after injection, lungs were perfused and homogenized in saline. Evans blue was extracted and quantified by dual wavelength at 620 and 740 nm using Bio-Rad Smartspec3000. Corrected pulmonary Evans blue absorbance at 620 nm was calculated as A_{620} nm ($1.426 \times A_{740}$ nm + 0.03). Permeability index was generated by dividing the corrected pulmonary Evans blue absorbance by the plasma Evans blue absorbance at 620 nm.

Pulmonary Endothelial Cell Culture

Adult lung microvascular endothelial cells were isolated and cultured as previously described.[36] In brief, lungs were perfused with 0.25% trypsin (Mediatech, Herndon, VA) and 2 μg/ml collagenase (Roche Applied Science. Indianapolis, IN). Perfused lungs were incubated at 37°C for 20 minutes. Then lung lobes were trimmed with a sterile scalpel and washed 10 to 20 times with 1 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Detached cells were collected and centrifuged at 2000 rpm. The cell pellet was washed once and resuspended in EGM2-MV medium supplemented with vascular endothelial growth factor (Cambrex, Walkersville, MD) and 2% fetal bovine serum. Cells were grown in six-well dishes (BD Falcon, Franklin Lakes, NJ) or coverslips (Fisher, Pittsburgh, PA) for 3 days before bleomycin treatment. Vascular endothelial growth factor and fetal bovine serum were removed during bleomycin incubation. All cells were maintained at 37°C and 5% CO_2 in a Hera cell incubator unit (Kendro Laboratories, Waltham, MA). Cells from triplicate wells were harvested for immunodetection by Western blotting as above.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL)

For cell death detection in lung tissue sections, paraformaldehyde-fixed paraffin sections were boiled in citrate buffer (pH 6.0) for 20 minutes, and apoptotic cells were detected by TUNEL using the In Situ cell death detection kit (Chemicon, Temecula, CA) according to the manufacturer's protocol. Detection was performed using a horseradish peroxidase-conjugated secondary antibody and visualized using the TSA Plus fluorescence system (Perkin-Elmer). Slides were subsequently double-stained with CD34 to mark endothelial cells.

For TUNEL staining of cultured endothelial cells, freshly isolated lung microvascular endothelial cells were grown in culture dishes for 3 days before treatment with 250 ng/ml bleomycin for 6 hours. The bleomycin-containing media were then removed and replaced with fresh EGM2-MV media for 3 hours. The cells were subsequently stained with TUNEL according to the manufacturer's protocol.

Quantification of Apoptotic Endothelial Cells by FACS Analysis

For FACS analysis on freshly isolated murine lung microvascular endothelial cells, saline- or bleomycin-treated lungs were perfused with 25 U/ml dispase (BD Biosciences) plus 2 μg/ml collagenase (Roche Applied Science) then incubated in digestive solution at 37°C for 20 minutes. The lungs were then minced, and a single-cell suspension was obtained by passing cells through a 40-μm cell strainer (BD Falcon). After centrifugation at 1000 rpm for 5 minutes, collected cells were resuspended in red cell lysis buffer[37] and incubated at room temperature for 10 minutes. The cells were then washed twice with PBS and stained with apoptotic marker using the annexin-5 apoptosis detection kit (Biovision, Mountain View, CA) according to the manufacturer's protocol.

For FACS analysis on cultured lung microvascular endothelial cells, freshly isolated lung microvascular endothelial cells were grown in a culture dish for 3 days before treatment with 250 ng/ml bleomycin for 6 hours. Cells were collected by dispase/collagenase digestion and labeled with annexin-5 to mark apoptotic cells. FACS sorting was performed at the Vanderbilt Flow Cytometry Facility.

Histology and Pathology Scoring

Lungs were perfused, inflated (20 cmH_2O), excised, and fixed in 4% paraformaldehyde at 4°C overnight. Subsequently, lungs were embedded in paraffin blocks, and 5-μm sections were collected and processed for hematoxylin and eosin (H&E). Slides of lung tissue were randomized and evaluated on 10 sequential, nonoverlapping fields (magnification, $\times$30) of lung parenchyma for each specimen. Evaluation of parenchymal distortion on H&E-stained lung sections was done by a pathologist blinded to the genotype and treatment group, using a 0 to 4 point scale, with a score of 0, normal architecture; 1, increased thickness of up to 50% of interalveolar septa; 2, thickening of >50% of interalveolar septa without formation of fibrotic foci; 3, thickening of the interalveolar septa with formation of isolated fibrotic foci; and 4, formation of multiple fibrotic foci with total or subtotal distortion of parenchymal architecture.[38]

Hydroxyproline Assay and Trichrome Staining

The left lungs of wild-type and *Id1*$^{-/-}$ mice, harvested at 2 weeks after bleomycin, were analyzed for hydroxyproline content as previously described.[39] In brief, the left lung lobes were weighed and homogenized in distilled water. The samples were mixed well and digested with 2 N sodium hydroxide in a total volume of 100 μl at 120°C for 20 minutes. After digestion, 900 μl of chloramine T (1.27 g of chloramine T, 20 ml of 50% *n*-propanol, and citrate-acetate buffer in 100 ml) was added to each sample, mixed, and left at room temperature for 25 minutes. Then, 1 ml of Ehrlich's solution (15 g of 4-dimethylaminobenzaldehyde in 100 ml of *n*-propanol and 70% perchloric acid at a volume ratio of 2:1) was added to each sample, mixed, and incubated for 20 minutes at 65°C. Samples were cooled for 10 minutes and then read at 550 nm on a spectrophotometer. Concentrations were calculated against a hydroxyproline standard curve. Trichrome staining of lung sections for collagen content was performed by the Vanderbilt Immunohistochemistry Core Laboratory.

Statistics

To assess differences among groups, statistical analyses were performed using a one-way analysis of variance with Microsoft Excel (Microsoft Corporation, Redmond, WA) and significance accepted at $P < 0.05$. Results are presented as mean ± SEM.

Results

Although Id1 is highly expressed in the embryonic lung mesenchyme during a period of epithelial-mesenchymal interaction,[17] its expression is not detectable in normal adult lung tissue sections by immunohistochemistry (Figure 1A, saline 1 week). By Western blot analysis, which is a more sensitive detection method using whole lung homogenates, we detected weak Id1 expression (Figure 1B, saline). To investigate whether Id1 expression is up-regulated on pulmonary insult, we treated 8-week-old adult wild-type mice with a single 0.08-U dose of bleomycin intratracheally and harvested lungs at 1 week after bleomycin for Id1 immunohistochemistry. Interestingly, we found significant induction of nuclear Id1 expression in the bleomycin-treated wild-type lung compared with saline control (Figure 1A, Bleo 1 week). The specificity of Id1 antibody staining was confirmed using *Id1*$^{-/-}$ lung as a negative control (Figure 1A; Bleo 1 week, *Id1*$^{-/-}$). To evaluate the level and time course of Id1 induction in wild-type mice, we examined Id1 protein levels from lung samples collected at 1, 2, and 3 weeks after bleomycin instillation ($n = 3$, Figure 1B). As shown by Western blotting, Id1 expression is significantly up-regulated at 1 week after bleomycin compared with saline control, and its up-regulation is maintained for 2 and 3 weeks after bleomycin (Figure 1B, data not shown).

We found that a large proportion of Id1 expression localized to lung endothelial cells as revealed by double immunolabeling with Id1 and endothelial marker CD34, which labels the capillary bed.[40] As shown in Figure 2A, Id1-positive cells displayed nuclear Id1 expression surrounded by membrane and cytoplasmic expression of CD34 (Figure 2A, arrows). There were only a few Id1-expressing cells that appeared to be CD34-negative (Figure 2A, arrowhead). To determine the fraction of Id1 protein expression level derived from endothelial cells, we performed FACS analysis of *Tie1Cre*-GFP-labeled endothelial cells to quantify the relative level of endothelial-derived Id1 expression by Western blotting. Tie-1 is a receptor tyrosine kinase expressed during early stages of vascular development, and *Tie-1* promoter-driven *Cre-GFP* reporter expression specifically marks endothelial cells in the adult mice.[32] By crossing *Tie1Cre* to *ZEG* mouse, which contains a transgene harboring a conditional GFP reporter that is activated in the presence of Cre recombinase,[41] mice were indelibly marked by GFP in all lung endothelial cells. The FACS-sorted endothelial fraction was confirmed by Western blotting with endothelial marker, PECAM-1 (Figure 2B). By Id1 Western blot analysis, we found that the GFP-positive endothelial cell population expressed a relatively much higher level of Id1 compared with the nonendothelial cell population. At 1 week after bleomycin, we determined that Id1 expression level in the lung endothelial population was up-regulated up to 11-fold compared with saline control based on normalized densitometric measurements (Figure 2B). We also determined that ~86% of total Id1 expression level was derived from the lung endothelial fraction (Figure 2B). In contrast, bleomycin-treated non-endothelial cells displayed only approximately threefold up-regulation. This significant contribution in Id1 expression level by the endothelial cell population persisted at 2 weeks after bleomycin (Figure 2C).

