THE FUNCTIONS OF INHIBITOR OF DNA-BINDING PROTEINS IN ENDOTHELIAL CELLS DURING LUNG DEVELOPMENT AND DISEASE

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LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ADD1	adipocyte determination and differentiation factor 1
AGS	Alagille Syndrome
ALI	Acute Lung Injury
Alk	activin receptor-like kinase
Ang-1	angiopoietin-1
ARDS	Acute Respiratory Distress Syndrome
ASM	Airway smooth muscle
bHLH	basic Helix-loop-helix
BMP	Bone morphogenic protein
BPD	Bronchopulmonary Dysplasia
BSA	bovine serum albumin
CDH	congenital diaphragmatic hernia
CDK	Cyclin-dependent kinase
CLP	cecal ligation and puncture
DMEM	Dulbecco‘s Modified Eagle‘s Medium
ECM	extracellular matrix
ELK1	Ets-like protein 1
EMC	extramicrochaetae
EPC	endothelial progenitor cells
ETS	E26 transformation-specific
FACS	fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
GFP	green fluorescent protein
H&E	Hematoxylin and Eosin
Herp	Hes-related repressor protein
HLH	Helix-loop-helix
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HUVEC	Human Umbilical Vein Endothelial Cells
Id	Inhibitor of DNA-binding/Differentiation
IPF	Idiopathic Pulmonary Fibrosis
LMVEC	lung microvascular endothelial cells
LPS	lipopolysaccharide
MIDA	mouse ID-associated protein 1
MMP	matrix metalloproteinases
MPO	myeloperoxidase
Net	New Ets

OCT	optimum cutting temperature
PAI	plasminogen activator inhibitors
PAVM	pulmonary arteriovenous malformations
PAX	Paired-domain homeobox
PBS	Phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	pigment epithelium-derived factor
PFA	paraformaldehyde
PPHN	persistent pulmonary hypertension of the newborn
pRB	phosphorylated retinoblastoma tumor supressor protein
RNS	reactive nitrogen species
ROS	reactive oxygen species
SAP1	serum response factor accessory protein 1
SBE	Smad-binding elements
Shh	sonic hedgehog
SMA	Smooth muscle α-actin
Smad	Sma- and Mad-related protein
SP-C	Surfactant Protein C
Tbx	T-box domain
TCF	ternary complex factors
TGF	transforming growth factor
TNF	Tumor Necrosis Factor
TUNEL	Terminal deoxynucleotidyl transferase–mediated dUTP Nick End Labeling
VEGF	vascular endothelial growth factor
vWF	von Willibrand Factor
WT	Wild type

CHAPTER I

GENERAL INTRODUCTION

One question that has attracted the interest of scientists for decades is how the vascular system of the lung is formed and maintained. To generate a structurally and functionally complex system with enough surface area and circulation for gas exchange, cells of the lung endothelium must undergo extensive temporally and spatially regulated developmental processes involving proliferation, differentiation and migration (Chuang and McMahon, 2003; Price and Stiles, 1996; Warburton et al., 1998) Furthermore, the adult lung is constantly under challenges of injury caused by environmental or intrinsic factors. In order to protect the endothelial barrier from extensive damage and maintain proper lung functions, the endothelial cells possess a highly sophisticated protective machinery and self-renewal abilities (Orfanos et al., 2004; Ryan, 1986). In the past few years, several key factors that mediate these processes have been identified and it is becoming clear that they are well-coordinated by cross-talk between endothelial and epithelial cells (Blume et al., 1998; Calabrese et al., 2005; Hermanns et al., 2004; Jakkula et al., 2000; Maeda et al., 2002; Parera et al., 2005; Wendt et al., 1994). However the precise molecular profile of lung organogenesis still remains to be established. This introduction reviews the

general processes of lung angiogenesis and summarizes what is known about endothelial damage and repair, as well as discusses the possibility that inhibitor of differentiation-1 (Id1) may be a key factor that mediates these processes.

Part I Development of the Pulmonary Vasculature

Most of our current understanding of mammalian lung development comes from studies using mouse models. In the mouse, the lung first originates from the laryngotracheal groove at embryonic day 9-9.5, and then divides laterally into two primordial lung buds, which invade the surrounding splanchnic mesenchyme. Starting at around E10.5, a sequence of highly ordered patterning events termed branching morphogenesis occur in the epithelium, generating the bronchial tree and the proximal-distal axis of the lung (Cardoso, 2006). Together with the formation of the bronchial tree, its surrounding mesenchyme differentiates into airway smooth muscle (ASM), which is juxtaposed around the tubules, and also gives rise to the vasculature and neural networks (Tollet et al., 2001). The formation of the vascular system begins as early as E9.0, when intercellular spaces are apparent in the lung mesenchyme. Yet precisely how the vasculature develops is not clear. There are three distinct lung vascular morphogenesis models (Fig1.1) (Parera et al., 2005). The first model (vasculogenesis & angiogenesis) proposes that lung mesenchymal cells firstly regroup to generate

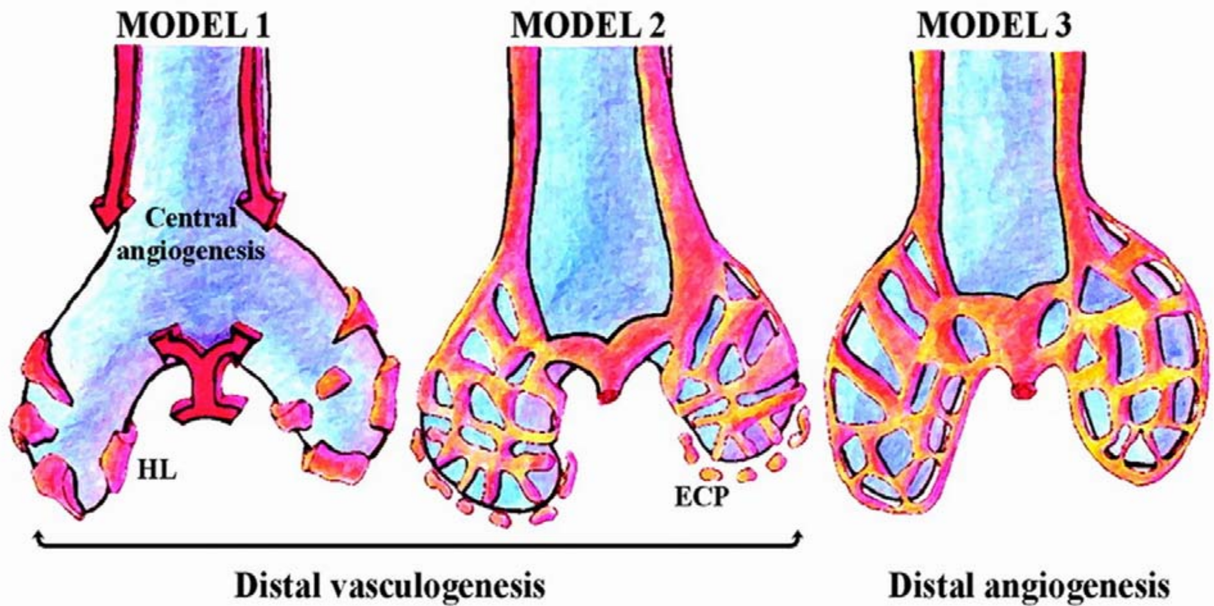


Fig 1.1 Proposed models for embryonic lung endothelial morphogenesis.

Reprinted from Parera et al. *Am J Physiol Lung Cell Mol Physiol* 2005

endothelial cells. Then sprouting of new vessels from the central pulmonary vascular trunks to the peripheral lung mesenchyme occurs and connections between peripheral and central vascular systems take place later on (deMello et al., 1997). The second model (vasculogenesis) proposes that distal vasculogenesis alone is the main mechanism that forms the lung vasculature (Hall et al., 2000). The third model, which is favored by more researchers than the others, claims that distal angiogenesis (formation and sprouting of new capillaries from pre-existing vessels accompanied by lung bud growth) is a major player in embryonic lung vascular development (Parera et al., 2005). Regardless of the differences between these proposed models, it is agreed that angioblast aggregation, endothelial differentiation and migration as well as formation of capillary plexus are the primary processes that take place during lung endothelial morphogenesis.

Defects in the developmental processes of embryonic lung endothelial morphogenesis may contribute to a variety of neonatal pulmonary disorders involving circulation problems. One particular type of human disease that attracted growing attention in recent years is called Bronchopulmonary Dysplasia (BPD). BPD is a chronic lung disease of infancy characterized by arrested lung growth with impaired vascular and alveolar development, which results in persistent respiratory problems (Stenmark and Abman, 2005). The

underdeveloped vessels in lungs of BPD patients often appear dilated and the microvascular structure disorganized. It is believed that disrupted vascular growth plays a key role in the pathogenesis of BPD (Lang et al., 2004; Stenmark and Balasubramaniam, 2005). However, detailed knowledge about the molecular mechanisms controlling prenatal vascular development is still needed to understand how BPD develops and to generate effective therapeutic modalities. Other examples of development-related pulmonary vascular diseases include pulmonary arteriovenous malformations (PAVMs), persistent pulmonary hypertension of the newborn (PPHN) and congenital diaphragmatic hernia (CDH) (Lang et al., 2004). Development of future therapies will rely primarily on the discovery of new cellular and molecular machineries responsible for the pathogenesis of these diseases.

Extensive studies have been done on the branching and differentiation of the respiratory epithelium, but relatively little is known about the mechanism underlying the regulation of lung mesenchyme development, particularly vascular development. It has been proposed that Notch signaling regulates multiple aspects of vascular morphogenesis through its primary effectors, the HERP family of bHLH proteins (Iso et al., 2003b). Other growth factors, such as the VEGF family (Gebb and Shannon, 2000; Healy et al., 2000), angiopoietin family (Koblizek et al., 1998; Maisonpierre et al., 1997) and ephrin family (Hall et al., 2002) are also involved in pulmonary vascular development. The detailed

mechanisms remain poorly understood and many issues wait to be addressed. Thus identification of novel key regulators controlling mesenchymal development of the lung is crucial to gain insight into the cellular processes governing pulmonary vascular development.

Part II Acute Lung Injury and Pulmonary Fibrosis

Acute Lung Injury (ALI) and Acute Respiratory Distress Syndrome (ARDS)

The terms Acute Lung Injury (ALI) and its more severe form, Acute Respiratory Distress Syndrome (ARDS), refer to a spectrum of increasingly severe syndromes of lung injury. This disease is characterized by widespread alveolar epithelial and capillary endothelial cell damage that occurs rapidly after external or internal insults, causing dyspnea, severe hypoxemia, decreased lung compliance and pulmonary edema (Matthay and Zimmerman, 2005; Matthay et al., 2003). ALI/ARDS have been diagnosed in adults as well as children, and often lead to acute respiratory failure with high mortality (30-40%) in critically ill patients. It is estimated that as much as 36,000 deaths per year in the U.S. are contributed by ARDS (Hudson and Steinberg, 1999). Currently the only effective treatment available for acute respiratory failure caused by ALI is mechanical ventilation (Matthay and Zimmerman, 2005). Considerable work is still needed to provide additional therapeutic approaches.

The insults leading to ALI can be either directly targeted to the lung, such as lung infection or aspiration, or indirectly by sepsis, trauma or large volume blood replacement and other systemic problems. The most common feature of ALI at early phase is pulmonary edema, largely due to increase in lung vascular permeability. Systemic conditions can injure cells of the pulmonary endothelial barrier more rapidly than other cell types in the lung, which makes the endothelium the initial mediator in permeability changes associated with ALI (Groeneveld, 2002) caused by sepsis or trauma. Epithelial damages, together with inflammatory responses are also major contributors of ALI pathogenesis (Abraham, 2003; Goodman et al., 2003).

Rodent have become the most widely used animal models in ALI research in recent years. Currently available models enable investigators to choose between cell-type specific and systemic approaches. Intratracheal administration of lipopolysaccharide (LPS) in mice triggers local infection that targets pulmonary epithelial cells (Thorn, 2001). Intravenous injection of bleomycin induces endothelial cell death directly, which leads to altered pulmonary vascular permeability (Azuma et al., 2000). Other more complex models include cecal ligation and puncture (CLP), which causes systemic sepsis in mice and ALI indirectly due to poly-microbial infection (Ebong et al., 1999; Guo et al., 2002; Laudes et al., 2004). The availability of mouse knockout and transgenic strains provides a great advantage to the study of the important roles of specific genes

and pathways involved in ALI. Tumor Necrosis Factor (TNF) alpha and interleukin (IL) signaling are two major players during the initial phase of ALI, mainly functioning by mediating and amplifying inflammatory responses in the lung epithelium and endothelium (Folkesson et al., 1995; Goldblum et al., 1989; Ortiz et al., 1998; Smith et al., 1998). It is believed that the key transcriptional factor involved in ALI pathogenesis is NF-κB, which induces the transcription of many injury-response genes such as cytokines, growth factors or adhesion molecules. And the transcriptional control of NF-κB is well coordinated through interaction with other transcription factors (Fan et al., 2001).

Idiopathic Pulmonary Fibrosis (IPF)

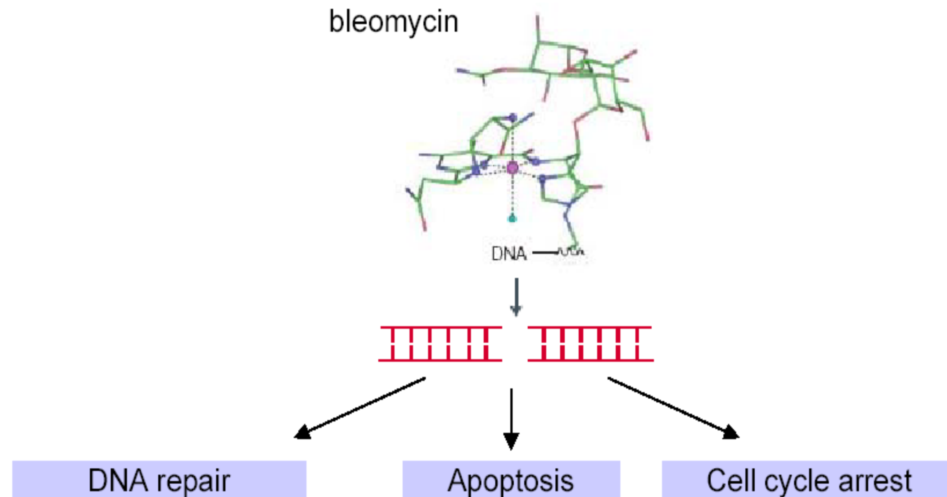
Idiopathic interstitial pneumonias consist of a group of six different types of devastating lung diseases of which idiopathic pulmonary fibrosis (IPF) is the most common type (Garantziotis et al., 2004). IPF is a progressive lung disease of unknown etiology with poor prognosis and no effective treatment. It is often diagnosed in individuals between 50 to 70 years of age, manifested as shortness of breath due to impaired gas exchange. The estimated annual incidence is 10 cases per 100,000 (Selman et al., 2001) although the true prevalence can vary depending on the population studied (Hodgson et al., 2002; Johnston et al., 1997). IPF is characterized by accumulation of inflammatory cells, epithelial and endothelial apoptosis, fibroblast proliferation and an abundance of myofibroblasts

in the lung interstitium, increased deposition of extracellular matrix proteins resulting in substantial loss of intact respiratory alveoli and irreversible distortion of the lung architecture (Kuhn et al., 1989; Phan, 2002; Phan, 2003; Selman et al., 2001; Tomasek et al., 2002; Zhang et al., 1994).

The disease is thought to result from persistent or recurrent episodes of lung injury triggering a pathophysiological response (Gross and Hunninghake, 2001; Katzenstein and Myers, 1998; Mason et al., 1999; White et al., 2003). IPF has been associated with identified mutations in the gene for surfactant protein C (Lawson et al., 2004), however, it is believed that adverse environmental conditions play a role in sporadic cases (Garantziotis et al., 2004; Green, 2002). Environmental factors can generate oxidant stress that has adverse effects in the lung (Gillissen and Nowak, 1998; Kim et al., 2000; Kinnula et al., 2005; Quinlan et al., 1994). IPF patients have higher levels of oxidant stress with elevated reactive free oxygen radical levels, reduced antioxidant defense and increased lipid peroxidation than control patients (Beeh et al., 2002; Gillissen and Nowak, 1998; Jack et al., 1996; Kinnula et al., 2005; Kurup and Kurup, 2003; Lenz et al., 1996; Rahman and Kelly, 2003; Rahman et al., 1999; Schunemann et al., 1997). Significant deficiency of the cellular antioxidant, reduced glutathione, has been reported in the lower respiratory tracts of IPF patients confirming the established role of oxidant/antioxidant imbalance as a major cause of cell damage in the pathogenesis of IPF (Beeh et al., 2002; Rahman and MacNee, 2000). Antioxidant

defense therapy has been suggested for lung diseases involving high alveolar oxidant burden such as IPF (Buhl et al., 1996; Gillissen and Nowak, 1998; Kinnula et al., 2005; Meyer et al., 1995).

Bleomycin is used clinically as a chemotherapeutic cancer drug (Fig1.2-A). However, it often causes detrimental toxic side effects including lung fibrosis (Hay et al., 1991). Intratracheal instillation of bleomycin is routinely used to induce pulmonary fibrosis in animal models. Mice treated with bleomycin generally develop fibrotic foci from the second week after injection (Fig1.2-B). The effect of bleomycin is, in part, mediated by TGF-β signaling, consistent with the established involvement of this important fibrogenic cytokine in wound healing by promoting myofibroblast differentiation, survival and persistence (Breen et al., 1992; Cutroneo and Phan, 2003; Desmouliere et al., 1993; Izbicki et al., 2002; Lawson et al., 2005b; Nakao et al., 1999; Phan and Kunkel, 1992; Zhang et al., 1996; Zhang et al., 1995). Alveolar epithelial cells in IPF are known to secrete, among other factors, cytokines such as TGF-βι and TNF-α, which have been shown to promote fibroblast activation (Kapanci et al., 1995; Khalil et al., 1991; Miyazaki et al., 1995). In bleomycin-induced lung fibrosis, it is believed that early inflammation occurs in response to epithelial injury (Adamson and Bakowska, 1999; Deterding et al., 1997; Guo et al., 1998; Sugahara et al., 1998; Yi et al., 1996). Release of chemoattractants by injured epithelial cells can promote recruitment of inflammatory cells, which adhere to and migrate across the lung



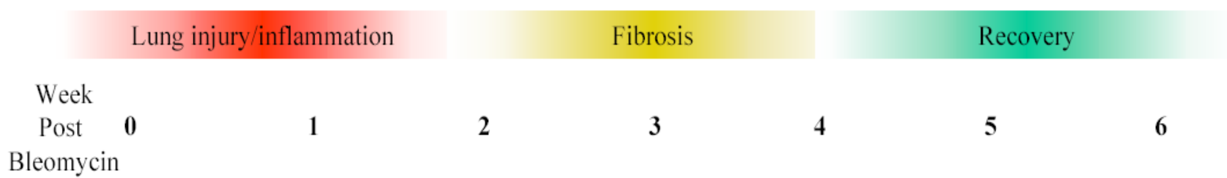


Fig 1.2 Intratracheal bleomycin induced mouse lung injury model *A.* Chemical structure of bleomycin molecule and mechanism of bleomycin induced cell apoptosis. Adapted form ***B.*** Time frame for acute (0-1 week) and chronic (2-6 week) phases of lung injury triggered by intratracheal administration of bleomycin.

endothelium into the airways (Liu et al., 1996). Epithelial injury and defects in re-epithelialization may lead to disruption of normal epithelial-fibroblast interactions, hence promoting the fibrotic process (Adamson et al., 1988; Chapman, 2004; Kasper and Haroske, 1996; Pardo and Selman, 2002a; Pardo and Selman, 2002b; Selman et al., 2001; Selman et al., 2004; Thannickal et al., 2004; White et al., 2003). Contributions by circulating fibroblasts as well as extracellular matrix (ECM) to the pathologic mechanisms have also been brought into consideration (Pardo and Selman, 2002a; Selman et al., 2001).

Pulmonary endothelium and response to injury

The adult lung microvascular endothelial structure originates from embryonic mesenchyme that gradually develops into a highly sophisticated vascular network system along with epithelial development. Besides providing a blood barrier and an interface for gas exchange, the endothelial cells also possess other important functional properties such as the secretion of enzymes and growth factors or participation in immune responses (Reviewed in Orfanos et al., 2004). Since the pulmonary endothelium is a major component of the adult lung structure and its integrity is crucial for maintaining lung homeostasis, its critical role in the pathogenesis of ALI is well recognized (Block, 1992; Orfanos et al., 2004; Wenzel et al., 2002). Insults affecting endothelial cell survival and functions often directly result in breakdown of the permeability balance, which is

an early pathological feature of acute lung injury. The pulmonary endothelium is not only a major target of insults, but also serves as a transmitter and amplifier of the damaging effects. Upon activation of endothelial cells by initial injury-induced signaling pathways, endothelial cells express E-selectin and ICAM-1, molecules that are essentially required for neutrophil recruitment, adhesion, transmigration and subsequent inflammatory responses (Albelda et al., 1994). On the other hand, activated endothelial cells also produce hemostatic modulators such as von Willibrand Factor (vWF) (Ware et al., 2001; Ware et al., 2004), thrombomodulin (TM) (Kawanami et al., 2000; MacGregor et al., 1997) and plasminogen activator inhibitors (PAI) (Wenzel et al., 2002). Release of these molecules contributes significantly to the decrease of hemofluidity in the circulation system of ALI patients. The role of pulmonary endothelial cells to mediate injury response is delicately counterbalanced by the protective and repair machineries they possess. Angiotensin-converting enzyme (ACE), for instance, is an ectoenzyme produced by endothelial cells that maintains endothelial barrier homeostasis. Reduction of ACE activity is one of the earliest signs of ALI and the protective role of ACE-2 against ALI has been established through examination of *ACE-2* knockout mice (Imai et al., 2005; Lazo et al., 1986; Newman et al., 1980). Plasminogen activators (PA) are also synthesized and released by pulmonary endothelial cells. They serve as inducers of endothelial fibrinolytic activities that are required during repair and restoration processes (Block, 1992; Idell, 2003).

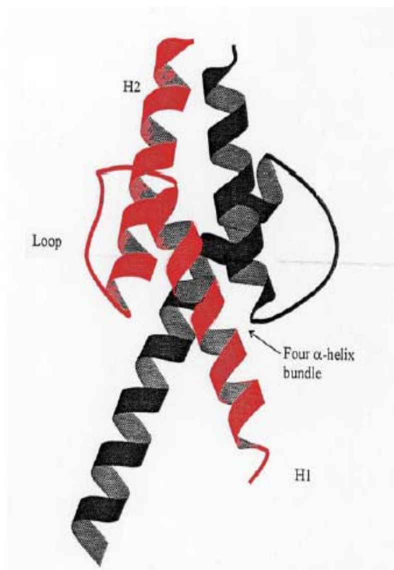
While recent focus has been on the role of epithelial dysfunction in IPF (Chapman, 2004; Pardo and Selman, 2002a; Selman et al., 2001; Thannickal et al., 2004; White et al., 2003), much less is known about the specific roles of the endothelium which is also a target tissue in lung injury. It has been suggested that aberrant vascular remodeling, resulting in net increase in angiogenesis, likely plays an indispensable role in the development and progression of lung fibrosis (Burdick et al., 2005; Keane et al., 1999; Keane et al., 2001). To date, the extent of neovacularization and its role in the pathogenesis of IPF remains contradictory and unresolved. Recent studies indicate elevated serum levels of endostatin, an anti-angiogenic factor, in IPF patients suggesting a correlation between endostatin and lung fibrosis (Sumi et al., 2005). Vascular heterogeneity and remodeling was found in patients with IPF (Ebina et al., 2004; Renzoni et al., 2003) and it has been suggested that functional defects of the lung microvessels may play a role in the pathogenesis of lung fibrosis (Koyama et al., 2002; Renzoni et al., 2003). The level of vascular endothelial growth factor (VEGF), an angiogenic factor, was significantly depressed (Meyer et al., 2000) while pigment epithelium-derived factor (PDGF), an angiostatic factor, was increased in IPF patients (Cosgrove et al., 2004). However, it remains unclear as to how vascular remodeling mechanistically contributes to lung fibrosis. Importantly, host responses to lung injury involving molecular cytoprotective mechanisms of the lung endothelium remain to be elucidated.

