NF-κB INTERACTS WITH SP3 TO LIMIT SP1-MEDIATED FGF-10 EXPRESSION IN THE DEVELOPING FETAL LUNG

By

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I dedicate this dissertation to my grandfather, Cecil.

He literally made the bricks on which I stand.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BMP4	Bone Morphogenetic Protein 4
BPD	Bronchopulmonary Dysplasia
BSA	Bovine Serum Album
ChIP	Chromatin Immunoprecipitation
CHO	Chinese Hamster Ovary
Chorio	Chorioamnionitis
cIKKβ	Constitutively Active IKKβ Mutant
COPD	Chronic Obstructive Pulmonary Disease
DAPI	4',6-Diamidino-2-Phenylindole
DNA	Deoxyribonucleic acid
dnIκB	Dominant-Negative Isoform of IκB
DTT	Dithiothreitol
E	Mouse Embryonic Day
Ecl	Eclampsia
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FGF-10	Fibroblast Growth Factor-10
FGF-9	Fibroblast Growth Factor-9
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GC box	Guanosine/Cytosine Box
GFP	Green Fluorescent Protein
HIP	Hedgehog Interacting Protein

HSPG	Heparin Sulfate Proteoglycans
IKKβ	IκB Kinase β
IL-1β	Interleukin-1β
IL-6	Interleukin-6
LPS	Lipopolysaccharide
MAPK	Mitogen Activated Protein Kinase
MCF-7	Michigan Cancer Foundation – 7
MCP-1	Monocyte Chemoattractant Protein 1
MIP1α	Macrophage Inflammatory Protein 1α
MMLV	Moloney Murine Leukemia Virus
mRNA	Messenger Ribonucleic Acid
NF-κB	Nuclear Factor κB
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PLA	Proximity Ligation Assay.
PVDF	Polyvinylidene Fluoride
RNA	Ribonucleic Acid
RTK	Receptor Tyrosine Kinase
SHH	Sonic Hedgehog
Sp1	Specificity Protein 1
Sp3	Specificity Protein 2
Sprty	Sprouty
TGFβ	Transforming Growth Factor β
TLR4	Toll-Like Receptor 4
TNFα	Tumor Necrosis Factor-α
UTR	Untranslated Regions

CHAPTER I

Introduction

Overview

The mammalian lung is a highly branched structure that facilitates gas exchange between the atmosphere and cardiovascular system. Proper development and function are required for life outside the womb. Children who are born early in gestation are at a greater risk of developing bronchopulmonary dysplasia (BPD), a disease that is characterized by large, dilated airways with thickened walls associated with decreased branching morphogenesis. This altered morphology is consistent with decreased surface area and capacity for gas exchange, and explains the respiratory distress experienced by infants with BPD. Beyond prematurity, chorioamnionitis is the most frequently-mentioned risk factor associated with BPD. The association of BPD with infected, inflamed fetal membranes potentially indicates a mechanism. The goal of this work is to assign a molecular mechanism to the BPD phenotype and gain a better understanding of how extracellular, pro-inflammatory signaling events might interact with the developmental program of the branching lung and distort the overall pattern of development.

Many genes have been implicated in facilitating the highly coordinated branching of the lung epithelium, but the FGF-10/FGFR2b signaling axis is potentially the most important pro-branching signaling event. An examination of fetal lung tissue from children with healthy lungs and children with BPD reveals less FGF-10 expression in the BPD-affected lungs. Loss of FGF-10 expression would explain the decreased epithelial complexity associated with BPD. Because bacterial endotoxin is a primary mediator in infection and inflammation, lung mesenchymal cells were treated with LPS to assay its effects on FGF-10 expression. LPS, along with other cytokines signaling through the NF-κB pathway, lowered FGF-10 expression. As deletion of the sole predicted NF-κB consensus sequence in the promoter fragment analyzed did not alter the ability of NF-κB to downregulate FGF-10, the mechanism appeared indirect. Further in silico analysis indicated that the FGF-10 promoter contains several conserved GC boxes, sequences associated with regulation by transcription factors of the Sp family. ChIP analysis revealed that Sp1 constitutively occupies the FGF-10 promoter, while Sp3 and RelA are enriched at the promoter after LPS treatment. FGF-10 reporter analysis indicated that Sp1 enhances FGF-10 expression, while Sp3 and NF-κB additively oppose this effect; some basal threshold level of NF-κB is absolutely required for this effect. In situ proximity ligation assays reveal that Sp3 and the NF-κB dimer interact constitutively, but that activating NF-κB heightens this interaction. These data strongly indicate that under basal conditions, Sp1 occupation of the FGF-10 promoter activates expression of the pro-branching growth factor, while inflammatory signaling through NF-κB