To examine Id1 expression in the alveolar epithelium, we took advantage of the *Sonic hedgehog* (*Shh*)-*Cre* mouse line that we had generated, which marks all lung cells of epithelial origin (Figure 3A).[33] *Shh* is expressed early in the epithelium of the embryonic lung primordium.[42–45] Mice generated by crossing *Shh-Cre* to *ROSA26R* mouse, which contains a transgene harboring a conditional *LacZ* reporter that is activated in the presence of Cre recombinase, are indelibly marked in all lung epithelial cells by LacZ reporter expression. Subsequently, all lung epithelial cells are marked by LacZ as shown at embryonic stages E11.5, E15.5, and in the normal adult bronchial and alveolar epithelium (Figure 3A). By double immunolabeling, we observed that Id1-expressing cells showed very few co-localization with LacZ^{+} epithelial cells both in the bronchial epithelium and in the alveolar bed (Figure 3B, arrow). Most Id1-expressing cells do not co-localize with epithelial LacZ expression (Figure 3B, arrowheads). By counting 10 nonoverlapping fields of Id1 and LacZ double-stained sections, we determined that only ~3% of all Id1-expressing cells were LacZ-positive. Similar to the strategy used in Figure 2, we used *ShhCre-ZEG* reporter mice to selectively identify GFP-positive epithelial cells in the lung by FACS analysis; the epithelial fraction was confirmed by Western blotting with epithe-

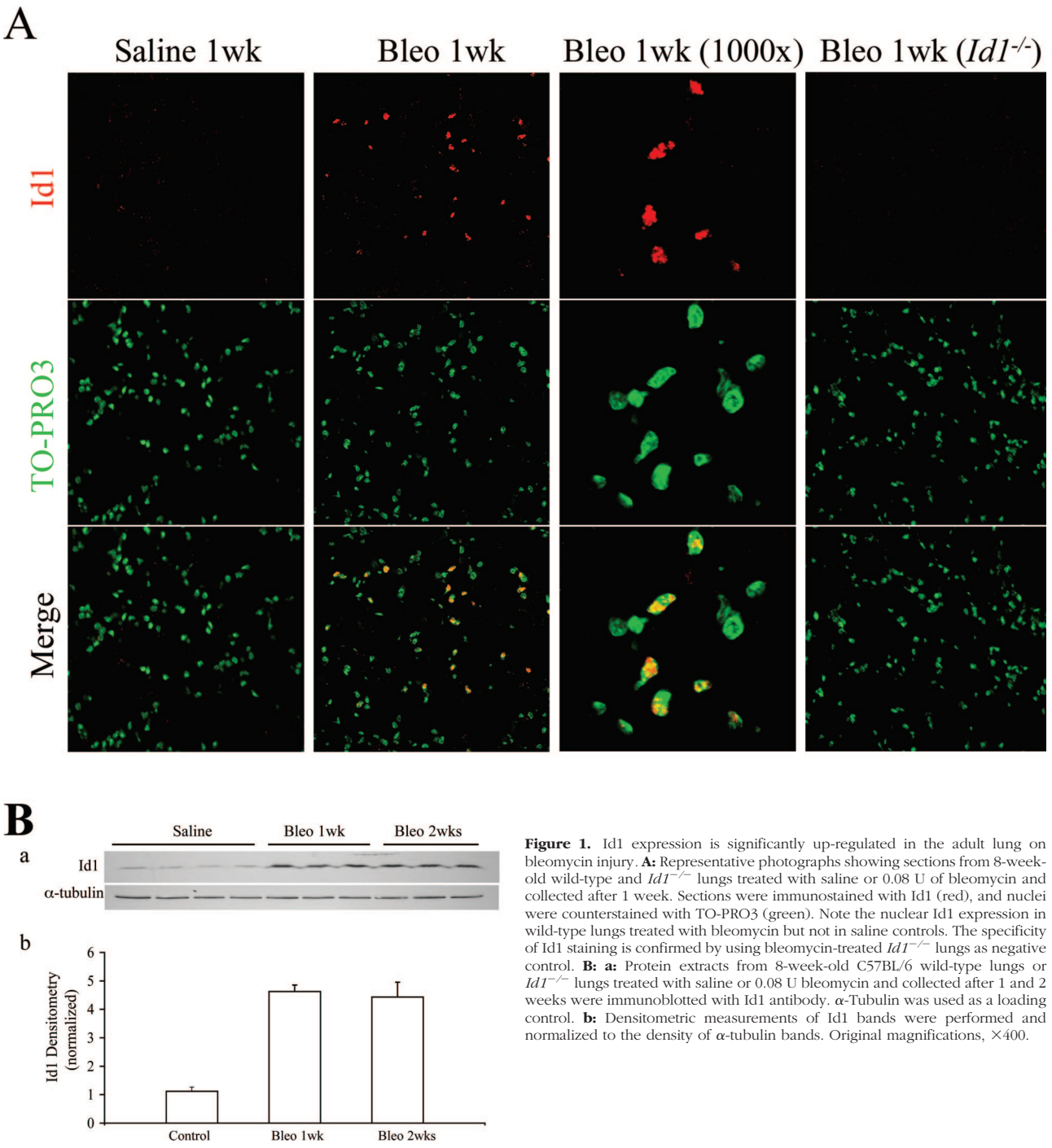

Figure 1. Id1 expression is significantly up-regulated in the adult lung on bleomycin injury. **A:** Representative photographs showing sections from 8-week-old wild-type and $Id1^{-/-}$ lungs treated with saline or 0.08 U of bleomycin and collected after 1 week. Sections were immunostained with Id1 (red), and nuclei were counterstained with TO-PRO3 (green). Note the nuclear Id1 expression in wild-type lungs treated with bleomycin but not in saline controls. The specificity of Id1 staining is confirmed by using bleomycin-treated $Id1^{-/-}$ lungs as negative control. **B: a:** Protein extracts from 8-week-old C57BL/6 wild-type lungs or $Id1^{-/-}$ lungs treated with saline or 0.08 U bleomycin and collected after 1 and 2 weeks were immunoblotted with Id1 antibody. α-Tubulin was used as a loading control. **b:** Densitometric measurements of Id1 bands were performed and normalized to the density of α-tubulin bands. Original magnifications, ×400.

lial marker TTF-1[46] (Figure 3C). As shown in Figure 3B, bleomycin-treated GFP-positive epithelial cells displayed less than twofold up-regulation of Id1, whereas the nonepithelial fraction displayed up to ninefold increase in Id1 expression level. These results indicate that Id1 is not significantly up-regulated in epithelial cells of bleomycin-treated lungs, which is consistent with the immunohistochemical data (Figure 3B).

We observed a few fibroblastic-like cells expressing Id1 as has been reported previously in the rat lung.[18] However, Id1 expression was not detected in the majority of α-SMA-positive cells located in fibrotic foci at 2 weeks after bleomycin (Figure 3D). Collectively, our data suggest that ~80% of total Id1 protein expression level in the lung, at 1 and 2 weeks after bleomycin exposure, is derived from the endothelial cell population, and ~20% is derived from other lung cell types, possibly immune cells that are involved in lung injury by bleomycin and have been shown to express Id1.[47] Our finding indicates that epithelial cells and α-SMA-positive fibroblasts do not contribute to the bulk of Id1 expression in bleomycin-injured lungs. In sum, our finding strongly supports a