Part III The Inhibitor of Differentiation family proteins

Id proteins as transcriptional repressors

The first member of the Id family of proteins was discovered in 1990, in a screen for cDNAs encoding novel helix-loop-helix (HLH) proteins in murine erythroleukemia cells (Benezra et al., 1990). The Id family is a distinct subfamily of HLH proteins that lack a DNA-binding region and function by dimerizing with other transcriptional regulators, primarily basic-HLH (bHLH) factors (Fig1.3-A). Basic-HLH transcription factors are well-known cell differentiation stimulators (Lee, 1997; Massari and Murre, 2000; Olson and Klein, 1994). In general, tissue-specific bHLH factors form dimers with ubiquitously expressed bHLH factors, bind to DNA and trigger tissue-specific gene expression that promotes cell differentiation. However, once bHLH proteins interact with Id proteins via their HLH domain, they are no longer able to bind to DNA or form functional heterodimers with other bHLH partners. Thus, Id proteins negatively regulate bHLH factors, leading to the inhibition of differentiation (Fig1.4) (Norton, 2000). In addition to bHLH factors, several other families and types of non-bHLH proteins also interact with and are functionally antagonized by Id proteins such as the ETS-domain transcription factors. Id proteins physically interact with the TCF subfamily of ETS proteins to inhibit their DNA binding and ability to induce immediate early genes (Yates et al., 1999). Other identified Id interacting partners

A



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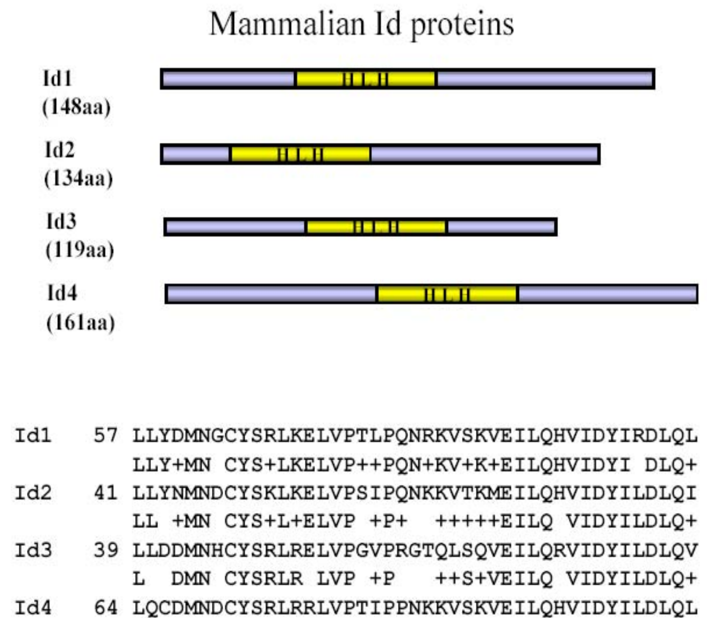


Fig 1.3 The inhibitor of DNA binding/differentiation family proteins. ***A.*** Biochemical structure of Helix-Loop-Helix (HLH) domain in Id family proteins and the conformation of the dimerization. Adapted from Wibley et al. *Biochimica Biophysical Acta* 1996 ***B.*** Mammalian Id proteins and their conserved HLH domain sequences.

include paired-domain homeobox (PAX) transcriptional factors (Roberts et al., 2001), Z-DNA-binding protein (Shoji et al., 1995) as well as the pRB tumor suppressor and related “pocket” proteins (Iavarone et al., 1994). A summary of identified interaction partners of Id proteins is outlined in Table 1.1.

Id proteins are highly conserved throughout evolution. Genes encoding Id-like proteins have been cloned in organisms such as *Drosophila* (Ellis et al., 1990; Garrell and Modolell, 1990), *Xenopus* (Wilson and Mohun, 1995), *Zebrafish* (Sawai and Campos-Ortega, 1997), mouse and human (Ellmeier et al., 1992). In *Drosophila*, a single locus termed *extramicrochaetae* (EMC), encodes an HLH protein showing high degree of structural and functional similarity to the murine Id proteins (Campuzano, 2001). EMC is apparently required for normal development, as *EMC* deficient flies die during embryogenesis. Studies on various partial loss- and gain-of-function mutants revealed that EMC is required for multiple processes in *Drosophila* development such as wing morphogenesis, neurogenesis and sex determination. In the *Drosophila* trachea, a functional counterpart of the mammalian lung, EMC is expressed in cells surrounding and including the invaginating tracheal pits. Interestingly, in EMC mutants, tracheal development is often affected; exhibiting structural alterations of the tracheal tree, similar to the phenotypes found in mutants for *Notch* and *Breathless* (Cubas et al., 1994). These studies may provide a paradigm for understanding how Id proteins function in mammalian lung development.

Table 1.1 The interaction partners of Id family proteins

Target Family	Examples	Id member	Interaction domain	Effects
bHLH transcription factors	E12, E47, E2-2, MyoD	Id1-4	HLH-HLH	Disrupt dimerization between Class A and Class B bHLH proteins, inhibit DNA binding and cell differentiation
Ets-domain transcription factors	Ets1-2, SAP-1, ELK-1	Id1-3	HLH-ETS	Disrupt formation of DNA-bound complexes by ETS proteins, block MAPK signaling
Paired-domain homeobox transcription factors	Pax2, Pax5, Pax8	Id1-3	HLH-Paired DNA binding domain	Disrupt DNA binding and transcriptional activation of Pax-responsive genes
pRB tumor suppressor protein and related “pocket” proteins	pRB, p107,p130	Id2, Id4	HLH-E1A/large T-binding pocket	Attenuate pRB suppression of E2F-DP1 transcription factors, potentiate S phase progression
Z-DNA binding protein	MIDA1	Id1	HLH-Zuotin homology region	Potentiate sequence-specific DNA binding of MIDA1,positive regulate cell growth
bHLH-Leucine-zipper transcription factor	ADD1	Id2, Id3	Unknown	Abrogate binding and transcriptional activation of adipocyte genes, regulate lipogenesis

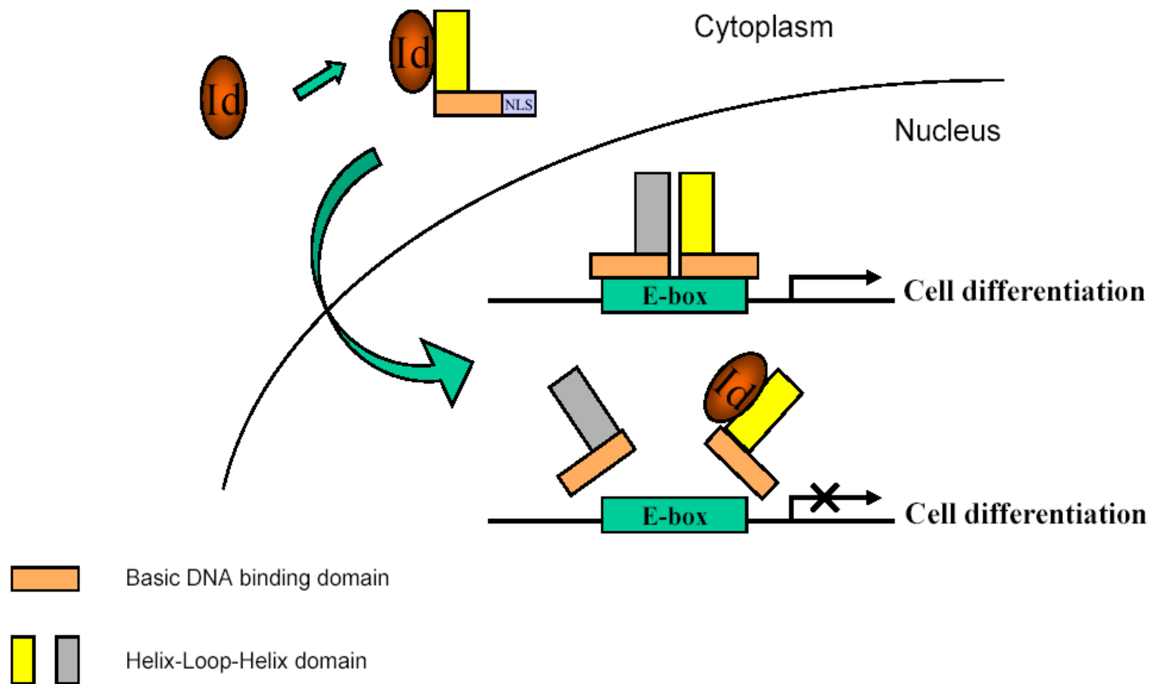


Fig 1.4 Schematical representation of the molecular mechanism of Id proteins' role as transcription inhibitor. Id proteins are produced in the cytoplasm and translocated into the nucleus upon binding to bHLH factors. The interaction between Id proteins and bHLH factors disrupted the formation of functional dimers of bHLH proteins that can recognize and bind to E-box DNA sequence. The subsequent gene activation is then inhibited.

Currently, four Id family members have been identified in mammals (Fig1.3-B). The sequences within the HLH domains are highly conserved among mammalian Id family members (Fig1.3-B), whereas the sequences outside the HLH domain display extensive divergence (Norton et al., 1998). The *Id* genes exhibit widespread and overlapping expression patterns in multiple tissues during mouse development. In general, *Id1, Id2* and *Id3* are readily detected in organs undergoing active morphogenetic activities, such as gut, lung, kidney, tooth and some glandular structures, whereas *Id4* expression is only restricted to neuronal tissues and stomach. In organs that arise from mesoderm-endoderm interactions, *Id1* and *Id3* are usually expressed in mesenchymal cells surrounding the endodermal epithelium, while *Id2* is located in the epithelium. In particular, strong *Id1* and *Id3* signals can be detected in the mesenchyme of the mouse lung, suggesting their importance in lung morphogenesis (Evans and O'Brien, 1993; Jen et al., 1996; Jen et al., 1997; Zhu et al., 1995).

Id proteins have distinct functions in development and disease, playing important roles in regulating proliferation and differentiation of various cell types including epithelial, endothelial, fibroblast and neural precursor cells (Benezra et al., 2001; Engel and Murre, 2001; Fong et al., 2003; Jen et al., 1997; Li et al., 2005; Lyden et al., 2001; Lyden et al., 1999; Ruzinova and Benezra, 2003; Sikder et al., 2003). One characteristic function of Id proteins is their ability to inhibit Smooth muscle alpha-actin-positive myofibroblast differentiation by disrupting

transcription complex formation between bHLH protein E2A and MyoD (Sun et al., 1991). 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 (Zebedee and Hara, 2001). Id proteins positively regulate cell proliferation mainly through repressing the expression of CDK inhibitors such as p15, p16 and p21 (Yokota and Mori, 2002; Zebedee and Hara, 2001). Ids exert these functions by directly binding to and disrupting transcriptional dimers formed by either bHLH proteins or ETS family proteins.

As in *Drosophila*, Id genes are essential for mouse development, although functional redundancy exists among the four Id members. Among Id single-knockout mice, only *Id2-/-* mice displayed obvious abnormalities such as retarded growth and neonatal morbidity (Yokota et al., 1999). However, all combinations of double knockouts of *Id1, Id2* and *Id3* are embryonic lethal (Fraidenraich et al., 2004; Norton et al., 1998). In addition, severe defects in neurogenesis and angiogenesis were observed in the brain when *Id1* and *Id3*, the two Id members showing extensive overlapping expression patterns during development, were inactivated simultaneously (Lyden et al., 1999). *Id1-/-Id3-/-* mice die at E13.5 due to intraventricular hemorrhage. Starting from E11.5, neuroblasts are prematurely withdrawn from the cell cycle in the mutant, accompanied by increased expression of CDK inhibitors and neural-specific differentiation markers. *Id1-/-Id3-/-* mice also exhibited vascular malformations in

the forebrain and absence of branching and sprouting of blood vessels into the neuroectoderm. Consistently, the expression of *VEGF, Flk-1* and Smooth muscle α-actin were reduced within the malformed vascular structures (Lyden et al., 1999). The apparent defects in proliferation and differentiation of the *Id1-/-Id3-/-* brain underscore the critical role of these genes in the endothelium as only *Id1* and *Id3* are detected in endothelial cells of the brain (Jen et al., 1996). A similar situation can be found in the lung mesenchyme, where only *Id1* and *Id3* are expressed (Jen et al., 1996), suggesting a potential role for these Id proteins in neurogenesis, myogenesis and vascular development, all of which occur in the lung mesenchyme.

The roles of Id proteins in vascular development and maintenance

Id proteins are prominently expressed in endothelial cells throughout development and in some adult organs. *Id1, Id2* and *Id3* are all expressed in blood vessels in most parts of the mouse embryo except for the embryonic brain where only *Id1* and *Id3* are expressed (Jen et al., 1997; Lyden et al., 1999). Although *Id* gene expression is normally downregulated or turned off in most adult organs, they are frequently upregulated exclusively in endothelial cells in most tumor tissues (Perk et al., 2006). Upregulation of *Id1* expression in endothelial cells is also detected during hypoxic vascular remodeling in pulmonary hypertension, suggesting a contributory role of Id1 in maintaining endothelial

homeostasis (Frank et al., 2005).

Increasing evidence suggests that Id proteins function as key players in endothelial function and homeostasis (Benezra et al., 2001). Mice lacking *Id1* and *Id3* functions display brain hemorrhage during development and defects in tumor-promoted angiogenesis (Lyden et al., 1999). Deregulated *Id1* expression in endothelial cells substantially affects angiogenesis and tumor growth in various tumor models (de Candia et al., 2004; Iavarone and Lasorella, 2004; Li et al., 2004a; Ling et al., 2005). *In vitro* studies demonstrated that overexpression of *Id* genes reduces human endothelial cell apoptosis rate (Nishiyama et al., 2005) (Valdimarsdottir et al., 2002) although the underlying molecular mechanisms governing this process remain unknown. Id1 has also been shown to delay endothelial senescence by suppressing the expression of CDK inhibitors p16 and p21. Thus Id function may be an important component of the cellular stress response pathway (Alani et al., 2001; Sharpless et al., 2001; Tang et al., 2002). Forced expression of *Id1* in Human Umbilical Vein Endothelial Cells (HUVECs) promotes angiogenic properties such as migration and tube formation (Nishiyama et al., 2005; Sakurai et al., 2004; Valdimarsdottir et al., 2002). Transplantation of *Id1*-overexpressing HUVECs into mice increased capillary density and limb salvage rate, indicating involvement of Id1 in endothelial repair (Nishiyama et al., 2005).

It has been proposed that the *in vitro* angiogenic activity of Id proteins may be partly mediated by induction of angiopoietin-1 (Ang-1) transcription, an endothelial survival factor that augments endothelial cell migration and reduces the rate of apoptosis. However no evidence is yet available to prove that the regulation of *Ang-1* by Id1 is direct. The function of Id1 in angiogenesis has also been associated with its ability to regulate the expression of matrix metalloproteinases (MMPs) and integrins. (Benezra et al., 2001; Coppe et al., 2004; Desprez et al., 1998; Sakurai et al., 2004). The MMPs are a family of Zn^{2+}- or Ca^{2+}-dependent endopeptides, which function primarily to degrade extracellular matrix. They are actively engaged in capillary formation and sprouting during endothelial morphogenesis and compromised MMP activity may result in defects in angiogenesis *in vivo* (Bergers et al., 2000; Hiraoka et al., 1998; Hotary et al., 2000; Vu et al., 1998). So far two MMP family genes, *MMP-2* and *MMP-9*, are shown to be transcriptionally regulated by Id proteins in different contexts. Forced expression of *Id1* and *Id3* in HUVEC cells induces increase in mRNA levels of both *MMP-2* and *MMP-9* (Sakurai et al., 2004). Knockdown of *Id1* and *Id3* by RNAi specifically abolished induction of *MMP-2* expression by VEGF, but not that of *MMP-9* (Sakurai et al., 2004). In addition, tumor endothelial cells lacking Id1 function also showed downregulation of *MMP-2* expression (Ruzinova et al., 2003). Although the detailed transcriptional controlling system is not yet established, it remains an interesting subject for future studies.

CHAPTER II

ID1 AND ID3 FUNCTIONS IN EMBRYONIC LUNG VASCULAR DEVELOPMENT

Introduction

Previous studies have suggested that Id proteins are crucial for vascular development both in the mouse forebrain and in tumors (de Candia et al., 2004; Iavarone and Lasorella, 2004; Li et al., 2004a; Ling et al., 2005; Lyden et al., 1999). As mentioned in the introduction, in the E12.5 lung mesenchyme, the expression domains of *Id1* and *Id3* overlap with the region where pecam-1 positive endothelial cells are generated (Jen et al., 1996), suggesting that these Ids may be involved in vascular development. Moreover, *in vitro* studies using cultured endothelial cells from various tissue origins revealed that the *Id* genes could facilitate endothelial cell proliferation, migration and tube formation during angiogenesis, indicating the critical influence of Id proteins on vascular development (Nishiyama et al., 2005; Sakurai et al., 2004; Valdimarsdottir et al., 2002). In this chapter, I will mainly address the potential roles of Id1 and Id3 in vascular development during embryonic lung mesenchymal cell differentiation and the molecular mechanisms contributing to those functions.

Experimental Procedures

In situ hybridization

Cryosection *in situ* hybridizations were performed as previously described (Litingtung et al., 1998). Id1 and Id3 cDNAs generated outside of the conserved HLH domain (gift of Dr. Benezra, (Jen et al., 1996)) were used as templates for synthesizing digoxygenin-labeled riboprobes.

Immunohistochemistry

Labeling using antibodies against Pecam/CD31 and MMP-2 were performed on 5-μm tissue sections from paraffin-embedded embryos fixed in 4% paraformaldehyde for 2 hours at 4°C. Paraffin sections were deparaffinized and rehydrated according to standard protocols. Endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol for 10 min at room temperature (RT). To reveal Pecam-1 and MMP-2 antigen, sections were antigen-retrieved by trypsin digestion using 0.75mg/ml at RT for 5 min. The antibodies used were rat anti-CD31 (BD Pharmingen, 1:10 dilution) and mouse anti-MMP2 (Neomarkers, 1:100 dilution). Alexa 488 (green)- or Alexa 568 (red)-conjugated secondary antibodies (Molecular Probes) were applied at 1:600 dilution for 1 h at room temperature.

For whole-mount immunohistochemistry of CD31, cultured embryonic lungs were collected and fixed in 4% paraformaldehyde for 2 hours at 4°C. After

staining, lungs were immersed in PBS solution before visualization. Confocal images were taken using the Zeiss LSM510 confocal microscope at the Vanderbilt Cell Imaging Core. Results were presented as screen captures of 3D z-stack overlays.

Terminal deoxynucleotidyl transferase–mediated dUTP Nick End Labeling (TUNEL)

For cell death detection in lung tissue sections, paraformaldehyde-fixed paraffin sections were treated with trypsin for 5 min and apoptotic cells were detected by TUNEL using the *In situ* Cell Death Detection Kit (Chemicon) according to manufacturer's protocol. Slides were subsequently double-stained with CD31 to mark endothelial cells.

Kidney Capsule

Kidney capsule implantation of embryonic lungs was performed as previously described (Vu et al., 2003). Briefly, adult male C57BL6 mice were anesthetized and a dorsal incision was made to expose the kidney on one side. Freshly dissected E11.5 lungs were placed underneath the membranous capsule through a small opening (1-2mm) using blunt-end glass needles. Two embryonic lungs were implanted in each kidney. The kidney was then placed back into position and the wound was resealed using surgical suture. Host mice were

sacrificed 6 days after surgery and grafted lungs were collected and fixed for analysis.

Lung organ culture under hypoxic condition

The lung culture method used was performed essentially as previously described (Li et al., 2004b). Briefly, wildtype or *Id1-/-Id3-/-* lungs were dissected out at E11.5 and cultured on nucleopore polycarbonate filters (8um pore size, Millipore) in standard DMEM medium with 10%FBS for 48 h in a hypoxia chamber with 3% oxygen before being collected and fixed for staining.

Results

Embryonic *Id1* and *Id3* expression are predominantly in the distal lung mesenchyme

Expression of *Id1* and *Id3* in the developing lung were reported more than a decade ago (Evans and O'Brien, 1993; Jen et al., 1996; Jen et al., 1997; Zhu et al., 1995), but detailed examination is lacking. In order to elucidate the role of Id proteins during lung mesenchymal development, we started by establishing the precise spatial expression patterns of *Id1* and *Id3* in the embryonic lung mesenchyme. The riboprobes for *Id1* and *Id3* genes were generated outside of the conserved HLH domain to ensure specificity (gifts of Dr. Benezra). RNA *in situ* hybridization was performed on E12.5 wildtype lung cryosections. As presented in Fig2.1, both *Id1* and *Id3* mRNA are localized mainly in the distal mesenchyme, with little expression in the cell layers immediately adjacent to the proximal epithelium (Fig2.1 arrows). Interestingly, when we correlated the expression patterns of *Id1* and *Id3* with that of endothelial marker CD31/Pecam-1 (Fig2.1-B), we observed that they have similar expression patterns in the lung. These results corroborate well with previous studies, which showed that *Id1* and *Id3* are frequently detected in endothelial cells throughout the mouse embryo. This finding also points to a potential role of Id proteins in lung endothelial morphogenesis.

Id1-/-Id3-/- lungs exhibit vascular defects *in vivo* and in culture

The interesting expression patterns of *Id* genes led us to investigate the

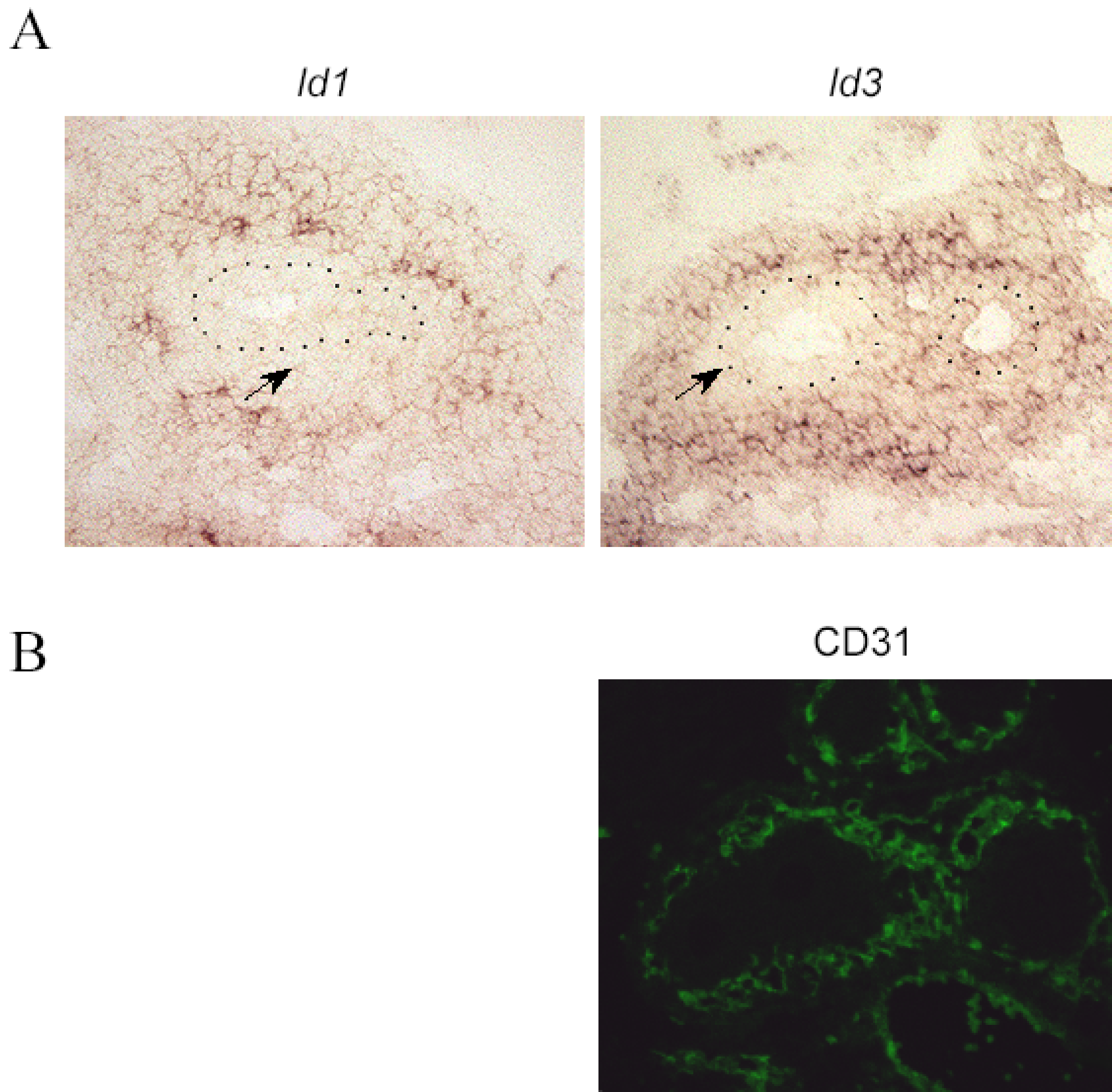


Fig 2.1 Expression domains of *Id1* and *Id3* overlap with that endothelial marker CD31.