stimulates an interaction between NF-κB and Sp3 that downregulates FGF-10 and halts further lung branching.

While Sp1, Sp3, and NF-κB have been well-characterized in their roles as individual regulators of gene transcription, their interaction and subsequent co-regulatory impact on FGF-10 is unique. There is no other published example of NF-κB and Sp3 interacting to downregulate Sp1-mediated gene expression. Indeed, NF-κB is almost universally understood to activate transcription. The mechanism proposed here could inform regulatory studies on developmental genes as well as the small subset of genes known to be downregulated by NF-κB activation. The relative ubiquity of Sp1, Sp3, and NF-κB expression strongly suggests that this mechanism could be responsible for regulating other genes in other tissues.

Diseases such as BPD disrupt normal airway morphogenesis and negatively affect epithelial function in the distal lung. Targeting master regulatory factors such as Sp1, Sp3, and NF-κB could restore FGF-10 to normal levels and protect the lungs from further damage associated with inflammation and infection, and aid in repair. This is especially true in light of the dual functions attributed to FGF-10 in regulating branching morphogenesis and guiding epithelial cell differentiation preferentially towards an alveolar cell fate. Treatments geared towards reestablishing normal FGF-10 levels would have the added benefit of preventing lung dysmorphia associated with excessive RTK signaling, such as improper branching and goblet cell dysplasia. Knowledge that Sp1, Sp3, and NF-κB could regulate other genes also presents the possibility

that any treatment geared towards their regulation could also be applied to other illnesses.

Development of the Mammalian Lung

The mammalian lung is a highly branched organ required for gas exchange between the cardiovascular system and the surrounding atmosphere. The mature organ is comprised of an endodermally-derived, highly complex epithelium surrounded by remnants of a mesodermally-derived mesenchymal compartment (Morrisey and Hogan 2010). A complex vascular network is closely associated with the highly specialized alveolar cells of the epithelium, allowing for more efficient gas exchange (Stenmark and Abman 2005). The highly distinct three-dimensional structure of the lung provides an intriguing model for complex organogenesis. Several distinct cell types must interact in a highly coordinated fashion for the vasculature and epithelium to develop in such a way that gas exchange can efficiently occur in the mature lung.

Lung development begins at embryonal day nine (E9) in mice (Kimura and Deutsch 2007) (Table 1.1). At this stage, two distinct domains develop in the anterior endoderm: a dorsal Sox2-expressing region and an Nkx 2.1 (also known as TTF1)-expressing domain in the ventral anterior endoderm. The Sox2 and Nkx 2.1 domains "split," forming two endodermally-derived tubes with separate lumina. The Sox2 domain forms the esophagus, while the Nkx 2.1-expressing domain forms the emerging trachea and lungs (Que, Luo et al. 2009). At E9.5,

dual evaginations emerge from the ventral anterior endoderm and begin to branch into the surrounding mesenchymal tissue. This marks the beginning of the pseudoglandular phase of lung development (Morrisey and Hogan 2010). The primary bronchi and major conducting airways are formed during the pseudoglandular phase. Interestingly, the branching process of the lung at this stage is incredibly stereotypical. Ordered branch points and relative geometry can be positively predicted and timed within congenic mouse strains (Metzger and Krasnow 1999). This remarkable phenomenon indicates that not only are the physiological processes governing lung branching morphogenesis tightly regulated, they are also highly regulated and conserved at a genetic level.