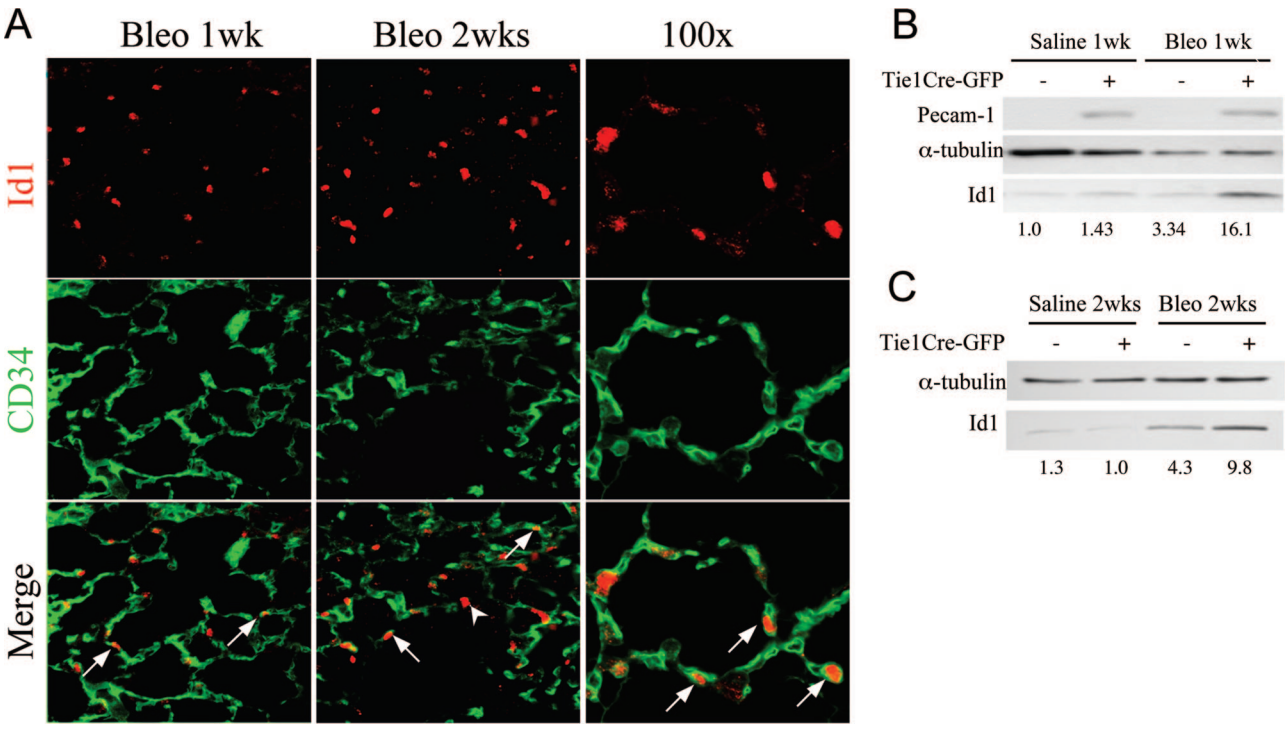

Figure 2. Id1 expression is up-regulated prominently in the injured lung endothelium. **A:** Representative photographs showing sections from 8-week-old wild-type lungs at 1 and 2 weeks after bleomycin. Sections were double-stained with Id1 (red) and endothelial marker CD34 (green) antibodies. **Arrows** in the figures point to representative Id1-expressing endothelial cells; note the membrane and cytoplasmic CD34 staining that wraps around the nuclear Id1 staining. **Arrowheads** point to Id1-expressing nonendothelial cells. **B:** Eight-week-old wild-type *Tie1Cre-ZEG* lungs were treated with saline or 0.08 U of bleomycin and collected after 1 week; lung cells were sorted into endothelial (GFP^+) and nonendothelial (GFP^-) group by FACS. Protein extracts were immunoblotted with Id1 and α-tubulin as the loading control. The endothelial fraction was confirmed by immunoblotting with endothelial marker, PECAM-1. Densitometric measurements of Id1 bands were performed and normalized to the density of α-tubulin bands and numeric values indicating differential levels of expression are shown at the bottom. Note the dramatic up-regulation of Id1 protein level in endothelial cells from bleomycin-treated lungs compared with the nonendothelial fraction and saline controls. **C:** Eight-week-old wild-type *Tie1Cre-ZEG* lungs were collected at 2 weeks after bleomycin or saline and then subjected to FACS sorting and Western blotting. Protein extracts were immunoblotted with Id1 and α-tubulin. Densitometric measurements of Id1 bands were performed and normalized to the density of α-tubulin bands. Original magnifications: ×400 (**A**, low magnification); ×1000 (**A**, high magnification).

critical role of Id1 in lung endothelial cells on bleomycin-induced injury; however, this study does address the potential role of Id1 in other cell types.

Because lung endothelial cells comprised a large fraction of Id1-expressing cells after bleomycin exposure, we investigated the functional role of Id1 in endothelial cells. To assess endothelial dysfunction in *Id1*$^{-/-}$ mutant lungs, we first evaluated change in vascular permeability by measuring the extravasation of Evans blue dye, which, when injected via the retro-orbital sinus, can immediately complex with circulating albumin (see Materials and Methods). Accumulation of albumin-dye complexes within the lung parenchyma was quantified spectrophotometrically and used as an indicator of vascular macromolecular leakage.[35] We found that wild-type lungs showed significant leakage of Evans Blue at 1 week after bleomycin when compared with saline-treated lungs (Figure 4A). However, vascular leakage is more pronounced in *Id1*$^{-/-}$ mutants, showing a 58% increase in dye content when compared with bleomycin-treated wild-type lungs ($n = 6$, $P < 0.001$; Figure 4A), indicating increased disruption of the endothelial barrier in the absence of Id1 function.

Id1 has been shown to attenuate apoptotic cell death in human umbilical vein endothelial cells.[12] Therefore, it is conceivable that an increase in endothelial permeability in *Id1*$^{-/-}$ lungs is, at least in part, contributed by an increase in endothelial cell death. To determine the level of endothelial cell death in *Id1*$^{-/-}$ and wild-type lungs at 1 week after bleomycin instillation, we first performed TUNEL and CD34 double immunolabeling. We found that there is a significantly higher number of apoptotic endothelial cells in *Id1*$^{-/-}$ compared with wild-type mice (Figure 4B). For quantitative analysis, we performed FACS to measure the percent increase in apoptotic endothelial cells that are double-positive for *Tie1*-GFP (endothelial) and annexin-5 (apoptotic) in *Id1*$^{-/-}$ lungs compared with wild type. In bleomycin-treated lungs, FACS analysis indicated that wild-type and *Id1*$^{-/-}$ mice contained, respectively, an average of 11.7% and 16.1% apoptotic endothelial cells that were double-positive for GFP and annexin-5 (Figure 4C). Therefore, there was an average increase of 37.6% in apoptotic endothelial cells in *Id1*$^{-/-}$ relative to the wild-type level ($n = 6$). Saline-treated lungs showed a relatively low level of apoptotic endothelial cells and were not significantly different in both genotypes (Figure 4C). This result clearly indicates a significant rise in apoptotic endothelial cells in *Id1*$^{-/-}$ mutant mice after bleomycin treatment, suggesting a key role of Id1 in attenuating endothelial cell death on lung injury.

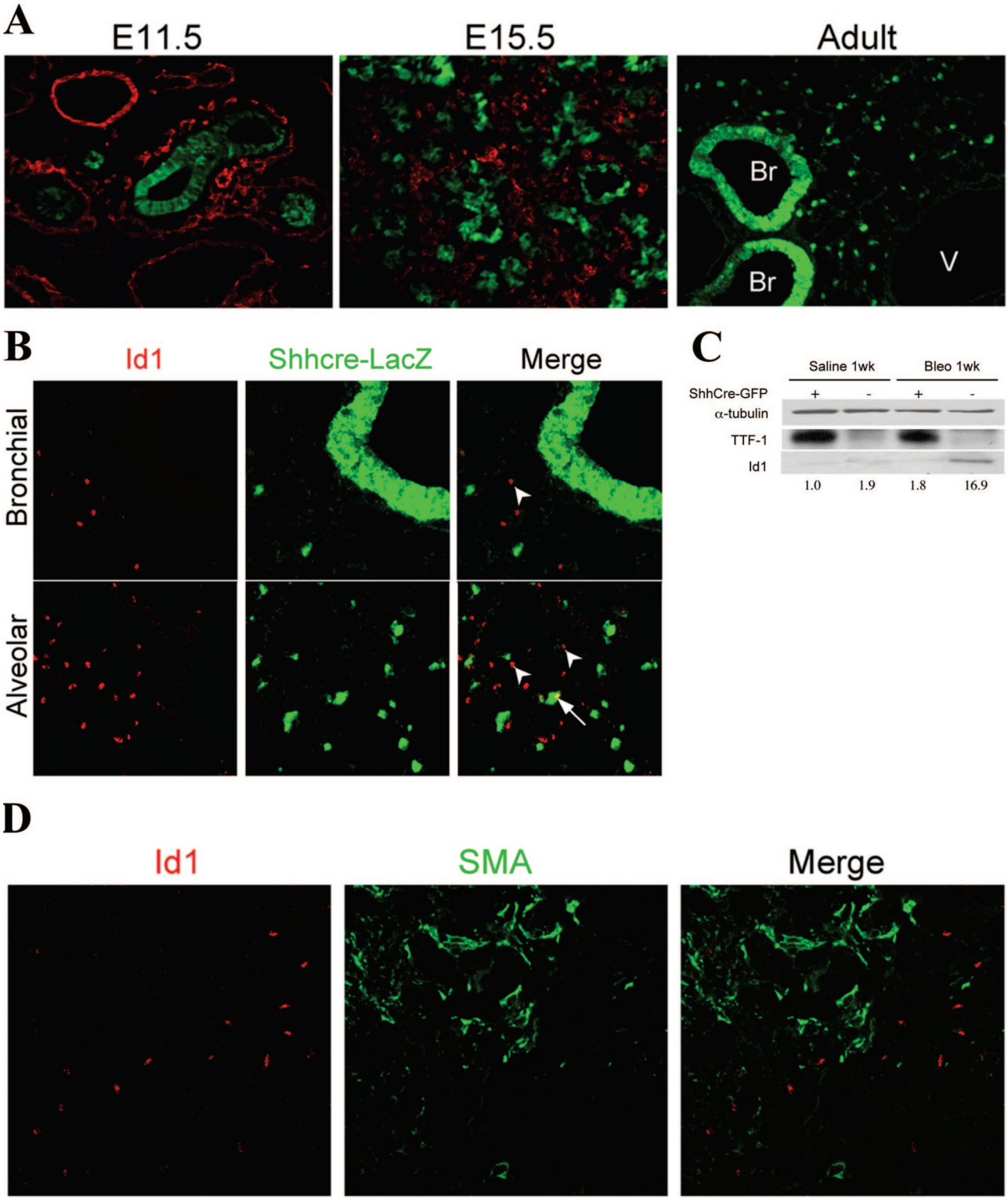

Figure 3. Id1 expression is not significantly up-regulated in bleomycin-exposed lung fibroblasts and *ShhCre*-GFP-marked epithelium. **A:** Distribution of GFP-labeled epithelial cells (green) in embryonic lungs at E11.5 and E15.5 and in the adult lung at 6 weeks. Embryonic lung sections were double-stained with endothelial marker PECAM-1 (red). Note that GFP expression is strictly confined in epithelial cells throughout development and is excluded from the PECAM-1-positive endothelial domain. GFP expression was detected in the adult bronchial epithelium (Br) and alveolar epithelial cells. Vessel (V) was negative. **B:** Representative photographs showing sections from 8-week-old wild-type *ShhCre-R26R* lungs at 1 week after bleomycin. Sections were double-stained with Id1 (red) and epithelial marker Shhcre-LacZ (green). **Arrows** in the figures point to representative Id1-expressing epithelial cells. **Arrowheads** point to Id1-expressing nonepithelial cells. **C:** Eight-week-old wild-type *ShhCre-ZEG* lungs collected at 1 week after bleomycin or saline were subjected to FACS sorting and Western blotting. Protein extracts were immunoblotted with Id1 and α-tubulin. Densitometric measurements of Id1 bands were performed and normalized to the density of α-tubulin bands. The epithelial fraction was confirmed by immunoblotting with epithelial marker, TTF-1. **D:** Representative photographs showing sections from 8-week-old wild-type lungs at 2 weeks after bleomycin. Sections were double-stained with Id1 (red) and myofibroblast marker, smooth muscle α-actin (SMA) (green). Original magnifications: ×200 (**A**); ×600 (**B**); ×400 (**D**).