A. Representative photographs showing RNA in situ hybridization of *Id1* and *Id3* transcripts in E12.5 wildtype lungs. Dotted circles outline boundary between mesenchyme and epithelial layer in the lung lobe. Magnification 400x. **B.** Endothelial cell distribution as illustrated by CD31 immunohistochemistry of E12.5 wildtype lung. Note that both the Id genes and CD31 are expressed in distal mesenchymal cells away from the epithelial layer. Magnification 400x.

functional roles of these genes in embryonic lung vascular development. We examined whether loss of *Id* gene function in embryonic lungs under normal developing conditions would result in detectable vascular defects. E11.75 wildtype and *Id1-/-Id3-/-* lungs were collected and alteration in Pecam-1 expression was examined by immunohistochemistry. Staining revealed that there is no obvious change in Pecam-1 expression level or patterns in *Id1-/-Id3-/-* double knockout lung mesenchyme compared to the wildtype (data not shown). However, since major vascular development happens later than E11.5, it is critical to obtain Id1 and Id3 double knockout lungs at later stage. Unfortunately, most *Id1-/-Id3-/-* embryos die before E12.5 due to brain hemorrhage (Lyden et al., 1999). To overcome this obstacle, we collected and cultured E11.5 *Id1-/-Id3-/-* double mutant lungs *in vitro* under hypoxic condition (van Tuyl et al., 2005) or *in vivo* under the renal capsule to allow recovery of more differentiated lung tissues for analysis. E11.5 wildtype and *Id1-/-Id3-/-* lungs were dissected out and cultured in hypoxic chambers with 3% oxygen for 48 hours. Lungs were then collected and expression of CD31/Pecam-1 was analyzed by immunohistochemistry. As shown by whole-mount CD31 immunostaining in Figure 2.2, the vascular density in cultured *Id1-/-Id3-/-* lungs is greatly reduced compared with wildtype lungs, suggesting impaired distal angiogenesis due to complete removal of Id function in lung endothelial cells. Moreover, section immunohistochemistry revealed that the capillary tubes in *Id1-/-Id3-/-* lungs appear to be more dilated and disrupted

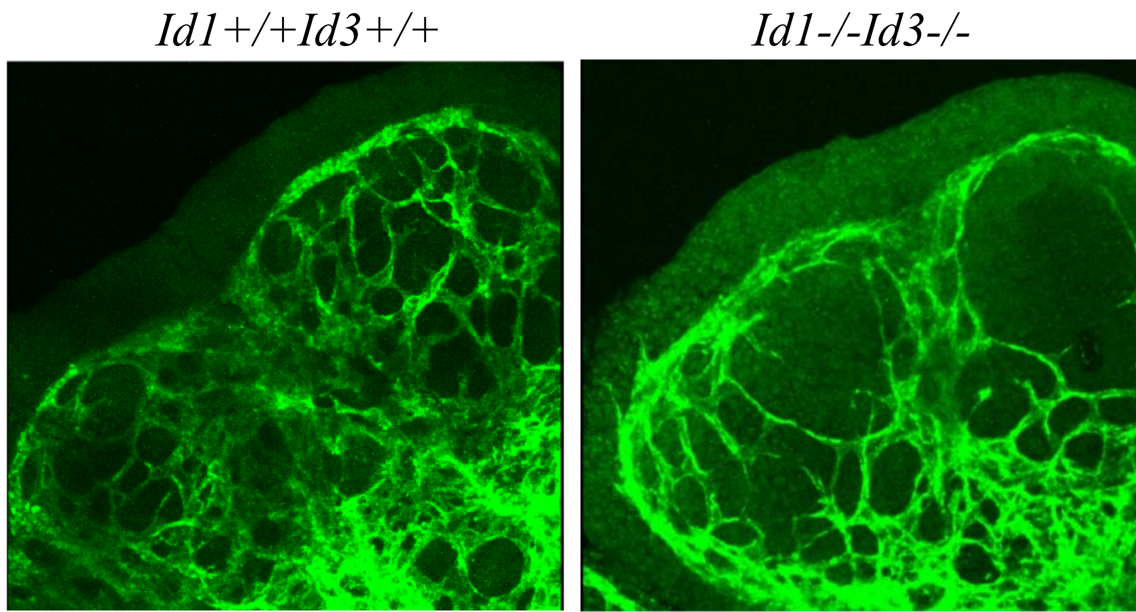


Fig 2.2 *Id1-/-Id3-/-* lungs display defects in endothelial morphogenesis under hypoxia culture condition*.*

Whole mount immunostaining of CD31 to show the morphology of vascular network in the lung. Representative photographs showing E11.5 wildtype and *Id1-/-Id3-/-* lungs at 48 hours post culture in 3% Oxygen. Magnification 400x. The lungs devoid of Id gene function display reduced vascular density compared with wildtype control.

compared with *Id1* single mutant (*Id1-/-Id3+/+*) lungs with two functional alleles of *Id3* (Fig2.3, arrows). I observed that the reduced vascular density defect is recapitulated in the renal capsule experiment. As shown by section immunostaining for CD31/Pecam-1, there is marked reduction of CD31-positive endothelial cells in the grafted *Id1-/-Id3-/-* double knockout lung compared to wildtype lungs within the same capsule (Fig2.3). This finding suggests that Id1 and Id3 appear to play an important role during vascularization of the embryonic lungs, consistent with their pivotal roles in brain vascularization.

_Id1-/-Id3-/-_ lungs show decreased MMP-2 expression in the mesenchyme _in vivo_ and in culture

Id1 has been shown to attenuate endothelial apoptosis rate in HUVEC cells (Nishiyama et al., 2005; Valdimarsdottir et al., 2002). In order to determine the contribution of apoptosis to the vascular defects observed in *Id1-/-Id3-/-* lungs, we examined the endothelial apoptotic profile in cultured wildtype and mutant lungs. Sections of cultured wildtype and *Id1-/-Id3-/-* lungs were stained with TUNEL (cell death marker) and CD31/Pecam-1 (endothelial marker). Double-labeling results showed that neither wildtype nor *Id1-/-Id3-/-* lung endothelial cells undergo extensive cell death, as very few TUNEL-positive cells co-stained with CD31 (Fig2.4). This observation suggests that endothelial cell death is not a major event during lung vascular development and does not contribute significantly to the defects of angiogenesis in *Id1* and *Id3* double knockout lungs.

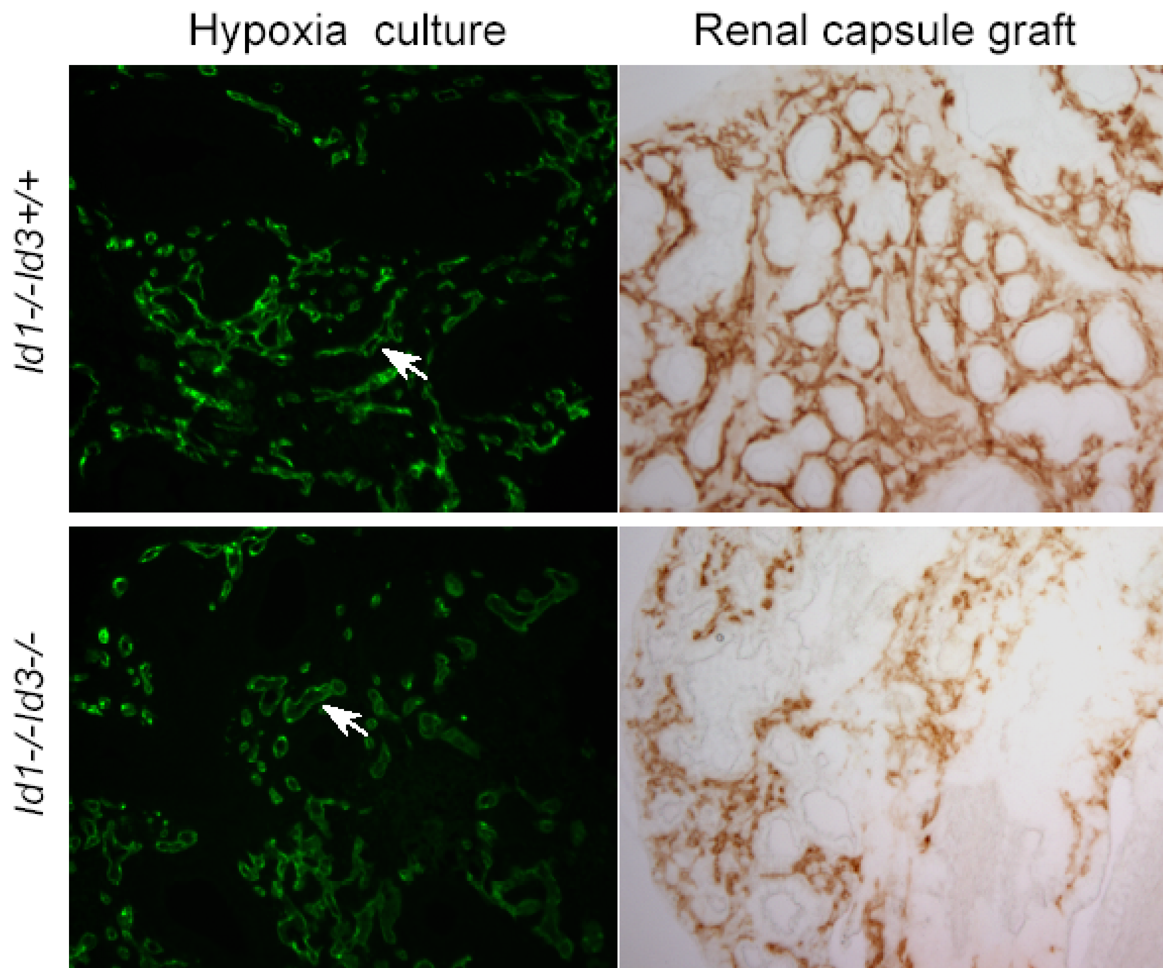


Fig 2.3 Impaired vascular development of *Id1-/-Id3-/-* lungs at both in vitro and in vivo culture conditions*.*

CD31 immunostaining of lung sections of E11.5 *Id1-/-* and *Id1-/-Id3-/-* lungs at 48 hours post hypoxia culture (left panel) or 6 days after renal capsule implantation. Magnification 200x.

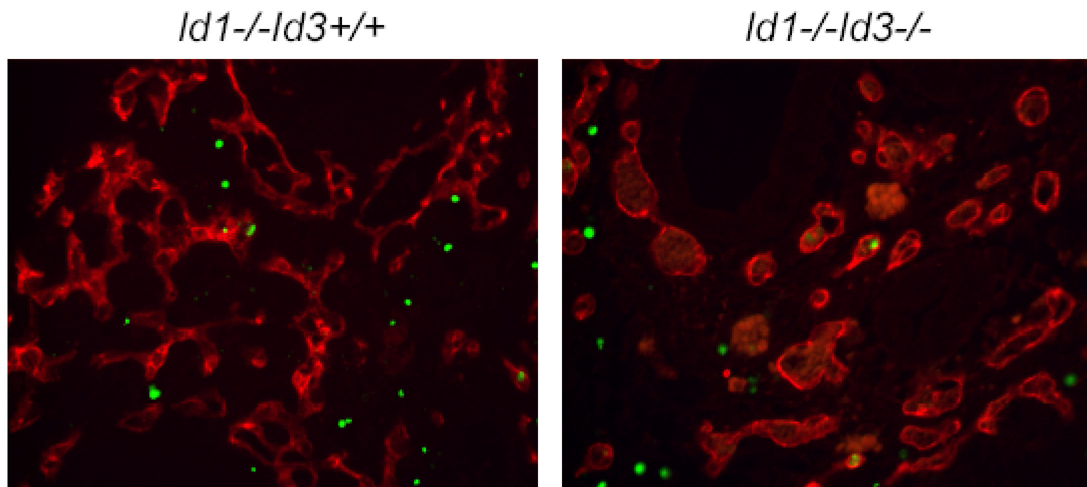


Fig 2.4 Endothelial apoptosis is not a major event during lung vascular development.
Endothelial cell death detection in *Id1-/-Id3+/+* and *Id1-/-Id3-/-* lungs by TUNEL (green) and CD31 (red) double-labeling. Representative sections are shown for E11.5 lungs at 48 hours post-hypoxia culture. Magnification 400x.

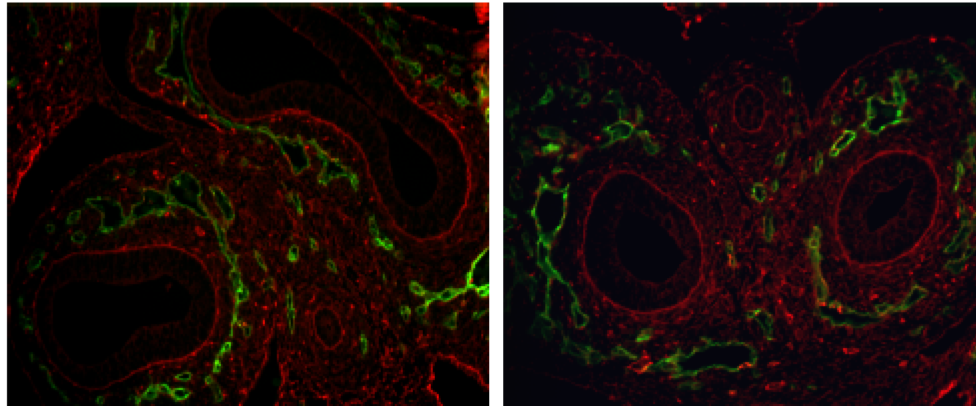
We reasoned that compromised vascular development caused by lack of Id function might also be linked to defects in endothelial cell migration. Id1 and Id3 has been shown to induce *MMP-2* and *MMP-9* expression, two pro-angiogenic MMP family genes that function to degrade ECM scaffolds during endothelial cell morphogenesis (Benezra et al., 2001; Coppe et al., 2004; Desprez et al., 1998; Sakurai et al., 2004). Hence we examined the expression of MMP-2 in cultured wildtype and *Id1-/-Id3 -/-* lungs by immunohistochemistry. Although we did not detect a significant change in MMP-2 level in freshly dissected E11.5 lungs (Fig2.5-A), MMP-2 expression was significantly upregulated after culturing for two days in hypoxic chambers (Fig2.5-B). Interestingly, we observed dramatic decrease of MMP-2 protein level in *Id1-/-Id3-/-* lung mesenchyme compared with *Id1* single mutant controls (Fig. 2.5-B). In addition, double labeling of MMP-2 and CD31 revealed that endothelial CD31 expression in *Id1-/-Id3-/-* lungs rarely co-localized with reduced MMP-2 expression, suggesting that loss of Id function in endothelial cells results in downregulated MMP-2 protein synthesis (Fig 2.5-B). Taken together, our results indicate that expression of Id1 and Id3 in embryonic lung mesenchymal cells are required to maintain MMP-2 expression, which may facilitate endothelial cell migration.

Discussion

Great emphasis has been focused on the functions of Id proteins in

A

Id1-/-Id3+/+ *Id1-/-Id3-/-*



B

Id1-/-Id3+/+ *Id1-/-Id3-/-*

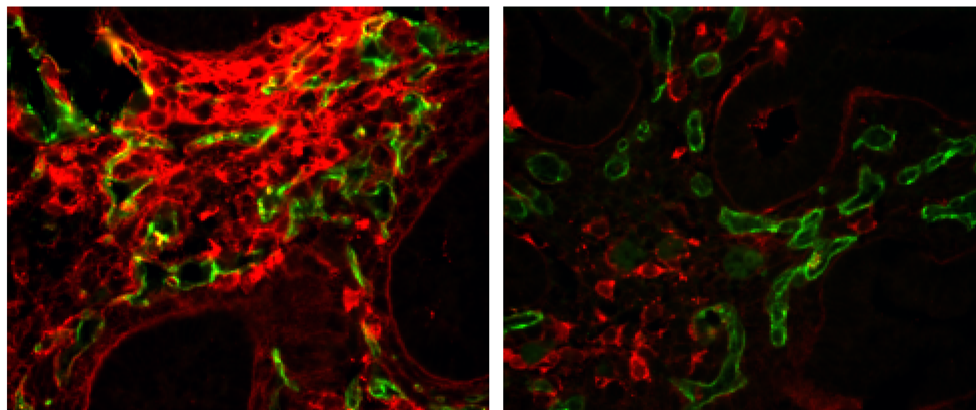


Fig 2.5 MMP-2 expression is compromised in *Id1-/-Id3-/-* lung mesenchyme under culture conditions.

Representative photographs MMP2 (red) and CD31(green) double-immunostaining of E11.5 Id1-/- and *Id1-/-Id3-/-* lungs freshly dissected **(A)** or cultured for 48 hours in 3% Oxygen **(B)**. Magnification 400x.

angiogenesis during embryonic brain development and during tumorigenesis, whereas little is known regarding their roles during endothelial morphogenesis in other organs. In this study, I have shown that *Id1* and *Id3* have overlapping expression patterns in embryonic lung mesenchyme and overlap with a region of active endothelial morphogenesis in the lung. Analysis of vascular defects in cultured or implanted *Id1-/-Id3-/-* lungs revealed that proper Id function is required for normal endothelial morphogenesis in the embryonic lung. The Id1 and Id3 double knockout lung serves as an ideal model for studying Id function in angiogenesis, given the fact that no other known Id family proteins are expressed in the lung mesenchyme. Furthermore, by culturing *Id1-/-Id3-/-* lungs *in vitro* under hypoxic condition or in renal capsules, I was able to overcome the early embryonic lethality problem and obtain more differentiated double knockout lungs representing late pseudoglandular stages. This model system may be valuable for future investigations into the other roles of Ids such as neurogenesis and myogenesis during lung development.

Id1 and Id3 are known to facilitate endothelial survival by either preventing senescence or apoptosis and by promoting proliferation (Alani et al., 2001; Nishiyama et al., 2005; Sharpless et al., 2001; Tang et al., 2002; Valdimarsdottir et al., 2002). In this study, I have shown that endothelial cells in the embryonic lung do not undergo extensive apoptosis (Fig2.4). Therefore it is unlikely that the anti-apoptotic function of Id proteins plays a key role during normal endothelial

morphogenesis in the embryonic lung. However since the endothelial cells are highly proliferative during development, it is reasonable to speculate that the presence of Id1 and Id3 is indispensable for maintaining proliferation of endothelial cells. Further examination of the association between loss of Id function and alteration in endothelial proliferation is needed to establish the role of Id proteins in endothelial cell proliferation.

The MMP family protein consists of 25 related endopeptidases. They are synthesized and secreted as precursor forms then activated upon proteolytic cleavage. The MMPs are primarily engaged in the regulation of ECM composition, with secondary functions in modulating growth factor bioactivities (Visse and Nagase, 2003). The crucial roles of MMP proteins in angiogenesis and metastasis have been well established through *in vitro* studies and tumor models (Bergers et al., 2000; Haas and Madri, 1999; Hiraoka et al., 1998; Pagenstecher et al., 2001; Pozzi et al., 2000; Vu et al., 1998). Numerous studies suggest that the MMPs may also be involved in lung vascular development, although detailed examination of the embryonic vascular network phenotypes in multiple MMP knockout mice is required to firmly establish the roles of MMPs in lung endothelial morphogenesis. *MMP-2*, *MMP-9* and *MMP-14* are all found to be highly expressed in the embryonic mouse lung mesenchyme during a period of extensive angiogenic activity (Kheradmand et al., 2002; Ryu et al., 2005). Mild defects in the alveolar space have been observed in *MMP-2* null mice, an indication of an impaired

alveolization process that involves migration of capillary endothelial cells (Kheradmand et al., 2002). A premature capillary structure, a double capillary network forming on both sides of the septa, was also documented in *MMP-14* null mice (Irie et al., 2005). My finding that loss of Id1 and Id3 function results in decreased MMP-2 expression in embryonic lungs underscores the pro-angiogenic role of Id proteins. MMP-2 has been shown to be transcriptionally regulated by Id proteins in both cultured endothelial cell lines and in mouse tumor grafts (Benezra et al., 2001; Coppe et al., 2004; Ruzinova et al., 2003; Sakurai et al., 2004). Sequence analysis revealed that *MMP-2* promoter activity is under direct repression by p16, a well-known downstream target of Id proteins. Therefore it is possible that the presence of Id1 and Id3 in the lung mesenchyme maintains MMP-2 expression by inhibiting its negative regulator p16 expression. Examination of alterations in p16 transcript level upon ablation of Id function in the lung may provide valuable information about the molecular connection between Id proteins and MMP-2.

CHAPTER III

ID1 FUNCTION IN ENDOTHELIAL DAMAGE DURING BLEOMYCIN-INDUCED ACUTE LUNG INJURY

Introduction

Although Id1 is highly expressed in the lung mesenchyme during embryogenesis, its expression is downregulated after birth and is barely detectable in the normal adult murine lung, suggesting that Id1 function is likely not essential in healthy adult lungs. In agreement, *Id1* loss-of-function mutant mice are viable and fertile under pathogen-free housing conditions (Yan et al., 1997). Upregulation of Id1 in bleomycin-treated rat lungs has been reported in previous studies although the detailed expression pattern and specific function of Id1 was not thoroughly investigated (Chambers et al., 2003). In this study, we found that upon bleomycin-induced injury, Id1 is upregulated in different lung cell types but predominantly in endothelial cells, suggesting a potential role of Id1 in these cell types upon lung injury. Loss of Id1 function in the lung endothelium resulted in increased vascular permeability and endothelial cell death after bleomycin instillation. Likewise, we found that *Id1-/-* lung microvascular endothelial cells showed decreased survival in culture. Taken together, our studies reveal a new function of Id1 as part of the self-defense response system in the lung, protecting

pulmonary endothelial cells from undergoing extensive damage upon bleomycin challenge.

Experimental Procedures

Mice and bleomycin treatment

Id1 null mice (*Id1-/-*) (gift of Dr. Robert Benezra) and *Tie1-Cre* mice (Gustafsson et al., 2001) were bred in the C57BL/6J background and *ShhCre-ZEG* mice (Li et al., 2006) were bred in the C57BL/6J;129 background. For the Id1 time-course study, C57BL/6J mice (8-10 week old) were purchased from the Jackson Laboratory. Mice were treated with either saline or bleomycin (0.08U) by intratracheal injection in a total volume of 50ul saline (Lawson et al., 2005b). 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% PFA for 5h at 4°C and embedded in OCT cryo embedding medium. 15um sections were cut and immunostained with Pecam-1 antibody (BD Pharmingen) followed by Alexa-568 conjugated secondary antibody (Molecular Probe) for signal visualization. *ShhCre-GFP* adult lungs were perfused using phosphate-buffered saline (PBS), then inflated and fixed in 4% PFA for 5h at 4°C. Subsequently, OCT was injected

intratracheally into fixed lung to preserve the lung architecture. Lungs were embedded in OCT and 15um sections were collected and GFP fluorescence visualized using an Olympus BX60F5 microscope.

