TGF-β1 IN FIBROSIS

ROLES FOR TGF-β

IN

PULMONARY DISEASE

By

THOMAS GALT, B.Sc.

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AUTHOR: Thomas Galt, B.Sc. (McMaster University)

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ABSTRACT

Fibrosis is a disease where the normally transitory wound healing response enters a chronic state. Bleomycin and Adenovector models of pulmonary fibrosis have implicated TGF-β1 in this disease. Concern regarding a synergistic combination of TGF-β1 with an adaptive immune response within the Adenovector model prompted its use within mice devoid of T Lymphocytes, Balb/c SCIDs. The lack of an adaptive immune response within these mice did not affect the severity of fibrogenesis, as compared to Balb/c data in a hydroxyproline assay. TGF-β1 is a pluripotent cytokine with key roles in wound healing, immune regulation, and development, making it a dangerous molecule to therapeutically modulate directly. Future strategies will likely focus on downstream fibrotic molecules uninvolved in immune regulation, such as CTGF. While CTGF has been associated with fibrosis and is likely activated by TGF-β1, no conclusive evidence is available within an animal model. TGF-β1 stimulates cells by binding its receptor and signaling through the Smad signal transduction pathway. Smad3 knockout mice were used to examine the regulation of CTGF by TGF-β1, and study its role in pulmonary fibrosis. We show that these mice produce dramatically less CTGF in response to TGF-β1 than littermates expressing Smad3, and they show protection against TGF-β1 induced pulmonary fibrosis, using the Adenovector system. TGF-β1 can alter lung development, and is thought to be a causative agent in Bronchopulmonary Dysplasia, a disease affecting immature lungs. Utilizing the Adenovector system, we developed a neonatal rat model of BPD that closely resembles the human disease, providing researchers with a system to study the disease course. TGF-β1 is part of a family of growth factors, of which TGF-β3 is also a member. What role TGF-β3 plays in pulmonary fibrosis has not been evaluated. To allow future *in vivo* studies on the effect of TGF-β3 on lung morphology, we constructed a replication deficient Adenovector expressing constitutively active TGF-β3.

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INTRODUCTION

Fibrosis is described as a state of altered wound healing, where the factors responsible for controlling inflammation, tissue repair, and scar formation fail to properly regulate these processes [1], leading to the extensive deposition of extracellular matrix, and destruction of tissue integrity within the structure of the lung. Idiopathic Pulmonary Fibrosis (IPF)[1], asbestosis, and hypersensitivity pneumonitis are all pulmonary fibrotic diseases induced by different agents, yet all are initiated by an inflammatory state developed in response to the inducing agent. For reasons that are as yet unknown, the acute inflammatory response, usually able to efficiently clear foreign agents, enters a chronic state [2]. What results is a prolonged, intense inflammatory infiltrate within the lung parenchyma that has been irreversibly altered by the marked deposition of extracellular matrix (ECM; Fig.1). ECM components deposited during fibrosis include types I and III collagen, fibronectin, laminin, elastin, and several proteoglycans [3].

Normal wound healing is a tightly controlled process that begins with an early stage inflammatory infiltrate to clear invading pathological agents. Cytokines characteristic of early stage wound repair include tumor necrosis factor (TNF)-α and granulocyte macrophage-colony stimulating factor (GM-CSF), both key components of the inflammatory cascade.

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[1] List of abbreviations: α-SMA, alpha smooth muscle actin; b-FGF, basic fibroblast growth factor; BPD, bronchopulmonary dysplasia; CTGF, connective tissue growth factor; ECM, extra cellular matrix; GM-CSF, granulocytes macrophage colony stimulating factor; Het, Heterozygote; IPF, idiopathic pulmonary fibrosis; IL-1, interleukin 1; KO, knockout; LAP, latency associated peptide; MMP, matrix metalloproteinases; MCP-1, monocyte chemoattractant protein 1; MAD, mothers against dpp; PDGF, platelet derived growth factor; PAI-1, plasminogen activator inhibitor 1; RDS, respiratory distress syndrome; TSP 1, thrombospondin 1; TIMP, tissue inhibitor of metalloproteinases; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; Wt, wildtype.

GM-CSF is a potent hematopoietic cytokine capable of inducing the migration and activation of granulocytes and monocytes [4] TNF-α is believed to be a prime inducer of transforming growth factor (TGF)-β_1 during wound healing [5], and like Interleukin (IL)-1 may be a growth factor for fibroblasts [6,7] Monocyte chemoattractant protein-1 (MCP-1) is produced during the fibrotic response possibly by eosinophils invading the lung [8] MCP-1 attracts and activates monocytes that can then serve as factories for secretion of activated forms of TGF-β.

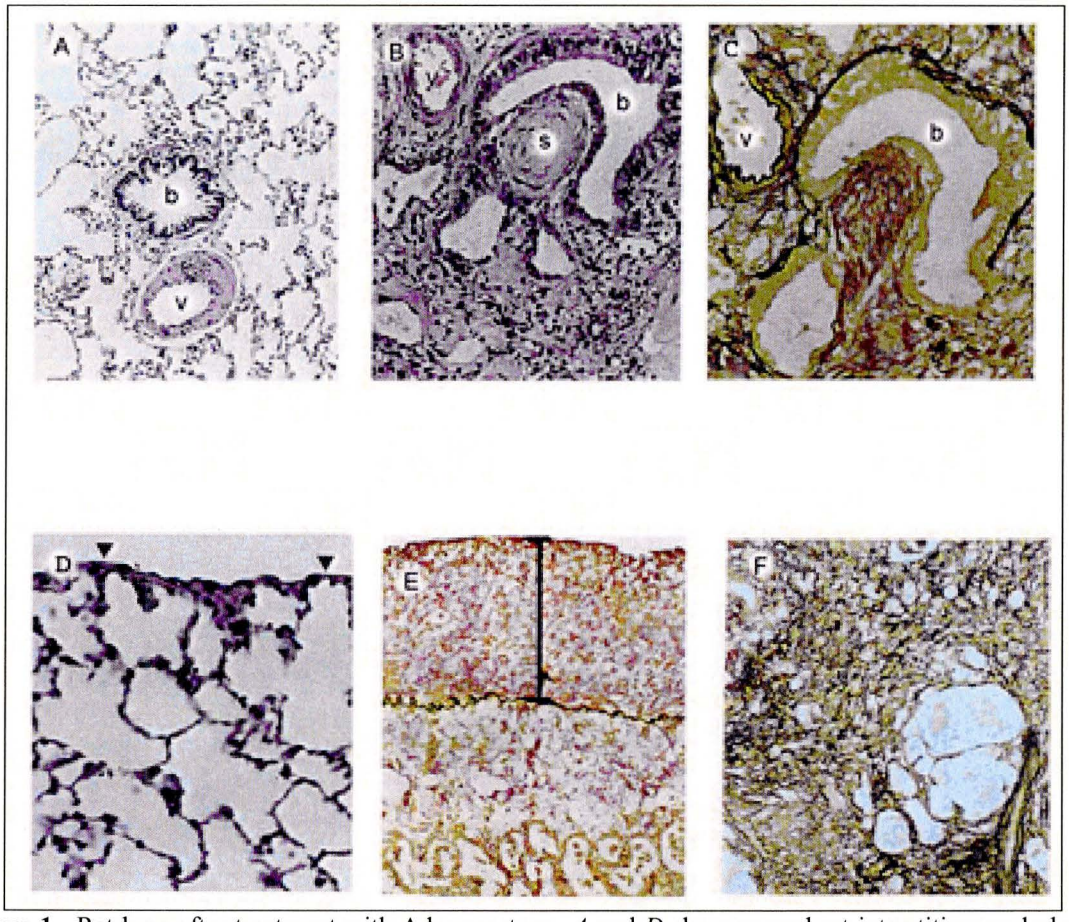

Figure 1. Rat lung after treatment with Adenovectors. *A* and *D* show normal rat interstitium and pleural areas 14 days after treatment with control virus (AdDL70-3), stained with H&E. *B* is a rat lung 14 days after treatment with AdTGF-$\beta1^{223/225}$, stained with H&E. *C* and *F* were also treated with AdTGF-$\beta1^{223/225}$, after 14 and 64 days, respectively *E* demonstrates the thickened pleura 14 days after AdTGF-$\beta1^{223/225}$ treatment. *C, E,* and *F* were all stained with EvG for matrix. *A,* and *F*, x250. *B, C, D,* and *E*, x350. *b,* bronchial structure. *v*, vascular structure. *s*, Scar Arrowheads in *D* denote pleural surface. Adapted from Sime *et al.*, 1997

In addition to clearing any remaining pathogens, cellular components of the inflammatory phase induce the production of additional cytokines, which take over the late stage of wound healing to repair the injured tissue. TGF-β1 is a major factor in wound repair, capable of carrying out several important functions. TGF-β_1 can itself serve as an inducer of several cytokines implicated in the pathology of fibrosis, including platelet-derived growth factor (PDGF), IL-1, basic fibroblast growth factor (b-FGF), TNF-α [9], Connective Tissue Growth Factor (CTGF, [10]), and MCP-1 (Zhang *et al.*, 1994). TGF-β_1 has also been shown capable of auto-inducing its own production [11,12], which likely plays a major role in the development of chronic fibrotic diseases. Sime *et al.* (1997, [13]) speculated that active TGF-β might induce the production of latent TGF-β, although this has not been conclusively shown. TGF-β is also responsible for the increase in the extracellular matrix content of the fibrotic lung, due to its abilities to increase matrix production while simultaneously decreasing the production of matrix metalloproteinases (MMPs) and increasing the production of tissue inhibitors of metalloproteinases (TIMPs) [3,14]. Although the importance of TGF-β in fibrotic diseases cannot be disputed, many of its effects on fibroblasts may be mediated by the cytokine Connective Tissue Growth Factor (CTGF). CTGF is a 38 kDa cysteine-rich protein [15] capable of replacing TGF-β_1 in stimulating fibroblast proliferation and collagen production [16], and to date is only inducible by TGF-β [10], primarily within fibroblasts [17]. The influences of TGF-β on other cell types such as immune cells and epithelial cells are not shared by CTGF [18]. CTGF is thought to be directly induced by TGF-β1, since its promoter has a unique TGF-β1 response element (TβRE) not seen in other TGF-β1 induced genes [10]. While the data suggests that TGF-β activates CTGF

production utilizing the Smad pathway, no direct evidence has been provided to date. Two other isoforms of TGF-β (TGF-β_2 and TGF-β_3) have also been implicated in fibrosis, although their roles are unclear [19-22]. The three TGF-βs exhibit a clear amino acid sequence homology, both within the family, and across species [14].

Why normal wound healing is sometimes diverted into a chronic state is currently the topic of much debate. However, it is clear that TGF-β1 is a primary effector of the chronic fibrotic response [13]. Many *in vivo* models have demonstrated the importance of TGF-β_1 in normal wound healing [23] and several types of fibrosis, including pulmonary [5,13], hepatic [24,25], and pancreatic [26] forms of the disease. In both the normal and disease states, TGF-β1 is produced by a number of cell types, including macrophages, neutrophils, eosinophils, fibroblasts, and epithelial cells [27-29]. TGF-β_1 is often termed a pluripotent cytokine. It plays a role in the elevation of the inflammatory response, thanks in part to its ability at low levels of expression to increase monocyte/macrophage migration and activation [14], and yet has the opposite effect on lymphocytes, preventing their activation [30], B cell immunoglobulin isotype switching [31], and also preventing epithelial cell proliferation [32]. In addition to the pro-inflammatory and anti-proliferative effects of TGF-β, it is also a very potent activator of fibroblasts. This cell type is the key producer of extracellular matrix components during a fibrotic response [33], and is thought to differentiate into an α-smooth muscle actin (αSMA)-expressing myofibroblast, also capable of producing ECM components in response to TGF-β1 induced activation [34].

The importance of TGF-β for normal immune function is clearly demonstrated in TGF-β_1 knockout mice. Maternally derived TGF-β_1 which crosses the placenta and

remains available for activation by adhering to matrix proteins within fetal organs, is sufficient to fuel early organ development, but excessive, uncontrolled inflammation disrupts organ function as this source dwindles [35]. Lethality within weeks of birth of $TGF\beta_1^{-/-}$ mice derived from heterozygous parents shows this cytokine as a key regulator of inflammatory processes, and possibly even some developmental processes as well.

TGF-β_1 is translated as a dimeric 390 amino acid precursor [36]. Cleavage of the amino portion produces the smaller, 25 kDa protein [14]. TGF-β_1 is most often found in an inactivated state, due to a re-association of the cleaved portion, termed the Latency Associated Peptide (LAP), with the mature peptide. The LAP is a homodimer of the amino-terminal portion of the TGF-β_1 precursor, held together by two disulfide bonds at positions Cys223 and Cys225. The LAP is noncovalently associated with TGF-β_1, and can be removed *in vitro* by extremes of pH, releasing the bioactive TGF-β dimer [37]. The mechanisms of TGF-β activation in a biological setting are unclear. Enzymes such as thrombospondin [38] and cathepsin [39] have been suggested. Thrombospondin 1 (TSP 1) is believed to be a major activator of latent TGF-β_1. Work by Crawford *et al.* (1998, [40]) using TSP 1 knockout mice showed a similar timecourse of rampant inflammation as the TGF-β_1 knockouts. The levels of tissue disruption were not as severe, nor were they lethal. Other methods of activation are known in addition to TSP 1, explaining the non-lethality of these knockout mice.

Several groups have demonstrated the ability of plasmin to activate latent TGF-β_1. Lyons and coworkers (1990, [41]) reported the activation of TGF-β_1 by plasmin in cell-free systems. Khalil and colleagues (1996, [20]) showed that alveolar macrophages producing TGF-β_1 in response to bleomycin also produced plasmin. The activation of

TGF-β_1 could be prevented by the addition of plasmin inhibitors to cultures of explanted macrophages. More recently, Godár *et al.* (1999, [42]) demonstrated that latent TGF-β_1 must be bound to the ubiquitous mannose-6-phosphate/insulin-like growth factor II receptor for its activation. The activation of TGF-β_1 in this manner first requires the activation of plasminogen to plasmin by urokinase plasminogen activator bound to its receptor. This prevents soluble plasmin from activating TGF-β_1, and allows the cells producing it to retain control of TGF activity.

Calpain, a calcium-activated neutral protease also is able to activate cell-associated latent TGF-β_1 [43]. Similar to plasmin, calpain must also be bound to the cell surface.

Epithelial tissues (such as lung and skin) undergoing fibrosis have an additional mechanism for activating TGF-β_1. In the bleomycin model of pulmonary fibrosis, epithelial cells upregulate the production of the transmembrane protein $\alpha v\beta 6$ integrin in response to TGF-β_1 [44]. $\alpha v\beta 6$ binds the TGF-β_1 LAP, but does not itself activate TGF-β_1. Integrin must first bind to a component of the cells actin cytoskeleton, inducing a conformational change in $\alpha v\beta 6$, ultimately exposing the TGF-β receptor binding site previously blocked by the LAP [44].

The existence of several cell and tissue-specific activators of TGF-β_1 demonstrate the importance of this cytokine, and suggests that the activation of latent TGF-β1 is a method for controlling its regulation. Indeed, in addition to the usual transcriptional control imposed on all cytokines, and the requirement for activation, TGF-β activity is also controlled by the expression of its receptors. Successful TGF-β signaling requires the direct interaction between the TGF-β receptor types I and II, (TGF RI, RII) as well as

with biglycan [45]. Many isoforms of the receptors exist, and are believed to vary in specificity for the three TGF-β isoforms [46]. The levels and sites of TGF RI and RII expression changes over the course of wound repair, and thus serves as an effective mechanism for controlling TGF-β activity [19,23].