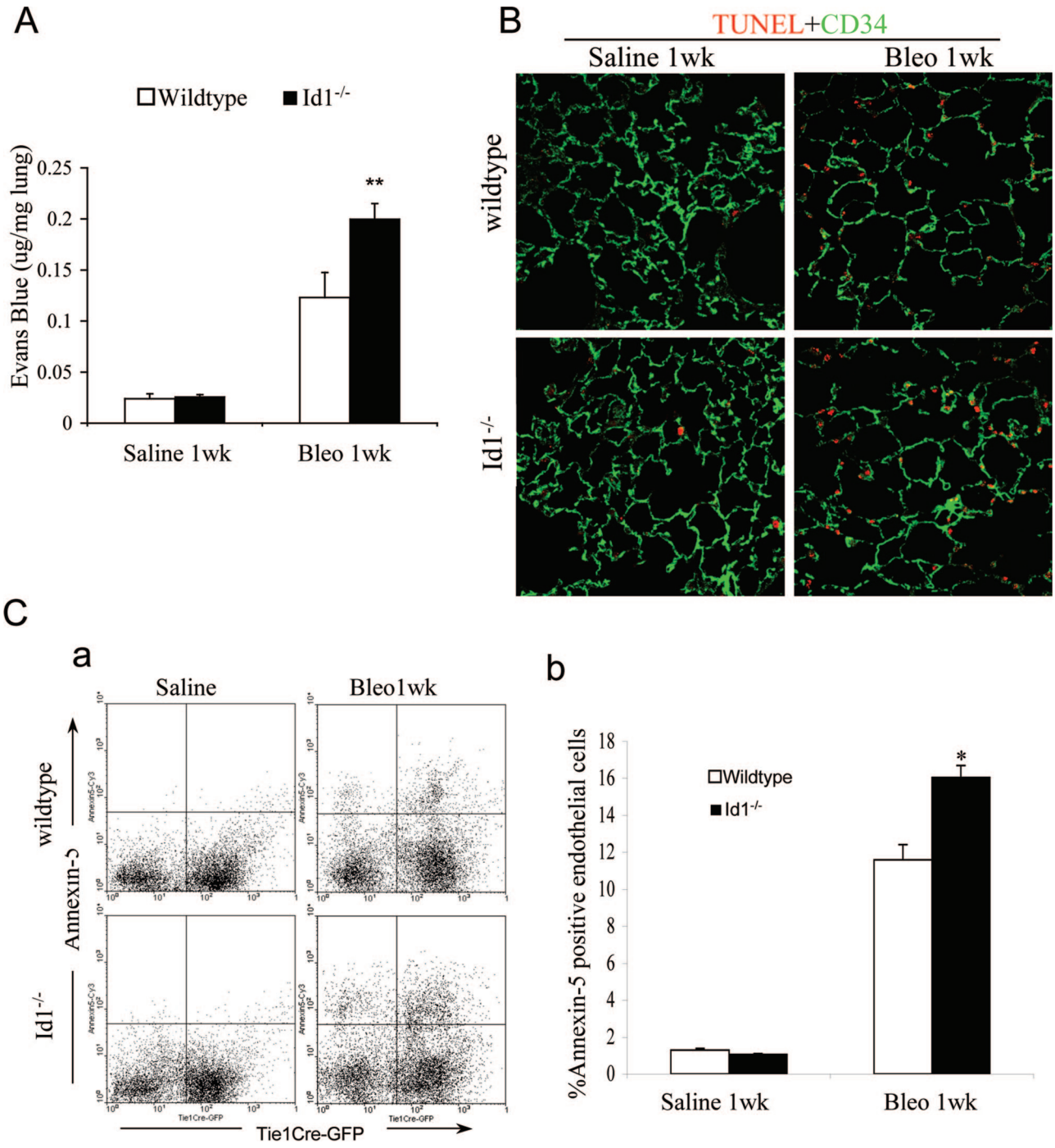

Figure 4. Bleomycin-injured *Id1*$^{-/-}$ lungs display increased endothelial barrier dysfunction and elevated endothelial apoptosis. **A:** Increased vascular permeability in bleomycin-challenged *Id1*$^{-/-}$ lungs. Pulmonary vascular permeability of saline or bleomycin-treated 8-week-old wild-type or *Id1*$^{-/-}$ lungs was determined by Evans blue extravasation assay ($n = 6$ for each group). Note 58% increase in Evans Blue dye leakage in injured *Id1*$^{-/-}$ lungs compared with wild-type lungs. **Asterisks** denote a significant difference ($P < 0.001$) between wild-type and *Id1*$^{-/-}$ lungs at 1 week after bleomycin. **B:** Endothelial cell death detection in wild-type and *Id1*$^{-/-}$ lungs by TUNEL (red) and CD34 (green) double-labeling. Representative sections are shown for lungs at 1 week after bleomycin or saline. **C:** Flow cytometric counting of apoptotic endothelial cells in wild-type or *Id1*$^{-/-}$ *Tie1Cre;ZEG* lungs at 1 week after bleomycin or saline. **a:** Representative dotspot graphs showing cell sorting results of 10,000 lung cells, using annexin-5 as the cell death marker and GFP as the endothelial marker. The top right quadrant represents apoptotic endothelial cells. **b:** Statistical representation of the sorting results from six independent experiments ($n = 6$). Note there is an average 37.6% increase in endothelial cell death in injured *Id1*$^{-/-}$ lungs compared with wild-type lungs. **Asterisks** denote a significant difference ($P < 0.05$) between wild-type and *Id1*$^{-/-}$ lungs. Original magnifications, ×400.

Because Id1 function has been linked to cell proliferation, we also examined the change in endothelial proliferation in wild-type and *Id1*$^{-/-}$ lungs after bleomycin treatment. Results from double labeling with proliferating cell nuclear antigen and CD34, to detect proliferating endothelial cells, revealed that the lung endothelium was relatively quiescent and did not appear to become highly proliferative on bleomycin treatment. In addition, we did

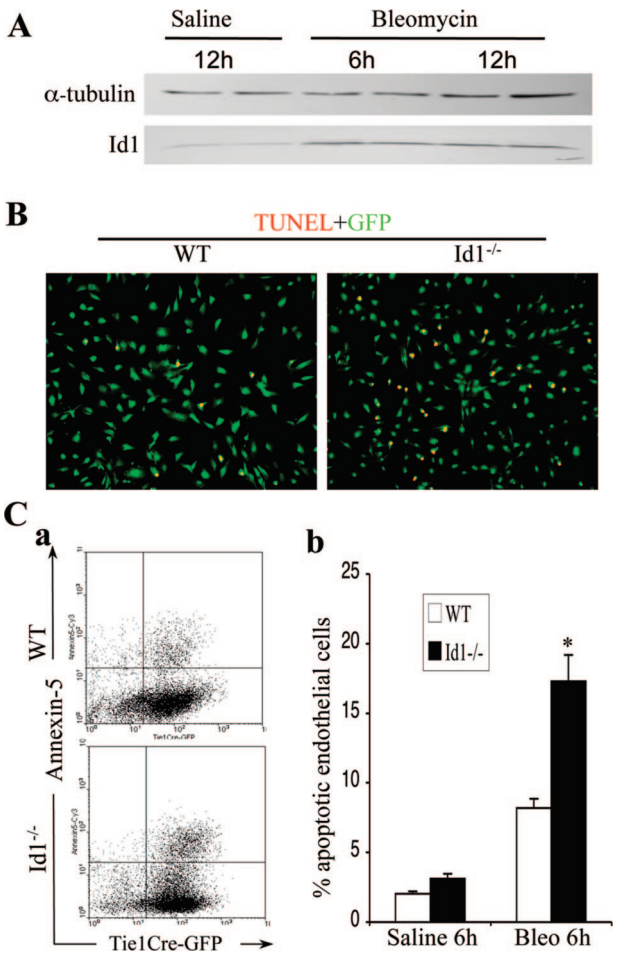

Figure 5. *Id1*$^{-/-}$ lung microvascular endothelial cells display reduced survival in culture. **A:** Id1 is up-regulated in endothelial cells by bleomycin *in vitro*. Western blotting showing Id1 level from wild-type primary LMVECs treated with saline or 250 ng/ml bleomycin for the indicated time points. Values represent relative fold change of Id1 protein level normalized to the density of α-tubulin bands. **B:** Cell death detection of wild-type and *Id1*$^{-/-}$ primary LMVECs by TUNEL (red) and *Tie1Cre*-GFP (green) double labeling. Representative photographs are shown for cell cultures treated with 250 ng/ml bleomycin for 6 hours followed by no bleomycin for 3 hours. **C:** Flow cytometric counting of apoptotic endothelial cells in wild-type or *Id1*$^{-/-}$ *Tie1Cre-ZEG* LMVECs treated with saline or 250 ng/ml bleomycin for 6 hours. **a:** Representative dotspot graphs showing the results of cell sorting, at 10,000 cells, each using annexin-5 as the cell death marker and GFP as the endothelial marker. The top right quadrant represents apoptotic endothelial cells. **b:** Statistical representation of the sorting results from six independent experiments ($n = 6$). Note that there is nearly twofold increase in endothelial cell death in *Id1*$^{-/-}$ LMVECs compared with wild type. **Asterisks** denote a significant difference ($P < 0.05$) between wild-type and *Id1*$^{-/-}$ cells. Original magnifications, ×100.

not find significant differences in endothelial proliferation in *Id1*$^{-/-}$ compared with wild-type lungs at 1 week after bleomycin, suggesting that Id1 is unlikely to play a major role in endothelial cell proliferation after lung injury (data not shown). Collectively, our findings indicate that Id1 plays a key role in the survival of cells of the lung endothelium after exposure to bleomycin, and loss of Id1 function results in increased endothelial apoptosis and damage to the lung endothelium.