Adult Lungs were perfused, inflated, excised, and fixed in 4% paraformaldehyde at $4^{o}C$ overnight. Subsequently, lungs were embedded in paraffin blocks and 5 um sections were collected and processed for immunolabeling. Antibodies against smooth muscle alpha-actin (Sigma, 1:300), CD34 (Labvision, 1:100) and β-galactosidase (LacZ) (Sigma, 1:2000) were used for immunostaining. For general immunolabeling, slides were antigen-retrieved using citrate buffer (pH6.0) and incubated at $4^{o}C$ overnight with primary antibody. Alexa-conjugated secondary antibodies or horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch) and HRP detection kit (Labvision) were used for signal visualization.

For Id1 immunolabeling, lungs were perfused with phosphate-buffered saline (PBS) and fixed in EFA solution (100% ethanol, 37% formaldehyde and 100% acetic acid at V/V ratio of 6:3:1) at 4°C for 5 hours. Subsequently, lungs were dehydrated and embedded in paraffin blocks and 5 um sections were collected and processed for immunolabeling. Slides were incubated at $4^{o}C$ overnight with primary antibody, Id1 (Santa Cruz Biotechnology), at 1:6000. Detection was performed using polymer-HRP secondary antibodies (Zymed) diluted at 1:4 and visualized using the TSA Plus Fluorescence System (Perkin

Elmer) diluted at 1:200. Slides were counterstained with TO-PRO-3 (Invitrogen) to highlight nuclei. For double-labeling involving Id1, sequential immunostaining were performed instead of a one-step double-labeling. Confocal images were taken using the Zeiss Upright LSM510 Confocal microscope at the Vanderbilt Cell Imaging Core. Regular images were taken using the Olympus BX60F5 microscope.

Western blotting

Left lungs of bleomycin-treated wildtype and *Id1-/-* mice were harvested and homogenized in RIPA lysis buffer at pH7.4. Protein lysates of 100ug each were resolved on SDS-polyacrylamide gels (Bio-Rad). Primary antibodies against Id1 were used for detection. Equal loading of protein samples was monitored by and normalized to the level of α-tubulin (Calbiochem-EMD Biosciences, 1:2000). Blots were analyzed using QuantityOne software (Bio-Rad). For western blotting using FACS-sorted cells, cells were immediately frozen in liquid nitrogen after collection. Sorted cells were lysed in RIPA buffer and loaded at 50ug/lane on SDS-polyacrylamide gels. Additional antibodies used for blotting were Pecam-1 (BD Pharmingen, 1:500), TTF-1 (labvision, 1:200), Bcl-2 (Santa Cruz Biotechnology, 1:500), Bcl-xL (Santa Cruz Biotechnology, 1:500), MEK1/2 (Cell Signaling, 1:1000), phospho-MEK1/2 (Cell Signaling, 1:1000), ERK1/2 (Cell Signaling, 1:1000) and phospho-ERK1/2 (Cell Signaling, 1:1000). 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 (Londhe et al., 2005). Briefly, Evans blue dye at 20mg/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 620nm and 740nm using Bio-Rad Smartspec3000. Corrected pulmonary Evans blue absorbance at 620nm was calculated as A620nm–(1.426xA740nm + 0.03). Permeability index was generated by dividing the corrected pulmonary Evans blue absorbance by the plasma Evans blue absorbance at 620nm.

Pulmonary endothelial cell culture

Adult lung microvascular endothelial cells were isolated and cultured as previously described (Pozzi et al., 2000). Briefly, lungs were perfused with 0.25% Trypsin (Mediatech) and 2ug/ml collagenase (Roche Applied Science). Perfused lungs were incubated at 37 ^{o}C for 20min. Then lung lobes were trimmed with a sterile scalpel and washed 10-20 times with 1ml DMEM medium containing 10% fetal bovine serum (FBS). Detached cells were collected and centrifuged at 2,000 rpm. Cell pellet was washed once and resuspended in EGM2-MV medium (Cambrex) with 2% FBS. Cells were grown on 6-well dishes (BD Falcon) or

coverslips (Fisher) for 3 days before application of treatment. All cells were maintained at 37^oC and 5% CO_2 in a Hera Cell incubator unit (Kendro Laboratories). Cells from triplicate wells were harvested for p21 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 (pH6.0) for 20min and apoptotic cells were detected by TUNEL using the *In situ* Cell Death Detection Kit (Chemicon) according to manufacturer's protocol. Detection was performed using an HRP-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 250ng/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 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 25units/ml dispase (BD Biosciences) plus 2ug/ml collagenase (Roche Applied Science) then incubated in digestive solution at 37°C for 20min. The lungs were then minced and a single cell suspension was obtained by passing cells through a 40um cell strainer (BD Falcon). After centrifugation at 1000rpm for 5min, collected cells were resuspended in Red Cell Lysis buffer (Lorimore et al., 2001) and incubated at room temperature for 10min. The cells were then washed twice with PBS and stained with apoptotic marker using the Annexin-5 Apoptosis Detection Kit (Biovision) according to the manufacturer's protocol.

For FACS analysis on cultured lung microvascular endothelial cells, freshly isolated lung microvascular endothelial cells were grown in culture dish for 3 days before treatment with 250ng/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 HHMI Flow Cytometry Facility.

Statistics

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

+/- SEM.

Results

Id1 expression is significantly upregulated in the lung upon bleomycin injury

Although Id1 is highly expressed in the embryonic lung mesenchyme during a period of epithelial-mesenchymal interaction (Jen et al., 1996), its expression is not detectable in normal adult lung tissue sections by immunohistochemistry (Fig. 3.1, Saline 1wk). By Western blot analysis, which is a more sensitive detection method using whole lung homogenates, we detected weak Id1 expression (Fig 3.2, Saline). To investigate whether Id1 expression is upregulated upon pulmonary insult, we treated 8 week-old adult wildtype mice with a single 0.08 unit dose of bleomycin intratracheally and harvested lungs at 1 week post-bleomycin for Id1 immunohistochemistry. Interestingly, we found significant induction of nuclear Id1 expression in the bleomycin-treated wildtype lung compared with saline control (Fig. 3.1, Bleo 1wk). The specificity of Id1 antibody staining was confirmed using *Id1-/-* lung as a negative control (Fig 3.1, Bleo 1wk, *Id1-/-*). To evaluate the level and time course of Id1 induction in wildtype mice, we examined Id1 protein levels from lung samples collected at 1, 2 and 3 weeks after bleomycin instillation (N=3, Fig. 3.2). As shown by Western blotting, Id1 expression is significantly upregulated at 1 week post-bleomycin compared with saline control, and its upregulation is maintained for 2 and 3 weeks post-bleomycin (Fig. 3.2).

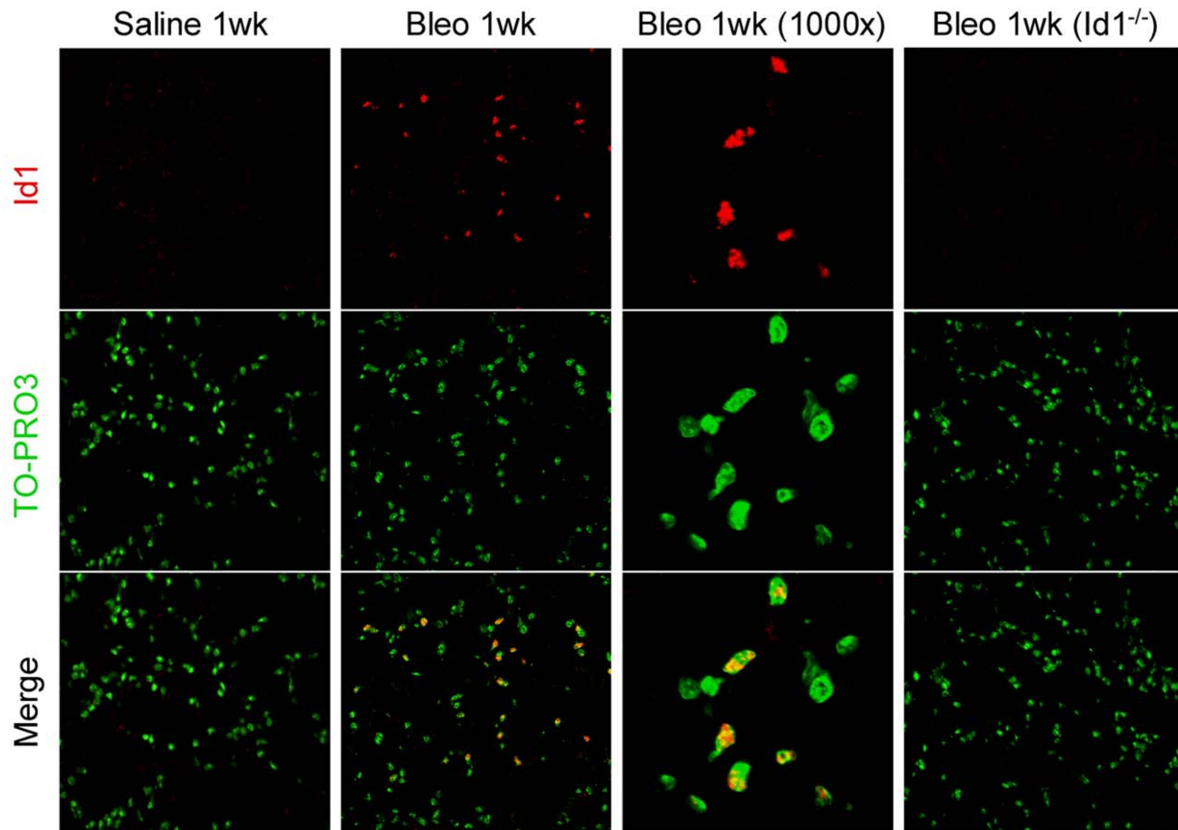
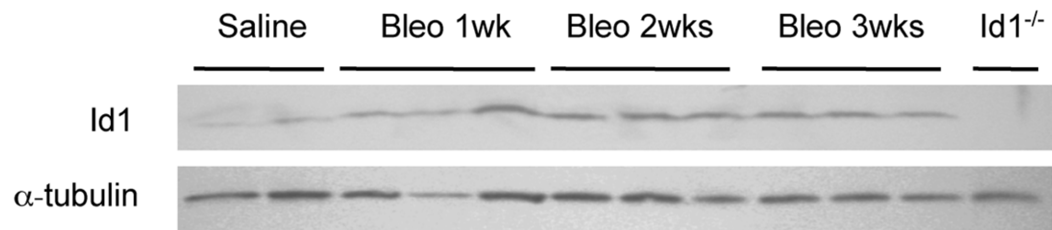


Fig 3.1 Id1 expression is significantly upregulated in the adult lung upon bleomycin injury. Representative photographs showing sections from 8 week-old wildtype and *Id1-/-* lungs treated with saline or 0.08U bleomycin and collected after 1 week. Sections were immunostained with Id1 (red) and nuclei were counterstained with TO-PRO3 (green). Note the nuclear Id1 expression in wildtype 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. Magnification 400x and 1000x.

A



B

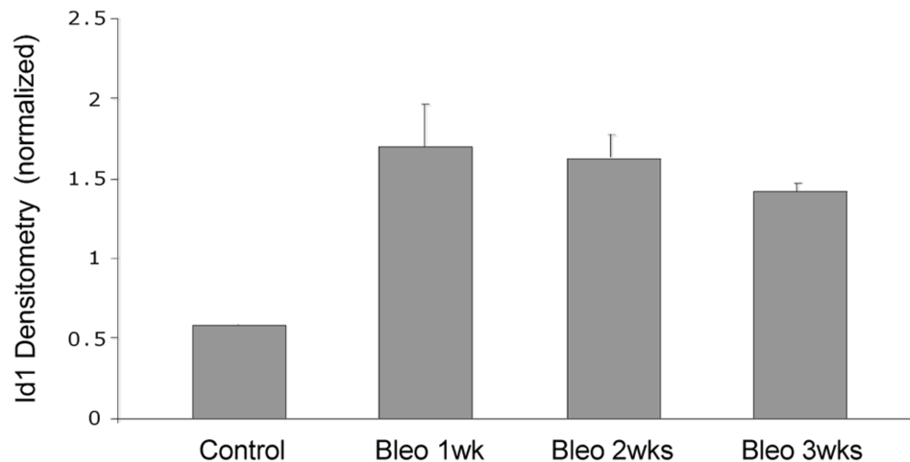


Fig 3.2 Id1 expression is significantly upregulated in the adult lung upon bleomycin injury.

A. Protein extracts from 8 week-old C57BL/6 wildtype lungs or *Id1-/-* lungs treated with saline or 0.08U bleomycin and collected after 1, 2 and 3 weeks (N=3 for each time point) 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.

Id1 protein expression is upregulated predominantly in the lung endothelium

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 (Balyasnikova et al., 2005). As shown in Fig. 3.3, Id1-positive cells displayed nuclear Id1 expression surrounded by membrane and cytoplasmic expression of CD34 (Fig. 3.3, arrows). There were only a few Id1-expressing cells that appeared to be CD34-negative (Fig. 3.3, arrowhead). To determine the fraction of Id1 protein expression level derived from endothelial cells, we performed fluorescence-activated cell sorting (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 (Gustafsson et al., 2001). By crossing *Tie1Cre* to *ZEG* mouse, which contains a transgene harboring a conditional Green Fluorescent Protein (GFP) reporter that is activated in the presence of Cre recombinase (Novak et al., 2000), mice were indelibly marked by GFP in all lung endothelial cells. The enrichment of the FACS-sorted endothelial cells was confirmed by Western blotting with endothelial marker, Pecam-1 (Fig. 3.6-A). By Id1 Western blot analysis, we found that the GFP-positive endothelial cell population expressed a

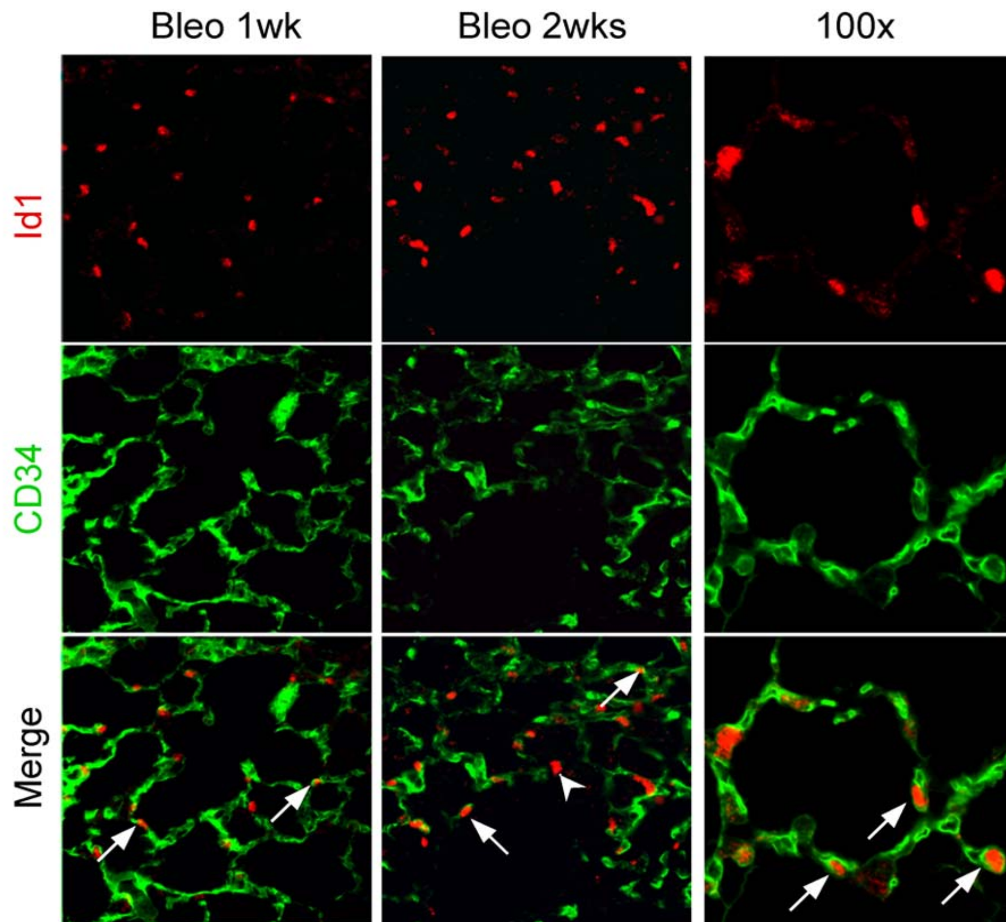
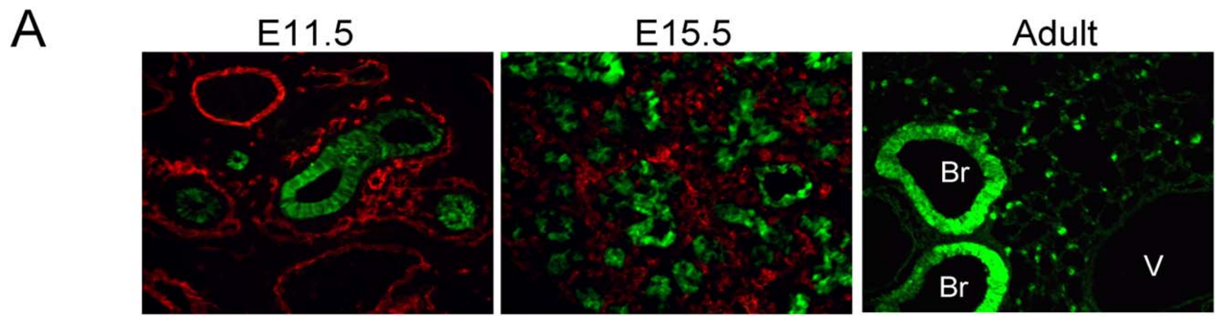


Fig 3.3 Id1 expression is upregulated prominently in the injured lung endothelium.

Representative photographs showing sections from 8 week-old wildtype lungs at 1 and 2 weeks post-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 which wraps around the nuclear Id1 staining. Arrowheads point to Id1-expressing non-endothelial cells. Magnification: low at 400x and high at 1000x.

relatively much higher level of Id1 compared with the non-endothelial cell population. At 1 week post-bleomycin, we determined that Id1 expression level in the lung endothelial population was upregulated up to 11-fold compared to saline control based on normalized densitometric measurements (Fig. 3.6-A). We also determined that about 86% of total Id1 expression level was derived from the lung endothelial fraction (Fig.3.6-A). In contrast, bleomycin-treated non-endothelial cells displayed only about 3-fold upregulation. This significant contribution in Id1 expression level by the endothelial cell population persisted at 2 weeks post-bleomycin, with approximately 80% of total Id1 expression level derived from endothelial cells (Fig. 3.6-C).

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 (Fig. 3.4-A) (Li et al., 2006). *Shh* is expressed early in the epithelium of the embryonic lung primordium (Bellusci et al., 1997; Bitgood and McMahon, 1995; Litingtung et al., 1998; Urase et al., 1996). 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 (Fig. 3.4-A). By double immunolabeling, we observed that Id1-expressing cells


A
E11.5
E15.5
Adult
Br
Br
V

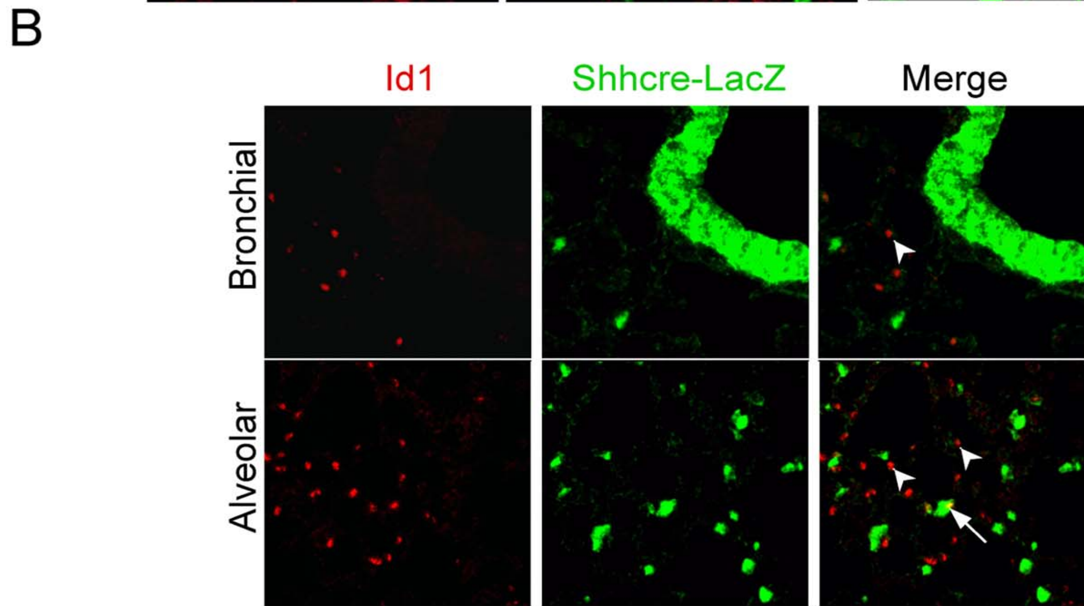

B
Id1
Shhcre-LacZ
Merge
Bronchial
Alveolar

Fig 3.4 Id1 expression is not significantly upregulated in bleomycin-exposed 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. Magnification 200x. ***B.*** Representative photographs showing sections from 8 week-old wildtype *ShhCre-R26R* lungs at 1 week post-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 non-epithelial cells. Magnification: 600x.

showed very little colocalization with LacZ+ epithelial cells both in the bronchial epithelium and in the alveolar bed (Fig. 3.4-B, arrow). Most Id1-expressing cells do not colocalize with epithelial LacZ expression (Fig. 3.4-B, arrowheads). By counting ten non-overlapping fields of Id1 and LacZ double-stained sections, we determined that only about 3% of all Id1-expressing cells were LacZ positive. Similar to the strategy used in Fig. 3.6-A, we utilized *ShhCre-ZEG* reporter mice to selectively identify GFP-positive epithelial cells in the lung by FACS analysis. The enrichment of epithelial cells was confirmed by Western blotting with an epithelial marker, TTF-1 (Nakamura et al., 2002). As shown in Fig. 3.6-B, bleomycin-treated GFP-positive epithelial cells displayed less than 2-fold upregulation of Id1 while the non-epithelial fraction displayed up to 9-fold increase in Id1 expression level. These results indicate that Id1 is not significantly upregulated in epithelial cells of bleomycin-treated lungs, which is consistent with the immunohistochemical data (Fig3.4).