TGF-β only binds to the type II receptor, but once bound this receptor can bind and activate the type I receptor through phosphorylation. Both receptor types contain a transmembrane serine-threonine kinase domain, although the type I receptor is responsible for initiating signal transduction through the Smad (related to the Drosophila gene *Mothers against dpp* (MAD), [47]) pathway. The Smad family of molecules is subdivided into three classes [48]. Class I (Smad 1-3, 5) Smads are receptor associated, becoming phosphorylated when in contact with a TGF-β1 bound receptor complex. Class I Smads dissociate from the receptor upon phosphorylation, allowing their association with Class II Smads (Smad 4; [49]). Class I and II heterodimers translocate to the nucleus, where they bind transcription factors allowing the transcription of fibrosis-associated gene products such as plasminogen activator inhibitor type I (PAI-1; [50]) and α2-I collagen [51]. Smad 4 is required for efficient DNA binding and transcriptional activation by the complex [52]. Class III Smads (Smad 6,7) are negative regulators of Smad signaling, preventing the phosphorylation of Class I Smads by the TGF-β1 receptor complex [53-55]. Once within the nucleus, Smad complexes can form with several different transcription factors, including C-Jun/c-Fos [56], FAST-1 [52], CBP/p300 [57], transcription factor E3 (TFE3; [58]), and Smad-interacting protein (SIP1; [59]). The broad number of transcription factors and transcriptional regulators that interact with the Smad pathway supports the wide array of functions carried out by TGF-β1.

Most studies investigating the pathway of TGF-β1 signaling have focused on events within lymphocytes using promoter constructs from their genes of interest. The immune regulatory functions of TGF-β1, and its target genes within lymphocytes no doubt involve a subset of transcriptional regulators distinct from those required during the mediation of wound healing, or a fibrotic response. Work on TGF-β1 signaling within the context of a fibrotic response has shown however that successful signaling through the Smad pathway is required for disease manifestation within animal models. Over expression of Smad7 prevents bleomycin-induced fibrosis [55], while mutations in Class I Smads similarly prevent correct TGF-β1 signaling [60]. Inhibiting the Smad pathway does not appear to inhibit the production of TGF-β1 itself [55] however, suggesting alternate mechanisms for the auto induction of TGF-β1.

Much of the knowledge obtained concerning TGF-β has been from *in vitro*, cell culture experiments [11, 16, 61, 62]. While the bleomycin models have been extremely effective at examining the pathology of disease and relative expression levels of implicated molecules [5, 8, 17, 19, 29, 63], they have been unable to confidently assign the function of specific cytokines in disease pathogenesis. Recombinant Adenoviral vectors expressing cytokines of interest can be utilized to assist in assigning *in vivo* functions to these molecules. Adenovectors injected intranasally or intratracheally infect bronchial epithelium [64], expressing their transgene to highly detectable levels over a transient but prolonged period (7 to 10 days) [13, 64, 65]. Adenovectors expressing both active TGF-β [13] and GM-CSF [66] injected intratracheally into rat lung showed that these cytokines are potent inducers of fibrotic reactions, GM-CSF likely through induction of TGF-β, and demonstrated the feasibility of cytokine-expressing adenovector

models of fibrosis. The TGF-β_1 produced in the Adenovector system was spontaneously active, generated by mutating the cysteines (Cys223Ser; Cys225Ser) responsible for disulfide bond formation within the LAP (Fig. 2). Incapable of dimerizing, the LAP is unable to inactivate the TGF-β_1 [13] The adenovectors used are engineered replication-deficient with the loss of the E1 region from their genome, thus infecting hosts only transiently Control viruses lacking a transgene are used to determine inflammation and immune responses to replication-deficient viruses, so these may be delineated from the changes seen through expression of the transgene.

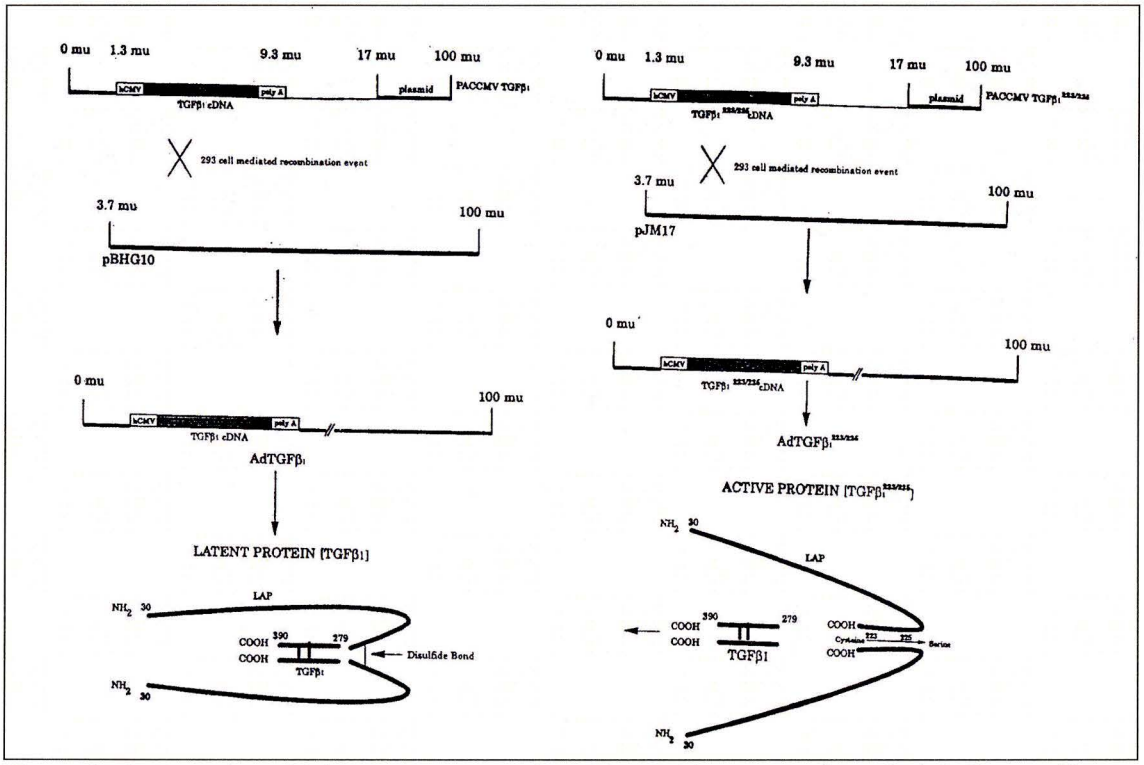

Figure 2. Construction of Adenovectors expressing active and latent TGF-β1 The left diagram illustrates how disulfide bonds in the LAP allow it to associate with the amino terminus of the TGF-β1 dimer, effectively inhibiting its activity On the right, the cysteines responsible for disulfide bond in the LAP have been mutated to serines (Cys 223,225 Ser). This prevents the LAP from dimerizing, and hence from associating with TGF-β1 When this construct is expressed by an Adenovector, constitutively active TGF-β1 is produced. Adapted from Sime *et al.*, 1997

RATIONALE

It was the purpose of these studies to further refine and develop the AdTGF-$\beta_1^{223/225}$ mediated pulmonary fibrosis model in rat designed by Dr. Patricia Sime, and to extend its use into other animal models and systems.

Several groups have conclusively shown that TGF-β_1 plays a central role in a variety of pulmonary fibrotic diseases. Due to its wide range of functions in development, lymphocytic and inflammatory cell mediation, and wound healing, TGF-β_1 is likely a dangerous cytokine to modulate directly: attempts to perturb one function may disastrously affect others. Future anti-fibrotic therapies will therefore likely be aimed at downstream mediators of TGF-β_1, specific to the fibrotic pathway. One such molecule that may have important pro-fibrotic effects is CTGF. While CTGF mRNA is up regulated during bleomycin induced pulmonary fibrosis, no evidence has yet been provided for a causative role of this cytokine in fibrosis. TGF-β is presumed to activate CTGF mRNA transcription by signaling through the Smad pathway, since over expression of Smad 7 can lessen a fibrotic response initiated by TGF-β_1 [55]. Further refining the flow of events in TGF-β1 induced fibrosis is of paramount importance, and so we sought answer the question does TGF-β1 induce fibrogenesis through the induction of CTGF, and is signaling through the Smad pathway a requirement for disease? To address these interrelated questions, and possibly identify an additional target for anti-fibrotic therapeutic strategies, we examined Smad3 knockout (S3 KO) mice (generously provided by Dr. Anita Roberts, NCI) to determine their susceptibility to AdTGF-$\beta_1^{223/225}$ induced pulmonary fibrosis, and whether they showed impairment in producing CTGF.

The success of the Adenovector system in adult rodents prompted us to develop similar models of human pulmonary fibrotic diseases, such as Bronchopulmonary Dysplasia (BPD) in newborns. Developing a model for this fatal disease will allow us to test and develop preventive strategies and further refine our knowledge of the disease course. The roles TGF-β plays in adult fibrogenic diseases prompted us to question whether TGF-β1 plays a role in the onset of BPD. Does TGF-β derived from an Adenovector have the ability to cause symptoms in newborn rats resembling those seen in human infants with BPD? To address this, we treated one-day-old (neonatal) rats with AdTGF-$\beta_1^{223/225}$ and examined its expression in the lung and effects at specific intervals over 28 days.

The pro-fibrotic actions of TGF-β are thought to be independent of an adaptive lymphocytic response, operating primarily through the deregulation of normal inflammatory and reparative processes. While the model designed by Sime and co-workers (1997, [13]) clearly demonstrated the pro-fibrotic actions of TGF-β_1 and the feasibility of the Adenovector as an *in vivo* pulmonary-delivery system, it was unable to determine the role of the adaptive immune response. Critics of the Adenoviral model suggested that active TGF-β_1 was not acting alone, but rather in concert with an adaptive response generated to the immunogenic backbone of the Adenoviral delivery system. To address the concerns regarding our cytokine delivery system, we asked whether Adenovector derived TGF-β1 induces fibrogenesis in conjunction with a required anti-adenovirus response, or is it independent of the antiviral response? To address the question, the fibrotic response to AdTGF-$\beta_1^{223/225}$ in normal Balb/c mice was compared to that generated in Balb/c SCID Beige mice. SCID Beige mice lack functional T Cells,

B Cells, and Natural Killer (NK) Cells, thus providing an ideal system for testing the role of the Adenovector backbone in the TGF-β_1 mediated fibrotic response.

The three TGF-β isoforms have been ascribed tentative roles during development and normal wound healing. Unclear and conflicting reports has prevented the description of specific roles, and their differential actions throughout a fibrotic response have also yet to be uncovered. The many potential roles for TGF-β3 in fibrotic diseases prompted us to ask the question does TGF-β3 induce gene regulation similar to that of TGF-β1? To begin answering this question, an Adenovector expressing constitutively active TGF-β_3 (Ad5E1TGF-$\beta_3^{223/225}$lox.6) was constructed. This Adenovector will allow us to examine the gene regulatory capacities of TGF-β3, and will possibly help us to identify a role of TGF-β_3 in fibrogenic and wound healing disorders.

MATERIALS AND METHODS

Construction of Ad5E1TGF-$\beta_3^{223/225}$lox.6.

cDNA coding for constitutively active TGF-β_3 cloned into pACCMV was kindly provided by Dr. John Groffen, Department of Pathology, Childrens Hospital of Los Angeles. The TGF-$\beta3^{223/225}$ transgene was excised from its shuttle plasmid using the restriction enzymes EcoRI and HindIII (Gibco BRL) in a simultaneous double digest, carried out at 37°C for 1 hour. After separation on a 1% agarose gel, the isolated transgene was purified using the Wizard DNA Isolation Kit (Promega), and their established protocol for isolation of DNA from high-melting temperature agarose. Purified transgene was then ligated using DNA Ligase (New England Biolabs) into the shuttle plasmid pDC104, containing the left end of the human adenovirus type 5 genome (0-17 mU) and a human cytomegalovirus promoter, a multicloning site, and an SV40 polyadenylation signal inserted into the E1 region [67]. Successful ligation was tested by restriction analysis using EcoRI and HindIII, followed by gel electrophoresis in a 1% agarose gel, and the orientation and identity of TGF-$\beta3^{223/225}$ was confirmed by DNA sequencing using a primer for the left end of the pDC104 multiple cloning site, 5'-GCG TCG GTA CCG TCG CAG TC-3' (conducted by Mobix, McMaster University, Hamilton ON, Canada). Recombinant plasmids were co-transfected together with pBHGloxΔE1,3 into 293 cells [68]. The Cre-Lox system utilizes site-specific recombination to increase the incidence of homologous recombination, and the successful integration of the crucial shuttle plasmid components into the correct location within the Adenoviral genome. The plasmids pBHGloxΔE1,3 and pDC104 were both kindly provided by the laboratory of Dr. Frank Graham, McMaster University.

Northern Blot Analysis of TGF-β3 mRNA Expression from Neonatal Murine Lung Fibroblasts Infected with Ad5E1TGF-β3$^{223/225}$.lox.

Confluent neonatal murine lung fibroblasts in 100 mm dishes were infected overnight with 100 µl unpurified Ad5E1TGF-β3$^{223/225}$.lox. Total mRNA was isolated and purified from the cells using TRIzol (Gibco, BRL). Purified mRNA was separated in a 1% formaldehyde agarose gel, and transferred overnight to a nylon membrane (ICN Pharmaceuticals, Montreal, Canada) in 25mM Sodium Phosphate, pH 6.5. Membrane was probed overnight at 45°C using a 1.3 kb TGF-β3$^{223/225}$ fragment excised from pDC104.TGF-β3$^{223/225}$. Probe was generated using the Quickprime Kit (BiochemPharma), and 50µCi ^{32}P-CTP. Blots were stringently washed, and exposed to Kodak XAR film (Kodak, Rochester, NY)

Luciferase Assay for Determination of Ad5E1TGF-β$_3^{223/225}$lox.6 Activity

To ensure that the Ad5E1TGF-β$_3^{223/225}$lox.6 construct was producing biologically active TGF-β$_3$, supernatants from infected *in vitro* cultures of murine fetal fibroblasts were assayed using an established Luciferase Assay protocol [69]. After an overnight infection, the supernatants were removed from the murine fetal fibroblasts and functional Adenovirus was inactivated under UV light for thirty minutes before it was assayed. Mink lung epithelial cells (MLEC, clone 32) with a stable transfection of a 800 bp fragment of the 5' end of the human plasminogen activator inhibitor-1 gene fused to the firefly Luciferase reporter gene were graciously provided by D. Rifkin, NY. MLECs were cultured in 6 well plates in 1ml DMEM supplemented with 1% penicillin/streptomycin, 1% L-glutamine, 10% FCS and 200 µg/ml Geneticin® (Sigma Chemicals, Oakville, ON, Canada). Upon confluency, media was removed and the cells were washed three times with 1ml PBS per wash. 1 ml of the supernatants from murine fetal fibroblasts infected with one of Ad5E1TGF-β$_3^{223/225}$lox.6,

Ad5E1TGF-$\beta_1^{223/225}$, or Ad5E1DL70-3 were added to the confluent MLECs, and incubated overnight at 37°C, 5% CO_2. 2 wells were assayed per data point. After 16 hours, the supernatant was removed, and the cells washed three times with PBS. 300µl of Cell Lysis Buffer (Promega) was added, and the cells scraped off. Cells in lysis buffer were transferred to 1.5ml eppendorf tubes, pelleted, and kept on ice until assayed. D(-)-Luciferin (Boehringer Mannheim, Germany) was used as the Luciferase substrate, and the reaction was assayed by luminometer (Lumat LB 9501, Berthold Systems Inc., Pittsburgh, PA). Data are presented as relative light units (RLU).

Genotyping Mice for Presence of the Smad3 Gene.

Exon 8 of the Smad 3 gene was disrupted in mice of background 129SVEV X C57BL/6 by Yang *et al.* (1999, [70]). Mice heterozygous for the Smad 3 null allele and their genotyping protocols were kindly provided by the laboratory of Dr. Anita Roberts, NCI, NIH. Immediately upon weaning (3 weeks old), mice were identified using an ear marking procedure. 1 cm of tail was cut, after being wiped with isopropanol. Tails were digested overnight at 55°C in 300µl Tail Lysis Buffer (200mM NaCl, 40 mM Tris (pH 8.0), 20 mM EDTA, 0.5% SDS, and 0.5% β-Mercaptoethanol). 20 mg/ml Proteinase K is added to the stock buffer just before use. Tail Homogenates are spun at 1,000 rpm for 4 minutes, and the DNA containing supernatant is transferred to a fresh tube. DNA is precipitated with the addition of approximately 2 volumes of 100% Ethanol. Inverting the tube several times condenses the DNA, forming long white strands. A pipette tip is used to remove the DNA from the ethanol, and transfer it to a fresh tube. DNA is resuspended in 100µl ddH_2O, and boiled for 5 minutes to completely dissolve it.