To examine further the role of Id1 in endothelial cell function and cell death induced by bleomycin, we performed cell culture experiments using freshly isolated lung microvascular endothelial cells (LMVECs) from both

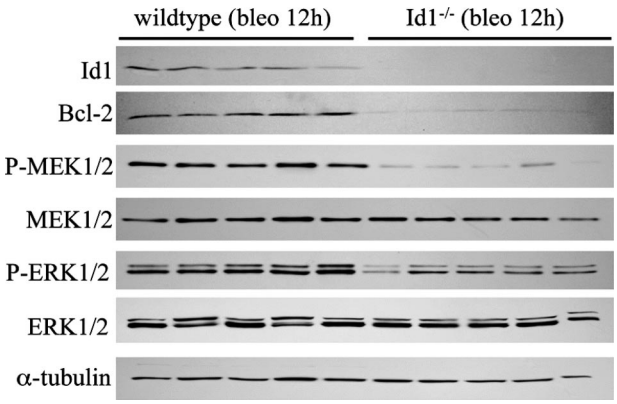

Figure 6. *Id1*$^{-/-}$ lung microvascular endothelial cells show reduced Bcl-2 level and MEK/ERK activity. Western blotting of cell lysates collected from wild-type or *Id1*$^{-/-}$ primary LMVECs treated with 250 ng/ml bleomycin for or 12 hours. Note that Bcl-2 level is significantly decreased in LMVECs in the absence of Id1 function. The MEK/ERK signaling pathway is also affected in *Id1*$^{-/-}$ endothelial cells, based on lower levels of phosphorylated MEK/ERK.

wild-type and *Id1*$^{-/-}$ lungs. Cultured wild-type LMVECs expressed a moderate level of Id1, and this expression was further increased after bleomycin challenge (Figure 5A). We also observed that although freshly dissociated wild-type LMVECs could be passaged three times in culture, it was not possible to passage *Id1*$^{-/-}$ LMVECs after first plating, which is probably because of their reduced growth potential. This is consistent with a slight increase in apoptotic cells in saline-treated Id1$^{-/-}$ LMVECs compared with Id1 wild type (Figure 5B, 3.11 versus 2.03%). Wild-type and *Id1*$^{-/-}$ LMVEC cultures grown for 3 days were treated with 250 ng/ml bleomycin for 6 hours and analyzed by TUNEL or double-labeled with *Tie1Cre*-GFP (endothelial reporter) and annexin-5-Cy3 (apoptotic marker) and sorted by FACS analysis to quantify the level of apoptotic endothelial cells in culture ($n = 6$). Consistent with TUNEL staining, which showed significantly more apoptotic cells in *Id1*$^{-/-}$ LMVECs (Figure 5B), *Id1*$^{-/-}$ LMVECs displayed an average of 17.3% apoptotic endothelial cells compared with 8.2% in wild type (Figure 5C). These results indicate that Id1 plays a crucial role in LMVEC survival in culture and are consistent with the functional role of Id1 in bleomycin-injured lungs *in vivo*.

We reasoned that increased endothelial cell apoptosis may be linked to decreased levels of Bcl-2 family proteins, specifically Bcl-2 and Bcl-xL, which have been shown to be regulated by Id1 in several cancer cell lines.[48–50] Because Bcl-2 proteins are well-known anti-apoptotic molecules, they may serve as a potential downstream effector of Id1 in the process of endothelial maintenance. Hence, we examined the expressions of Bcl-2 and Bcl-xL in bleomycin-exposed wild-type and *Id1*$^{-/-}$ LMVECs by Western blotting. Although we did not detect a significant difference in Bcl-xL levels in *Id1*$^{-/-}$ endothelial cell population compared with wild type, Bcl-2 expression was significantly higher in wild-type LMVECs as early as 6 hours after bleomycin exposure (data not shown). This difference was even more pronounced after 12 hours of bleomycin treatment (Figure 6). This finding strengthens the notion that Id1 can inhibit cell apoptosis by modulating Bcl-2 expression. In addition, we exam-

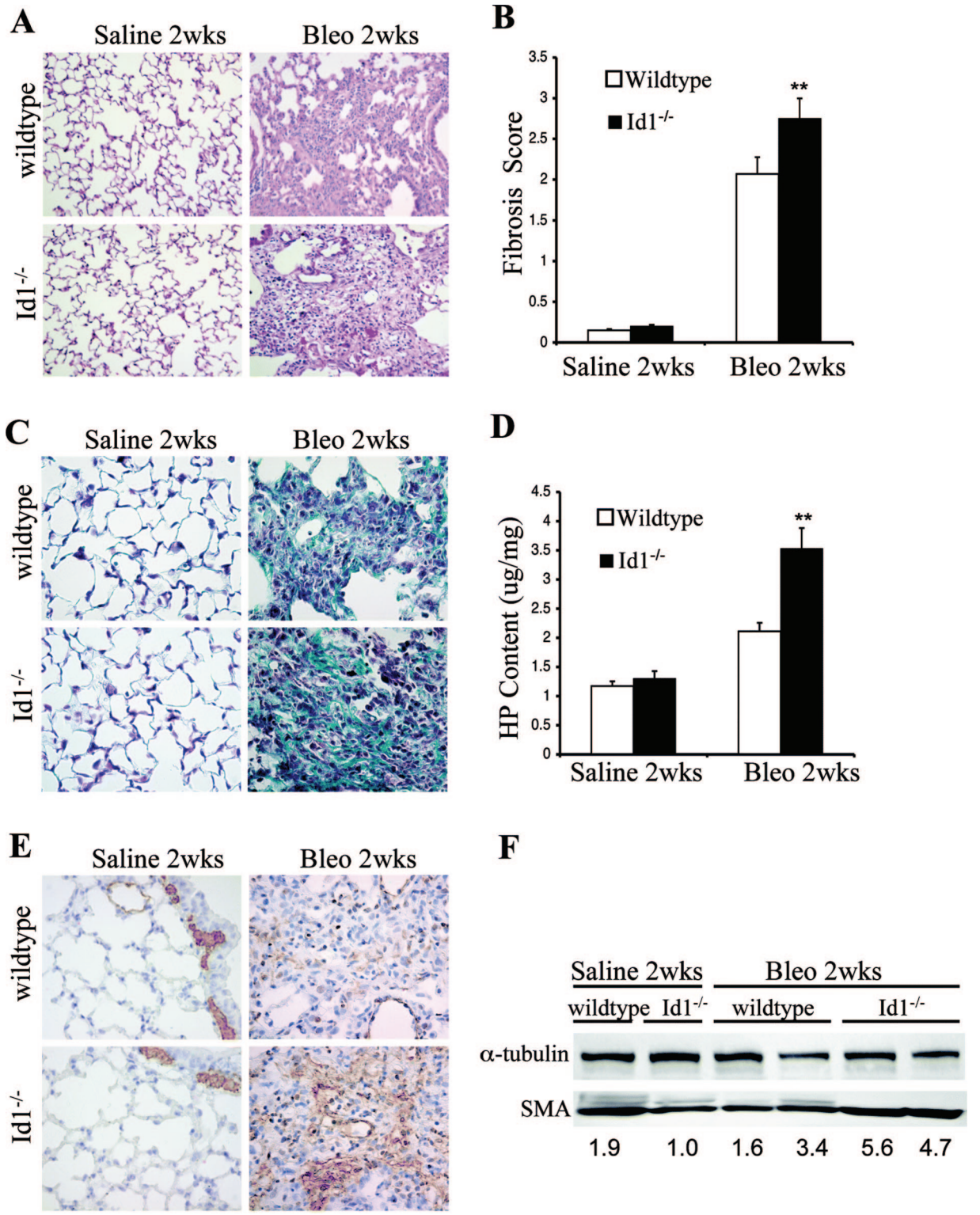

Figure 7. *Id1*$^{-/-}$ lungs are more susceptible to bleomycin-induced fibrogenesis. **A:** H&E staining of lung sections to show architectural changes. Representative photographs showing foci formation in wild-type and *Id1*$^{-/-}$ lungs at 2 weeks after bleomycin. **B:** Increase in the index of architectural distortion in *Id1*$^{-/-}$ lungs compared with wild-type lungs after bleomycin challenge. **Double asterisks** denote a significant difference ($P < 0.001$) between wild-type and *Id1*$^{-/-}$ lungs at 2 weeks after bleomycin ($n = 9$ for each group). **C:** Trichrome staining showing increased collagen deposition in lungs of *Id1*$^{-/-}$ mice after bleomycin challenge. Representative photographs reveal increased collagen accumulation (blue) in *Id1*$^{-/-}$ lungs compared with wild type at 2 weeks after bleomycin. **D:** Quantification of collagen deposition in lungs of wild-type and *Id1*$^{-/-}$ mice after bleomycin challenge by hydroxyproline assay. **Asterisks** denote a significant difference ($P < 0.01$) between wild-type and *Id1*$^{-/-}$ lungs at 2 weeks after bleomycin ($n = 8$ for each group). **E:** Immunostaining of wild-type and *Id1*$^{-/-}$ lungs with antibodies specific for α-SMA. Representative sections are shown for lungs at 2 weeks after bleomycin or saline. Sections were counterstained with hematoxylin to highlight all nuclei. Note elevated expression of α-SMA in fibrotic foci of *Id1*$^{-/-}$ lungs compared with wild type. **F:** Western blotting showing expression level of α-SMA in wild-type and *Id1*$^{-/-}$ lungs at 2 weeks after bleomycin or saline. Values represent the relative fold change of α-SMA protein level normalized to the density of α-tubulin bands. Original magnifications, ×400.

ined the MEK/ERK pathway, which is involved in maintaining cell survival by regulating Bcl-2 expression in diverse cell types[51] and during lung injury.[52] By Western blotting using antibodies against phosphorylated-MEK and phosphorylated-ERK, we found that MEK/ERK pathway activity was also decreased, concomitant with reduced Bcl-2 protein level (Figure 6, A and B). Collectively, our results indicate that up-regulation of Id1 expression seems to attenuate lung endothelial cell death by inducing Bcl-2 expression, which may be modulated by the MEK/ERK pathway.