We observed a few fibroblastic-like cells expressing Id1 as has been reported previously in the rat lung (Chambers et al., 2003). However, Id1 expression was not detected in the majority of lung myofibroblasts located in fibrotic foci at two weeks post-bleomycin (Fig. 3.5, arrowheads). We observed expression of Id1 in few smooth muscle actin-positive myofibroblasts (Fig. 3.5, arrow). Collectively, our data suggest that over 80% of total Id1 protein expression level in the lung, at 1 and 2 weeks after bleomycin exposure, is derived from the

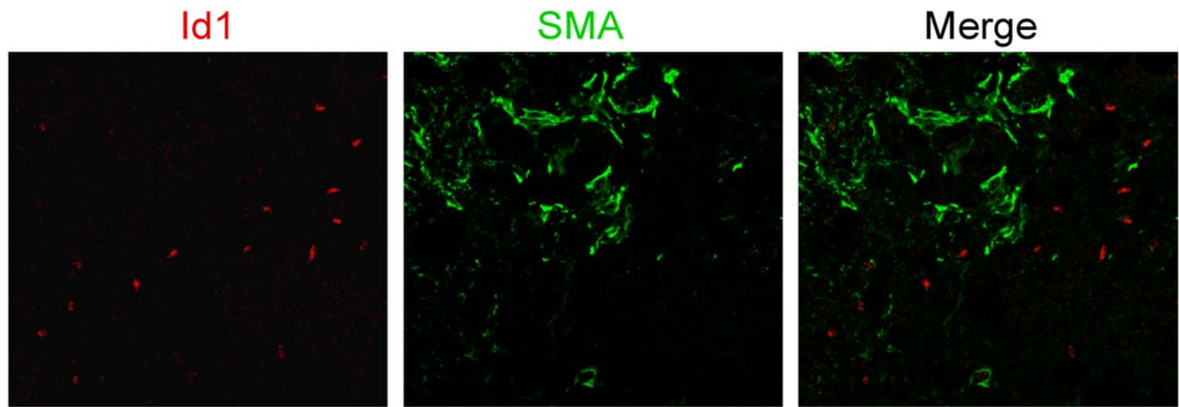
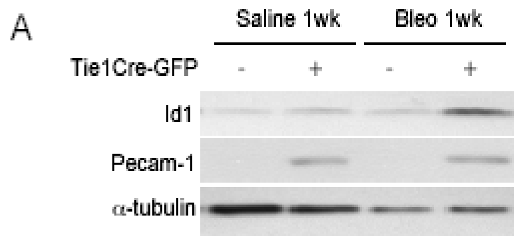
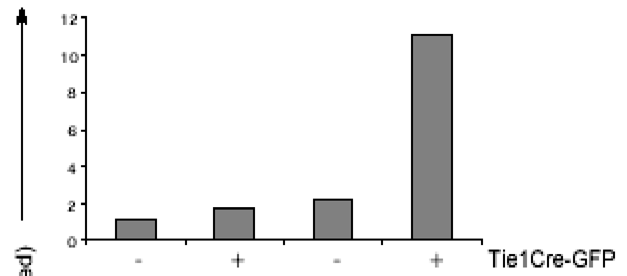
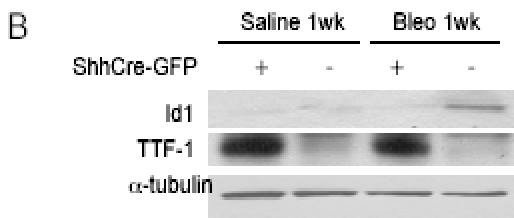


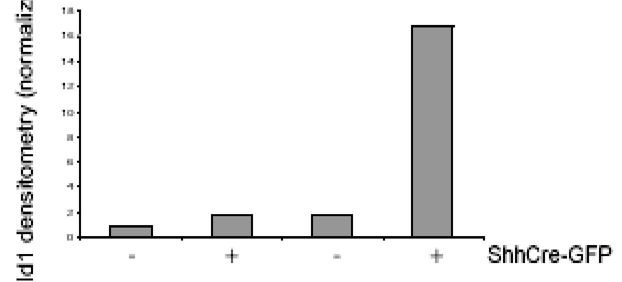
Fig 3.5 Id1 expression is not significantly upregulated in bleomycin-exposed lung fibroblasts

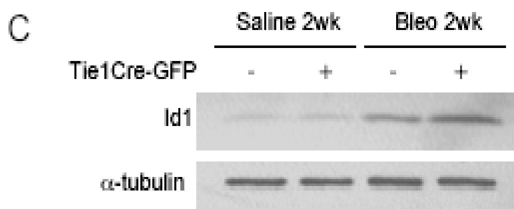
Representative photographs showing sections from 8 week-old wildtype lungs at 2 weeks post-bleomycin. Sections were double-stained with Id1 (red) and myofibroblast marker, Smooth muscle actin (SMA) (green). Arrows in the figures point to Id1-expressing myofibroblast cell. Arrowheads point to Id1-expressing non-myofibroblast cells. Magnification: 400x.


A
Saline 1wk
Bleo 1wk
Tie1Cre-GFP
-
+
-
+
Id1
Pecam-1
α-tubulin


12
10
8
6
4
2
0
-
+
-
+
Tie1Cre-GFP


B
Saline 1wk
Bleo 1wk
ShhCre-GFP
+
-
+
-
Id1
TTF-1
α-tubulin


Id1 densitometry (normalized)
-
+
-
+
ShhCre-GFP


C
Saline 2wk
Bleo 2wk
Tie1Cre-GFP
-
+
-
+
Id1
α-tubulin

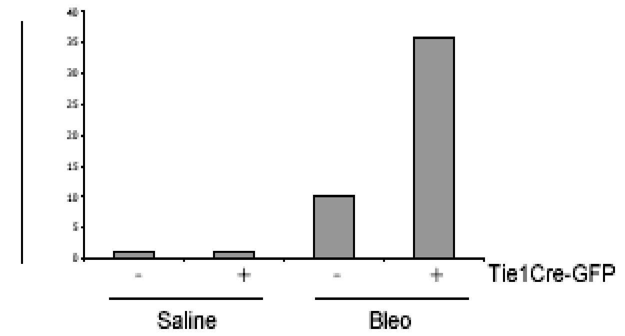

-
+
-
+
Tie1Cre-GFP
Saline
Bleo

Fig 3.6 Id1 expression is upregulated prominently in the injured lung endothelium.

Eight week-old wildtype *Tie1Cre-ZEG* or ShhCre-ZEG lungs were treated with saline or 0.08U bleomycin and collected after 1 week or 2 weeks; lung cells were sorted into GFP+ and GFP- group by FACS. Protein extracts were immunoblotted with Id1 and α-tubulin as the loading control. Purity of the fractions was confirmed by immunoblotting with endothelial marker Pecam-1 or epithelial marker TTF-1. Densitometric measurements of Id1 bands were performed and normalized to the density of α-tubulin bands and values indicating differential levels of expression are shown as columns on the right. Note the dramatic upregulation of Id1 protein level in endothelial cells from bleomycin-treated lungs compared with the non-endothelial fraction and saline controls.

endothelial cell population and less than 20% is derived from other lung cell types, possibly immune cells which are involved in lung injury by bleomycin and have been shown to express Id1 (Leeanansaksiri et al., 2005). Our finding indicates that epithelial and fibroblastic cells contribute minimally to the Id1 expression level in bleomycin-injured lungs. In sum, these findings point to potential roles of Id1 in different lung cell populations, in particular, the endothelial cells, upon bleomycin-induced injury.

Id1 is required for survival of cells of the lung endothelium exposed to bleomycin

Since 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 Methods). Accumulation of albumin-dye complexes within the lung parenchyma was quantified spectrophotometrically and used as an indicator of vascular macromolecular leakage (Londhe et al., 2005). We found that wildtype lungs showed significant leakage of Evans Blue at 1 week post-bleomycin when compared to saline-treated lungs (Fig. 3.7). However, vascular leakage is more pronounced in *Id1-/-* mutants, showing a 58% increase

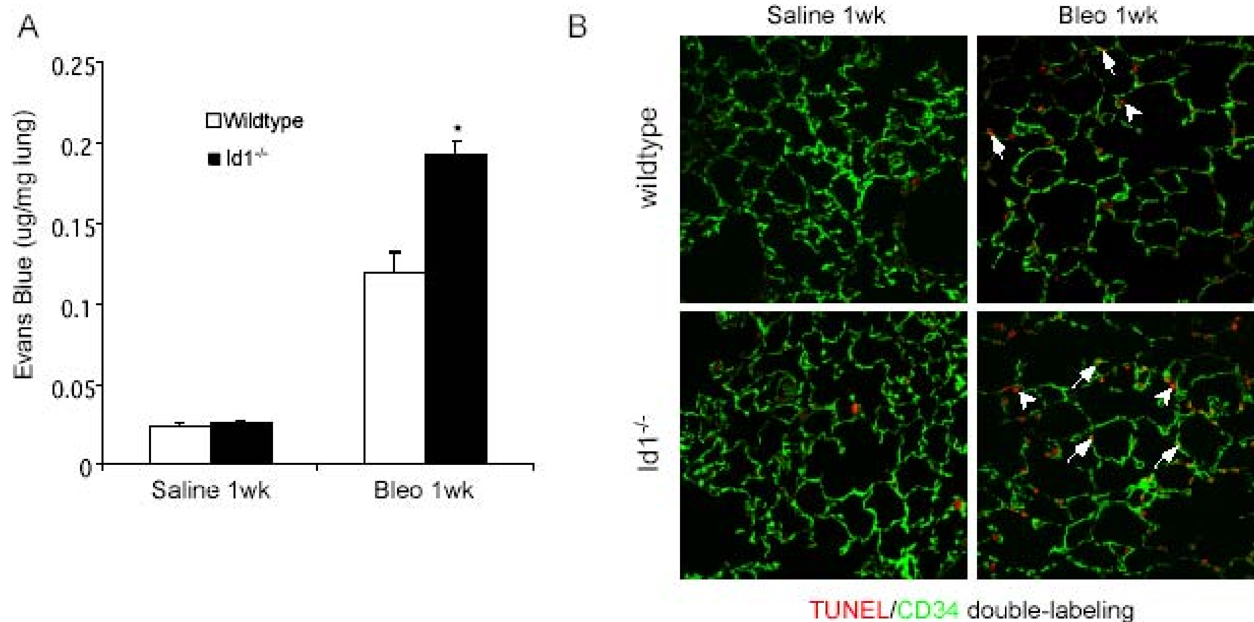


Fig 3.7 Bleomycin-injured *Id1-/-* lungs display increased endothelial barrier dysfunction, elevated endothelial apoptosis. ***A.*** Increased vascular permeability in bleomycin-challenged *Id1-/-* lungs. Pulmonary vascular permeability of saline or bleomycin-treated 8 week-old wildtype or *Id1-/-* lungs was determined by Evans blue extravasation assay (n=3 for each group). Note 58% increase in Evans Blue dye leakage in injured *Id1-/-* lungs compared with wildtype lungs. Asterisks denote a significant difference ($p<0.05$) between wildtype and *Id1-/-* lungs at 1 week post-bleomycin. ***B.*** Endothelial cell death detection in wildtype and *Id1-/-* lungs by TUNEL (red) and CD34 (green) double-labeling. Representative sections are shown for lungs at 1 week post-bleomycin or saline. Magnification 400x.

in dye content when compared to bleomycin-treated wildtype lungs (N=3, Fig. 3.7-A), 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 (Nishiyama et al., 2005). Therefore, it is conceivable that increase in endothelial permeability in *Id1-/-* lungs is, at least in part, contributed by increase in endothelial cell death. To determine the level of endothelial cell death in *Id1-/-* and wildtype lungs at 1 week after bleomycin instillation, we first performed TUNEL and CD34 double immunolabeling. We found that there was significantly higher number of apoptotic endothelial cells in *Id1-/-* compared with wildtype (Fig. 3.7-B). For quantitative analysis, we performed FACS to measure the percentage increase in apoptotic endothelial cells that are double positive for *Tie1*-GFP (endothelial) and annexin-5 (apoptotic) in *Id1-/-* lungs compared with wildtype. In bleomycin-treated lungs, FACS analysis indicated that wildtype and *Id1-/-* contained, respectively, an average of 11.7% and 16.1% apoptotic endothelial cells that were double positive for GFP and annexin-5 (Fig. 3.8). Therefore, there was an average increase of 37.6% in apoptotic endothelial cells in *Id1-/-* relative to the wildtype level (N=3). Saline-treated lungs showed relatively low level of apoptotic endothelial cells and were not significantly different in both genotypes (Fig. 3.8). This result clearly indicates a significant rise in apoptotic

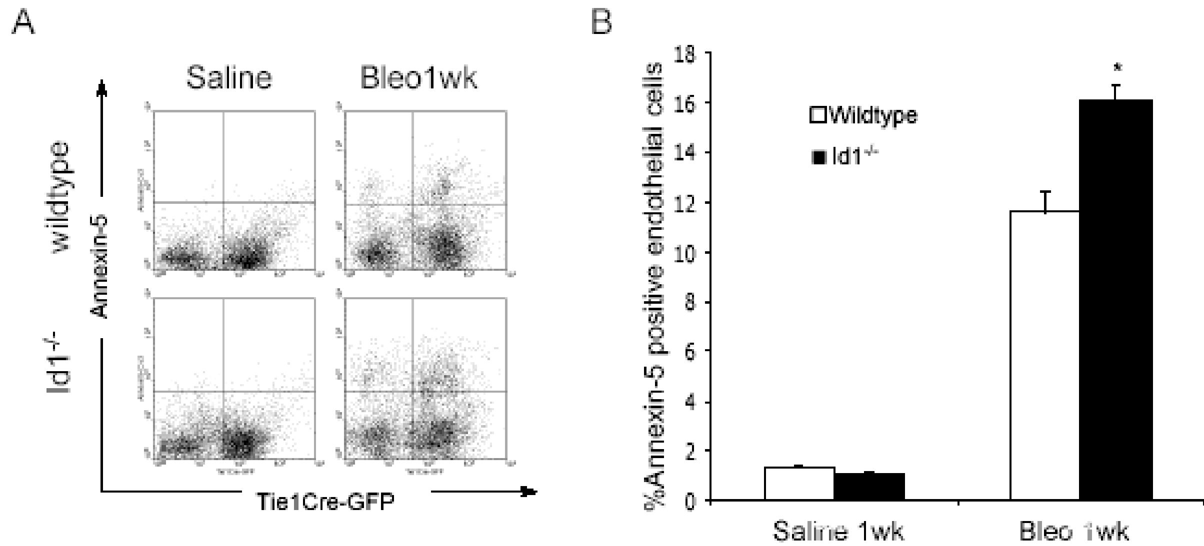


Fig 3.8 Bleomycin-injured *Id1-/-* lungs display increased endothelial apoptosis.

Flow cytometric counting of apoptotic endothelial cells in wildtype or *Id1-/- Tie1Cre;ZEG* lungs at 1 week post-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 upper right quadrant represents apoptotic endothelial cells. **B.** Statistic representation of the sorting results from 3 independent experiments (N=6). Note there is an average of 37.6% increase in endothelial cell death in injured *Id1-/-* lungs compared with wildtype lungs. Asterisks denote a significant difference ($p<0.05$) between wildtype and *Id1-/-* lungs.

endothelial cells in *Id1-/-* mutant mice after bleomycin treatment, suggesting an important role of Id1 in preventing endothelial cell death upon injury.

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 apoptotic cell death which leads to severe endothelial damage.

***Id1-/-* lung microvascular endothelial cells display increased cell death in the presence of bleomycin in culture**

To further examine the role of Id1 in endothelial cell function and cell death induced by bleomycin, we carried out cell culture experiments using freshly isolated lung microvascular endothelial cells (LMVECs) from both wildtype and *Id1-/-* lungs. Cultured wildtype LMVECs expressed a moderate level of Id1 and this expression was further increased after bleomycin challenge (Fig. 3.9-A). We also observed that while freshly dissociated wildtype LMVECs could be passaged three times in culture, it was not possible to passage *Id1-/-* LMVECs after first plating which is likely due to their reduced growth potential. Wildtype and *Id1-/-* LMVEC cultures grown for 3 days were treated with 250ng/ml bleomycin for 6 hours and analyzed by TUNEL or double-labeled with *Tie1Cre*-GFP (endothelial reporter) and annexin5-Cy3 (apoptotic marker) and sorted by FACS analysis to quantify the level of apoptotic endothelial cells in culture (N=3). Consistent with

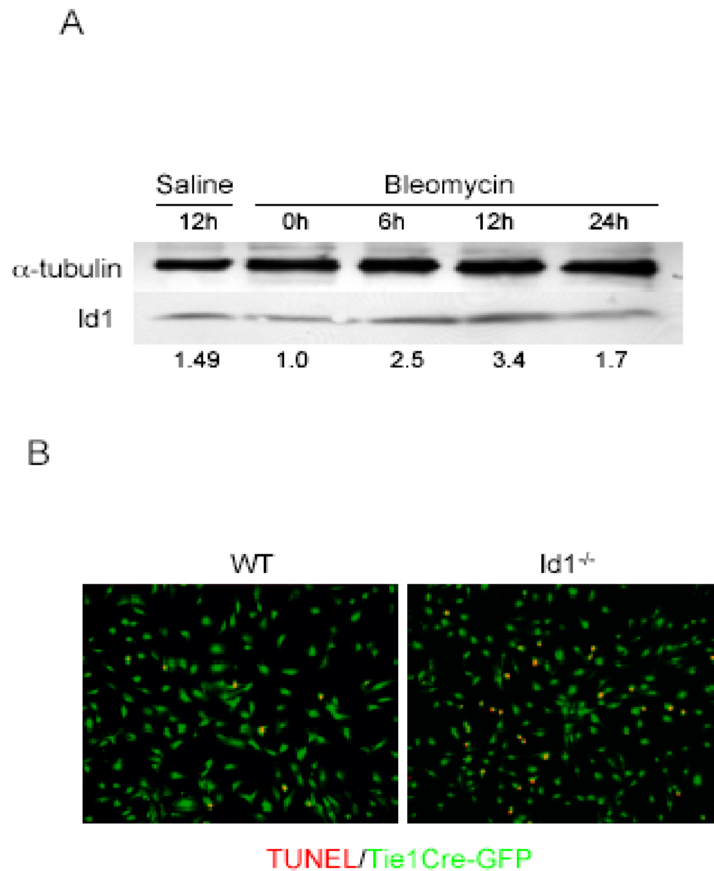


Fig 3.9 *Id1-/-* lung microvascular endothelial cells display reduced survival in culture

A. Id1 is upregulated in endothelial cells by bleomycin in vitro. Western blotting showing Id1 level from wildtype primary LMVECs treated with saline or 250ng/ml bleomycin for the indicated time points. Values represent relative fold change of Id1 protein level normalized to the density of a-tubulin bands. ***B.*** Cell death detection of wildtype and *Id1-/-* primary LMVECs by TUNEL (red) and *Tie1Cre*-GFP (green) double labeling. Representative photographs are shown for cell cultures treated with 250ng/ml bleomycin for 6 hours followed by no bleomycin for 3 hours. Magnification 100x.

TUNEL staining which showed significantly more apoptotic cells in *Id1-/-* LMVECs (Fig. 3.9-B), *Id1-/-* LMVECs displayed an average of 17.3% apoptotic endothelial cells compared with 8.2% in wildtype (Fig. 3.10). 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 decrease in the 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 (Ling et al., 2003; Cheung et al., 2004; Hui et al., 2006). Since 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 expression of Bcl-2 and Bcl-xL in bleomycin-exposed wildtype and *Id1-/-* LMVECs by Western blotting. Although we did not detect significant difference in Bcl-xL level in *Id1-/-* endothelial cell population compared with wildtype, Bcl-2 expression was significantly higher in wildtype LMVECs after bleomycin exposure. (Fig. 3.11) This finding strengthens the notion that Id1 can inhibit cell apoptosis by modulating Bcl-2 expression. Previous studies suggest that upregulation of Bcl-2 by Id1 may indirectly go through Ras/MEK/ERK pathway (Chang et al., 2003; Rivo et al., 2007). By Western blotting of phosphor-MEK and phosphor-ERK, we found that the activity of Ras/MEK/ERK pathway was also decreased, in concomitant with the reduction of Bcl-2 protein level. Taken together, our results

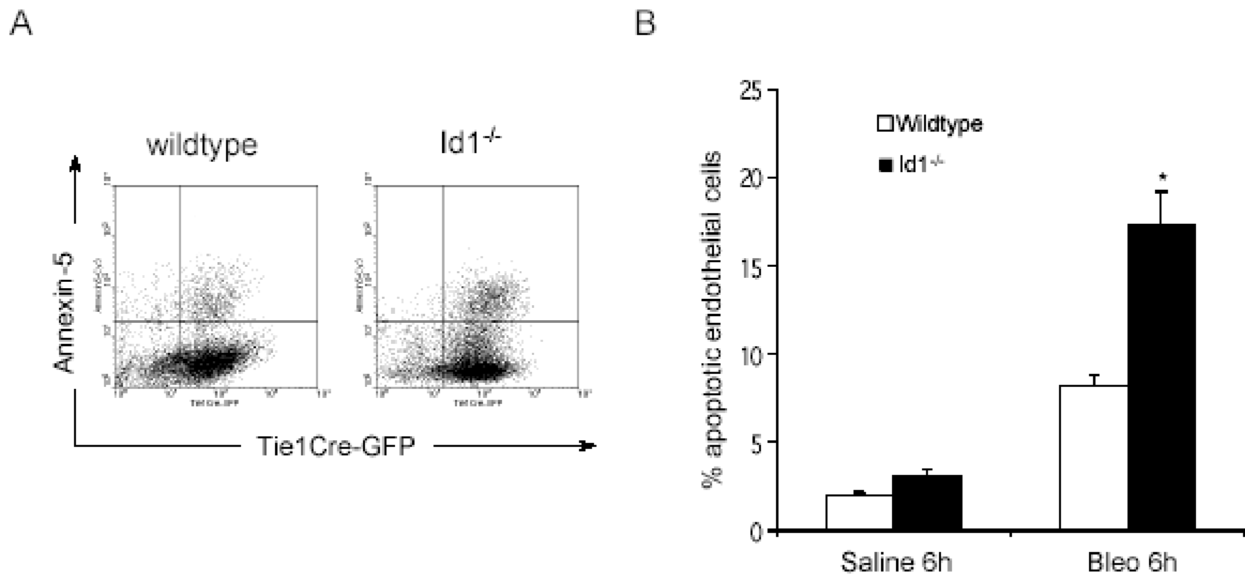


Fig 3.10 *Id1-/-* lung microvascular endothelial cells display reduced survival in culture.

Flow cytometric counting of apoptotic endothelial cells in wildtype or *Id1-/- Tie1Cre-ZEG* LMVECs treated with saline or 250ng/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 upper right quadrant represents apoptotic endothelial cells. **B.** Statistic representation of the sorting results from 3 independent experiments (N=3). Note that there is nearly 2-fold increase in endothelial cell death in *Id1-/-* LMVECs compared with wildtype. Asterisks denote a significant difference ($p<0.05$) between wildtype and *Id1-/-* cells.

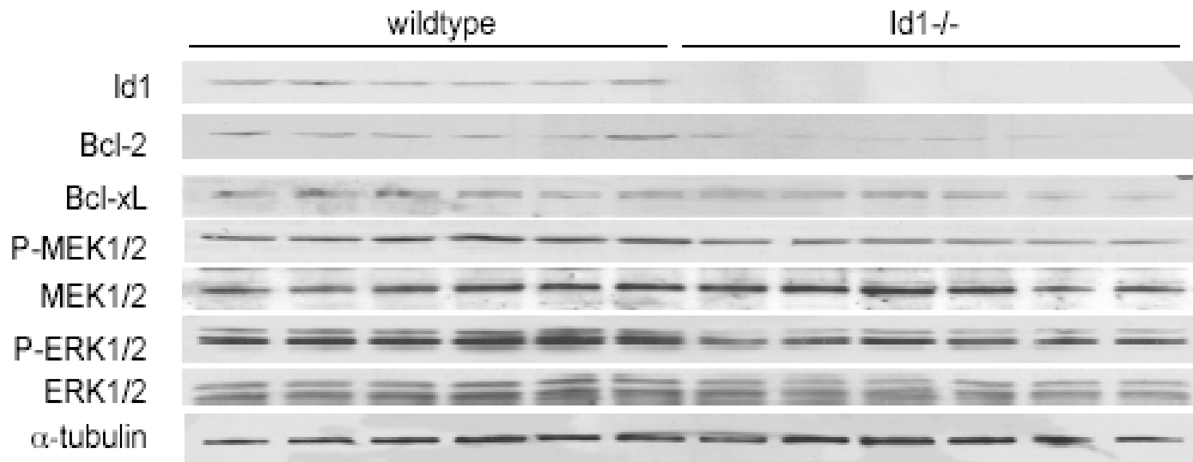


Fig 3.11 ***Id1-/-*** **lung microvascular endothelial cells show reduced Bcl-2 level and MEK/ERK activity.**

Western blotting of cell lysates collected from wildtype or *Id1-/-* primary LMVECs (N=6 lungs for each genotype) treated with 250ng/ml bleomycin for 6 hours. Note that Bcl-2 level is significantly decreased in LMVECs in the absence of Id1 function as opposed to the level of Bcl-xl, which remains unaltered. The MEK/ERK signaling pathway is also affected in *Id1-/-* endothelial cells, based on lower levels of phosphorylated MEK/ERK.

indicate that upregulation of Id1 expression appears to inhibit lung endothelial cell death by activating Ras/MEK/ERK pathway and in turn inducing anti-apoptotic protein Bcl-2 expression.