The Smad3 wild-type and mutant alleles were identified with PCR, using 3 to 5 µg DNA, Vent DNA Polymerase (New England Biolabs), 10 mM dNTPs, and 10 mM of each of

the following primers. To identify the wild-type allele, Smad3 primer 1 (S3p1, 5'-CCACTTCATTGCCATATGCCCTG-3') and Smad3 primer 2 (S3p2 5'-CCCGAACAGTTGGATTCACACA-3') were used, amplifying a fragment of about 400 bp. To identify the mutant Smad3 allele, S3p1 was used with Smad3 primer 3 (S3p3 5'-CCAGACTGCCTTGGGAAAAGC-3') to amplify a fragment of 250 bp. PCR began at 50°C for 10 minutes, followed by 2 minutes at 94°C. Amplification was carried out for 30 cycles of 94°C for 30s, 60°C for 30s, and 72°C for 2 min. A final elongation step at 72°C for 2 minutes ended the PCR. Products were separated in a 2% agarose gel, stained with ethidium bromide.

Administration of Adenovector to Neonatal Rat Lung and Mouse Lung.

Lightly anaesthetized, one-day-old neonatal Sprague-Dawley rats were injected intranasally (i.n.) with $1x10^8$ plaque forming units (pfu) of either Ad5E1DL70-3 (control virus, no transgene), or Ad5E1TGF-$\beta1^{223/225}$ diluted in 10 µl sterile PBS. Prior to handling the rat pups, and intermittently throughout the injections, we rubbed our hands in the bedding and feces from their cage in an effort to prevent the mother from detecting our scents on her pups. Holding the anaesthetized rat upright, the Adenoviral solution was injected into the nasal passages using a P10 Pipette. Using the thumb, slight pressure was placed on the bottom of the jaw to keep the mouth closed and force the animal to inhale the solution through its nose. The rats were held upright for a short time to maximize delivery, and then allowed to recover under our supervision prior to returning them to their mother.

Smad3 KO adult (4 –6 weeks) mice and Wildtype (Wt) littermate controls were treated in a similar manner, except $1x10^9$ pfu of Adenovector was injected in a total volume of 20 µl with PBS. Balb/c and Balb/c SCID Beige mice were injected with a dose of $5x10^8$ pfu diluted in 20 µl PBS. Mice are held by the scruff of the neck, with the tail held between the

pinky finger and the palm of the hand. The Adenoviral solution is carefully pipetted to form drops at the end of the pipette tip. Single drops are placed on the nostrils until they are inhaled, and repeated until the full volume has been administered.

Detection of Transgene Expression

3-5 Adult mice injected with control Adenovirus or those expressing active TGF-β_1 were sacrificed after 3 days, and their lungs excised. Bronchoalveolar Lavage (BAL) was performed, where 250 µl of PBS was injected intratracheally and subsequently removed while massaging the lung. This was repeated using fresh PBS for a total BAL volume of 500 µl. BAL fluid was spun at 4°C for 5 minutes to pellet cells and other debris, and the cytokine containing supernatant was transferred to a fresh tube where it was stored at –70°C until the time of assay. TGF-β_1 levels were examined using a human TGF-β_1 ELISA kit (R & D Systems, Inc., Minneapolis, MN). Total TGF-β1 levels were measured by acid-activating samples as per the manufacturers protocol. Levels of active TGF-β1 were measured by assaying samples that were not acid-activated.

Due to the small and delicate nature of neonatal rat lungs, BAL could not be performed to assess the presence of TGF-β_1 transgene. To accomplish this, 3-5 neonatal rats per group were sacrificed 2 days after Adenovector injection. Prior to excising their lungs, the right ventricle of the heart was perfused with 3 ml ice cold PBS to remove all blood from the lungs. The lungs were then excised and kept in ice cold PBS. Shortly thereafter, they were transferred to 2 ml fresh PBS, and homogenized. The homogenate was pelleted by centrifugation at 12,000 rpm for 10 minutes at 4°C. The cytokine rich supernatant was transferred to a new tube and stored at –70°C until assayed. TGF-β_1 levels were determined as described above, and expressed as pg of active TGF-β_1 per mg wet lung weight.

Lung Hydroxyproline Determination

A modified Woessners protocol [71] was used. Tissue was prepared by immediately snap freezing the right lobe of excised lungs in liquid nitrogen, then transferring the samples to –70°C for storage until the time of assay. Samples were homogenized in 5 ml ddH_2O. 1 ml of the resultant homogenate was hydrolyzed in 2 ml 6 N HCl for 16 hours at 110°C. Samples were adjusted to pH 7.0, and then 400 µl was diluted into 2.0 ml with ddH_2O. The colorimetric assay was initiated with the addition of 1 ml chloramines-T solution to the diluted sample at pH 7.0. 1 ml of 70% perchloric acid was added, followed by 1 ml of a dimethylbenzaldehyde solution. After 20 minutes at 60°C, the samples were returned to room temperature, and the OD at 557 nm was determined. Using hydroxyproline standards (Sigma Chemicals), results were expressed as µg hydroxyproline per mg wet lung weight.

Lung Fixation and Histological Examination

Upon lung excision, the left lobe was perfused with 10% formalin, and fixed for 24 hours before processing and paraffin embedding. All histological sections were stained with Hematoxylin and Eosin (H&E), a general nuclear stain, and Elastin van Gieson (EVG), which stains collagen and elastin. Select sections also underwent immunohistochemical staining for α-Smooth Muscle Actin, as previously described [13].

Smad3 Lung Stromal Cell Line Establishment and CTGF mRNA Analysis

Lungs from naïve Smad3 KO and Wt mice were excised and immediately placed in ice cold F15 media containing 10%FBS, 1% Penicillin/Streptomycin, and 0.1% Fungizone. Lungs were finely minced, and placed in 100 mm Tissue Culture plates without media and incubated for 1 hour at 37°C, 5% CO_2. 5 ml F15 media (as above) was then added, and cells

incubated overnight. The following day, the media was removed, and the adherent lung sections gently washed twice with 5 ml PBS. 10 ml fresh media was then added. Cells were washed as above once every two days. After 7 days, the lung sections were removed from the culture, and the stromal cell outgrowth was allowed to continue, with passaging at 80-90% confluency.

Cells between passages 3 and 5 (p3-p5) were cultured in 6 well plates in 2 ml F15 media (as above). When confluent, the media was removed and the cells were washed once with 1 ml PBS, infected at a multiplicity of infection (m.o.i.) of 100 with AdTGF-$\beta_1^{223/225}$ or AdDL70-3, diluted in 100 µl PBS. After a 1 hour incubation, 1ml of F15 media with 1% FBS was added, and cells were returned to the incubator. Cells were harvested at 4 hours, 16 hours, 24 hours, and 48 hours. Each data point was achieved in 2 wells.

At the time of harvesting, the supernatant was frozen at –70°C, and the cells were washed once with 1 ml PBS. The PBS was removed, and 500 µl TRIzol (Gibco BRL) was added to each well. Cells in TRIzol were harvested, and duplicate wells were combined into 10 ml polypropylene tubes. The total cellular mRNA was isolated according to the manufacturers established protocol.

3 µg of mRNA from each data point was reverse transcribed using the Reverse Transcriptase Superscript II system (Gibco BRL), utilizing the manufacturers established protocol. The resultant cDNA was used in a PCR analysis for CTGF mRNA expression. The CTGF PCR primers 5'-GAGCTTTCTGGCTGCACC-3' and 5'-TCTCCGTACATCTTCCTG-3' amplify a fragment of 250 bp, and were originally developed by Lasky *et al.* (1998, [17]), and synthesized by Mobix (McMaster University, Hamilton ON). GADPH was amplified from the same samples for use as a loading control. The primers 5'-CCTGCACCACCAACTGCTTAGCCC-3' and 5'-GATGTCATCATATTTGGCAGGTT-3' amplify a 310 bp fragment.

Data Analysis

Data were expressed as mean±SEM. Statistical significance was determined using the Students two-tailed t test, assuming unequal variances. Differences were considered statistically significant if $p < 0.05$.

RESULTS

Smad3 is required for TGF-β1-induced Pulmonary Fibrosis, and for Induction of CTGF mRNA through TGF-β1.

BAL fluid from adult Smad3 KO and wt. littermates injected with either Ad5E1DL70-3 or Ad5E1TGF-$\beta1^{223/225}$ was examined for active TGF-β1 levels by ELISA. Both the KO and wt. Smad3 mice confirmed the efficacy of the i.n. Adenovector injections, with the Ad5E1TGF-$\beta1^{223/225}$ treated mice having higher BAL TGF-β1 levels than those injected with Ad5E1DL70-3 three days after Adenovector administration (data not shown). Unfortunately, groups contained between 1-5 animals, so statistical significance could not be determined.

The characteristic early-stage pulmonary inflammatory response to Adenovector injection (regardless of the treatment) was observed in the Smad3 KO and Wildtype mice when they were examined 2 days post-injection (data not included).

Injecting wt. mice with the TGF-β1 expressing Adenovectors gave similar results that we saw when Balb/c mice were treated with the same Adenovector. By 14 days, treatment with Ad5E1TGF-$\beta1^{223/225}$ had caused a persistent, dense cellular infiltrate within the lung parenchyma that was not restricted to the peribronchial or perivascular areas (Fig. 3). The lung interstitium is nearly completely filled with invading cells 28 days post injection, and these areas are also occluded by an abnormal amount of extracellular matrix within the peribronchial areas, the alveolar septa, and around the pleural surface (Fig. 4). Wt. mice injected with control virus did not develop a fibrotic response, but did demonstrate a minor inflammatory response that by 14 and 28 days was localized to the peribronchial layers.

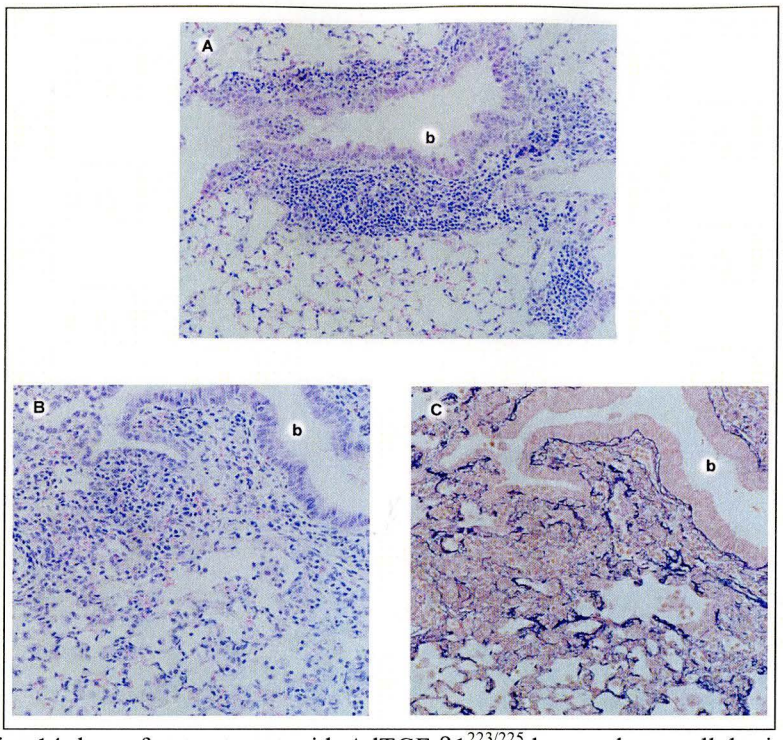

Figure 3. Smad3 wt. mice 14 days after treatment with AdTGF-$\beta 1^{223/225}$ have a dense cellular infiltrate (B) that has resulted in the deposition of ECM components (C, matrix stains dark purple). Treatment with AdDL70-3 (A) causes only sparse inflammation. A and B were stained with H&E, and C was stained with EvG. b, bronchial structure. A, 160x magnification. B and C, 200x.

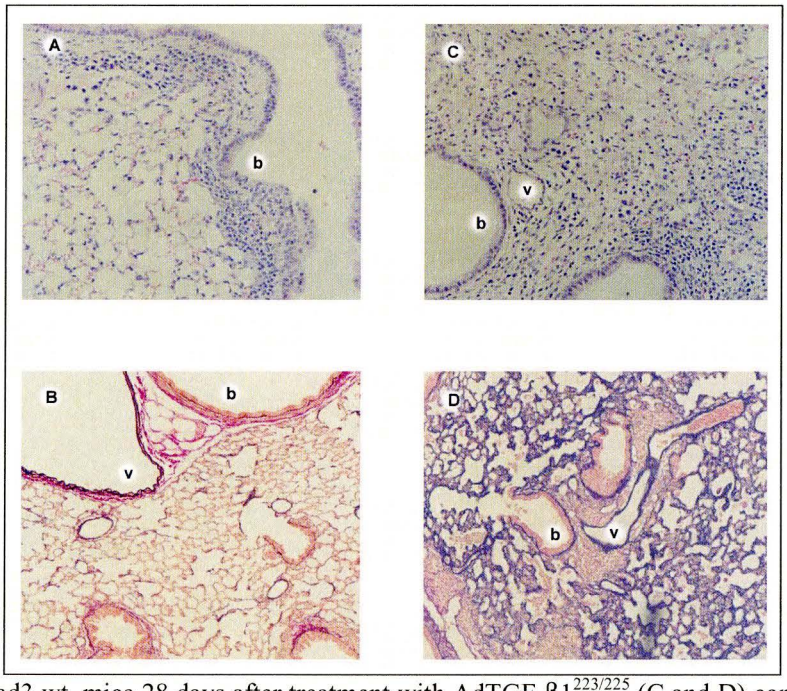

Figure 4. Lungs of Smad3 wt. mice 28 days after treatment with AdTGF-$\beta 1^{223/225}$ (C and D) continue to have a dense cellular infiltrate (C, H&E stain) and a large amount of ECM deposition (D, EvG). Mice treated with AdDL70-3 have only a sparse peribronchial and perivascular infiltrate after 28 days (A, H&E stain) that is not associated with a fibrotic response (B, EvG stain). b, bronchial structure. v, vasculature. A and C 160x magnification. B and D 100x.

Smad3 KO mice injected with Ad5E1TGF-β1$^{223/225}$ did not have the same pathological response as the wt. mice. After 14 days, the lungs of KO mice still contained infiltrating cells within their parenchyma, but not to the same extent as in the wt. mice. As well, the infiltrate residing within the Smad3 KO lungs did not result in the excess deposition of ECM (Fig. 5). The infiltrate is greatly reduced by 28 days post-injection, restricted to the perivascular and peribronchial areas of the lung (Fig. 6). There is no difference in the ECM content between 14 and 28 days within the Smad3 KO mice treated with Ad5E1TGF-β1$^{223/225}$, nor does this content appear to differ from the Smad3 KO group treated with control virus.

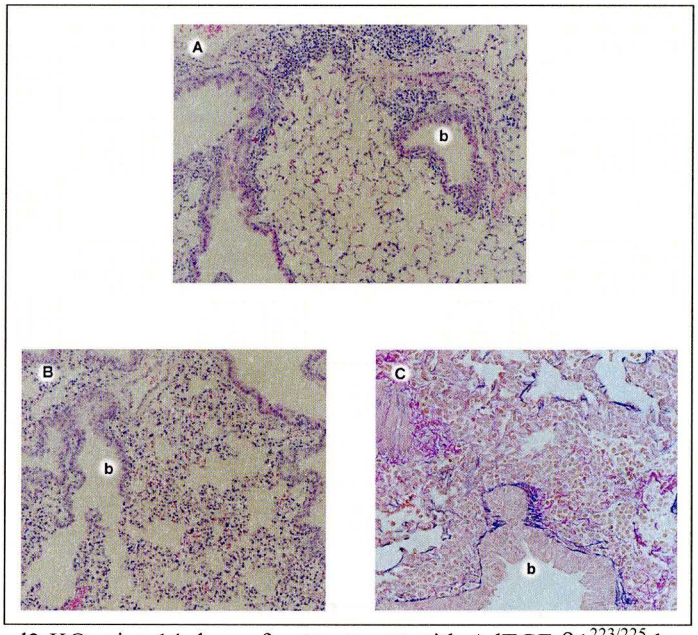

Figure 5. Lungs of Smad3 KO mice 14 days after treatment with AdTGF-β1$^{223/225}$ have a cellular infiltrate (B, H&E stain), but an apparent lack of ECM deposition (C, EvG). Treatment with AdDL70-3 results in only a peribronchial and perivascular cellular infiltrate (A, H&E). b, bronchial structure. A and B are 100x magnification. C is 200x.