Because endothelial injury and the resulting increase in vascular permeability may be involved in the pathogenesis of pulmonary fibrosis,[53–57] we examined the susceptibility of *Id1*$^{-/-}$ lungs to bleomycin-induced fibrogenesis. First, we performed morphological analysis of *Id1*$^{-/-}$ and wild-type lungs by histological staining with H&E of lung sections harvested at 2 weeks after bleomycin instillation to assess the alveolar architecture. As indicated in Figure 7A, *Id1*$^{-/-}$ lungs showed more parenchymal distortion and fibrotic foci compared with bleomycin-treated wild-type lungs. For semiquantitative analysis of lung fibrosis, H&E-stained sections were evaluated by a pathologist blinded to the genotypes and treatment groups. As shown in Figure 7B, *Id1*$^{-/-}$ lungs displayed a higher parenchymal distortion, ~35% increase, compared with wild type ($n = 9$, $P < 0.001$).

One of the hallmarks of lung fibrosis is the accumulation of collagen secreted by the large fibroblast population within foci. Indeed, we observed more collagen deposition in *Id1*$^{-/-}$ lungs at 2 weeks after bleomycin by Trichrome staining (Figure 7C). In agreement, we detected ~90% increase in collagen deposition in *Id1*$^{-/-}$ lungs compared with wild type, 2 weeks after bleomycin as measured by hydroxyproline content ($n = 8$, $P < 0.01$; Figure 7D). Consistent with elevated collagen secretion, *Id1*$^{-/-}$ lungs also showed significant increase in the population of myofibroblasts as revealed by α-SMA immunolabeling (Figure 7E). Western blotting of *Id1*$^{-/-}$ lung homogenates revealed increased levels of α-SMA expression compared with wild type (Figure 7F). Collectively, we demonstrated that *Id1*$^{-/-}$ mutant lungs are more susceptible to bleomycin-induced fibrogenesis as evidenced by increased lung parenchymal distortion, collagen and hydroxyproline content, and myofibroblast cell population.

Discussion

In this study, we have shown that, although Id1 was expressed at a basal level in the normal adult lung, its protein expression was significantly up-regulated predominantly in cells of the lung endothelium in response to bleomycin injury. This new finding is consistent with the endothelial-specific induction of Id1 in tumors of a variety of tissue origins[58] and prompted us to investigate the contributory role of Id1 in bleomycin-injured lung endothelium.

Bleomycin-induced lung injury and fibrogenesis (Figure 8), like many other types of lung injury, is almost always accompanied by an increase in vascular permeability, suggesting that vascular endothelial integrity and function are compromised. Our finding that bleomycin exposed *Id1*$^{-/-}$ lungs showed increased vascular permeability suggests that Id1 may play a critical role in maintaining endothelial integrity and function. Consistent with this notion, we demonstrated decreased endothelial cell survival in *Id1*$^{-/-}$ lungs injured by bleomycin compared with wild type. In addition, bleomycin-treated lung microvascular endothelial cells from *Id1*$^{-/-}$ lungs also showed significantly reduced survival potential compared with wild-type lung endothelial cells. Furthermore, our results indicate that up-regulation of Id1 expression seems to attenuate lung endothelial cell death by inducing the expression of the anti-apoptotic molecule Bcl-2, which may be modulated by the MEK/ERK pathway. Activated ERK1/2 has been shown to inhibit apoptosis in injured endothelial cells.[59,60] Reduction in Bcl-2 expression and MEK/ERK signaling has been documented in acute and chronic phases of injured lungs, respectively.[52,61] In addition, enhanced MEK/ERK signaling reduces ischemia/reperfusion (I/R)-induced lung injury, together with an increase in Bcl-2 expression.[52] Therefore, MEK/ERK and Bcl-2 pathway may serve as a promising downstream effector of Id1 function in the lung.

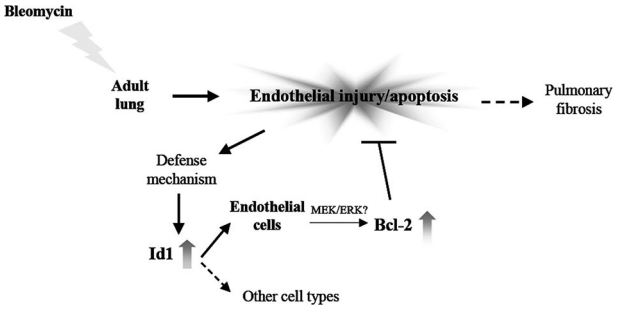

Figure 8. Schematic representation of Id1 function in the lung endothelium in acute lung injury. Injury caused by bleomycin results in extensive endothelial apoptosis. The lung responds to tissue damage by up-regulating Id1 expression in endothelial cells (and other cell types). Up-regulation of Id1 in endothelial cells activates the MEK/ERK pathway and elevates expression of anti-apoptotic protein Bcl-2. Increased Bcl-2 protein level reduces the extent of endothelial cell apoptosis, thus alleviating endothelial damage. Acute lung injury often results in pulmonary fibrosis in the chronic phase, which may be attenuated by Id1 function.

The lung microvasculature is intimately associated with the alveolar epithelium for efficient blood-gas exchange and it has been suggested that functional defects of microvessels may play a role in the pathogenesis of lung fibrosis. Pulmonary microvascular lesions and increased vascular permeability have been linked to the pathogenesis of pulmonary fibrosis therefore limiting endothelial cell injury and cell death may alleviate fibrogenesis.[54–57,62–66] Several other studies have also implicated a role of the microvasculature in lung fibrosis.[53,67–73] Conceivably, increased microvascular damage in *Id1*$^{-/-}$ lungs may lead to increased extravasation of plasma that may promote fibroblast proliferation. In addition, stimulated/injured endothelial cells can also secrete fibrogenic molecules that may affect fibroblast migration and proliferation.[74] Our findings indicate that Id1 plays a critical role in promoting endothelial survival after bleomycin-induced injury and

are consistent with a role of the endothelium in lung fibrogenesis. However, less than 20% of Id1 expression was derived from nonendothelial cells in bleomycin-exposed lungs. Our study, therefore, does not exclude the possibility that loss of Id1 function in other lung cell populations may also contribute to increased lung fibrogenesis. Interestingly, Id1 has been shown to be expressed in a subgroup of immune cells such as the macrophages and granulocytes.[47] Therefore, it is possible that Id1 may also be involved in the injured lung inflammatory response that may contribute to fibrogenesis. Increasing evidence suggests that rare lung stem cells reside in the bronchioalveolar junction and might be involved in lung repair.[75,76] It remains to be determined whether Id1 is involved in the maintenance of rare lung progenitor cells as has been suggested for hematopoietic stem cells.[77]

Bmp signaling has been strongly implicated in Id gene induction in a number of cell types including endothelial cells,[78–80] and vascular endothelial growth factor has also been shown to induce Id1 and Id3 in bone marrow-derived endothelial precursor and hematopoietic cells.[81] Both signaling pathways are promising candidates for activating Id1 expression in the lung endothelium on bleomycin injury, although the actual *in vivo* signals remain to be identified. Signaling mediated by transforming growth factor-β1 and tumor necrosis factor-α are known to play key roles in many types of lung diseases including bleomycin-induced pneumopathy in animal models.[21,23,82–86] Interestingly, transforming growth factor-β1 can exert long-term repression of Id1 expression in a number of cell types,[18,87,88] suggesting that transforming growth factor-β could potentially antagonize Id1 expression in the course of lung pathogenesis. Tumor necrosis factor-α has also been implicated in the regulation of Id expression in astrocytes during inflammatory injury of the central nervous system.[88–90] Thus, there may be a balance between Id1-inducing and Id1-repressing mechanisms in the bleomycin-injured lung. Therefore, future identification of signals that modulate Id1 expression in bleomycin-induced lung injury will improve our understanding of the regulatory mechanisms underlying Id1 function in lung injury and fibrosis.

Acknowledgments

We thank Dr. Robert Benezra for providing *Id1* mutant mice, Dr. Scott Baldwin for providing the *Tie1-Cre* mice, Dr. Jin Chen for her advice on retro-orbital injection of Evans blue, and the Vanderbilt University Medical Center Cell Imaging Shared Resource for use of the confocal microscope.