Discussion

In this study, we demonstrated that although Id1 was barely detectable in the normal adult lung, its protein expression was significantly upregulated predominantly in cells of the lung endothelium in response to bleomycin exposure. This new finding prompted us to investigate the contributory role of Id1 in bleomycin-injured lung endothelium. To this end, in addition to morphological and immunohistochemical approaches, we performed FACS analysis, which is a more rigorous and quantitative method, to specifically isolate endothelial or epithelial lung cell population from whole lungs by taking advantage of available transgenic reporter mouse lines.

Bleomycin-induced lung injury and fibrogenesis, like many other types of lung injury, is almost always accompanied by 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 wildtype. In addition, cell culture experiments using freshly dissociated lung microvascular endothelial cells from *Id1-/-* lungs also showed significantly reduced survival potential compared with wildtype lung endothelial cells. Taken together, these observations revealed that one critical role of Id1 is to maintain

pulmonary endothelial integrity by preventing endothelial cell death upon insult.

Since Id1 function has been linked to cell proliferation, we also examined the change in endothelial proliferation in wildtype and *Id1-/-*lungs after bleomycin treatment. Results from double labeling with PCNA and CD34, to detect proliferating endothelial cells, revealed that the lung endothelium is relatively quiescent and did not appear to become highly proliferative upon bleomycin treatment. In addition, we did not find significant differences in endothelial proliferation in *Id1-/-* compared with wildtype at 1 week after bleomycin (N=3), suggesting that Id1 is unlikely to play a major role in endothelial cell proliferation after lung injury (data not shown).

FACS and double immunohistochemical labeling demonstrated that upregulation of Id1 expression was infrequently detected in epithelial and fibroblast cells, in contrast with Id1 upregulation in cells of the lung endothelium exposed to bleomycin. We determined that less than 20% of Id1 expression in bleomycin-exposed lungs was derived from non-endothelial cells; the identity of this lung cell population and the role of Id1 in that population remain to be determined. Id1 was shown to be moderately expressed in a subgroup of immune cells such as the macrophages and granulocytes (Leeanansaksiri et al., 2005). Therefore, Id1 may also be involved in the inflammatory response of lungs upon injury, which in turn can contribute to fibrogenesis. Therefore, it remains possible that loss of Id1 function in different lung cell populations and circulating cells may

also contribute to increased lung fibrogenesis induced by bleomycin in *Id1-/-* mutant lungs.

Bmp signaling has been strongly implicated in Id gene induction in a number of cell types including endothelial cells (Hollnagel et al., 1999; Itoh et al., 2004; Valdimarsdottir et al., 2002) and vascular endothelial growth factor (VEGF) has also been shown to induce Id1 and Id3 in bone marrow-derived endothelial precursor and hematopoietic cells (Lyden et al., 2001). Both signaling pathways are promising candidates for activating Id1 expression in the lung endothelium upon bleomycin injury, although the actual *in vivo* signals remain to be identified. Signalings mediated by TGF-β1 and TNF-α are known to play key roles in many types of lung diseases including bleomycin-induced pneumopathy in animal models (Bartram and Speer, 2004; Cutroneo and Phan, 2003; Nakao et al., 1999; Pittet et al., 2001; Santana et al., 1995; Wang et al., 2002; Zhao et al., 2002). Interestingly, TGF-β1 can exert long-term repression of Id1 expression in a number of cell types (Chambers et al., 2003; Kang et al., 2003; Kowanetz et al., 2004) , suggesting that TGF-β could potentially antagonize Id1 expression in the course of lung pathogenesis. TNF-α has also been implicated in the regulation of Id expression in astrocytes during inflammatory injury of the central nervous system (Goumans et al., 2002; Kang et al., 2003; Tzeng et al., 1999). 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.

CHAPTER IV

THE INDUCTION AND FUNCTION OF ID1 IN OTHER ACUTE LUNG INJURY MODELS

Introduction

Our finding that Id1 plays a critical role in endothelial survival in the mouse endotracheal bleomycin injection model also implicates a key role of Id1 and the lung endothelium in the pathogenesis of acute lung injury (ALI). In order to obtain a better understanding of the general involvement of Id1 in ALI, we extended the study of Id1 function in endothelial damage using other ALI models. Cecal ligation & puncture (CLP) and intravenous injection of bleomycin are both broadly used methods to induce acute lung injury. By generating inflammation or cell death in blood vessels, these methods of introducing insults directly target the endothelium (Azuma et al., 2000; Ebong et al., 1999; Guo et al., 2002; Laudes et al., 2004). These alternative approaches could circumvent the possible side effects of injured lung epithelium on endothelial injury as is the case with the endotracheal bleomycin model. Intratracheal injection of lipopolysaccharide (LPS) is another commonly used method to investigate the effect of a localized insult in the generation of ALI (Thorn, 2001). By examining Id1 expression and analyzing its function in several ALI models, we were able to complement our previous

observations and thus provide strong support that Id1 is upregulated upon insult to the lung endothelium and may serve to promote lung endothelial survival in ALI.

Experimental Procedures

Mouse models and treatment

Wildtype mice used in this study were bred in the C57BL/6J background. For tail-vein injection of bleomycin, the 6 week-old mouse was placed in a 50 ml conical tube with its tail exposed through an opening in the cap. The dorsal tail vein was visualized by brief warming at 42°C using a heating lamp. Saline or bleomycin (2.5U per animal) was then administrated by intravenous injection into the tail vein using a total volume of 300ul solution (Lawson et al., 2005b). For intratracheal injection of LPS, mice were treated with either saline or LPS (50ug per animal) injected into the surgically-exposed trachea using a total volume of 50ul saline (Lawson et al., 2005b). Cecal ligation and puncture was performed as described previously (Baker et al., 1983). Briefly, mice were anesthetized and a midline incision was made to expose the cecum and adjoining intestine. The cecum was then tightly ligated at the base and punctured twice with a 19-gauge needle. The incision was closed afterwards and mice were left for the indicated number of hours before sample collection. The experimental protocol was reviewed and approved by the Institutional Animal Care and Utilization Committee at Vanderbilt University.

Immunohistochemistry

Lungs were perfused with phosphate-buffered saline (PBS) and fixed in 4% PFA solution at 4°C for 5 hours. Subsequently, lungs were dehydrated and embedded in paraffin blocks and 5 um sections were collected and processed for immunolabeling. Slides were incubated at 4°C overnight with primary antibody Id1 (1:1500) or MPO (1:300). Detection was performed using polymer-HRP secondary antibodies (Zymed) diluted at 1:2 and HRP detection kit (Labvision) were used for signal visualization. Slides were counterstained with Hematoxylin (Sigma) to highlight nuclei. Regular images were taken using the Olympus BX60F5 microscope.

Pulmonary vascular permeability assay

Vascular permeability was examined using the Evans blue extravasation method as previously described (Londhe et al., 2005). Briefly, Evans blue dye at 20mg/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 620nm and 740nm using Bio-Rad Smartspec3000. Corrected pulmonary Evans blue absorbance at 620nm was calculated as A620nm–(1.426xA740nm + 0.03). Permeability index was generated by dividing the corrected pulmonary Evans blue absorbance by the plasma Evans blue absorbance at 620nm.

Neutrophil Counting

Slides of MPO-stained lung tissue were randomized and evaluated on ten sequential, non-overlapping fields (magnification, 400X) of lung parenchyma for each specimen. Counting of MPO-positive cells was performed blinded to the genotype and treatment groups. Results were presented as the average number of MPO-positive cells (neutrophils) per 400x field.

Results

Id1 expression is significantly upregulated in the lung in different lung injury models

To investigate whether Id1 expression is upregulated upon pulmonary insult caused by different insults, we treated adult wildtype mice using three different methods (Cecal ligation-puncture, Tail-vein injection of bleomycin and intratracheal injection of lipopolysaccharide) that are known to induce acute lung injury. Interestingly, we found significant induction of nuclear Id1 expression in the treated lungs compared with control groups in all three treatment methods (Fig. 4.1). The expression pattern of Id1 appears to be in endothelial-like cells, consistent with what we observed in the intratracheal injection of bleomycin model. This interesting finding suggests that Id1 is generally involved in acute lung injury regardless of the pathological cause.

Loss of Id1 function is associated with increased lung vascular permeability in CLP-induced lung injury

Since we obtained similar expression patterns of Id1 in CLP-treated lungs compared to intratracheal-bleomycin treated lungs, we hypothesized that the upregulated Id1 in the lung in CLP models may also have a cytoprotective role in endothelial cells. To examine the extent of endothelial damage in *Id1-/-* mutant lungs, we evaluated the change in vascular permeability by measuring the extravasation of Evans blue dye, the same method utilized in the intratracheal

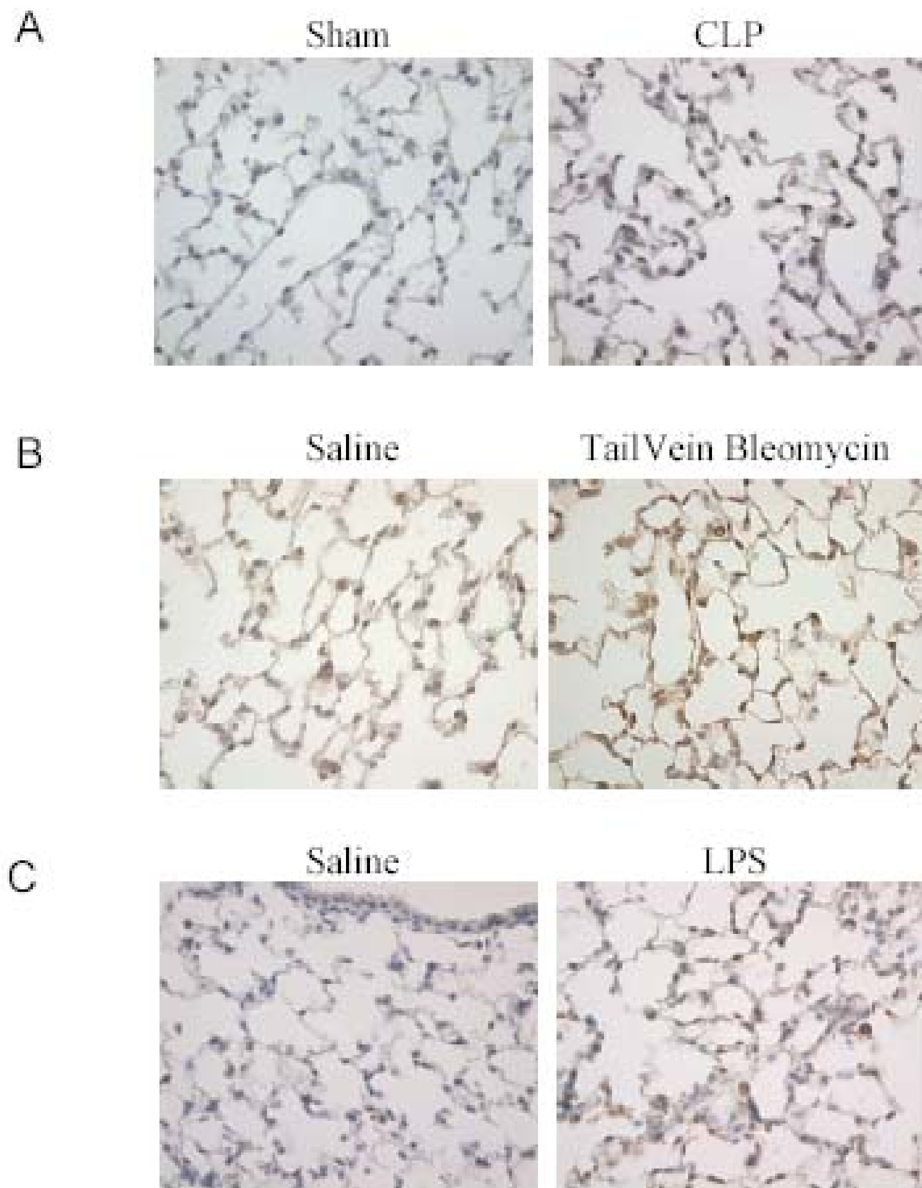


Fig 4.1 Id1 expression is significantly upregulated in the adult lung upon injury of different causes.
Representative photographs showing Id1 immunohistochemistry of lung sections from wildtype lungs with different treatment. Sections were counterstained with hematoxylin to highlight nuclei. Note the nuclear Id1 expression in treated wildtype lungs but not in saline controls. Magnification 400x.

bleomycin model. We found that *Id1-/-* lungs showed significantly more leakage of Evans Blue at 8-hour post-CLP treatment when compared to wildtype lungs (Fig 4.2, N=3), This result indicates that increased disruption of the endothelial barrier may be the consequence of ablated Id function, supporting the notion that Id1 has a cytoprotective role in endothelial cells.

Loss of Id1 function in CLP-treated lungs results in increased neutrophil accumulation

Since the inflammatory response in CLP-challenged mice contributes significantly to increased lung injury, we further analyzed the population of inflammatory cells in *Id1-/-* lung alveolar space and interstitium compared with wildtype at 4 and 8 hours after CLP treatment. We performed immunostaining of myeloperoxidase (MPO) to identify infiltrated neutrophils in the alveolar space. As shown in Fig 4.3-A, we detected significant difference in the inflammatory response between wildtype and *Id1-/-* lungs at 8 hours post-CLP treatment, indicated by increased MPO-positive cells in *Id1-/-* lungs. For semi-quantitative analysis, cell counts were performed on multiple fields and the degree of inflammation was determined as the number of MPO-positive cells per field. As illustrated in Fig 4.3-B, although no difference was observed at the 4-hour time point with the highest level of neutrophil attachment, *Id1-/-* lungs showed significant increase in MPO-positive cells at 8-hour post-CLP treatment compared

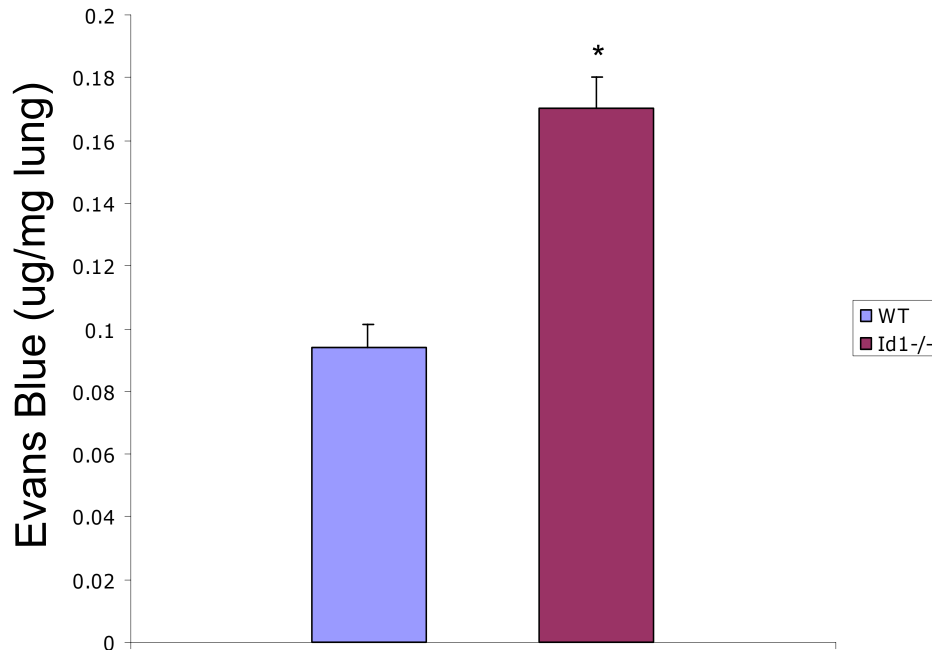


Fig 4.2 *Id1-/-* lungs from CLP-treated mice display increased endothelial barrier dysfunction,.

Evans blue extravasation assay showing increased vascular permeability in injured *Id1-/-* lungs. Pulmonary vascular permeability of 8 hour CLP-treated wildtype or *Id1-/-* lungs was determined by Evans blue extravasation assay (n=3 for each group). Note nearly two-fold increase in Evans Blue dye leakage in injured *Id1-/-* lungs compared with wildtype lungs. Asterisks denote a significant difference ($p<0.05$) between wildtype and *Id1-/-* lungs.

to wildtype lungs. This observation indicates that absence of Id1 function in lung endothelial cells can lead to increased immune cell recruitment.

Discussion

Structural and functional alterations of the pulmonary endothelium are among the characteristic features of ALI pathogenesis (Groeneveld, 2002). The finding that Id1 is upregulated in pulmonary endothelial cells in different lung injury models suggests that Id1 may function as an important component in maintaining adult lung endothelial homeostasis. It appears that when endothelial cells are injured, by DNA-damaging chemicals or cytokines produced during systemic inflammation, Id1 expression is activated. The upstream signals that trigger Id1 upregulation in these different lung injury models, however, remain to be investigated. One might speculate that a common stress-response signal may be activated to induce Id1 when the endothelial barrier is compromised. It also remains possible that different upstream signals may be involved in Id1 upregulation in different types of ALI.

Our finding that CLP-treated *Id1-/-* lungs showed increased vascular permeability compared to wildtype controls suggests that Id1 may play a critical role in maintaining endothelial integrity and function. In addition, immunostaining of neutrophil marker MPO revealed that the degree of inflammation, as demonstrated by increased neutrophil infiltration, is also elevated in CLP-injured

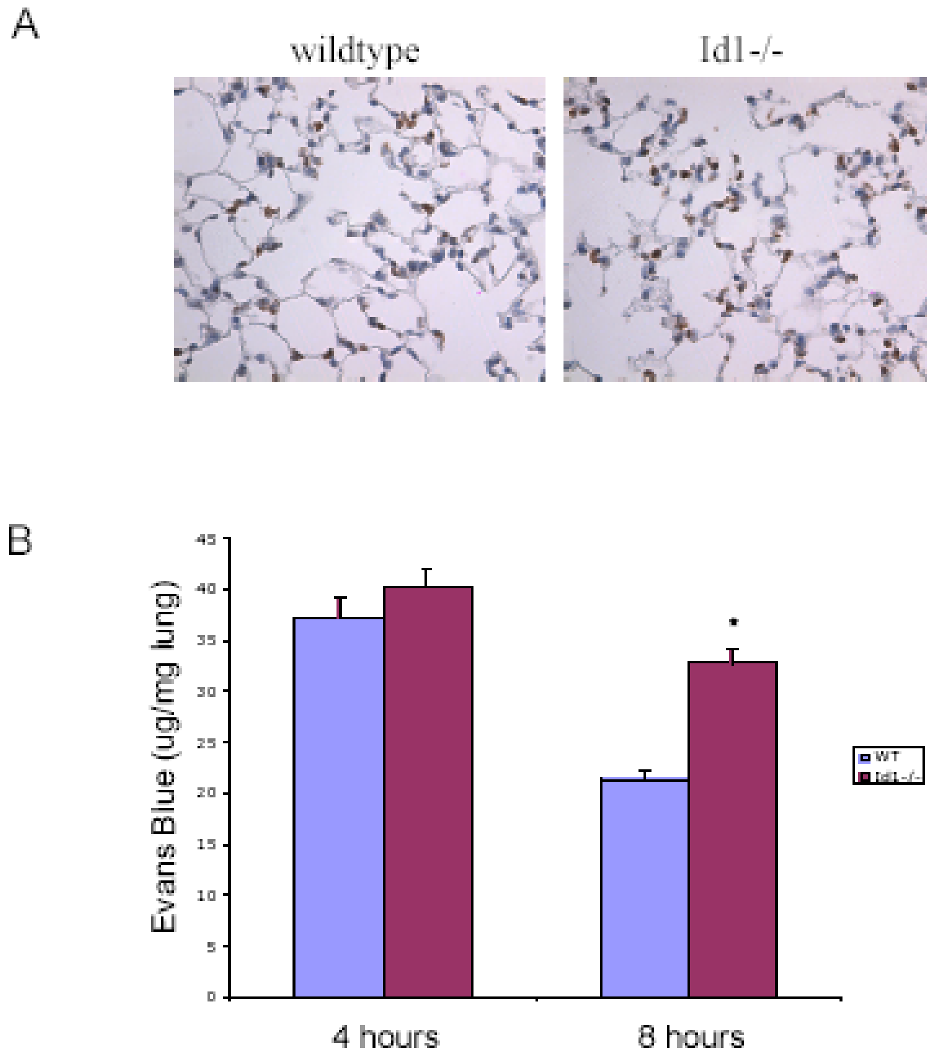


Fig 4.3 Loss of Id1 function in CLP-treated lungs results in increased neutrophil accumulation/inflammation.
A. Representative photographs showing MMP2 immunohistochemistry of lung sections from wildtype and Id1-/- lungs with 19G CLP treatment for 8 hours. Sections were counterstained with hematoxylin to highlight nuclei. Magnification 400x. **B.** Neutrophil counting of wildtype and Id1-/- sections from lungs with 19G CLP treatment for indicated hours. Results are obtained from 10 non-overlapping 400x fields of each group and shown as the number of MPO-positive cells per field. Asterisk denote a significant difference ($p<0.05$) between wildtype and *Id1-/-* lungs.

Id1-/- lungs. However, it remains to be seen whether the influence of Id1 protein function in pulmonary inflammation is direct or indirect. Redox (cellular reduction and oxidation state) balance is recognized as a key component in maintaining proper lung microvascular permeability and junctional integrity (Zhao et al., 2001). Following initial exposure to an inflammatory stimulus, injured endothelial cells produce reactive oxygen and nitrogen species (ROS and RNS, respectively) due to increased oxidant burden (Orfanos et al., 2004). Oxidative stress and ROS production mainly contribute to endothelial cell injury and death. Increase in the number of damaged endothelial cells due to oxidant-induced injury in turn can lead to the expression of damage-responsive proteins such as E-selectin and ICAM, which further facilitate recruitment of immune cells like neutrophils (Albelda et al., 1994). We speculate that the role of Id1 in protecting endothelial cells against excessive apoptosis and endothelial damage could alleviate the signaling cascade that ultimately leads to an increased inflammatory response. It would be interesting to examine the requirement of Id1 in injury-response gene expression to elucidate the link between Id1 function and lung inflammation processes.

CHAPTER V

THE FUNCTION OF ID1 DURING CHRONIC PHASE OF LUNG INJURY

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive chronic lung disease of unknown etiology and 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 (Kuhn et al., 1989; Phan, 2002; Phan, 2003; Selman et al., 2001; Tomasek et al., 2002; Zhang et al., 1994). Fibrotic lung diseases can be caused by a multitude of factors resulting in complex heterogeneous pathological conditions, and they contribute to the chronic events after acute lung injury (Matthay and Zimmerman, 2005). Our findings that Id1 expression is induced in adult mouse lung endothelial cells upon bleomycin exposure and that abrogating Id1 function results in increased vascular permeability and endothelial cell death in lung microvessels implicate a cytoprotective role of Id1 in the pulmonary endothelium. In this chapter, we will emphasize the critical role of the lung endothelium, which has been implicated but not rigorously studied, in lung fibrogenesis. In addition, our proposed studies implicate a critical function of Id1 protein in the lung during the fibrotic process.

Experimental Procedures

Mice and bleomycin treatment

Id1 null mice (*Id1-/-*) (gift of Dr. Robert Benezra) and *Tie1-Cre* mice (Gustafsson et al., 2001) were bred in the C57BL/6J background and *ShhCre-ZEG* mice (Li et al., 2006) were bred in the C57BL/6J;129 background. For the Id1 time-course study, C57BL/6J mice (8-10 week old) were purchased from the Jackson Laboratory. Mice were treated with either saline or bleomycin (0.08U) by intratracheal injection in a total volume of 50ul saline (Lawson et al., 2005b). The experimental protocol was reviewed and approved by the Institutional Animal Care and Utilization Committee at Vanderbilt University.