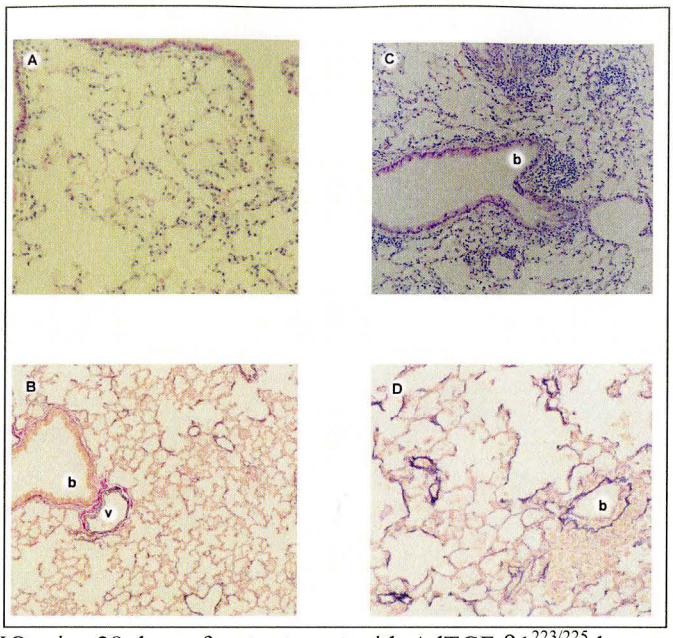

Figure 6. Lungs of Smad3 KO mice 28 days after treatment with AdTGF-$\beta1^{223/225}$ have only a small localized cellular infiltrate (C, H&E) that is not associated with the production of ECM components (D, EvG). 28 days after treatment with AdDL70-3, the lungs of Smad3 KO mice have returned to normal, lacking any significant cellular infiltration (A, H&E) or ECM deposition within the lung parenchyma (B, EvG). b, bronchial structure. v, vasculature. A and D are 200x magnification. B and C are 100x.

Quantifying the hydroxyproline contents of these lungs validates the histological data. Only the wt. mice treated with a TGF-β1 expressing Adenovector had an increase in lung hydroxyproline content, while the Smad3 KO mice injected with the same Adenovector had lung hydroxyproline levels equivalent to those groups treated with control virus (Fig. 7).

To further evaluate TGF-β1 functionality in the absence of Smad3, lung stromal cells were isolated, and a cell line was established for each of the three Smad3 genotypes. Lung stromal cells from Smad3 KO, het., and wt. mice were treated with Ad5E1TGF-$\beta1^{223/225}$ or Ad5E1DL70-3 for 4, 12, 24, and 48 hour intervals, and the production of CTGF mRNA was examined using RT-PCR (Fig. 8). Ad5E1TGF-$\beta1^{223/225}$ stimulated cells derived from Smad3 wt. mice to produce detectable levels of CTGF mRNA within 4 hours of starting the incubation, and continued up to the latest time point of 48 hours. Expression of CTGF mRNA peaks between 12 and 24 hours in the wt. cells. Smad3 KO stromal cells are still able

to produce CTGF mRNA but expression falls to undetectable levels after 24 hours, and never reaches the levels produced by the Smad3 wt. cell line. In fact, the levels produced by the Smad3 KO cells when stimulated with Ad5E1TGF-$\beta 1^{223/225}$ did not differ much than when they were stimulated with Ad5E1DL70-3 As would be expected, the lung stromal cells derived from Smad3 het. mice showed an intermediate ability to produce CTGF mRNA. It was still detectable after 48 hours, but at lower levels than that produced by the Smad3 wt. lung stromal cells. Stimulating the lung stromal cells with Ad5E1DL70-3 resulted in the stimulation of only background levels of CTGF mRNA. To confirm that equal levels of RNA was used in each RT-PCR reaction, mRNA for GAPDH was reverse transcribed, PCR amplified and run on an agarose gel.

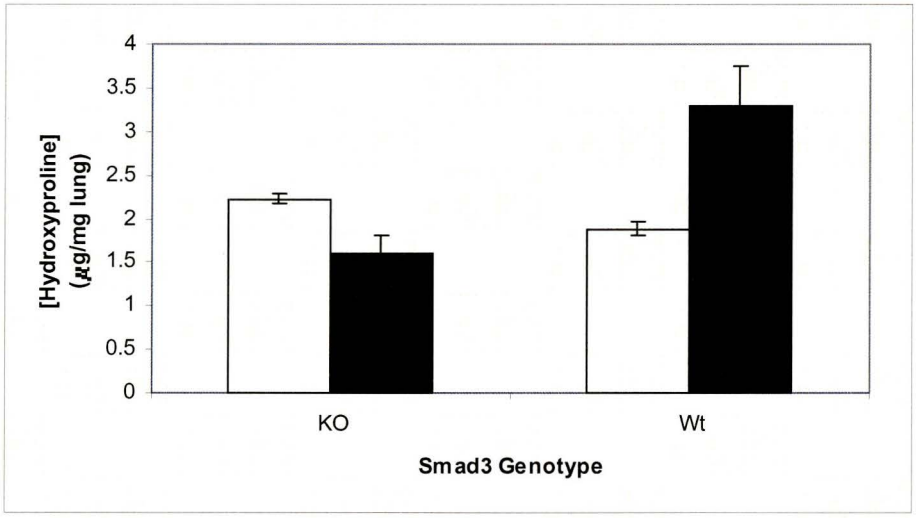

Figure 7 Analysis of lung hydroxyproline content in Smad3 KO and wt. mice, 28 days after injection of Ad5E1TGF-$\beta 1^{223/225}$ (black bars) or Ad5E1DL70-3 (white bars). Data are expressed as µg hydroxyproline per mg wet tissue. wt. mice treated with TGF-β1 have significantly more hydroxyproline than all other groups ($p<0.025$).

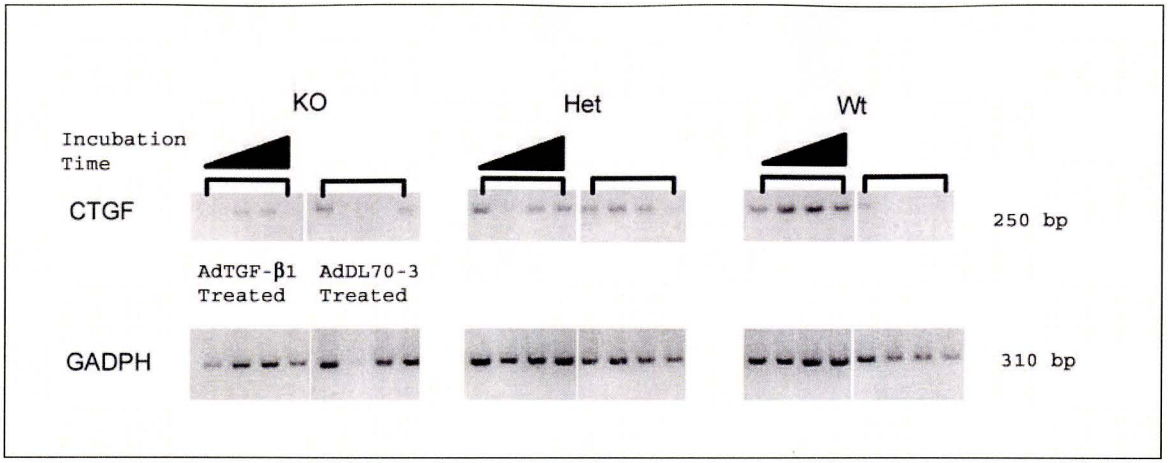

Figure 8. Smad3 is required for TGF-β1 to induce CTGF mRNA. Lung stromal cells explanted from Smad3 KO, het, and wt. mice were infected with 100 moi of AdTGF-β1 or AdDL70-3 for 4, 12, 24 and 48 hour intervals. Total mRNA was isolated and reversed transcribed. The resultant cDNA was subjected to PCR to amplify CTGF (250 bp; top row). GADPH (310 bp; bottom row) was also amplified as a control. PCR products separated in a 2% agarose gel, and stained with ethidium bromide.

TGF-β1 Causes Bronchopulmonary Dysplasia in Newborn Rats.

Injecting 1-day-old rat pups intranasally with Adenovector is an inherently difficult task, due to their frailty and small size. The efficacy of the i.n. injection method was assessed by measuring the amount of bioactive TGF-β1 within the lungs of pups 2 days after infection with the control Adenovirus or active TGF-β1-expressing Adenovirus. While BAL fluid is generally examined to identify cytokines important in the lung micro-environment, the small size and frailty of the neonatal rat trachea prevented accurate BAL fluid recovery As an alternative, lungs from 3 day-old pups (2 days post-injection) were excised, perfused free of blood using ice-cold PBS, and quickly homogenized. After brief centrifugation, supernatants were examined using ELISA (Fig. 9). Expressed as pg of TGF-β1 per mg of lung tissue, the pups injected with AD5E1TGF-β1$^{223/225}$ showed significantly higher levels of TGF-β1 than untreated controls or those treated with control virus ($p<0.012$).

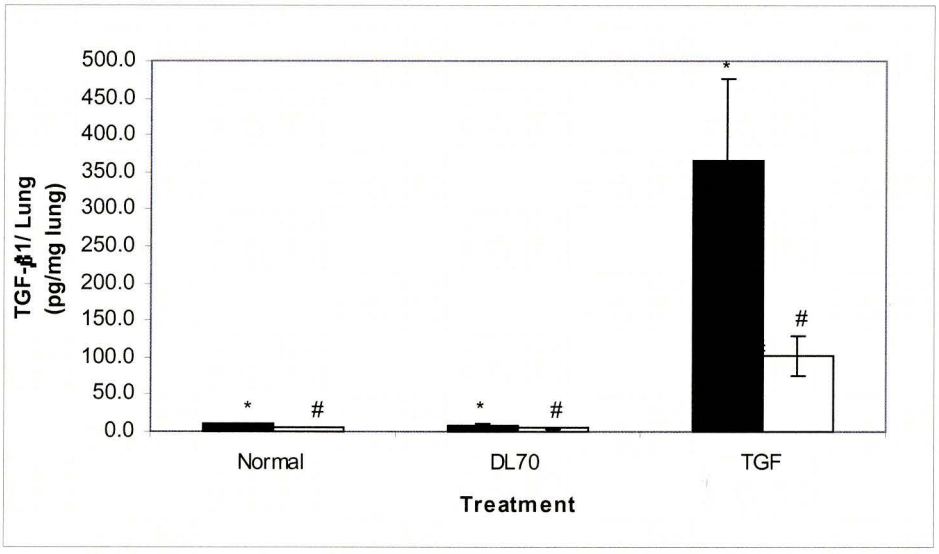

Figure 9. TGF-β1 levels in 3 day old rat lung homogenate, untreated, or 2 days post i.n. injection of $1x10^8$ pfu Ad5E1DL70-3 or Ad5E1TGF-β1$^{223/225}$ Levels of total TGF-β1 (black bars) and active TGF-β1 (white bars) as measured by ELISA, are expressed as pg of TGF-β1 per mg of lung. Differences between the TGF-β1 treated group and the control groups for levels of active TGF-β1 (#, $p<0.014$) and total TGF-β1 (*, $p<0.012$).

Lungs examined histologically at the early time points, 2 days and 7 days post-injection, showed an intense inflammatory response (Fig. 10). This was not exclusive to those pups injected with TGF-β1-expressing Adenovectors, however. The lungs of both the untreated pups and those treated with control virus were congested with an inflammatory infiltrate at these time points, in some cases at levels equivalent to the TGF-β1 treated group. Inflammation subsided throughout the course of the experiment in all groups, and by 29 days old (28 days post-injection) the lungs of both control groups demonstrated fundamentally normal neonatal lung architecture when examined histologically (Fig. 11). Alveoli have divided and sub-divided into their saccular network, providing a much larger area for gas exchange than was available at birth when the alveoli had not yet fully compartmentalized. At 29 days old, rat lungs are not quite fully developed. The distal portions of the lung are often incompletely alveolarized, demonstrating the elastin caps on budding alveolar septa characteristic of ongoing alveolar septation. The alveolar spaces are significantly smaller than those seen in the neonatal rats injected with Ad5E1TGF-$\beta 1^{223/225}$.

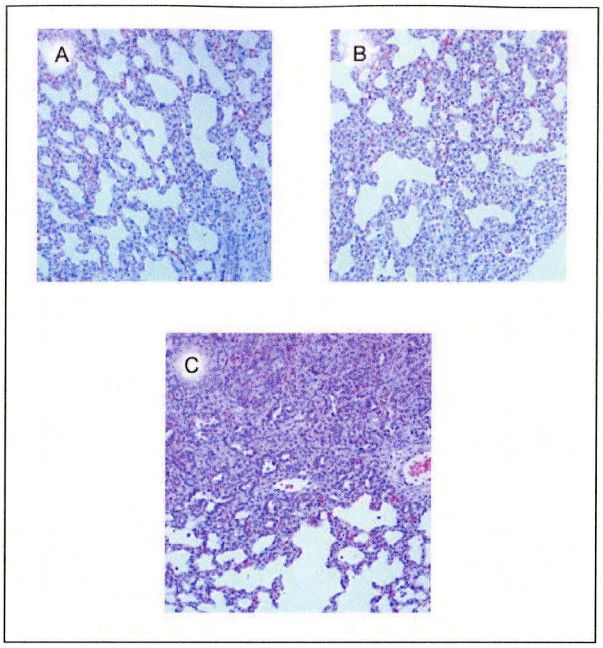

Figure 10. Neonatal rat lungs 7 days post treatment (8 days old). A, control lung, untreated. B, Lung treated with AdDL70-3. C, lung treated with AdTGF-$\beta 1^{223/225}$ All stains are H&E, at x100 magnification.

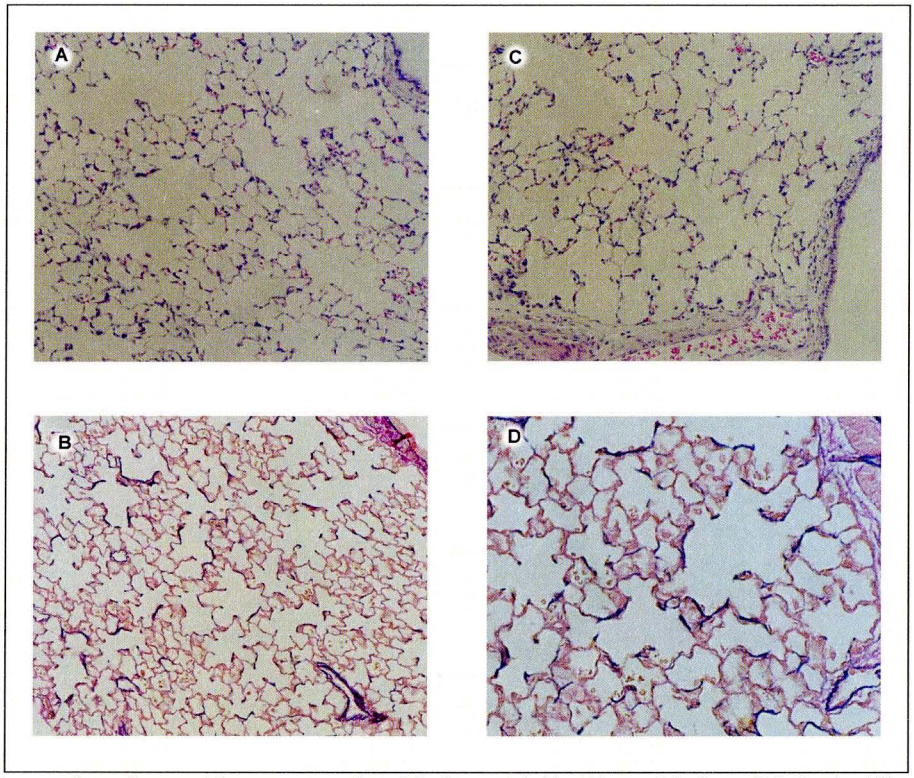

Figure 11. Neonatal rat lung, either untreated at 29 days old (A, B), or 28 days (29 days old) after treatment with AdDL70-3 (C, D). A and C were stained with H&E, while B and D were stained with EvG. Lungs of both groups are histologically normal. A, B, and C are x100. D, x200.