The pseudoglandular period lasts until approximately E16.5 in the mouse, and is followed by the canalicular, saccular, and alveolar stages of development (Warburton, El-Hashash et al. 2010). The canalicular phase is marked by expansion of bronchial lumina and development of the capillary network closely associated with the airway epithelium. Once the primary epithelial tree is complete and the associated vascular network has properly developed, the distal airways begin the process of saccularization. This saccular stage of development begins at E17 and continues until shortly after birth at postnatal day five (P5). During this phase, the terminal epithelium begins to thin and fenestrate, and alveolar cells terminally differentiate. The alveoli are comprised primarily of two kinds of cells, type I cells and type II cells (Joshi and Kotecha 2007; Warburton, El-Hashash et al. 2010). Most of the surface area of the alveoli is occupied by large, flat type I cells, which are primarily responsible for

exchanging O_2 and CO_2. The smaller but more numerous type II cells are responsible for secreting factors that aid in gas exchange, such as surfactant proteins (Herzog, Brody et al. 2008). Type II cells are also thought to serve as resident stem cells that can repopulate the alveoli in the event of injury (Fehrenbach 2001). These changes in lung anatomy during the saccular stage allow them to adequately exchange gas to support life. Importantly, it is during the saccular stage that human fetuses become viable if preterm labor occurs. Finally, alveolar septation occurs during the alveolar stage of development. Terminal saccules septate to become mature alveoli during this stage of development, which occurs largely postnatally in humans and almost entirely postnatally in mice (Warburton, El-Hashash et al. 2010).

Stage	Human Gestation (Deutsch and Pinar 2002)	Mouse Gestation (Warburton, El-Hashash et al. 2010)	Description
Embryonic	3 – 7 Weeks	E8.5 – 9.5	Initial specification of the lung anlagen in the endoderm. Initial budding of the primary bronchi.
Pseudoglandular	7 – 16 Weeks	E9.5 – 16.6	Stereotypical branching of the conducting airways and terminal bronchioles. Cartilage formation occurs in the major airways.
Canalicular	16 – 24 Weeks	E16.6 – 17.4	Appearance of pulmonary acini – prospective units of gas exchange. Development of the capillary network adjacent to the pulmonary acini. Early differentiation of alveolar cells type II cells.
Saccular	24 – 36 Weeks	E17.4 – P5	“Saccules” for, which represent future terminal airways and alveoli. Mesenchymal tissue compresses to form interstitium, while epithelium thins to support gas exchange.
Alveolar	36 Weeks - Infancy	P5 – 30	Epithelial cells in the terminal saccules coordinate with endothelial cells to septate into distinct alveoli.

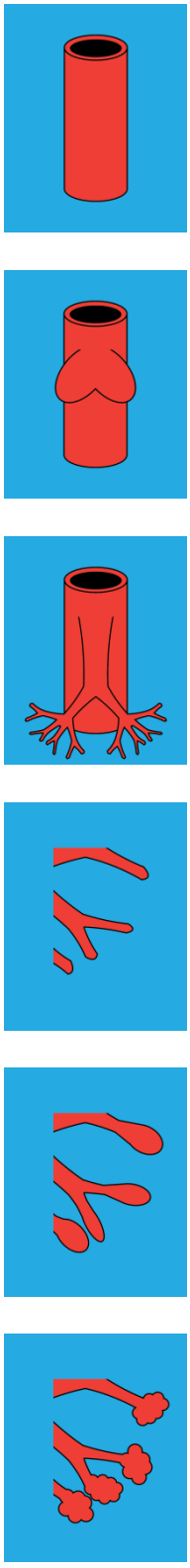


Table 1.1. Overview of key stages in lung development.