References

1. Ruzinova MB, Benezra R: Id proteins in development, cell cycle and cancer. Trends Cell Biol 2003, 13:410–418
2. Sun XH, Copeland NG, Jenkins NA, Baltimore D: Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins. Mol Cell Biol 1991, 11:5603–5611
3. Zebedee Z, Hara E: Id proteins in cell cycle control and cellular senescence. Oncogene 2001, 20:8317–8325
4. Benezra R, Rafii S, Lyden D: The Id proteins and angiogenesis. Oncogene 2001, 20:8334–8341
5. Wong YC, Wang X, Ling MT: Id-1 expression and cell survival. Apoptosis 2004, 9:279–289
6. Lyden D, Young AZ, Zagzag D, Yan W, Gerald W, O'Reilly R, Bader BL, Hynes RO, Zhuang Y, Manova K, Benezra R: Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. Nature 1999, 401:670–677
7. Engel I, Murre C: The function of E- and Id proteins in lymphocyte development. Nat Rev Immunol 2001, 1:193–199
8. Iavarone A, Lasorella A: Id proteins in neural cancer. Cancer Lett 2004, 204:189–196
9. Li H, Gerald WL, Benezra R: Utilization of bone marrow-derived endothelial cell precursors in spontaneous prostate tumors varies with tumor grade. Cancer Res 2004, 64:6137–6143
10. de Candia P, Benera R, Solit DB: A role for Id proteins in mammary gland physiology and tumorigenesis. Adv Cancer Res 2004, 92:81–94
11. Ling MT, Lau TC, Zhou C, Chua CW, Kwok WK, Wang Q, Wang X, Wong YC: Overexpression of Id-1 in prostate cancer cells promotes angiogenesis through the activation of vascular endothelial growth factor (VEGF). Carcinogenesis 2005, 26:1668–1676
12. Nishiyama K, Takaji K, Kataoka K, Kurihara Y, Yoshimura M, Kato A, Ogawa H, Kurihara H: Id1 gene transfer confers angiogenic property on fully differentiated endothelial cells and contributes to therapeutic angiogenesis. Circulation 2005, 112:2840–2850
13. Alani RM, Young AZ, Shifflett CB: Id1 regulation of cellular senescence through transcriptional repression of p16/Ink4a. Proc Natl Acad Sci USA 2001, 98:7812–7816
14. Sharpless NE, Bardeesy N, Lee KH, Carrasco D, Castrillon DH, Aguirre AJ, Wu EA, Horner JW, DePinho RA: Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. Nature 2001, 413:86–91
15. Tang J, Gordon GM, Nickoloff BJ, Foreman KE: The helix-loop-helix protein id-1 delays onset of replicative senescence in human endothelial cells. Lab Invest 2002, 82:1073–1079
16. Frank DB, Abtahi A, Yamaguchi DJ, Manning S, Shyr Y, Pozzi A, Baldwin HS, Johnson JE, de Caestecker MP: Bone morphogenetic protein 4 promotes pulmonary vascular remodeling in hypoxic pulmonary hypertension. Circ Res 2005, 97:496–504
17. Jen Y, Manova K, Benezra R: Expression patterns of Id1, Id2, and Id3 are highly related but distinct from that of Id4 during mouse embryogenesis. Dev Dyn 1996, 207:235–252
18. Chambers RC, Leoni P, Kaminski N, Laurent GJ, Heller RA: Global expression profiling of fibroblast responses to transforming growth factor-beta1 reveals the induction of inhibitor of differentiation-1 and provides evidence of smooth muscle cell phenotypic switching. Am J Pathol 2003, 162:533–546
19. Chua F, Gauldie J, Laurent GJ: Pulmonary fibrosis: searching for model answers. Am J Respir Cell Mol Biol 2005, 33:9–13
20. Zhang HY, Gharaee-Kermani M, Zhang K, Karmiol S, Phan SH: Lung fibroblast alpha-smooth muscle actin expression and contractile phenotype in bleomycin-induced pulmonary fibrosis. Am J Pathol 1996, 148:527–537
21. Cutroneo KR, Phan SH: TGF-beta1-induced Smad 3 binding to the Smad 7 gene: knockout of Smad 7 gene transcription by sense phosphorothioate oligos, autoregulation, and effect on TGF-β1 secretion: bleomycin acts through TGF-β1. J Cell Biochem 2003, 89:474–483
22. Desmoulière A, Geinoz A, Gabbiani F, Gabbiani G: Transforming growth factor-β1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J Cell Biol 1993, 122:103–111
23. Nakao A, Fujii M, Matsumura R, Kumano K, Saito Y, Miyazono K, Iwamoto I: Transient gene transfer and expression of Smad7 prevents bleomycin-induced lung fibrosis in mice. J Clin Invest 1999, 104:5–11
24. Bonniaud P, Margetts PJ, Kolb M, Schroeder JA, Kapoun AM, Damm D, Murphy A, Chakravarty S, Dugar S, Higgins L, Protter AA, Gauldie J: Progressive transforming growth factor β1-induced lung fibrosis is blocked by an orally active ALK5 kinase inhibitor. Am J Respir Crit Care Med 2005, 171:889–898

25. Chen J, Stubbe J: Bleomycins: towards better therapeutics. Nat Rev Cancer 2005, 5:102–112
26. Phan SH: Fibroblast phenotypes in pulmonary fibrosis. Am J Respir Cell Mol Biol 2003, 29:S87–S92
27. Phan SH: The myofibroblast in pulmonary fibrosis. Chest 2002, 122:286S–289S
28. Kuhn III C, Boldt J, King Jr TE, Crouch E, Vartio T, McDonald JA: An immunohistochemical study of architectural remodeling and connective tissue synthesis in pulmonary fibrosis. Am Rev Respir Dis 1989, 140:1693–1703
29. Zhang K, Rekhter MD, Gordon D, Phan SH: Myofibroblasts and their role in lung collagen gene expression during pulmonary fibrosis. A combined immunohistochemical and in situ hybridization study. Am J Pathol 1994, 145:114–125
30. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA: Myofibroblasts and mechano-regulation of connective tissue remodeling. Nat Rev Mol Cell Biol 2002, 3:349–363
31. Selman M, King TE, Pardo A: Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. Ann Intern Med 2001, 134:136–151
32. Gustafsson E, Brakebusch C, Hietanen K, Fassler R: Tie-1-directed expression of Cre recombinase in endothelial cells of embryoid bodies and transgenic mice. J Cell Sci 2001, 114:671–676
33. Li Y, Zhang H, Litingtung Y, Chiang C: Cholesterol modification restricts the spread of Shh gradient in the limb bud. Proc Natl Acad Sci USA 2006, 103:6548–6553
34. Lawson WE, Polosukhin VV, Zoia O, Stathopoulos GT, Han W, Plieth D, Loyd JE, Neilson EG, Blackwell TS: Characterization of fibroblast-specific protein 1 in pulmonary fibrosis. Am J Respir Crit Care Med 2005, 171:899–907
35. Londhe VA, Belperio JA, Keane MP, Burdick MD, Xue YY, Strieter RM: CXCR2 is critical for dsRNA-induced lung injury: relevance to viral lung infection. J Inflamm (Lond) 2005, 2:4
36. Pozzi A, Moberg PE, Miles LA, Wagner S, Soloway P, Gardner HA: Elevated matrix metalloprotease and angiostatin levels in integrin α1 knockout mice cause reduced tumor vascularization. Proc Natl Acad Sci USA 2000, 97:2202–2207
37. Lorimore SA, Coates PJ, Scobie GE, Milne G, Wright EG: Inflammatory-type responses after exposure to ionizing radiation in vivo: a mechanism for radiation-induced bystander effects? Oncogene 2001, 20:7085–7095
38. Lawson WE, Polosukhin VV, Stathopoulos GT, Zoia O, Han W, Lane KB, Li B, Donnelly EF, Holburn GE, Lewis KG, Collins RD, Hull WM, Glasser SW, Whitsett JA, Blackwell TS: Increased and prolonged pulmonary fibrosis in surfactant protein C-deficient mice following intratracheal bleomycin. Am J Pathol 2005, 167:1267–1277
39. Reddy GK, Enwemeka CS: A simplified method for the analysis of hydroxyproline in biological tissues. Clin Biochem 1996, 29:225–229
40. Balyasnikova IV, Visintine DJ, Gunnerson HB, Paisansathan C, Baughman VL, Minshall RD, Danilov SM: Propofol attenuates lung endothelial injury induced by ischemia-reperfusion and oxidative stress. Anesth Analg 2005, 100:929–936
41. Novak A, Guo C, Yang W, Nagy A, Lobe CG: Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. Genesis 2000, 28:147–155
42. Urase K, Mukasa T, Igarashi H, Ishii Y, Yasugi S, Momoi MY, Momoi T: Spatial expression of Sonic hedgehog in the lung epithelium during branching morphogenesis. Biochem Biophys Res Commun 1996, 225:161–166
43. Litingtung Y, Lei L, Westphal H, Chiang C: Sonic hedgehog is essential to foregut development. Nat Genet 1998, 20:58–61
44. Bitgood MJ, McMahon AP: Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. Dev Biol 1995, 172:126–138
45. Bellusci S, Furuta Y, Rush MG, Henderson R, Winnier G, Hogan BL: Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. Development 1997, 124:53–63
46. Nakamura N, Miyagi E, Murata S, Kawaoi A, Katoh R: Expression of thyroid transcription factor-1 in normal and neoplastic lung tissues. Mod Pathol 2002, 15:1058–1067
47. Leeanansaksiri W, Wang H, Gooya JM, Renn K, Abshari M, Tsai S, Keller JR: IL-3 induces inhibitor of DNA-binding protein-1 in hemopoietic progenitor cells and promotes myeloid cell development. J Immunol 2005, 174:7014–7021
48. Ling MT, Wang X, Ouyang XS, Xu K, Tsao SW, Wong YC: Id-1 expression promotes cell survival through activation of NF-kappaB signalling pathway in prostate cancer cells. Oncogene 2003, 22:4498–4508
49. Hui CM, Cheung PY, Ling MT, Tsao SW, Wang X, Wong YC, Cheung AL: Id-1 promotes proliferation of p53-deficient esophageal cancer cells. Int J Cancer 2006, 119:508–514
50. Cheung HW, Ling MT, Tsao SW, Wong YC, Wang X: Id-1-induced Raf/MEK pathway activation is essential for its protective role against taxol-induced apoptosis in nasopharyngeal carcinoma cells. Carcinogenesis 2004, 25:881–887
51. Chang F, Steelman LS, Shelton JG, Lee JT, Navolanic PM, Blalock WL, Franklin R, McCubrey JA: Regulation of cell cycle progression and apoptosis by the Ras/Raf/MEK/ERK pathway. Int J Oncol 2003, 22:469–480
52. Rivo J, Zeira E, Galun E, Einav S, Linden J, Matot I: Attenuation of reperfusion lung injury and apoptosis by A2a adenosine receptor activation is associated with modulation of Bcl-2 and Bax expression and activation of extracellular signal-regulated kinases. Shock 2007, 27:266–273
53. Azuma A, Takahashi S, Nose M, Araki K, Araki M, Takahashi T, Hirose M, Kawashima H, Miyasaka M, Kudoh S: Role of E-selectin in bleomycin induced lung fibrosis in mice. Thorax 2000, 55:147–152
54. Takabatake N, Arao T, Sata M, Abe S, Inoue S, Shibata Y, Takeishi Y, Kubota I: Involvement of pulmonary endothelial cell injury in the pathogenesis of pulmonary fibrosis: clinical assessment by 123I-MIBG lung scintigraphy. Eur J Nucl Med Mol Imaging 2005, 32:221–228
55. Brown LF, Dvorak AM, Dvorak HF: Leaky vessels, fibrin deposition, and fibrosis: a sequence of events common to solid tumors and to many other types of disease. Am Rev Respir Dis 1989, 140: 1104–1107
56. Magro CM, Waldman WJ, Knight DA, Allen JN, Nadasdy T, Frambach GE, Ross P, Marsh CB: Idiopathic pulmonary fibrosis related to endothelial injury and antiendothelial cell antibodies. Hum Immunol 2006, 67:284–297
57. Renzoni EA, Walsh DA, Salmon M, Wells AU, Sestini P, Nicholson AG, Veeraraghavan S, Bishop AE, Romanska HM, Pantelidis P, Black CM, Du Bois RM: Interstitial vascularity in fibrosing alveolitis. Am J Respir Crit Care Med 2003, 167:438–443
58. Perk J, Gil-Bazo I, Chin Y, de Candia P, Chen JJ, Zhao Y, Chao S, Cheong W, Ke Y, Al-Ahmadie H, Gerald WL, Brogi E, Benezra R: Reassessment of id1 protein expression in human mammary, prostate, and bladder cancers using a monospecific rabbit monoclonal anti-id1 antibody. Cancer Res 2006, 66:10870–10877
59. Khan TA, Bianchi C, Ruel M, Voisine P, Li J, Liddicoat JR, Sellke FW: Mitogen-activated protein kinase inhibition and cardioplegia-cardiopulmonary bypass reduce coronary myogenic tone. Circulation 2003, 108(Suppl 1):II348–II353
60. Yue TL, Wang C, Gu JL, Ma XL, Kumar S, Lee JC, Feuerstein GZ, Thomas H, Maleeff B, Ohlstein EH: Inhibition of extracellular signal-regulated kinase enhances ischemia/reoxygenation-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart. Circ Res 2000, 86:692–699
61. Yoshida K, Kuwano K, Hagimoto N, Watanabe K, Matsuba T, Fujita M, Inoshima I, Hara N: MAP kinase activation and apoptosis in lung tissues from patients with idiopathic pulmonary fibrosis. J Pathol 2002, 198:388–396
62. Magro CM, Allen J, Pope-Harman A, Waldman WJ, Moh P, Rothrauff S, Ross P Jr: The role of microvascular injury in the evolution of idiopathic pulmonary fibrosis. Am J Clin Pathol 2003, 119:556–567
63. Wang Q, Hyde DM, Giri SN: Abatement of bleomycin-induced increases in vascular permeability, inflammatory cell infiltration, and fibrotic lesions in hamster lungs by combined treatment with taurine and niacin. Lab Invest 1992, 67:234–242
64. Kaplan JD, Trulock EP, Anderson DJ, Schuster DP: Pulmonary vascular permeability in interstitial lung disease. A positron emission tomographic study. Am Rev Respir Dis 1992, 145:1495–1498
65. Peterson LM, Evans ML, Graham MM, Eary JF, Dahlen DD: Vascular response to radiation injury in the rat lung. Radiat Res 1992, 129:139–148
66. Slosman DO, Donath A, Alderson PO: ^{131}I-Metaiodobenzylguanidine and 125I-iodoamphetamine. Parameters of lung endothelial cell function and pulmonary vascular area. Eur J Nucl Med 1989, 15:207–210