Rat pups injected with the TGF-β1-expressing Adenovector developed fibrotic lesions within their lungs, although not to the extent demonstrated in experiments using adult rats (Fig. 12). Even so, analysis of Hydroxyproline content showed a statistically significant increase in matrix accumulation within the lungs of the Ad5E1TGF-$\beta1^{223/225}$ treated pups compared to the control groups (Fig. 13, $p<0.013$). These differences were not evident until 28 days post-injection, there was no difference after only 21 days.

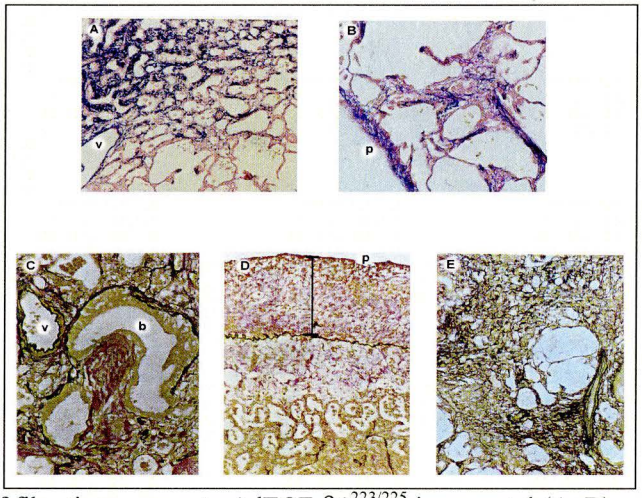

Figure 12. Comparison of fibrotic response to AdTGF-$\beta1^{223/225}$ in neonatal (A, B) and adult (C, D, and E) rats. A and B are lungs 28 days post treatment, while C and D are after 14 days, and E is after 64 days. All sections were stained with EvG. b, bronchial structure. v, vasculature. p, pleural surface. A, x100. B x200. C, D x350. E x250. C, D, E are adapted from Sime *et al.*, 1997).

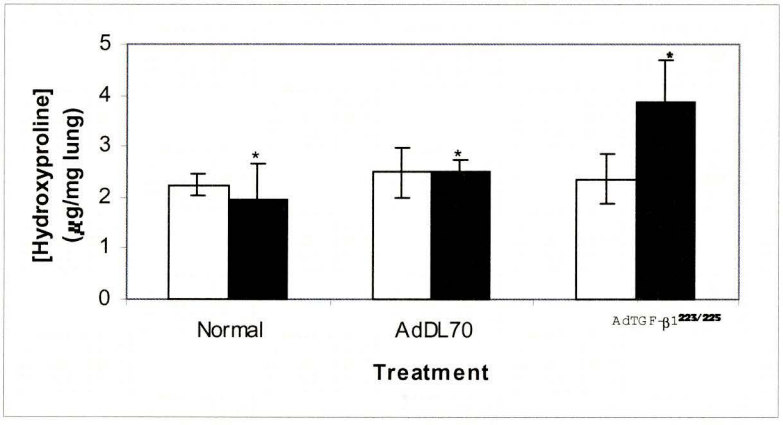

Figure 13. Hydroxyproline content of rat lungs left untreated, or after i.n. injection of $1x10^8$ pfu Ad5E1DL70-3 or Ad5E1TGF-$\beta1^{223/225}$ Hydroxyproline content was analyzed 21 days post-injection (white bars) and 28 days after injection (black bars) when the pups were 22 and 29 days old, respectively TGF-β1 treated group had significantly more hydroxyproline than the control groups after 28 days (*, $p<0.013$), expressed as µg Hydroxyproline per mg of wet tissue.

Fibrotic sequaeli was not the only pathology seen in the Ad5E1TGF-$\beta1^{223/225}$ treated lungs, however Even more striking are the large, apparently undeveloped alveoli saccules that predominate the lungs of pups from this group (Fig. 14). First evident at 21 days, the differences are even more apparent 28 days post-injection compared to controls. In some cases, inflammatory cells persist within the alveolar septa, but more often the septa resemble those of a normal lung, without inflammatory infiltrate or are thickened from an inter-alveolar fibrotic process. Examining these areas under high magnification, we observed many examples of undeveloped alveolar septa, identified by their elastin caps (Fig 15). The fibrotic responses observed were generally spatially distinct from areas exhibiting an inhibition of alveolar development (Fig. 16).

The underdeveloped alveoli and localized fibrotic lesions that developed after TGF-β1 exposure are both hallmarks of BPD in newborns, and they were not seen after treating pups with the control virus. Thus, using replication deficient Adenovectors expressing constitutively active TGF-β1, we have successfully developed a pathologically accurate model of BPD that can be used in the search for therapeutic inhibitors of the disease.

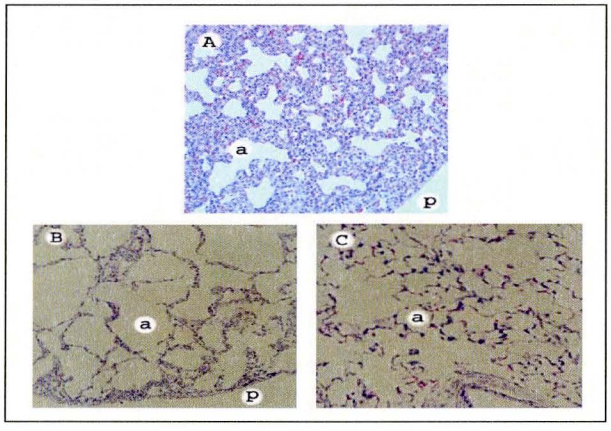

Figure 14. Neonatal rat lung untreated at 7 days old (panel A), 28 days (29 days old) after treatment with AdTGF-$\beta1^{223/225}$ (Panel B), and normal rat lung at 29 days old (Panel C). a, alveoli. p, pleural surface. All sections stained with H&E, and are shown at 100x magnification.

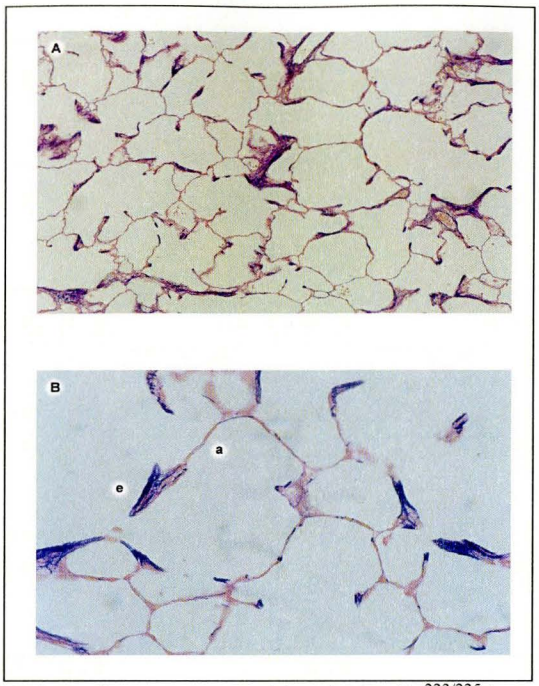

Figure 15. Neonatal rat lungs 28 days after treatment with AdTGF-$\beta 1^{223/225}$ have elastin caps, a characteristic of developing alveoli. A and B were stained with EvG. a, alveolar septation. e, elastin cap (stained dark purple). A, x100. B, x400.

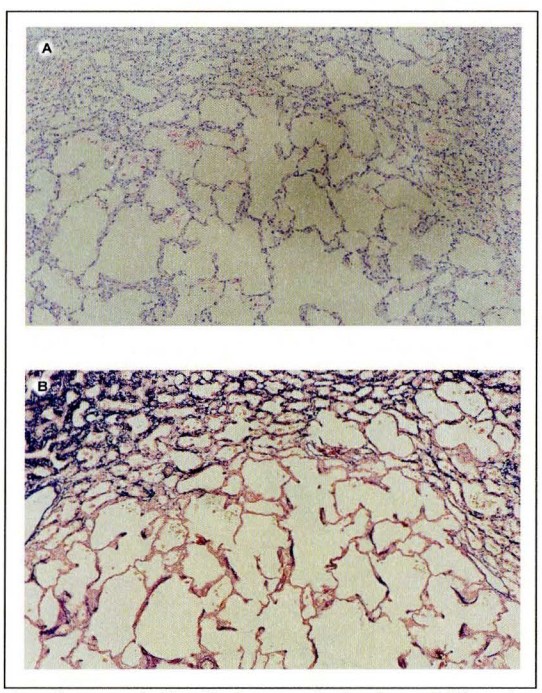

Figure 16. Neonatal rat lungs develop spatially distinct pathologies of fibrosis and inhibited alveolarization 28 days after treatment with AdTGF-$\beta 1^{223/225}$ A was stained with H&E, and shows the dense cellular infiltrate within the fibrotic area at the top. B is the same section stained with EvG (matrix stains dark purple) and shows the fibrotic area corresponds with the cellular infiltrate, yet is not associated with the underdeveloped alveoli. A and B 100x magnification.

TGF-β_1 Induced Lung Fibrosis on a SCID Background.

Balb/c mice injected i.n. with Adenovector expressing active TGF-β1 develop fibrotic lesions 14 days post-injection. The fibrosis progresses from the site of infection, the bronchial epithelium, occurring throughout the lung parenchyma and even affecting the pleural surface by 28 days (Fig. 17). Although the cellular infiltrate appears to have lessened after 28 days in the sections examined, the ECM that has been deposited throughout the parenchyma is evidence of a previously occurring fibrotic event. Balb/c SCID Beige mice injected with Ad5E1TGF-β1$^{223/225}$ show an equivalent level of inflammation, and exhibit identical histopathologic lesions as the Balb/c mice, with a similar cellular infiltrate 14 and 28 days after treatment (Fig. 18).

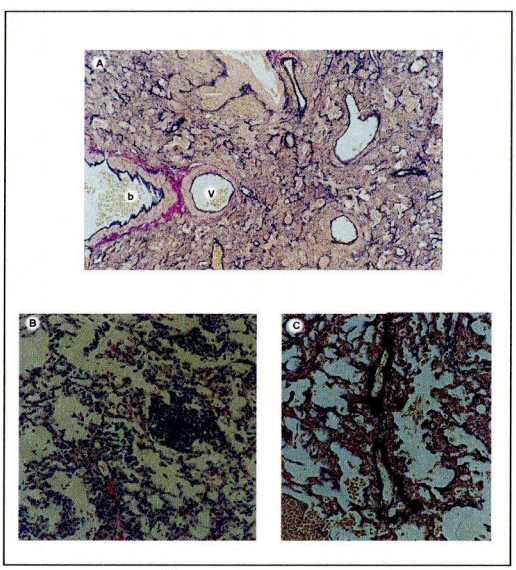

Figure 17 Effect of treating Balb/c mice i.n. with AdTGF-β1$^{223/225}$ The lungs after 14 (A) and 28 (B,C) days already show the hallmarks of a fibrotic response to TGF-β, with the deposition of ECM (A and C; EvG stains matrix pink and dark purple). B is an H&E stain. b, bronchial structure. v, vasculature. A, x100. B and C x320.

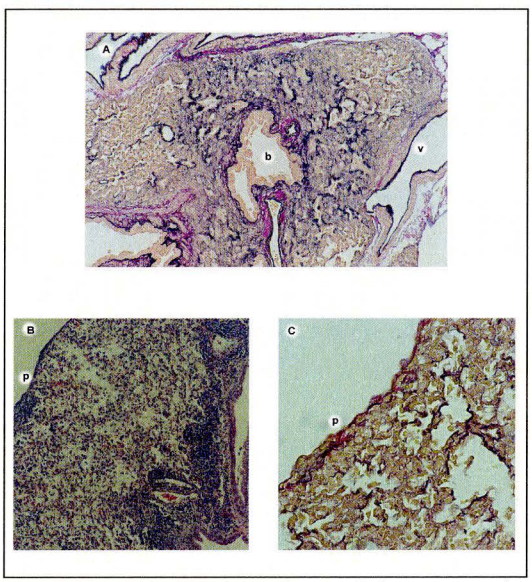

Figure 18. Effects of treating Balb/c SCID Beige mice i.n. with AdTGF-β1$^{223/225}$ Lungs after 14 (A) and 28 (B,C) days are undergoing a fibrotic response. A and C are stained with EvG, and demonstrate the excessive deposition of ECM (stained pink and dark purple) that occurs after treatment with with AdTGF-β1$^{223/225}$ B is an H&E stain. b, bronchial structure. v, vasculature. p, pleural surface. A and B x100. C x200.

The pathology that over expression of TGF-β1 causes is even more striking when compared to the histology of a normal lung (Fig. 19). Normal lungs have an extensive saccular network, with alveolar walls no more than a few cells thick that when combined with a generally low level of cellular infiltration, allow efficient gas exchange to occur

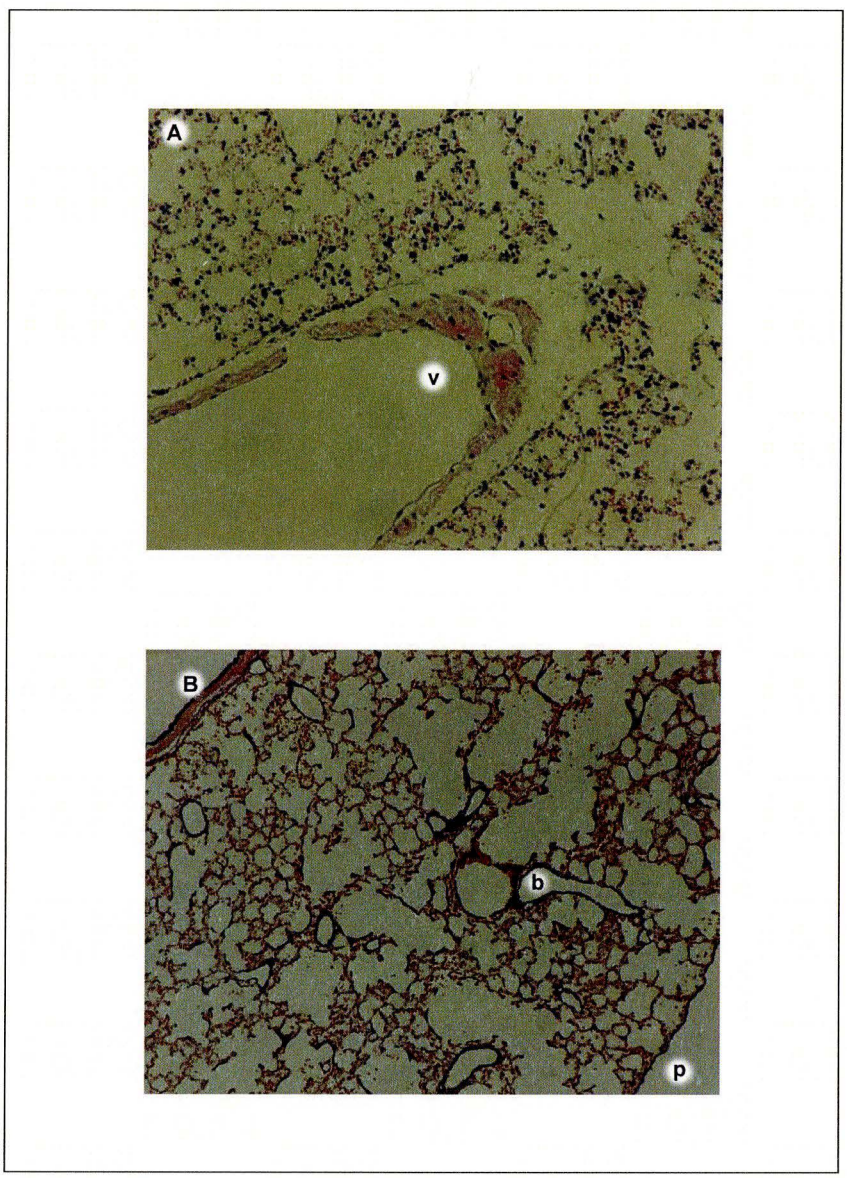

Figure 19. Histology of a normal lung from a Balb/c mouse, 6 to 8 weeks old. A, is an H&E stain demonstrating the absence of a cellular infiltrate. B is an EvG stain showing a lack of extracellular matrix throughout the lung interstitium, with the exception of that seen around the bronchial structures. b, bronchial structure. v, vasculature. p, pleural surface. A and B x100.