Molecular Signaling Associated with Lung Branching Morphogenesis

Much of the signaling required for proper lung development has been previously described (Warburton, Schwarz et al. 2000). In order to produce functional lungs capable of mediating gas exchange, highly regulated reciprocal signaling between the epithelium and mesenchyme dictates which subdomains of the epithelium should branch, and which should not (Hogan and Yingling 1998) (Figure 1.1). The highly systematic and predictable nature of early lung branching morphogenesis indicates how precise this interplay must be. Perturbations in known signaling pathways associated with lung branching morphogenesis further reveal that even slightly altering expression of these morphogens can have deleterious effects on respiratory anatomy and physiology (Litingtung, Lei et al. 1998; Min, Danilenko et al. 1998; Goss, Tian et al. 2009; Wang, Deimling et al. 2011).

Signaling events can be divided into roughly two categories: pro-branching and anti-branching. During much of the branching process, bifurcation at the termini of nascent airways is the primary means of expanding and complicating the epithelium (Menshykau, Kraemer et al. 2012; Schnatwinkel and Niswander 2013). Signaling by the mesenchymally-derived growth factor FGF-10 through the epithelially-expressed receptor FGFR2b is among the best understood and most important factors in lung budding, outgrowth, and branching (Bellusci, Grindley et al. 1997; Park, Miranda et al. 1998). FGF-10 is a member of the fibroblast growth factor family of growth factors. These molecules play important roles in many different physiological processes, and are key in the development

of several different tissues (Goldfarb 1996). FGF-10 in particular has been implicated in normal branching morphogenesis of the lung, development of the thyroid, salivary, and pituitary glands, limb bud outgrowth, and in wound healing (Xu, Weinstein et al. 1999; Ohuchi, Hori et al. 2000; Braun, auf dem Keller et al. 2004). FGF-10 activates the FGFR2b receptor tyrosine kinase, activating a downstream mélange of signaling pathways including MAP kinase, PI3 Kinase, PLCγ and others typical of tyrosine kinase signaling. FGF-10 signaling is an extremely localized event owing to the affinity FGF-10 ligand has for heparin sulfate proteoglycans (HSPGs) in the extracellular matrix. This interaction is thought to limit the diffusion potential of FGF-10, so that only the branching tips respond (Luo, Ye et al. 2006; Makarenkova, Hoffman et al. 2009). FGF-7, a closely related factor which binds HSPGs with less affinity than FGF-10, elicits a very different response from explants lung buds than FGF-10: proliferation and expansion of the epithelium instead of outgrowth and branching (Bellusci, Grindley et al. 1997). Fascinatingly, switching the HSPG binding domains of FGF-10 and FGF-7 converts FGF-7 into an FGF-10 mimetic, precisely replicating the effects of FGF-10 on pulmonary epithelium (Makarenkova, Hoffman et al. 2009). This indicates that spatial distribution is crucial for FGF-10 to mediate proper lung development, and that the extracellular matrix plays a role beyond physical support in directing lung branching.

The importance of FGF-10 signaling for proper lung development has been demonstrated in murine models null for the ligand or receptor. Attenuation of either FGF-10 or FGFR2b severely disrupts lung bud branching and proper

cell differentiation, resulting in poorly developed lung anlagen arrested immediately after budding (Min, Danilenko et al. 1998; Sekine, Ohuchi et al. 1999; De Moerlooze, Spencer-Dene et al. 2000; Fairbanks, Kanard et al. 2004; Fairbanks, Kanard et al. 2005). Other FGF ligands typically associated with tissue growth and migration are also expressed in the lung mesenchyme, including FGF-2, FGF-7, and FGF-9 (among others) (Buch, Han et al. 1995; Charafeddine, D'Angio et al. 1999; White, Xu et al. 2006; White, Lavine et al. 2007). While these ligands have not been expressly implicated in branching morphogenesis of the epithelium, they are thought to play a role in proliferation and survival of epithelial cells in the developing lung.