Immunohistochemistry

Lungs were perfused, inflated, excised, and fixed in 4% paraformaldehyde at $4^{o}C$ overnight. Subsequently, lungs were embedded in paraffin blocks and 5 um sections were collected and processed for immunolabeling. Antibody against Smooth muscle alpha-actin (SMA, Sigma Chemical) was used for immunostaining. For regular immunolabeling, slides were antigen-retrieved using citrate buffer (pH6.0) and incubated at $4^{o}C$ overnight with primary antibody. Regular Alexa-conjugated secondary antibodies or HRP-conjugated secondary antibodies (Jackson ImmunoResearch) and HRP detection kit (Labvision) were used for signal visualization.

Western blotting

Left lungs of bleomycin-treated wildtype and *Id1-/-* mice were harvested and homogenized in RIPA lysis buffer at pH7.4. Protein lysates of 100ug each were resolved on SDS-polyacrylamide gels (Bio-Rad). Primary antibody against Smooth muscle alpha-actin (SMA, Sigma Chemical) was used for detection. Equal loading of protein samples was monitored by and normalized to the level of α-tubulin (Calbiochem-EMD Biosciences). Blots were analyzed using QuantityOne software (Bio-Rad).

Histology and pathology scoring

Lungs were perfused, inflated, excised, and fixed in 4% paraformaldehyde at 4°C overnight. Subsequently, lungs were embedded in paraffin blocks and 5 um sections were collected and processed for H&E staining. Slides of lung tissue were randomized and evaluated on ten sequential, non-overlapping fields (magnification, 30X) of lung parenchyma for each specimen. Evaluation of parenchymal distortion on H&E-stained lung sections was done by Dr. Vasiliy Polosukhin, a pathologist in the Department of Allergy, Pulmonary and Critical Care Medicine at Vanderbilt University, 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 (Lawson et al., 2005a).

Hydroxyproline assay and Trichrome staining

The left lungs of wildtype and *Id1-/-* mice, harvested at 2 weeks after bleomycin, were analyzed for hydroxyproline content as previously described (Reddy and Enwemeka, 1996). Briefly, the left lung lobes were weighed and homogenized in distilled water. The samples were mixed well and digested with 2N sodium hydroxide in a total volume of 100ul at 120°C for 20min. After digestion, 900ul of chloramine T (1.27g chloramine T, 20 ml of 50% n-propanol and citrate-acetate buffer in 100ml) was added to each sample, mixed, and left at room temperature for 25 min. Then 1 ml of Ehrlich's solution (15g of 4-dimethylaminobenzaldehyde in 100ml n-propanol and 70% perchloric acid at a volume ratio of 2:1) was added to each sample, mixed and incubated for 20 min at 65°C. Samples were cooled for 10 min 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 (ANOVA) with Microsoft Excel (Microsoft Corporation) and significance accepted at $p<0.05$. Results are presented as mean +/- SEM.

Results

Id1-/- mice display increased lung fibrogenesis during chronic phase of bleomycin-induced lung injury

Since endothelial injury and the resulting increase in vascular permeability are often considered a pathological trigger of pulmonary fibrosis (Brown et al., 1989; Magro et al., 2006; Renzoni et al., 2003; Takabatake et al., 2005), we examined the susceptibility of *Id1-/-* lungs to bleomycin-induced fibrogenesis. First, we performed morphological analysis of *Id1-/-* and wildtype lungs by histological staining with hematoxylin and eosin (H&E) of lung sections harvested at two weeks after bleomycin instillation to assess the alveolar architecture. As indicated in Figure 5.1-A, *Id1-/-* lungs showed more parenchymal distortion and fibrotic foci compared to bleomycin-treated wildtype lungs. For semi-quantitative analysis of lung fibrosis, H&E-stained sections were evaluated by a pathologist (see Methods) blinded to the genotypes and treatment groups. As shown in Figure 5.1B, *Id1-/-* lungs displayed a higher parenchymal distortion, approximately 35% increase, compared with wildtype (N=5).

Id1-/- lungs display more collagen accumulation in bleomycin-induced fibrogenesis

One of the hallmarks of lung fibrosis is the accumulation of collagen that is secreted by the large fibroblast population within foci. By Trichrome staining, which specifically highlights extracellular collagen, we observed significantly more

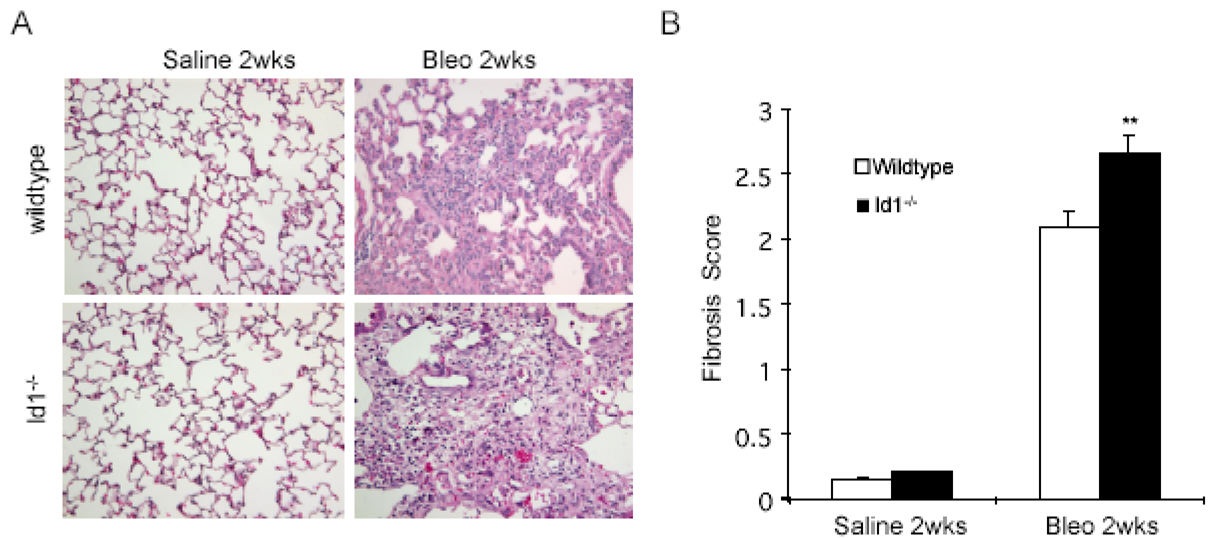


Fig 5.1 *Id1-/-* lungs are more susceptible to bleomycin-induced fibrogenesis.

A. Hematoxylin and eosin staining of lung sections to show architectural changes. Representative photographs showing foci formation in wildtype and *Id1-/-* lungs at 2 weeks post-bleomycin. Magnification 40x. ***B.*** Increase in the index of architectural distortion in *Id1-/-* lungs compared to wildtype lungs after bleomycin challenge. Double asterisks denote a significant difference ($p<0.01$) between wildtype and *Id1-/-* lungs at 2 weeks post-bleomycin ($n=5$ for each group).

collagen accumulation in the fibrotic foci of *Id1-/-* lungs at 2 weeks post-bleomycin compared with wildtype (Fig. 5.2-A, collagen as bright blue). In agreement with the Trichrome staining result, we detected about 90% increase in collagen deposition in *Id1-/-* lungs compared with wildtype, two weeks post-bleomycin as measured by hydroxyproline content assay (N=4, Fig. 5.2-B).

***Id1-/-* lungs display more myofibroblast differentiation in bleomycin-induced fibrogenesis**

Consistent with elevated collagen secretion, *Id1-/-* lungs also showed significant increase in the population of myofibroblasts within developing foci as revealed by Smooth muscle α-actin (SMA) immunolabeling (Fig. 5.3-A) of lungs two weeks post-bleomycin treatment. Accordingly, Western blotting of *Id1-/-* lung homogenates revealed increased levels of SMA compared with wildtype. (Fig. 5.3-B). Taken together, we demonstrate that *Id1-/-* mutant lungs are more susceptible to bleomycin-induced fibrogenesis as evidenced by increased lung parenchymal distortion, elevated collagen and hydroxyproline content and larger myofibroblast cell population. These findings implicate a critical role of the pulmonary endothelium in mediating the fibrotic response upon lung injury and underscore Id1 as a key molecular participant.

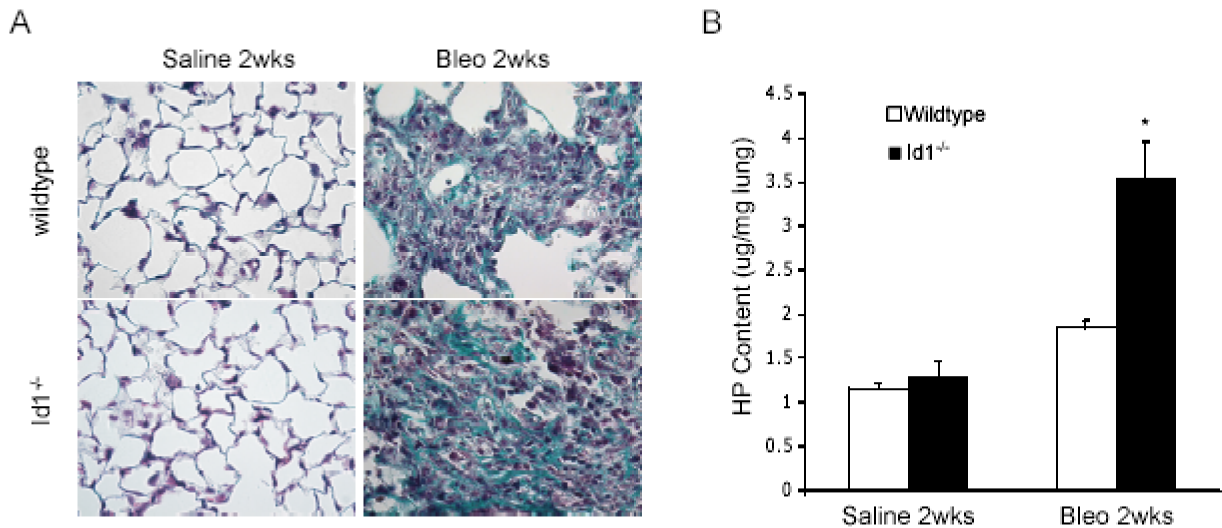


Fig 5.2 *Id1-/-* lungs display more ECM accumulation in bleomycin-induced fibrogenesis.

A. 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 to wildtype at 2 weeks post-bleomycin. Magnification 40x. ***B.*** Quantification of collagen deposition in lungs of wildtype and *Id1-/-* mice after bleomycin challenge by hydroxyproline assay. Asterisks denote a significant difference ($p<0.05$) between wildtype and *Id1-/-* lungs at 2 weeks post-bleomycin (n=4 for each group).

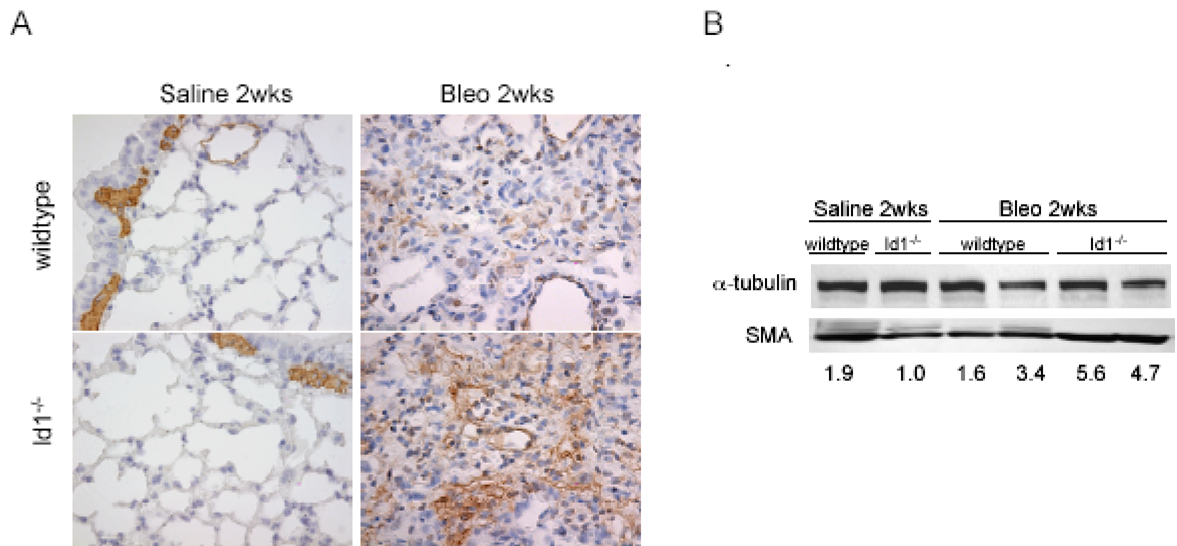


Fig 5.3 *Id1-/-* lungs display more myofibroblast differentiation in bleomycin-induced fibrogenesis.

***A*.** Immunostaining of wildtype and *Id1-/-* lungs with antibodies specific for a-smooth muscle actin (SMA). Representative sections are shown for lungs at 2 weeks post-bleomycin or saline. Sections were counterstained with hematoxylin to highlight all nuclei. Note elevated expression of SMA in fibrotic foci of *Id1-/-* lungs compared with wildtype. Magnification 40x. ***B*.** Western blotting showing expression level of SMA in wildtype and *Id1-/-* lungs at 2 weeks post-bleomycin or saline. Values represent the relative fold change of SMA protein level normalized to the density of α-tubulin bands.

Discussion

Numerous reports using animal models indicate that antioxidants, drugs, biological factors or specific genetic alterations can provide protection against lung injury suggesting that the pathogenesis of ALI involves complex cellular and molecular mechanisms (Aoki et al., 2005; Brewer et al., 2003; El-Medany et al., 2005; Hamaguchi et al., 2002; Manoury et al., 2005; Murakami et al., 2004; Otsuka et al., 2004; Ozyurt et al., 2004; Serrano-Mollar et al., 2003; Sogut et al., 2004; Zhao et al., 2002; Zuo et al., 2002). In this study, we have uncovered a critical function of Id1 in the lung endothelium upon bleomycin-induced lung injury that results in fibrosis. We show significant upregulation of Id1 protein expression during the first week after exposure to bleomycin and expression is maintained for several weeks. Compared with wildtype, lungs lacking Id1 function displayed increased bleomycin-induced vascular permeability and endothelial cell death and more pronounced lung architectural distortion and fibrosis that are associated with significant increase in fibroblast and myofibroblast populations and collagen deposition.

Together with the results obtained in Chapter 3, our findings support a critical role of Id1 as a protective molecule induced in the lung endothelium upon injury and underscore a key role of the lung endothelium during fibrogenesis. Injury caused by bleomycin results in extensive apoptosis in the endothelium and other lung cell types (Hamada et al., 2005). Pulmonary endothelial cells respond to

tissue damage by upregulating Id1 expression through unknown stress response signals. I found that upregulation of Id1 in endothelial cells activates the MEK/ERK pathway and elevates expression of anti-apoptotic protein Bcl-2 (Fig 3.11). It is reasonable to suggest that increased Bcl-2 protein level can reduce the extent of endothelial apoptosis. Acute lung injury often results in pulmonary fibrosis in the chronic phase, which appears to be attenuated indirectly by Id1 function due to decreased endothelial cell death (Fig5.4). 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 (Brown et al., 1989; Kaplan et al., 1992; Magro et al., 2003; Magro et al., 2006; Peterson et al., 1992; Renzoni et al., 2003; Slosman et al., 1989; Takabatake et al., 2005; Wang et al., 1992). Several other studies have also implicated a role of the microvasculature in lung fibrosis (Azuma et al., 2000; Burdick et al., 2005; Drab et al., 2001; Fichtner et al., 2004; Kasper et al., 1996; Kawanami et al., 1995; Piguet and Vesin, 1994; Ward et al., 1989). Conceivably, increased microvascular damage in *Id1-/-* lungs may lead to increased extravasation of plasma that could promote fibroblast proliferation. In addition, stimulated/injured endothelial cells can also secrete fibrogenic molecules that may affect fibroblast migration and

proliferation (Calabrese et al., 2005). Our findings indicate that Id1 plays a critical role in promoting endothelial survival after bleomycin-induced injury and further suggest a role of the lung endothelium in lung fibrogenesis.

Although upregulation of Id1 protein is predominantly found in lung endothelial cells, the potential roles of Id1 in other cells types contributing to the pathogenesis of lung injury and fibrogenesis need to be further investigated (Fig5.4). For example, a previous report by Chambers et. al., (Chambers et al., 2003) and our study showing Id1 and SMA double-immunolabeling indicate that some Id1-expressing cells display fibroblastic features. With new discoveries of fibroblast-specific molecular markers that label all the fibroblast population within the lung, it will be possible to investigate the role of Id1 in fibroblast proliferation and differentiation during fibrogenesis. In addition, Id1 has been shown to have moderate expression in a sub-group of immune cells such as macrophages and granulocytes (Leeanansaksiri et al., 2005). Therefore, Id1 may also be involved in the inflammatory response of lungs upon injury that results in fibrogenesis.

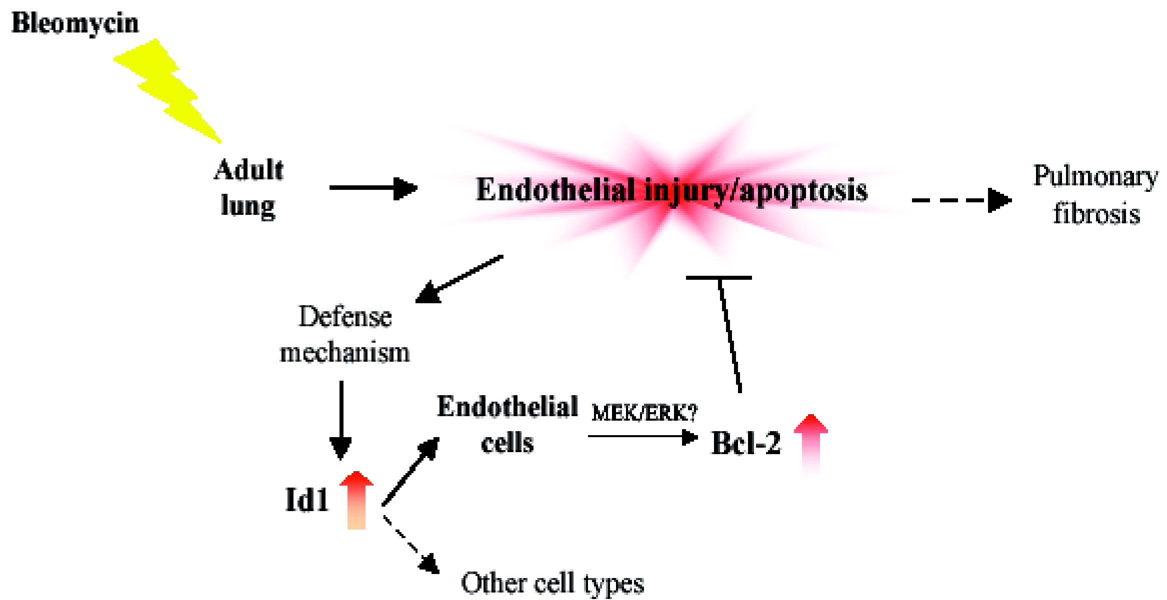


Fig 5.4 Schematic representation of Id1 function in the lung endothelium in acute lung injury.

Injury caused by bleomycin results in extensive endothelial apoptosis. The lung responds to tissue damage by upregulating Id1 expression in endothelial cells (and other cell types). Upregulation of Id1 in endothelial cells activates the MEK/ERK pathway and elevate 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.

CHAPTER VI

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Since the first Id protein was discovered more than a decade ago, the Id family of transcription inhibitors has attracted the attention of investigators from diverse research fields. Numerous studies focused on Id family proteins revealed that their functions encompass an extremely wide range of biological processes including myogenesis (Katagiri et al., 2002), angiogenesis (Benezra et al., 2001), neurogenesis (Lyden et al., 1999), hematopoietic lineage commitment (Benezra et al., 2001; Leeanansaksiri et al., 2005; Norton, 2000), spermatogenesis (Chaudhary and Skinner, 1999; Sablitzky et al., 1998), proliferation (Lasorella et al., 2001; Yokota and Mori, 2002; Zebedee and Hara, 2001) and apoptosis (Wong et al., 2004).

In this project, we investigated the roles of *Id* genes in modulating pulmonary endothelial cell function during lung development and in adult acute lung injury. We uncover the importance of *Id1* gene function in attenuating endothelial cell apoptosis and dysfunction in the adult lung upon insult by intratracheal instillation of bleomycin. We also provide data to support the potential roles of Id1 and Id3 in endothelial morphogenesis during embryonic lung development. We utilized the *Id1* and *Id3* single or double knockout mice as they

are ideal genetic tools for studying the functions of theses genes.

The finding that Id1 expression is significantly upregulated in adult mouse lung endothelial cells upon bleomycin-induced injury and abrogating Id1 function results in increased vascular permeability and endothelial apoptosis, hence contributing to increased lung injury, underscores the importance of Id1 function in maintaining endothelial survival and homeostasis in response to stress. This study also emphasizes the critical role of the lung endothelium in both acute and chronic phases of lung injury. However, Id1 also functions in other cell types such as endothelial progenitor cells and immune cells under conditions of cellular stress (Benezra et al., 2001; Leeanansaksiri et al., 2005; Lyden et al., 2001; Norton, 2000). In this chapter, I will contemplate the other possible functions of Id1 in the pathogenesis of acute lung injury and fibrosis. I will also discuss potential upstream and downstream signaling pathways regulating Id protein expression in the context of development and disease.

The role of Id proteins in lung angiogenesis and endothelial survival

I have carried out several studies to investigate the contribution of Id protein functions in endothelial cell growth, differentiation and injury response. These studies are in agreement with published reports that Id proteins are key regulators of endothelial function. In my study using *Id1-/-Id3-/-* double knockout

embryonic lungs, I showed that Id1 and Id3 may promote angiogenesis and they may be required for maintaining MMP-2 expression in endothelial cells.

Recent studies suggest that Notch signaling may play an essential role in dictating cell fate determination in the lung, particularly during vascular development (Iso et al., 2003a). *Notch-1* and its ligand *Jagged-1* are specifically expressed in endothelial cells of pulmonary vessels, a pattern not seen in other organs (Taichman et al., 2002). Humans with Alagille syndrome (AGS), which is caused by mutation of *Jagged-1*, display major abnormalities in pulmonary arteries (Oda et al., 1997). *Herp3*, a primary effector of Notch signaling, also shows strong expression level in pulmonary endothelial cells (Chin et al., 2000). These findings support the notion that Notch signaling is involved in pulmonary vascular development. Interestingly, the Herp proteins belong to the bHLH family of transcription factors. Thus, I speculate that Id1 and/or Id3 may physically interaction with Herp in regulating Notch signaling activity. Ets transcription factors also comprise a group of interaction partners for Id proteins (Yates et al., 1999). The Ets-domain transcription factor *Net* is highly expressed in the lung mesenchyme during embryogenesis (Ayadi et al., 2001a). Interestingly, mice deficient in Net function exhibit severe vascular defects in the embryonic lung (Ayadi et al., 2001b), suggesting a potential role of Net during lung angiogenesis. Therefore, Net may serve as another potential candidate Id target in the embryonic lung endothelial cells.