Hydroxyproline analysis of lungs from both strains of Balb/c mice shows an increase in extracellular matrix content after 14 days (Fig. 20).By 28 days hydroxyproline levels are still equivalent between the strains supporting the histological analysis (Fig. 21). Injection of control virus lacking a transgene (Ad5E1DL70-3) failed to create fibrotic lesions in either Balb/c or Balb/c SCID Beige mice. An inflammatory response to the Adenovector was observed 3 days after injection, but was cleared by 7 days. The Hydroxyproline content of mice injected with Ad5E1DL70-3 was significantly less than that in mice injected with Ad5E1TGF-$\beta 1^{223/225}$ at all time points measured, with a $p<0.05$.

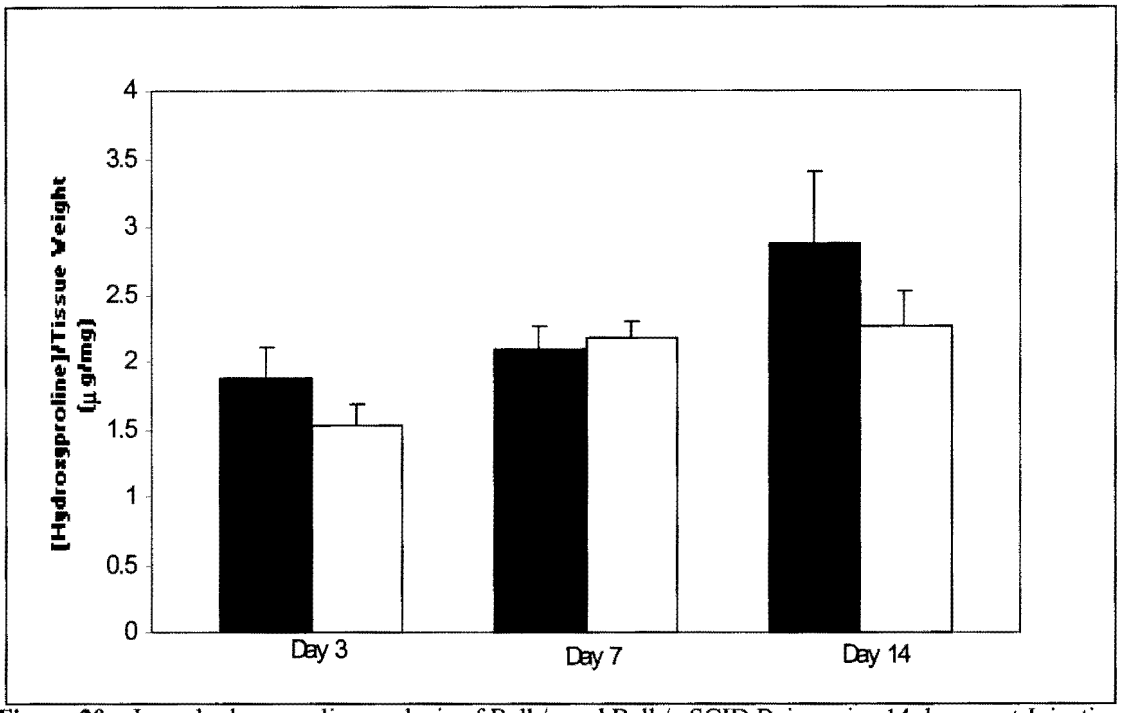

Figure 20. Lung hydroxyproline analysis of Balb/c and Balb/c SCID Beige mice 14 days post-Injection of Ad5E1TGF-$\beta1^{223.225}$. Balb/c (white bars) and Balb/c SCID Beige (black bars) were anaesthetized and injected i.n. with a viral dose of $5x10^8$ pfu in 20 µl PBS. After 3, 7, and 14 days, the right lobe was excised and frozen until the time of assay. Lung hydroxyproline content was determined, and expressed as µg of hydroxyproline per mg of wet tissue.

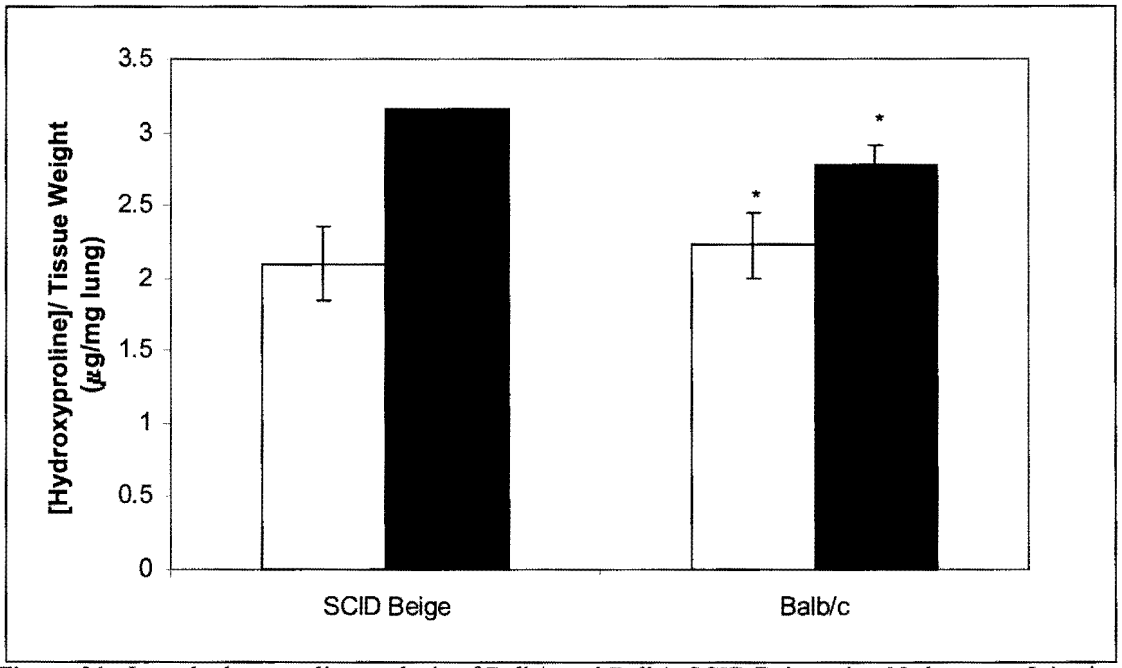

Figure 21. Lung hydroxyproline analysis of Balb/c and Balb/c SCID Beige mice 28 days post-Injection of Ad5E1TGF-$\beta1^{223.225}$ (black bars) or Ad5E1DL70-3 (white bars). Anaesthetized mice were injected i.n. with a viral dose of $5x10^8$ pfu in 20µl PBS. After 28 days the right lobe was excised and frozen until the time of assay. Lung hydroxyproline content was determined, and expressed as µg of hydroxyproline per mg of wet tissue. * $p<0.05$.

Production of an Adenovector Expressing Constitutively Active TGF-β3, Using the Cre-Lox Site-Specific Recombination System.

Dr. John Groffen provided the cDNA encoding constitutively active form of TGF-β3, with Cys223 and Cys225 both mutated to serines (TGF-$\beta3^{223/225}$), ligated within the pACCMV plasmid. Co-transfections using this construct with pBHG10.2 were unsuccessful. The co-transfections using these constructs resulted in the generation of 21 viral plaques, and amplification of the plaques and analysis of their DNA by Southern blotting revealed the presence of the TGF-$\beta3^{223/225}$ gene, although TGF-$\beta3^{223/225}$ mRNA was undetectable by Northern blot analysis (data not shown). Presumably, the virus was not efficiently transcribing the TGF-$\beta3^{223/225}$ transgene. It was then decided to excise the TGF-$\beta3^{223/225}$ cDNA using EcoRI and HindIII, then ligate it using the same sites into the multiple cloning site of the pDC104 shuttle plasmid, for use in the Cre/*loxP* system in conjunction with pBHGloxΔE1,3 (Fig. 22). DNA sequencing confirmed that the TGF-$\beta3^{223/225}$ transgene was ligated properly into the pDC104 shuttle plasmid (data not shown). Co-transfection of the recombinant pDC104.TGF$\beta3^{223/225}$ plasmid with pBHGloxΔE1,3 plasmid within 293 cells resulted in the production of Adenoviral plaques after 7 to 21 days. 6 plaques were picked and expanded in 293 cells, and northern blot analysis confirmed the ability of these 6 Adenovectors to produce detectable levels of TGF-$\beta3^{223/225}$ mRNA (Fig. 23). The nylon membrane stained with ethidium bromide does not show equivalent mRNA between the lanes. Despite there being more mRNA derived from AdDL70-3 treated cells than from any of the picks, the results are still conclusive, since there only appears to be background binding of the mRNA in that lane to the TGF-β3 cDNA probe.

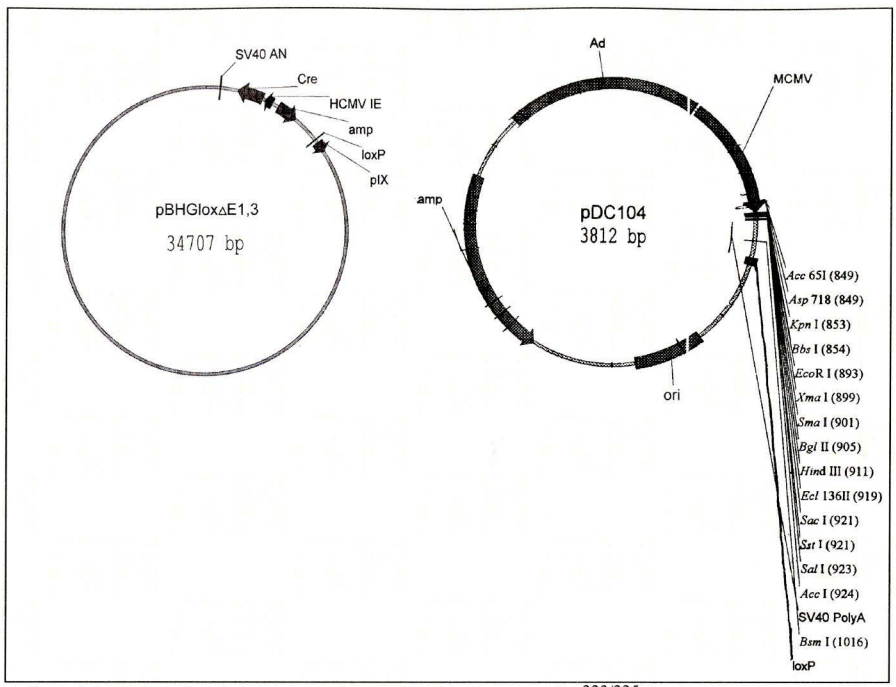

Figure 22. Plasmids used in the generation of Ad5E1TGF-β3$^{223/225}$.lox. pBHGloxΔE1,3 contains the majority of the Adenoviral genome (unlabelled sections), while pDC104 contains a small section (top) used for homologous recombination between it and the same sequence in pBHGloxΔE1,3. Once inserted into multiple cloning site of pDC104, the transgene is under control of a MCMV promoter Cre is produced by pBHGloxΔE1,3, and directs the site-specific recombination between the loxP sites on both plasmids. Both plasmids and their maps were kindly provided by the laboratory of Dr. Frank Graham, McMaster University

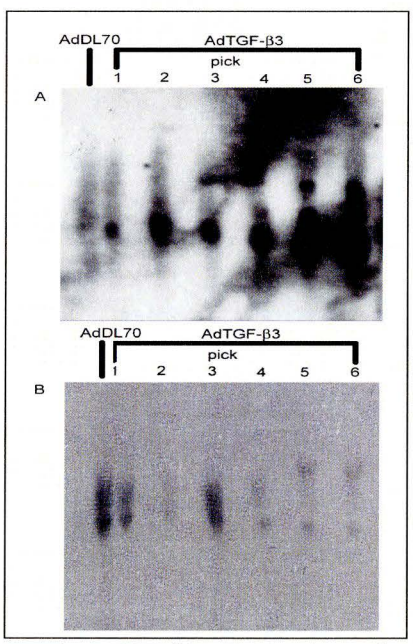

Figure 23. 6 AdTGF-β3$^{223/225}$.lox picks produce TGF-β3 mRNA. A, northern blot of mRNA isolated from cells infected with either AdDL70 or one of 6 AdTGF-β3$^{223/225}$.lox picks. B, nylon membrane after transfer showing ribosomal mRNA. The membrane was probed O/N, stringently washed, and Kodak X-AR film was exposed for 24 hours.

To ascertain whether the TGF-$\beta3^{223/225}$ mRNA was being translated into a bioactive product, a bioassay for TGF-β was performed on each of the 6 Adenovectors we had isolated. Supernatants from Ad5E1TGF-$\beta3^{223/225}$ infected murine fetal lung fibroblasts were added to the mink lung epithelial cells stably transfected with the Luciferase gene under control of the Plasminogen Activator Inhibitor-1 (PAI-1) promoter. While commonly used to assay TGF-β1 bioactivity, TGF-β3 has been previously shown to activate PAI-1 through this promoter as well (Dr. John Groffen, personal communication). All 6 Ad5E1TGF-$\beta3^{223/225}$.lox picks induced the production of Luciferase to levels significantly higher than those induced by Ad5E1DL70-3, yet lower than the levels seen after Ad5E1TGF-$\beta1^{223/225}$ stimulation (Fig. 24). After the large-scale isolation of Ad5E1TGF-$\beta3^{223/225}$.lox.6 was complete, the Luciferase assay was repeated using three 10-fold dilutions of the freshly prepared virus. Ad5E1TGF-$\beta3^{223/225}$.lox.6 induced the production of Luciferase in a dose-dependant manner (Fig. 25). This confirmed the successful production and bioactivity of the Ad5E1TGF-$\beta3^{223/225}$.lox.6 Adenovector.