67. Kasper M, Schobl R, Haroske G, Fischer R, Neubert F, Dimmer V, Muller M: Distribution of von Willebrand factor in capillary endothelial cells of rat lungs with pulmonary fibrosis. Exp Toxicol Pathol 1996, 48:283–288
68. Kawanami O, Jiang HX, Mochimaru H, Yoneyama H, Kudoh S, Ohkuni H, Ooami H, Ferrans VJ: Alveolar fibrosis and capillary alteration in experimental pulmonary silicosis in rats. Am J Respir Crit Care Med 1995, 151:1946–1955
69. Ward WF, Sharplin J, Franko AJ, Hinz JM: Radiation-induced pulmonary endothelial dysfunction and hydroxyproline accumulation in four strains of mice. Radiat Res 1989, 120:113–120
70. Piguet PF, Vesin C: Pulmonary platelet trapping induced by bleomycin: correlation with fibrosis and involvement of the β2 integrins. Int J Exp Pathol 1994, 75:321–328
71. Drab M, Verkade P, Elger M, Kasper M, Lohn M, Lauterbach B, Menne J, Lindschau C, Mende F, Luft FC, Schedl A, Haller H, Kurzchalia TV: Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. Science 2001, 293:2449–2452
72. Fichtner F, Koslowski R, Augstein A, Hempel U, Rohlecke C, Kasper M: Bleomycin induces IL-8 and ICAM-1 expression in microvascular pulmonary endothelial cells. Exp Toxicol Pathol 2004, 55:497–503
73. Burdick MD, Murray LA, Keane MP, Xue YY, Zisman DA, Belperio JA, Strieter RM: CXCL11 attenuates bleomycin-induced pulmonary fibrosis via inhibition of vascular remodeling. Am J Respir Crit Care Med 2005, 171:261–268
74. Calabrese F, Giacometti C, Rea F, Loy M, Valente M: Idiopathic interstitial pneumonias: Primum movens: epithelial, endothelial or whatever. Sarcoidosis Vasc Diffuse Lung Dis 2005, 22(Suppl 1): S15–S23
75. Kim CB: Advancing the field of lung stem cell biology. Front Biosci 2007, 12:3117–3124
76. Gomperts BN, Strieter RM: Stem cells and chronic lung disease. Annu Rev Med 2007, 58:285–298
77. Jankovic V, Ciarrocchi A, Boccuni P, DeBlasio T, Benezra R, Nimer SD: Id1 restrains myeloid commitment, maintaining the self-renewal capacity of hematopoietic stem cells. Proc Natl Acad Sci USA 2007, 104:1260–1265
78. Hollnagel A, Oehlmann V, Heymer J, Ruther U, Nordheim A: Id genes are direct targets of bone morphogenetic protein induction in embryonic stem cells. J Biol Chem 1999, 274:19838–19845
79. Valdimarsdottir G, Goumans MJ, Rosendahl A, Brugman M, Itoh S, Lebrin F, Sideras P, ten Dijke P: Stimulation of Id1 expression by bone morphogenetic protein is sufficient and necessary for bone morphogenetic protein-induced activation of endothelial cells. Circulation 2002, 106:2263–2270
80. Itoh F, Itoh S, Goumans MJ, Valdimarsdottir G, Iso T, Dotto GP, Hamamori Y, Kedes L, Kato M, ten Dijke Pt P: Synergy and antagonism between Notch and BMP receptor signaling pathways in endothelial cells. EMBO J 2004, 23:541–551
81. Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, Witte L, Wu Y, Hicklin D, Zhu Z, Hackett NR, Crystal RG, Moore MA, Hajjar KA, Manova K, Benezra R, Rafii S: Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 2001, 7:1194–1201
82. Pittet JF, Griffiths MJ, Geiser T, Kaminski N, Dalton SL, Huang X, Brown LA, Gotwals PJ, Koteliansky VE, Matthay MA, Sheppard D: TGF-β is a critical mediator of acute lung injury. J Clin Invest 2001, 107:1537–1544
83. Santana A, Saxena B, Noble NA, Gold LI, Marshall BC: Increased expression of transforming growth factor beta isoforms (β1, β2, β3) in bleomycin-induced pulmonary fibrosis. Am J Respir Cell Mol Biol 1995, 13:34–44
84. Bartram U, Speer CP: The role of transforming growth factor β in lung development and disease. Chest 2004, 125:754–765
85. Zhao J, Shi W, Wang YL, Chen H, Bringas Jr P, Datto MB, Frederick JP, Wang XF, Warburton D: Smad3 deficiency attenuates bleomycin-induced pulmonary fibrosis in mice. Am J Physiol 2002, 282: L585–L593
86. Wang Q, Hyde DM, Gotwals PJ, Giri SN: Effects of delayed treatment with transforming growth factor-beta soluble receptor in a three-dose bleomycin model of lung fibrosis in hamsters. Exp Lung Res 2002, 28:405–417
87. Kowanetz M, Valcourt U, Bergstrom R, Heldin CH, Moustakas A: Id2 and Id3 define the potency of cell proliferation and differentiation responses to transforming growth factor beta and bone morphogenetic protein. Mol Cell Biol 2004, 24:4241–4254
88. Kang Y, Chen CR, Massague J: A self-enabling TGFβ response coupled to stress signaling: smad engages stress response factor ATF3 for Id1 repression in epithelial cells. Mol Cell 2003, 11:915–926
89. Goumans MJ, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P: Balancing the activation state of the endothelium via two distinct TGF-β type I receptors. EMBO J 2002, 21:1743–1753
90. Tzeng SF, Kahn M, Liva S, De Vellis J: Tumor necrosis factor-α regulation of the Id gene family in astrocytes and microglia during CNS inflammatory injury. Glia 1999, 26:139–152