Although the relationship between Id proteins and apoptosis in cancer cells has been expansively studied (Wong et al., 2004), little information is available regarding the relationship between Id1 and apoptosis of endothelial cell. In our study, we demonstrated that *Id1-/-* lung endothelial cells, when challenged with bleomycin, display increased apoptosis *in vivo* and *in vitro*. Our results also suggest that Id1 may exert its protective function by maintaining Bcl-2 expression, possibly through activation of Ras/MEK pathway. However, our preliminary observation suggests that other mechanisms might also contribute to the protection against endothelial cell death by Id1. We have found that the transcript and protein levels of p21, a cyclin-dependent kinase (CDK) inhibitor, were higher in bleomycin-exposed *Id1-/-* lung endothelium compared with wildtype. This finding correlates with increased p21 protein level observed in *Id1-/-* LMVECs challenged with bleomycin (Fig. 6.1). This result is consistent with several previous studies implicating the upregulation of p21 in induction of endothelial apoptosis (Kurosawa et al., 2002; Lin et al., 2002; Pendergraft et al., 2004; Yang et al., 1996). Our finding supports a role of p21 as another downstream effector of Id1, which can modulate the transcriptional repression of the *p21* gene promoter containing E-box binding sequences (Prabhu et al., 1997). Although some reports suggest that p21 favors inhibition of apoptosis under certain conditions (Gartel and Tyner, 2002), our findings indicate that repression of p21 by Id1 may be one mechanism by which Id1 suppresses apoptosis. Another possible target of Id1 is

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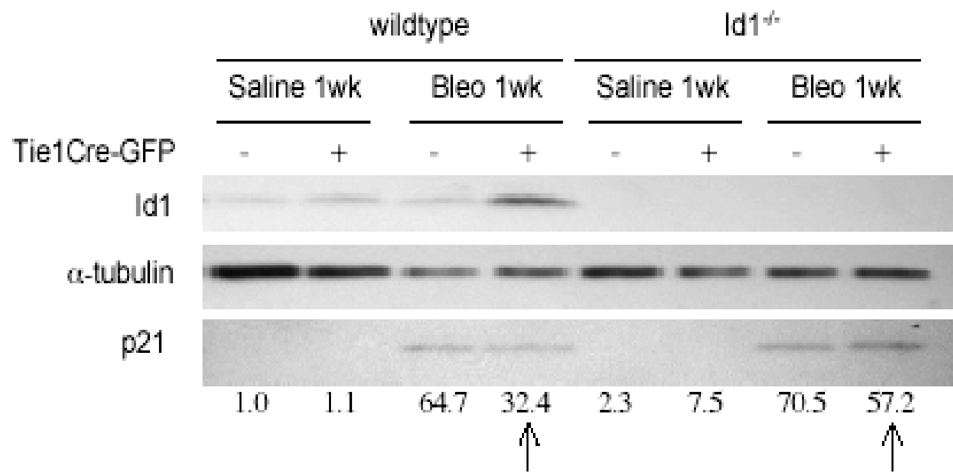

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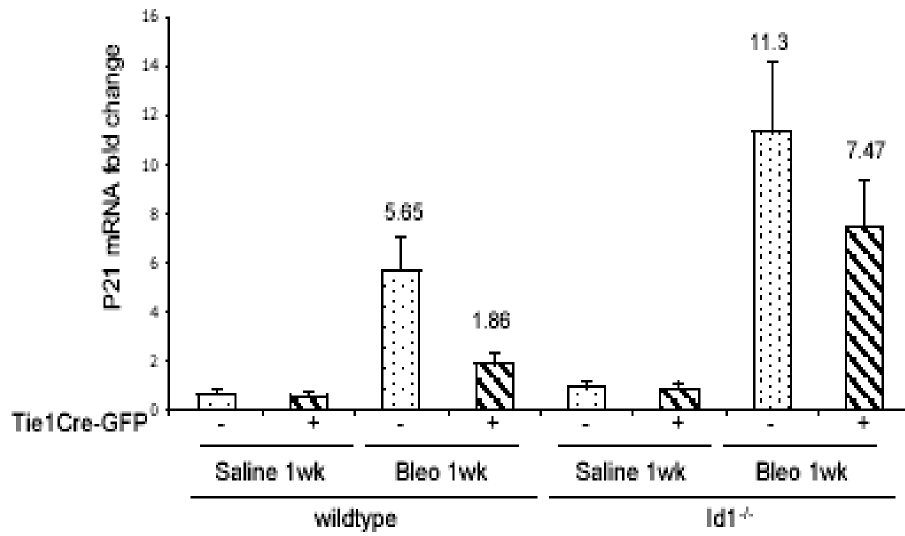

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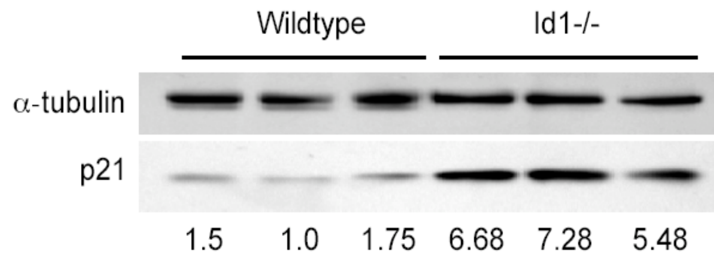

Fig 6.1 Bleomycin-injured *Id1-/-* lungs display increased endothelial barrier dysfunction, elevated endothelial apoptosis and p21$^{CIP/WAF-1}$ level

A. Western blotting of FACS-sorted endothelial (GFP+) and non-endothelial cells (GFP-) from wildtype or *Id1-/-* lungs collected at 1 week post-bleomycin or saline. Values showing relative fold change of p21 protein level normalized to the density of α-tubulin bands. Note that p21 expression was further increased in the endothelial population in the absence of Id1 function (lane 8 versus lane 4, arrows). **B.** Real Time PCR analysis using total RNA extracted from FACS-sorted endothelial (GFP+) and non-endothelial cells (GFP-) of wildtype or *Id1-/-* lungs collected at 1 week post-bleomycin or saline (N=2). Results show the average fold change of p21 mRNA level from two independent experiments. **C.** Western blotting of triplicate cell lysates from wildtype or *Id1-/-* primary LMVECs treated with saline or 250ng/ml bleomycin for 6 hours. Values showing relative fold change in p21 protein level normalized to the density of a-tubulin bands. Note that p21 level is significantly increased in LMVECs in the absence of Id1 function (lanes 4, 5, 6 versus lanes 1, 2, 3).

E2F-1, a basic HLH domain-containing transcription factor that is essential to mediate DNA damage-induced apoptosis (Norbury and Zhivotovsky, 2004). Since a prominent feature of Id1 function is to inhibit the transcriptional activities of basic HLH proteins, Id1 might prevent cell death by binding to E2F-1 or its dimerization partners thus disrupting E2F-1 function.

The functions of Id1 in other lung cell types

Besides the processes of vascular development presented in Chapter 2, Id proteins are also involved in the development of the nervous system and the immune system, as revealed by analysis of *Id1* and *Id3* knockout mice (Lyden et al., 1999). Therefore, in order to expand our knowledge on Id proteins in lung development, efforts should be geared toward investigating the requirement of Id proteins in the generation and/or maintenance of other lung cell types during development. Neural network development in the embryonic lung progresses in parallel with endothelial morphogenesis (Tollet et al., 2001). I found elevated *Id1* and *Id3* expression in the lung mesenchyme where neurogenesis occurs, suggesting that Id functions may be required for the development of the nervous system in the lung, similar to the embryonic brain (Fig 2.1). Examination of neural cell distributions in *Id1-/-Id3-/-* double knockout lungs using different neuronal markers may reveal the importance of Id proteins in neurogenesis during lung development.

The lung endothelium, when subjected to acute injury, undergoes extensive cell apoptosis, as revealed by our studies in Chapter 3. However, little or no proliferative activity was detected in resident lung endothelial cells of the injured lung. Therefore, I reason that in order for the lung tissue to survive the acute damage, which can potentially lead to respiratory failure, the lung endothelium must possess a mechanism to replenish depleted endothelial cells from sources outside of the lung. Recent studies have shown that repair of lung endothelium can be achieved by recruitment of blood-borne endothelial progenitor cells (EPCs) which are unique types of cells with angiogenic properties that home to sites of endothelial injury (Asahara et al., 1999; Burnham et al., 2005; Chinoy et al., 2005; Khakoo and Finkel, 2005; Takahashi et al., 1999; Yamada et al., 2005). The potential role of circulating EPCs in acute lung injury and pulmonary fibrosis has opened up a new paradigm for investigative research in acute lung injury and fibrotic lung diseases. However, the mechanism of EPC cell recruitment remains poorly understood. I contemplate the possibility that increased endothelial injury in *Id1-/-* lung may require enhanced recruitment of circulating EPCs for vascular repair, however, lack of Id1 function in EPCs may also affect the repair mechanism (Lyden et al., 2001). Recently, by using a GFP-marked EPC engraftment procedure in mice followed by treatment with bleomycin, it was demonstrated that EPCs derived from outside of the lung do contribute to pulmonary endothelial repair (Asahara et al., 1999). Using a similar approach to

introduce GFP-marked wildtype or *Id1-/-* EPCs into injured lungs, we may reveal novel functions of Id1 protein in endothelial progenitor cell recruitment during acute lung injury.

Delineating the origins of effector fibroblast populations in the lung will provide important insights into the pathogenesis of lung fibrosis. Currently, two sources for lung fibroblasts have been shown to significantly contribute to lung fibrosis: circulating fibrocytes (Garantziotis et al., 2004) and proliferating resident fibroblasts. Although the lung alveolar epithelial cell can transdifferentiate into fibroblast, its relative contribution to lung fibrosis remains to be determined (Kasai et al., 2005; Willis et al., 2005). As shown in previous studies, endothelial cells have the capacity to differentiate into smooth muscle cells (Arciniegas et al., 1992; Frid et al., 2004; Frid et al., 2002; Ishisaki et al., 2003), suggesting that endothelial cells may be another potential source of lung fibroblast contributing to fibrogenesis. In light of the role of Id1 as an inhibitor of myofibroblast differentiation, I contemplate the possibility that endothelial expression of Id1 upon injury may also help prevent endothelial-to-fibroblast transdifferentiation. Using lung microvascular endothelial cells (LMVEC) isolated from wildtype or *Id1-/-* mice, we can examine the potential of *Id1-/-* endothelial cells to differentiate into α-SMA-positive cells upon TGF-β stimulation. Furthermore, using *Tie1Cre-GFP* transgenic mouse line that faithfully marks cells of endothelial origin (Gustafsson et al., 2001), we can assess the extent of endothelial-to-fibroblast

transdifferention in intact wildtype and *Id1-/-* lungs under pathological conditions and the contribution of Id1 in this process.

Id1 has been shown to be moderately expressed in a subgroup of immune cells such as the macrophages and granulocytes (Leeanansaksiri et al., 2005). An association between loss of Id function and deregulated inflammatory cell differentiation has also been reported (Benezra et al., 2001; Leeanansaksiri et al., 2005; Norton, 2000) Therefore, although our current studies focus on the role of Id1 protein in endothelial cell integrity and survival, the possibility remains that altered inflammatory response in challenged *Id1-/-* mice, can contribute, at least in part, to increased lung injury and fibrosis. In order to address this question, it is possible to analyze the population of inflammatory cells in *Id1-/-* lung alveolar space and interstitium compared with wildtype at 3, 7,14 and 21 days after bleomycin instillation. Differential cell counts in the fraction of eosinophils, neutrophils and macrophage/monocytes will reveal possible contribution of Id1 function in immune response processes during bleomycin-induced injury.

Potential upstream signaling pathways regulating expression of Id proteins

The upstream signaling pathways that regulate *Id* gene expression during lung development and, in particular, in disease progression remain largely unknown. However, considerable work has been focused on the signals controlling expression of the *Id* genes in diverse cell types *in vitro*. Although the

expression of *Id* genes are likely stimulated by various factors, Bone morphogenetic proteins (Bmps) play a major role in regulating Id expression (Miyazono and Miyazawa, 2002). Bmps can induce the expression of *Id1, Id2* and *Id3* in a Smad-dependent manner in several established cell lines (Hollnagel et al., 1999; Miyazono and Miyazawa, 2002; Ogata et al., 1993). Analysis of the Id1 promoter revealed that it contains two Bmp-responsive sites, one of which contains CGCC sequence elements flanked by CAGC motifs and the other one harbors two Smad-binding elements (SBEs). Activation of the *Id1* promoter upon treatment with Bmps demonstrated that Id1 is a direct target of Bmp signaling (Korchynskyi and ten Dijke, 2002). During embryonic lung development, Bmp4, a member of the Bmp protein family that is most extensively studied in lung morphogenesis, is expressed in the distal epithelium at high level and subjacent mesenchyme at lower level (Bellusci et al., 1996). These *Bmp4* signaling domains overlap extensively with *Id1* and *Id3* expression in the mesenchyme and Id2 expression in the epithelium. In addition, our Bmp4-bead implantation experiment also revealed that addition of Bmp4 protein in embryonic lung mesenchyme significantly upregulates *Id1* and *Id3* expression (Fig. 6.2). Taken together, these lines of evidence indicate that the Id proteins are likely under direct regulation by Bmp proteins in the embryonic lung to transduce Bmp-regulated biological effects. Although direct correlation between Bmp signaling and lung injury and fibrogenesis has not been established, related studies suggest that Bmps may be

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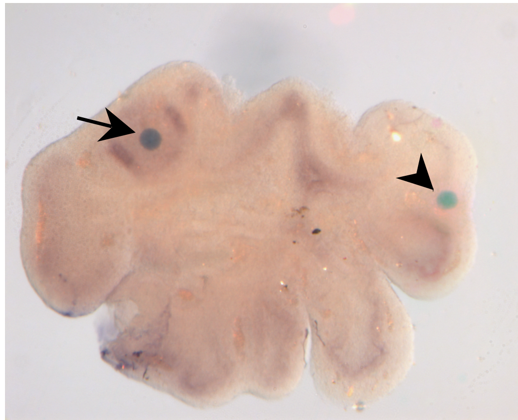

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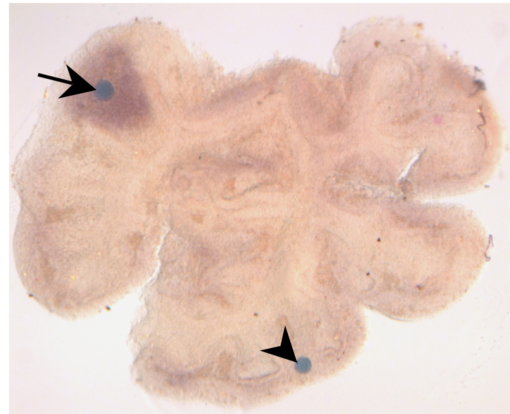

Fig 6.2 Ectopic Bmp4 signaling in distal lung mesenchyme induces *Id1* and *Id3* gene expression.
Human recombinant BMP4 protein soaked bead (arrows) or BSA soaked bead (arrowheads) were implanted into mesenchymal layers at similar locations of E11.5 WT lungs. Lungs were collected 24 hours after culture and analyzed for *Id1* **(A)** and *Id3* **(B)** expression by whole-mount *in situ* hybridization.

actively engaged in the pathogenesis of fibrosing diseases. Bmp7 signaling has been implicated in the attenuation of kidney fibrosis by restoring and maintaining renal epithelial phenotype via down-modulation of epithelial-to-mesenchymal transition induced by TGF-β1 (Morrissey et al., 2002; Zeisberg et al., 2003). Therefore Bmp-mediated signaling may be a critical component pathway involved in lung injury and fibrosis. Vascular endothelial growth factor (VEGF) has also been shown to induce *Id1* and *Id3* in human umbilical vascular endothelial cells (HUVECs) (Sakurai et al., 2004) and hematopoietic cells (Lyden et al., 2001). Since VEGF is renowned for its role in endothelial survival and its involvement in endothelial injury (Fehrenbach et al., 1999; Gupta et al., 1999; Hamada et al., 2005; Koyama et al., 2002; Meyer et al., 2000), VEGF may be another potential *Id1* inducer. Another example is fluvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor with pleiotropic effects on cellular metabolism. Several reports indicate that fluvastatin can act as antioxidant and an effective inhibitor against lipid peroxidation (Suzumura et al., 2001; Suzumura et al., 1999; Yamamoto et al., 1998; Yamamoto et al., 2001). Interestingly, *Id1* has been shown to be upregulated by fluvastatin in human dermal microvascular endothelial cells (Pammer et al., 2004). However, it remains to be determined if fluvastatin can upregulate Id1 in the lung endothelium and since this drug is known for its pleitropic effects, its cell-specific mechanism of action should be carefully examined.

Wildtype mice display endothelial damage and fibrosis, albeit less severe than *Id1-/-* mice, suggesting that increase in Id1 level above baseline in bleomycin-challenged wildtype lungs is not sufficient to provide full protection. This point raises an important issue as to whether there exists a competition or balance *in vivo* between Id1-inducing and Id1-repressing mechanisms upon lung injury. It is possible that the level of Id1 upregulation we observed upon challenge does not reflect maximally induced Id1 levels due to coexisting Id1-repressing signals. While the Id1-inducing and protective mechanism promotes the maintenance of endothelial cell function, antagonistically, the persistence of pro-inflammatory cytokines may downregulate Id1 expression, thus promoting endothelial dysfunction, vascular leakage and fibrosis. Signaling mediated by TGF-β1 and TNF-α are known to play key roles in many types of lung diseases including bleomycin-induced pneumopathy in animal models (Bartram and Speer, 2004; Cutroneo and Phan, 2003; Nakao et al., 1999; Pittet et al., 2001; Santana et al., 1995; Wang et al., 2002; Zhao et al., 2002). TGF-β1 activated Smad2/3 and stress-induced signalings are believed to mediate lung fibrogenesis in bleomycin-treated animals (Phan and Kunkel, 1992; Wang et al., 2002; Zhao et al., 2002). Interestingly, TGF-β1 can exert long-term repression of Id1 expression in a number of cell types including endothelial cells, suggesting that TGF-β can potentially contribute to the downregulation of Id1 expression during lung fibrosis.

TNF-α signaling has also been implicated in the regulation of Id expression

in astrocytes during inflammatory injury of the central nervous system (Goumans et al., 2002; Kang et al., 2003; Tzeng et al., 1999). One of the key downstream components of TNF-α is mediated by p38 MAPK, which has been shown to directly downregulate *Id1* expression in epithelial cells (Kang et al., 2003). Activation of p38 MAPK has been demonstrated in various disease models involving vascular injury and pulmonary fibrosis and p38 MAPK inhibitors can reduce the severity of the disease condition (Ju et al., 2002; Mackay and Mochly-Rosen, 1999; Matsuoka et al., 2002). p38 MAPK activation in endothelial cells of IPF patients was found to occur at an intermediate stage of fibrosis (Yoshida et al., 2002). Activation of p38 MAPK has been reported in human pulmonary microvascular endothelial cells (HMVEC) upon exposure to bleomycin indicating a key role of stress-induced p38 MAPK in lung microvascular endothelium (Fichtner et al., 2004). p38 MAPK can mediate TNF-α-induced microtubule rearrangements and increase in lung vascular permeability and endothelial barrier dysfunction and SB203580, a p38 MAPK inhibitor, can rescue TNF-α-induced cellular changes (Goldblum et al., 1993; Goldblum et al., 1994; Petrache et al., 2003). p38 MAPK has also been implicated in lung endothelial barrier dysfunction by modulating microtubule disassembly (Birukova et al., 2005). These observations indicate a key role of p38 MAPK in lung endothelium in response to stress. Speculatively, one consequence of TNFα signaling and p38 MAPK activation in the course of lung injury is the downregulation of Id1, which

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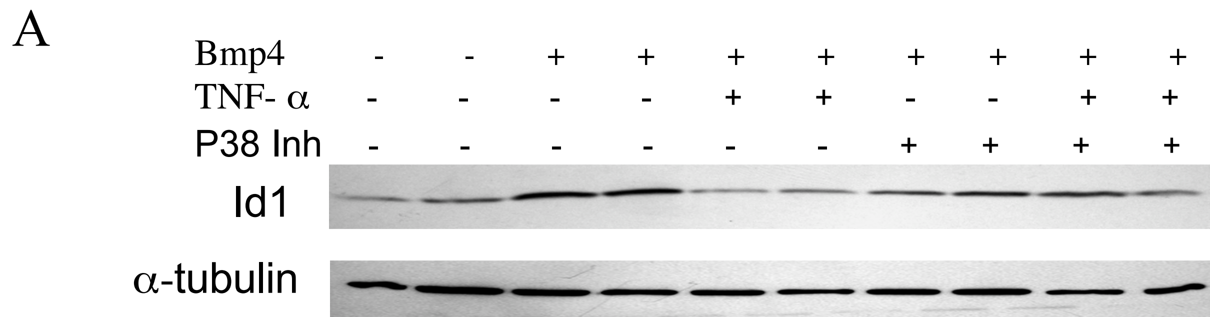

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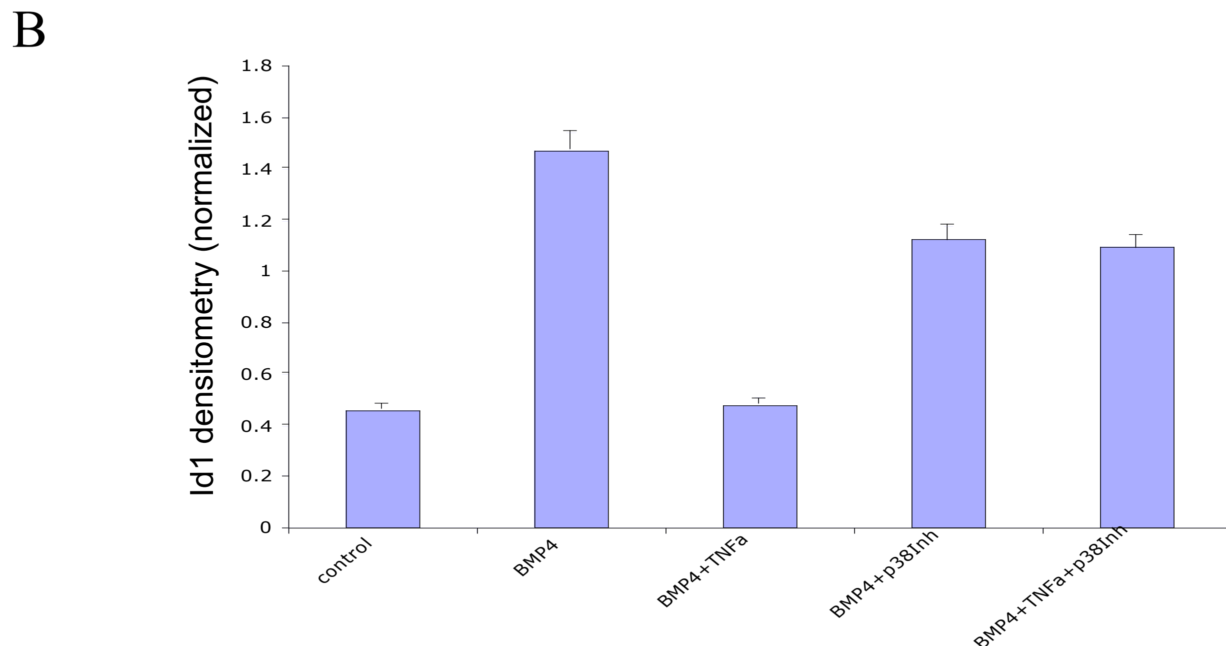

Fig 6.3 TNF-alpha downregulates Id1 in endothelial cells in a p38-MAPK dependent manner.

A. Western blot analysis showed that Bmp4-induced Id1 expression in LMVEC cells is rapidly repressed to baseline by TNF-alpha. This downregulation of Id1 is largely mediated by p38-MAPK, as its specific inhibitor SB203580 can reverse the effect of TNF-alpha. **B.** Densitometric measurements of Id1 bands were performed and normalized to the density of α-tubulin bands.

must be counteracted and counterbalanced by inducers of Id1 expression. My preliminary studies indicating that TNF-α mediated signaling can downregulate Id1 expression in cultured LMVECs in a p38 MAPK-dependent manner (Fig. 6.3) set the stage for investigating the role of p38 MAPK signaling in regulating Id1 expression *in vivo*. Future studies using a combination of biochemical, molecular and genetic approaches will advance our understanding of the regulatory mechanisms underlying Id1 expression and function in the pathogenesis of acute lung injury and fibrosis.

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