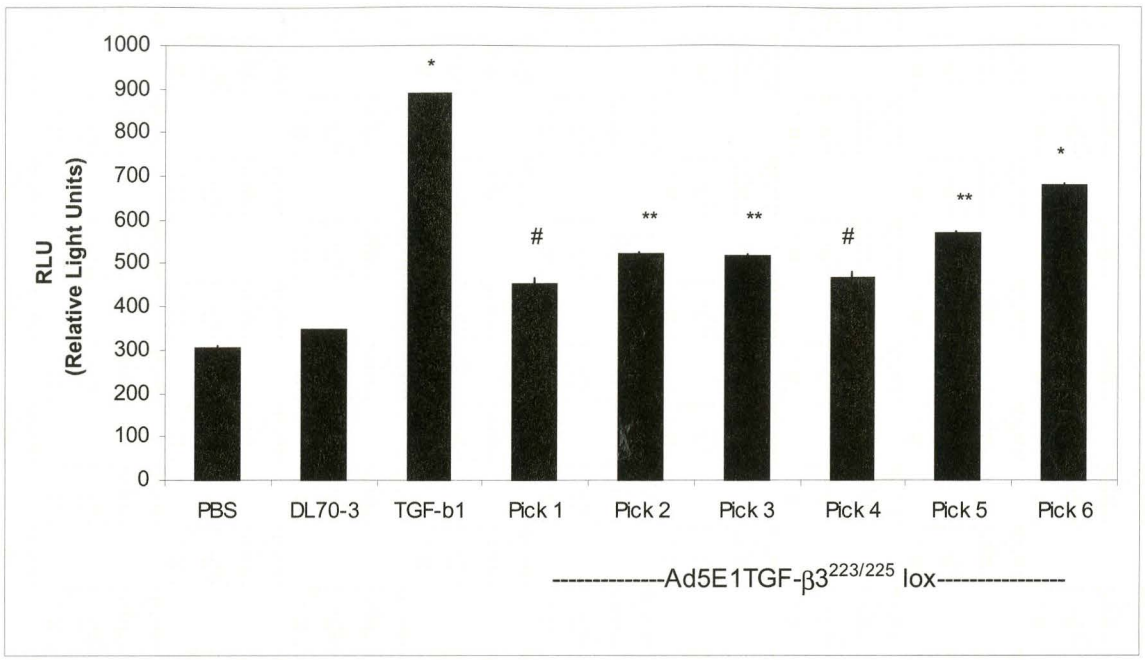

Figure 24. Bioassay with PAI-1 promoter, luciferase reporter cells to determine the activity of TGF-β3 expressed by 6 different Adenovector picks (Ad5E1TGF-$\beta3^{223/225}$.lox.1-6, 1ml). Positive control was TGF-β1 expressed by purified Adenovector (Ad5E1TGF-$\beta1^{223/225}$, moi=100), and negative control was Ad5E1DL-70-3 (moi=100) or PBS (1ml) untreated cells. Data are expressed as RLU *, $p<0.00024$ compared to Ad5E1DL70-3. **, $p<0.0028$ compared to Ad5E1DL70. #, $p<0.013$ compared to Ad5E1DL70.

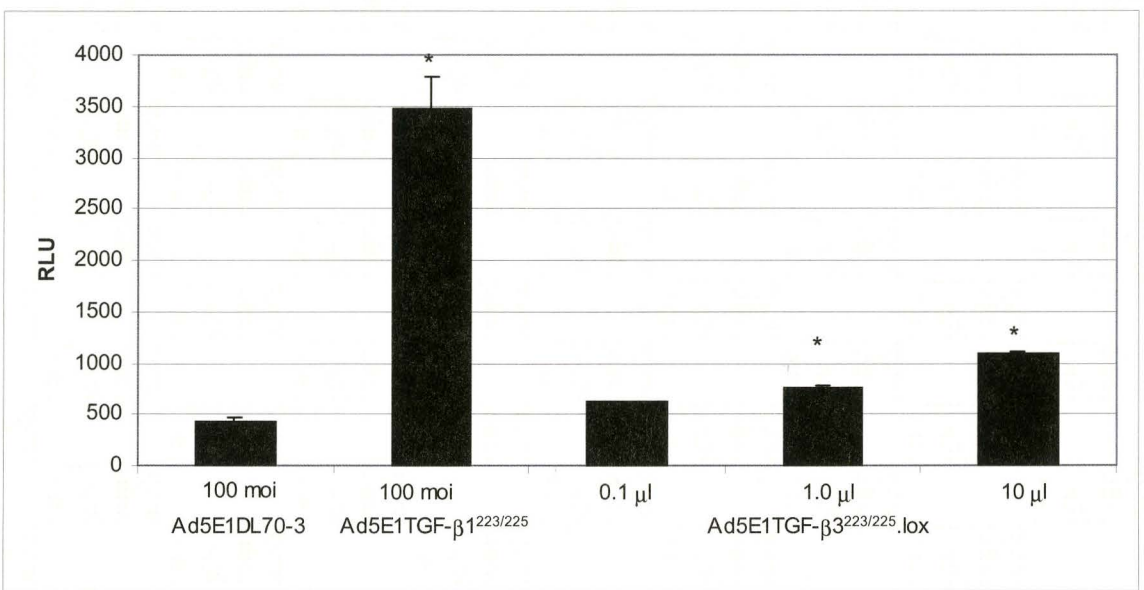

Figure 25. Bioassay with PAI-1 promoter, luciferase reporter cells to confirm activity of purified Ad5E1TGF-$\beta3^{223/225}$.lox.6 (Volume diluted in 100µl PBS). 100 moi of virus in 100µl PBS was used for the positive (Ad5E1TGF-$\beta1^{223/225}$) and negative (Ad5E1DL70-3) controls. Data are expressed in RLU *, $p< 0.05$ compared to AD5E1DL70-3.

DISCUSSION

Smad3 is required for TGF-β1-induced Pulmonary Fibrosis, and for Induction of CTGF mRNA through TGF-β1.

TGF-β1 signals through the Smad pathway to regulate its target genes (for a brief review, see [72]), whether during immune regulation, wound healing, or fibrosis. Presumably, TGF-β1 also utilizes the Smad pathway to activate CTGF, an inducer of fibroblast activation and proliferation acting downstream of TGF-β1, but until now this has never been proven. CTGF has been linked to pulmonary fibrosis, but its importance has not yet been described. The desire to extend our models of pulmonary fibrosis to include a role for CTGF prompted us to study the ability of TGF-β1 to induce fibrosis in mice devoid of Smad 3, using the Adenovector delivery system within an intranasally injected pulmonary fibrosis model.

We have previously shown that TGF-β1 delivered via Adenovector can cause pulmonary fibrosis in several rat and mouse strains (SCID studies, above; unpublished results; [13]), and we were successful in reproducing these results in wild-type mice within the current study. In studies of embryonic branching morphogenesis, the negative effects of TGF-β1 could only be abolished when both Smad2 and Smad3 gene expression was abrogated [73].

In the current study, a fibrotic response to exogenous TGF-β1 was completely abrogated in littermates homozygous for the mutated, nonfunctioning Smad3 gene. Like Smad3, Smad2 is a Class I Smad capable of transmitting signals from an activated TGF-β receptor complex. A role for Smad2 in signaling during a fibrotic response cannot be ruled out even after our study, since inactivating mutations in exogenously added Smad3

can prevent the activation of functional, endogenous Smad2 [60], and in our model using the Smad3 knockout mice, the mutant Smad3 gene product could be inhibiting activation of Smad2 in a similar manner. Smad7 is an intracellular inhibitor of TGF-β1 that when over expressed can reduce a fibrotic response [55], by preventing the phosphorylation of both Smad2 and Smad3 by the TGF-β receptor complex. Once activated, both are able to bind Smad4, transmitting a signal from the cytoplasm into the nucleus [49], and little work has been done to describe the role either Smad2 or Smad3 plays in fibrosis. In other systems, Smad2 has recently been shown to be induced in T lymphocytes, and interestingly, this occurs after either TGF-β1 stimulation or ligation of the T cell receptor complex, which traditionally has been known to signal through the mitogen activated protein (MAP) Kinase pathway. [74]. Other studies have also shown a link between the Smad and MAP Kinase pathways (for a review of the MAP Kinase pathway, see [75]). Studies have found that TGF-β1 binding to its receptor activates the MAPKK Kinase TAB1, which can then activate the MAPK Kinase TAK1 [76, 77]. TAK1 is in turn able to phosphorylate two different MAP kinases, ATF-2 and p38. ATF-2 binds Smad3/4 complexes, and the Smad and MAP kinase pathways are believed to be synergistic in the activation of ATF-2 [78, 79]. TAB1 is the earliest signaling molecule identified in the TGF-β1 induced MAP Kinase cascade to date, yet no studies have shown an association between it and the TGF-β1 receptor complex. Although more research is required, it seems that within T lymphocytes Smad2 may function as the bridge between the Smad and MAP Kinase Pathways, since both are induced upon T cell receptor ligation. Regardless of a potential role for Smad2, we can conclude from our studies that TGF-β1 requires functional Smad3 for intracellular signaling, and the loss of Smad3 is sufficient

to disrupt the development of pulmonary fibrosis in response to exogenously added TGF-β1.

Efforts to treat fibrotic diseases have in the past focused on TGF-β1 as a therapeutic target. Several extracellular inhibitors of TGF-β1 such as decorin and dominant-negative forms of the receptor have been successfully used by us and others to prevent or reduce the degree of pulmonary fibrosis within several model systems [80-82], while Smad7 acts at the intracellular level by associating with the TGF-β type II receptor to prevent signaling, and this has been shown to similarly reduce the degree of fibrosis in bleomycin models [53, 55]. The many roles TGF-β1 plays throughout development and in adults makes it a potentially dangerous cytokine to modulate, since altering its functionality within the context of wound healing likely has adverse effects on its ability to regulate other immune functions [14].

CTGF is an attractive alternative, since it mediates TGF-β induced activation of fibroblasts [83], and can replace TGF-β in activating fibroblasts and myofibroblasts *in vitro* [84], and does not share the anti-proliferative effects TGF-β1 has on lymphocytes and epithelial cells [18]. It is not yet clear if CTGF by itself is sufficient to cause pulmonary fibrosis, but it is produced during the fibrotic response [15, 85-88].

In the current study, we stimulated naïve lung stromal cells isolated from wild-type and Smad3 knockout mice with TGF-β1 expressing Adenovirus, and using RT-PCR show that Smad3 is required for the successful transcription of CTGF mRNA. This provides mounting evidence that the activation of CTGF by TGF-β1 is a critical step in the development of fibrosis, and preventing CTGF production is partially responsible for the amelioration of fibrosis that we observed in the Smad3 knockout mice. Abolishing

CTGF production is likely not sufficient to completely prevent fibrosis, since TGF-β1 can also activate fibroblasts directly [89]. CTGF is also a potent inducer of endothelial adhesion and migration [90], induces angiogenesis in adults [91, 92], and possibly during development [93], and may assist in the adhesion of activated platelets during thrombosis [94]. Angiogenic chemokines are up regulated during pulmonary fibrosis [95], and anti-angiogenic molecules can reduce the degree of fibrosis within the bleomycin model [96]. CTGF activation within a fibrotic response may not only induce ECM deposition, but as the above data suggests, it may also induce angiogenesis within affected areas, supplying them with a source of nutrients.

An Adenovector expressing CTGF can be used within the established model to determine if it is sufficient to induce fibrosis in the absence of functional TGF-β1 signaling. This will provide evidence for a role of CTGF in fibrosis, although more studies will need to be carried out to determine what role CTGF plays in fibrosis, be it through angiogenesis or ECM deposition or a combination thereof.

TGF-β1 is an Inducer of Bronchopulmonary Dysplasia in Newborn Rats.

Branching morphogenesis is an early stage of lung development where the respiratory epithelium invades surrounding mesodermal mesenchyme to form a primitive bronchial tree. Bronchial outgrowths continue to branch and develop throughout gestation, eventually forming the bronchial network of the lung (for a review, see [97]).

Upon birth, neonatal lungs resemble adult lungs in their external structure, with the lungs divided into discrete lobes, each networked by an almost fully developed bronchial tree. Internally however, the lung is not yet mature. Extensive alveolarization greatly increases the surface area available for gas exchange by dividing and sub-dividing the large, premature alveoli already formed. During this period of growth, the infant lung is particularly sensitive to perturbations in the developmental signals. Bronchopulmonary Dysplasia (BPD) is a neonatal disease caused by such an alteration, and is particularly prevalent in premature, often low birth weight newborns that are recovering from Respiratory Distress Syndrome (RDS). Infants suffering from BPD exhibit a dramatically reduced lung volume [98], lower levels of alveolarization [99], and much larger alveolar spaces [100] compared to normal infants. Chronic inflammation can lead to pulmonary fibrosis in mature lungs, and similarly BPD in infants may be caused by inflammation, illustrated by the positive correlation between the occurrence of BPD and high levels of the inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8 in amniotic fluid [101]. Similar inflammatory mediators are also found in neonatal lungs that have been hyperventilated, and infants exposed to excessive mechanical ventilation have high O_2 levels that can be correlated with an increased chance of developing BPD [102]. While high O_2 levels may directly contribute to BPD, it has been shown that over

inflating the lung during ventilation can lead to the production of pro-fibrotic cytokines such as bFGF and TGF-β_1 and extracellular matrix components [103] Combined, these data indicate that BPD begins with an inflammatory insult much like pulmonary fibrosis is initiated in adults, followed by the over expression of TGF-β1 and extracellular matrix components (Fig. 26). The early inflammation can be caused by either a hyperventilation of the lung with O_2, or by exposure of the lung to inflammatory mediators within the amniotic fluid, which may be present due to invading microbes within the mother [99, 101]

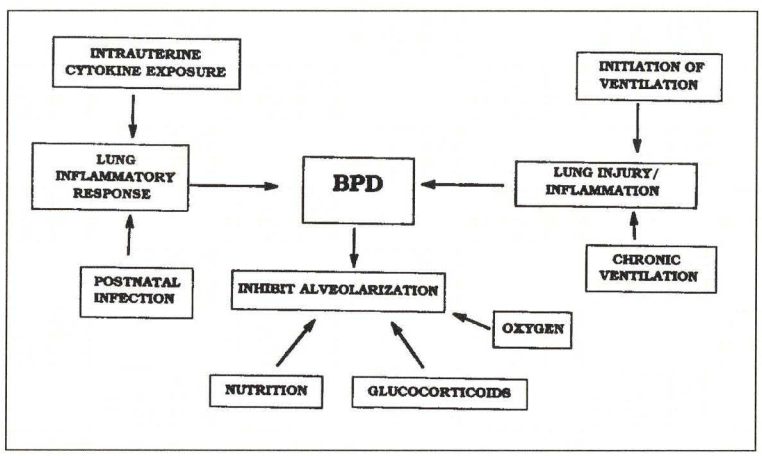

Figure 26. BPD can be initiated by an inflammatory response from a number of sources. It may begin at the embryonic stage by an exposure to cytokines or infectious agents residing in the mother's amniotic environment, or at the neonatal stage after excessive hyperventilation. Adapted from Jobe *et al.*, 1998.

To investigate the theory that over expressing TGF-β1 plays a role in BPD, we injected neonatal rats with Ad5E1TGF-$\beta1^{223/225}$, and compared their lung histology and collagen content with that seen in controls, either injected with Ad5E1DL70-3, or uninjected naïve neonatal lungs. TGF-β1 induced a pathology that closely mimics that seen in the human disease, and its delivery using the Adenovector system provides us with an excellent model to study the disease course.

In a study of 46 lung autopsy specimens from infants that suffered negative effects of RDS, Erickson *et al* (1987, [104]) defined two distinct pathologic features of BPD, separated into three groups. Patients in Group 1 have interstitial fibrosis, with extensive scar formation, and a marked inflammatory infiltrate. Group 1 BPD occurs in the very young infant (less than two months), and is often called early BPD. At the other end of the spectrum, seen in older infants (up to 9 months post birth) are Group 3 patients. The lungs of these infants don't show evidence of interstitial fibrosis, but their lungs have reduced numbers of alveolar spaces, resulting in fewer but much larger terminally branched air spaces. Group 2 BPD has features from both Group 1 and Group 3, with distal areas of the lung experiencing the different pathologies. The progression from interstitial fibrosis to large, underdeveloped airspaces with normal septa is believed to be the hallmark of the BPD disease course.

Normal rat lungs examined 4 days after birth demonstrate an incompletely developed lung with large airspaces and some interstitial cellular infiltrate. Evaluation of high-power fields for granulocytes characteristic of an inflammatory infiltrate failed to detect abnormally high numbers (data not shown), suggesting the infiltrate has a developmental function. Rats of an equivalent age that were injected with control Adenovector or Ad5E1TGF-$\beta 1^{223/225}$ have similar large alveolar spaces, but their lungs have a significantly greater cellular infiltrate. The lungs of rats treated were indistinguishable after only 3 days post-injection, and the reaction at this stage is largely in response to the virus injection. After 7 days (8 days old), the lungs treated with Ad5E1TGF-$\beta 1^{223/225}$ are even more occluded, and begin to resemble the lungs of infants with early BPD. Normal lungs, while still containing some infiltrate within the alveolar

septa, have been undergoing branching morphogenesis, and their alveolar spaces have successfully divided to a much greater extent.

Once naïve rats have reached 29 days old, their lungs are nearly completely developed, with the exception of some inflammatory foci. Rats injected with control virus had also recovered, and had normally developed lungs at this time, although some lung specimens did exhibit large interstitial infiltrates, that have probably persisted since the injection. It would not be unlikely that the small inflammatory insult caused by the Adenovector itself is enough to cause this limited pathology. Nonetheless, the hydroxyproline content is nearly identical between the two control groups and there is no histological evidence of fibrosis.

Neonatal rats injected with Ad5E1TGF-$\beta 1^{223/225}$ showed pathology closely resembling either mid-stage BPD (Fig. 27) or late stage BPD (Fig. 28) after 28 days post-injection (29 days old). Areas with interstitial fibrosis demonstrate marked matrix deposition, leading to a significantly higher hydroxyproline content. Often adjacent to the fibrotic areas are large, underdeveloped alveolar spaces surrounded by normal septa with only a limited infiltrate, characteristic of a more advanced stage of the disease progression.

At 29 days old, the rat lungs are still developing, and even the naïve infants were continuing their alveolar differentiation. Protruding alveolar septations end in elastin caps, which is a feature characteristic of budding septa (Dr. D. Warburton, personal communication). They seem to be equally prevalent in all groups, and show that the rats are not yet fully developed.

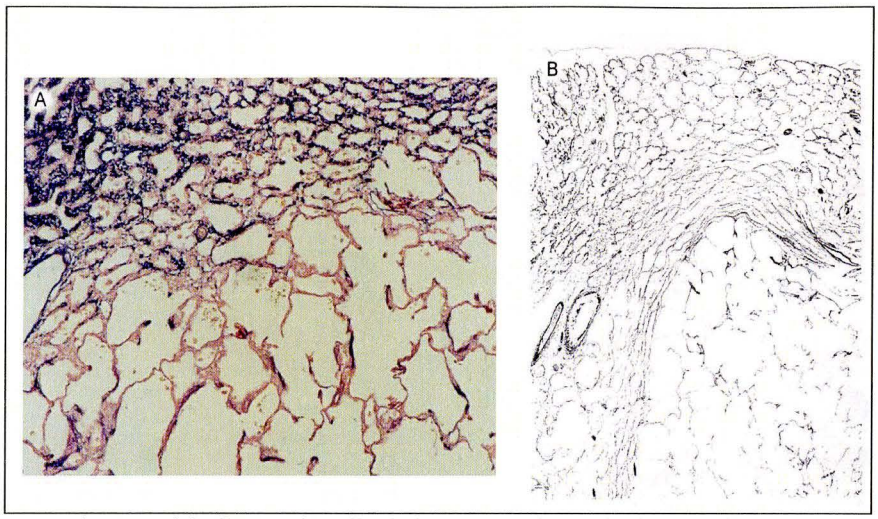

Figure 27 Neonatal rat model of BPD described above reproduces the human disease. A, an EvG stain of rat lung 28 days after treatment with AdTGF-$\beta 1^{223/225}$, demonstrating a combination of fibrosis (dark purple stain, upper portion) and an inhibition of alveolarization. B, a human infant 120 days old suffering from mid-stage BPD, characterized by the large air spaces surrounded by fibrotic tissue. A, 100x magnification. B, 20x. Panel B was adapted from Erickson *et al.*, 1987

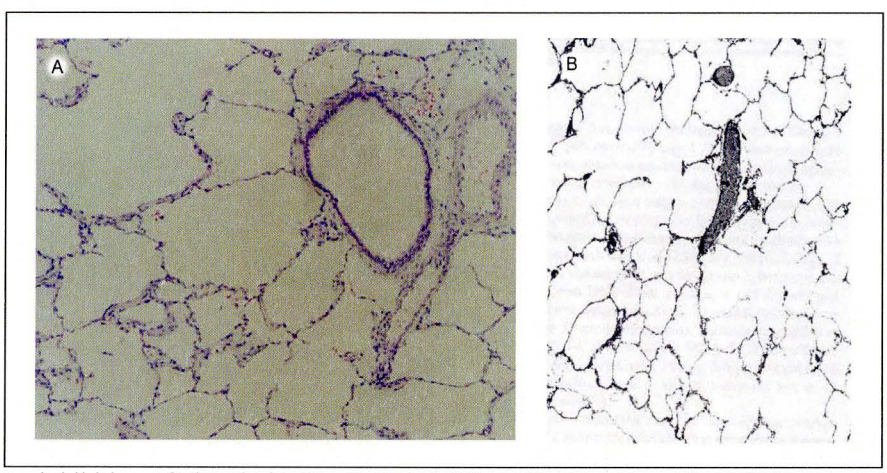

Figure 28. An inhibition of alveolarization is seen in the neonatal rat model of BPD described above. A, an H&E stain of rat lung 28 days after treatment with AdTGF-$\beta 1^{223/225}$ The alveolar spaces are abnormally large with a definite lack of any interstitial cellular infiltrate. Alveolarization is similarly inhibited in the human disease (B), shown here in the lungs of a 790 day old child with late stage BPD. A, 100x magnification. B, x50. Adapted from Erickson *et al.*, 1987

At the last time point examined, 28 days post-injection, the rats were progressing through mid or late stage BPD. The heterogeneity of the response in terms of the disease course would likely evolve over a longer time frame. It would be interesting to observe the final stage of the disease course at a later time point, and if the hydroxyproline levels decrease in accordance with the resolution of interstitial fibrosis, as predicted by Erickson *et al* (1987, [104]).

Our results show that TGF-β1 is a negative regulator of normal alveolarization, and it has been shown previously to act specifically on the embryonic bronchial epithelium to prevent branching morphogenesis [73] within embryonic lungs, possibly by negatively regulating the positive signaling through other growth factor receptors [105].

The Adenovector delivery of constitutively active TGF-β1 to neonatal lungs was successful in initiating a disease course that closely resembles Bronchopulmonary Dysplasia in newborns. The development of an efficient intranasal delivery method makes this a promising model to further evaluate the role of other growth factors in normal and altered lung development. Ideally, this model will be useful in the development of therapeutic strategies that lessen or prevent the negative effects of hyperventilation.

TGF-β_1 Induced Fibrosis Occurs in the Absence of a Lymphocytic Response

Many cytokines that characterize the inflammatory response have been shown to be proficient at initiating fibrosis when they are over expressed, or their inhibitors are under expressed. TNF-α is a pluripotent cytokine with potent pro-inflammatory roles [106] whose over expression can also induce fibrosis [5], apparently through its ability to instigate a robust inflammatory response [107]. IL-5 recruits eosinophils to the fibrotic site, and without their presence, the fibrotic response is greatly diminished in a bleomycin model of fibrosis [108]. Eosinophils are likely important for their ability to produce MCP-1 [8], which in turn activates monocytes and attracts them to the fibrotic area [109]. GM-CSF also attracts and induces the proliferation of monocytic cells and granulocytes [110], and its macrophage chemoattractant ability likely plays a primary role in animal fibrotic models and in human pulmonary fibrotic diseases [66, 111]. In light of the above data, the onset of pulmonary fibrosis may be attributed to chronic alterations in inflammatory responses

The pro-inflammatory cytokines are crucial for their abilities to recruit cellular factories for the production of TGF-β1. Invading eosinophils and macrophages are primary producers of TGF-β1 during a fibrotic response, along with resident stromal cells, such as the bronchial epithelium and fibroblasts/myofibroblasts [29, 111-113]. While pro-inflammatory cytokines are important for the early recruitment of cells to a wound healing or fibrotic site, the actions of TGF-β1 are essential for the development and prolongation of the fibrotic reaction, illustrated in animal models of bleomycin-induced fibrosis [5] and Adenovector expressing TGF-β1 induced fibrosis [13].

TGF-β1 has functions outside wound healing, and these include the regulation of adaptive immune responses through effects on both T and B lymphocytes [114, 115]. TGF-β1 delivered to rodent lung via replication deficient Adenovectors, combined with the numerous functions of TGF-β1 prompted issues around the validity of fibrotic models utilizing the Adenovector as a cytokine delivery vehicle [13]. Indeed, the possibility exists that adaptive immune responses may exacerbate fibrotic reactions, either by T lymphocytes producing TGF-β1 and other pro-fibrotic mediators directly, or by recruiting eosinophils or macrophages that will produce extra TGF-β1 [116]. The presence of immunogenic Adenovectors may affect the fibrotic outcome in such a manner. Adenovectors expressing active TGF-β1 are potent inducers of fibrosis, both in rats [13], and in mice (this study). To determine if this was caused in part by an adaptive T lymphocytic response, we also injected SCID Beige mice with Adenovectors expressing active TGF-β1. The absence of T lymphocytes, B lymphocytes, and NK cells did not affect the fibrotic outcome, when compared to immune-competent mice of the same strain (Balb/c). The onset of matrix deposition was similar for both strains, as they had statistically similar hydroxyproline levels early on. There was no difference in the lung hydroxyproline content between strains after 14 days, and remained so after 28 days post-injection. Both strains showed an increase over control virus injected mice after 28 days. Both strains had extensive infiltration of the lung parenchyma 3 days after injection, and the lack of functional lymphocytes did not appear to limit the early stage inflammatory response. T lymphocytes thus do not appear to play a role in TGF-β1 induced pulmonary fibrosis. These data confirm the validity and usefulness of the Adenovector-mediated delivery of cytokines to rodent lungs, permitting us to discount a

role of an adaptive immune response targeted to the Adenovector backbone within the model. More importantly, we can confidently argue that the fibrotic effects we observe are initiated solely by the over expression of TGF-β1 in the lung.

While T lymphocytes do not appear to have a role in the disease initiation, they may be involved in restoring tissues to homeostasis. Working on scleroderma, Shäffer *et al* (1998, [117]) has shown that during the course of injury repair, T cells are suppressed, and their activation altered. While it was suggested that re-instigating normal T cell function will help prevent excessive repair [117], it may be that the suppression on T cells is a bystander effect caused by the up regulation of TGF-β1 at the repair site. Consistent with this notion, after 28 days post-injection the presence of T lymphocytes in Balb/c mice did not lessen the degree of fibrosis when compared to the SCID Beige mice.

Production of an Adenovector Expressing Constitutively Active TGF-β3, Using the Cre/loxP Site-Specific Recombination System.

Evaluating the roles that individual cytokines play in biological processes allows us to draw more complete pictures of biological systems, be they developmental, immune, or disease related. Over expressing culprit cytokines is a powerful method for evaluating its role in a disease, especially when the site of expression is an important factor in the disease. Within the context of pulmonary fibrosis, efficiently delivering cytokines into the lung while avoiding their systemic over expression is paramount. The Adenovector delivery system allows us to obtain this organ specificity, while maintaining efficient transfer of the vector and high levels of transgene expression in the organ. We have previously generated a replication-deficient Adenovector expressing constitutively active TGF-β1, and used it to develop a model of TGF-β1 induced pulmonary fibrosis [13]). Given the tremendous success of the Adenovector-mediated approach in all the model systems where it has been used, and to broaden our understanding of the TGF-β family, we attempted to produce a replication-deficient Adenovector expressing constitutively active TGF-β3 for use in several of our models of fibrosis.

TGF-β3 is produced as a latent molecule in the same manner as TGF-β1 [14], and was made constitutively active by mutating the same cysteine residues to serine within the LAP that were mutated to produce constitutively active form of TGF-β1 (Cys223, 225Ser; Dr. John Groffen, personal communication).

After several unsuccessful attempts relying on *in vivo* homologous recombination between overlapping viral sequences in the transgene-containing shuttle plasmid and Adenoviral sequence containing plasmids within 293 cells to generate recombinant

Adenovectors, we switched to a Cre/loxP-based system. The Cre/loxP system relies on *in vivo* site-specific recombination to generate the Adenovector, greatly increasing the yield of recombinant Adenovirus over the homologous recombination method. Derived from bacteriophage P1, the Cre recombinase catalyzes recombination between two *loxP* sites [118]), one cloned 3' of the transgene within the shuttle plasmid (pDC104), and another within the plasmid containing the majority of the Adenoviral sequences (pBHGlox •E1,3). An Adenoviral sequence 5' of the transgene in pDC104 undergoes homologous recombination with the same sequence in pBHGlox •E1,3; the frequency of recombination is greatly increased since the sites are lined up after recombination at the *loxP* sites. The Cre recombinase was originally supplied by stably transfecting 293 cells [67], but it has since been cloned into pBHGlox •E1,3.

Once Adenoviral plaques were successfully isolated, we demonstrated that the transgene was being transcribed through northern blotting, and showed that functional TGF-β3 protein was biologically active using a PAI-1 reporter construct within a Luciferase assay. Traditionally, the PAI-1 bioassay has been used to evaluate the biological activity of TGF-β1 [80], but TGF-β3 is also capable of activating the PAI-1 reporter construct in this system (Dr. John Groffen, personal communication). TGF-β1 was able to stimulate more Luciferase activity than TGF-β3 in both assays conducted. TGF-β1 may be a more efficient activator of the PAI-1 promoter, but this can't be evaluated since the concentration of Ad5E1TGF-$\beta3^{223/225}$.lox was undetermined in either assay. Adenovectors are generally not titered until after they have been purified (Duncan Chong, personal communication).

TGF-β1 plays fundamental roles in immune regulation, development, and wound healing. TGF-β3 shares a high degree of homology with TGF-β1 [14], but whether it also functions in these processes is the topic of much debate in the scientific community. TGF-β3 exhibits both spatial and temporally distinct modes of expression during mouse embryogenesis [119], suggesting each plays a different role during development. Supporting this is *in vitro* work by Coker and colleagues (1997, [61]) on human fetal lung fibroblast cell lines showing differential induction levels of pro-collagen by the TGF-β isoforms. While TGF-β3 may stimulate collagen production within the fetal fibroblast, lung development throughout embryogenesis does not require TGF-β3 [120]. Differential expression characteristics have also been shown during epidermal wound healing in adult rodents [21]. Indeed, several groups have suggested that while TGF-β_1 serves to increase scar formation in healing epidermal wounds, TGF-β_3 actually serves to inhibit scar formation [19, 121]. While it is interesting to speculate that an inability of TGF-β_3 to override TGF-β_1 and -β_2 may be partially responsible for fibrosis, such distinct roles for the isoforms in pulmonary fibrosis have not been uncovered. Several groups have examined the characteristics of their expression during bleomycin-induced fibrosis, but have failed to uncover any roles played by TGF-β_2 or -β_3. Two independent studies have shown that TGF-β_2 and TGF-β_3 are expressed at equivalent levels, and within identical cell types after both bleomycin and saline instillation into rodent lung [20, 63]. Interestingly, these same studies both demonstrated that alveolar macrophages were primary producers of TGF-β_1 during the inflammatory phase, while bronchial epithelial cells were the main producers during the later, fibrotic stages. TGF-β1 increases the total collagen content in the fibrotic lung by increasing collagen production, decreasing the

production of collagenases, and increasing the production of collagenase inhibitors [14]). Recent work has found that both TGF-β1 and TGF-β3 stimulate lung fibroblasts equivalently to increase collagen production, decrease matrix metalloproteinase (MMP)-1 production, and increase tissue inhibitor of metalloproteinase (TIMP)-1 production [122].

The replication-deficient Adenovector expressing constitutively active TGF-β3 that we produced provides us with the perfect tool to study the functions of this growth factor in a number of model systems. The ability of TGF-β3 to induce pulmonary fibrosis in adults, or its potential roles in BPD can both be easily tested, and a model of scleroderma we have developed using the TGF-β1 expressing Adenovector (unpublished results) can easily be adapted for use with the vector expressing TGF-β3. These cases will help further refine our knowledge on the extracellular matrix inducing properties of TGF-β3, but using Ad5E1TGF-$\beta3^{223/225}$.lox, we can also examine the potential anti-fibrotic functions that several groups have suggested TGF-β3 may have. To accomplish this, it can be applied to the bleomycin model of pulmonary fibrosis in the same manner that Kolb *et al* (in print, [101]) used an Adenovector expressing decorin to demonstrate its anti-fibrotic effects. Adenovector can be applied topically to excisional skin wounds, or injected intradermally to study the effects TGF-β3 has on wound healing in the skin.

The uses of an Adenovector expressing constitutively active TGF-β3 will make this an extremely useful tool in our work to unravel the complexities of many biological processes, with studies on wound healing and fibrosis sure to benefit from this powerful vector of gene transfer.

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