To prevent development of inappropriate lateral branching of the “trunk” of the branching airway, several pathways are employed to work against ectopic expression and response to FGF-10. Three of the most well characterized “anti-branching” signaling pathways utilized in the developing lung are Bone Morphogenetic Protein 4 (BMP4), Sonic Hedgehog (SHH), and Transforming Growth Factor β (TGFβ) signaling (Serra, Pelton et al. 1994; Hyatt, Shangguan et al. 2002; Chuang and McMahon 2003). BMPs are ligands within TGFβ superfamily that signaling through their own series of receptors and SMADs; BMP4 specifically signals through the BMPR1 in the lung. BMP4 expression is dynamic in the branching lung epithelium, and is apparently stimulated by FGF-10 signaling from the abutting mesenchyme (Weaver, Dunn et al. 2000; Gleghorn, Kwak et al. 2012). This feedback system is thought to be important to temporally limit the epithelial response to FGF-10, preventing excessive

outgrowth of new branches. SHH is expressed throughout the pulmonary epithelium, and generally acts to decrease FGF-10 expression in the mesenchyme (Bitgood and McMahon 1995; Urase, Mukasa et al. 1996). Expression of Hedgehog-Interacting Protein (HIP1) at the branching tips of the growing epithelium significantly decreases available SHH ligand in the immediate surroundings, allowing expression of FGF-10 in those loci (Chuang, Kawcak et al. 2003). SHH signaling persists in the non-branching areas of the epithelium, preventing FGF-10 in those localities and thus limiting ectopic branching. Finally, epithelial expression of TGFβ1 and TGFβ2 activates TGFβR2 in the mesenchyme, decreasing FGF-10 expression and stimulating mesenchymal cells to secrete extracellular matrix (ECM) components along the non-branching component of the epithelium (Heine, Munoz et al. 1990; Serra, Pelton et al. 1994; Gleghorn, Kwak et al. 2012). This ECM could physically inhibit inappropriate branching, and might also serve as a sink for excess growth factors (especially FGF-10 which binds tightly to heparin sulfate proteoglycans in the ECM) (Izvolsky, Shoykhet et al. 2003; Izvolsky, Zhong et al. 2003).

Finally, signaling through FGFR2b activates a series of intracellular feedback mechanisms to prevent excessive signaling. One of the most well-studied groups of intracellular repressors of FGF signaling are the Sprouty proteins. Sprouty proteins work to dampen signaling from FGF receptors and other RTKs through mechanisms that are not well understood, though several potential pathways have been proposed. For instance, Sprouty proteins are known to bind to proteins that bind to active RTKs and recruit signaling

molecules, interrupting the downstream signaling cascade (Gross, Bassit et al. 2001). Another proposed mechanism suggests that Sprouty proteins can stimulate release of secondary secreted molecules that interrupt signaling at the ligand/receptor level (Glienke, Fenten et al. 2000). Sprouty 2 and Sprouty 4 are both expressed in epithelial tissue in the expanding lung, often directly adjacent to areas of high FGF-10 expression (Mailleux, Tefft et al. 2001). Tightly controlled expression of these genes is required for proper lung morphogenesis. Increased Sprouty 2 expression in the lung epithelium, through either adenovirus or transgenic allele, results in smaller lungs and decreased branching (Mailleux, Tefft et al. 2001). Blocking Sprouty 2 with antisense oligonucleotides causes increased branching and eventual lung hyperplasia (Tefft, Lee et al. 1999). Overexpressing Sprouty 4 also leads to smaller lungs and epithelium, but no difference in lung size is noted in the lungs of mice null for Sprouty 4 (Perl, Hokuto et al. 2003; Taniguchi, Ayada et al. 2007). The Drosophila homologue, Sprouty, plays a integral role in stimulating bifurcation and branching of the fly endotracheal system, and it has been suggested that Sprouty 2 and 4 could play similar roles in the mammalian lung (in addition to other branches organs) (Hogan 1999; Metzger and Krasnow 1999).

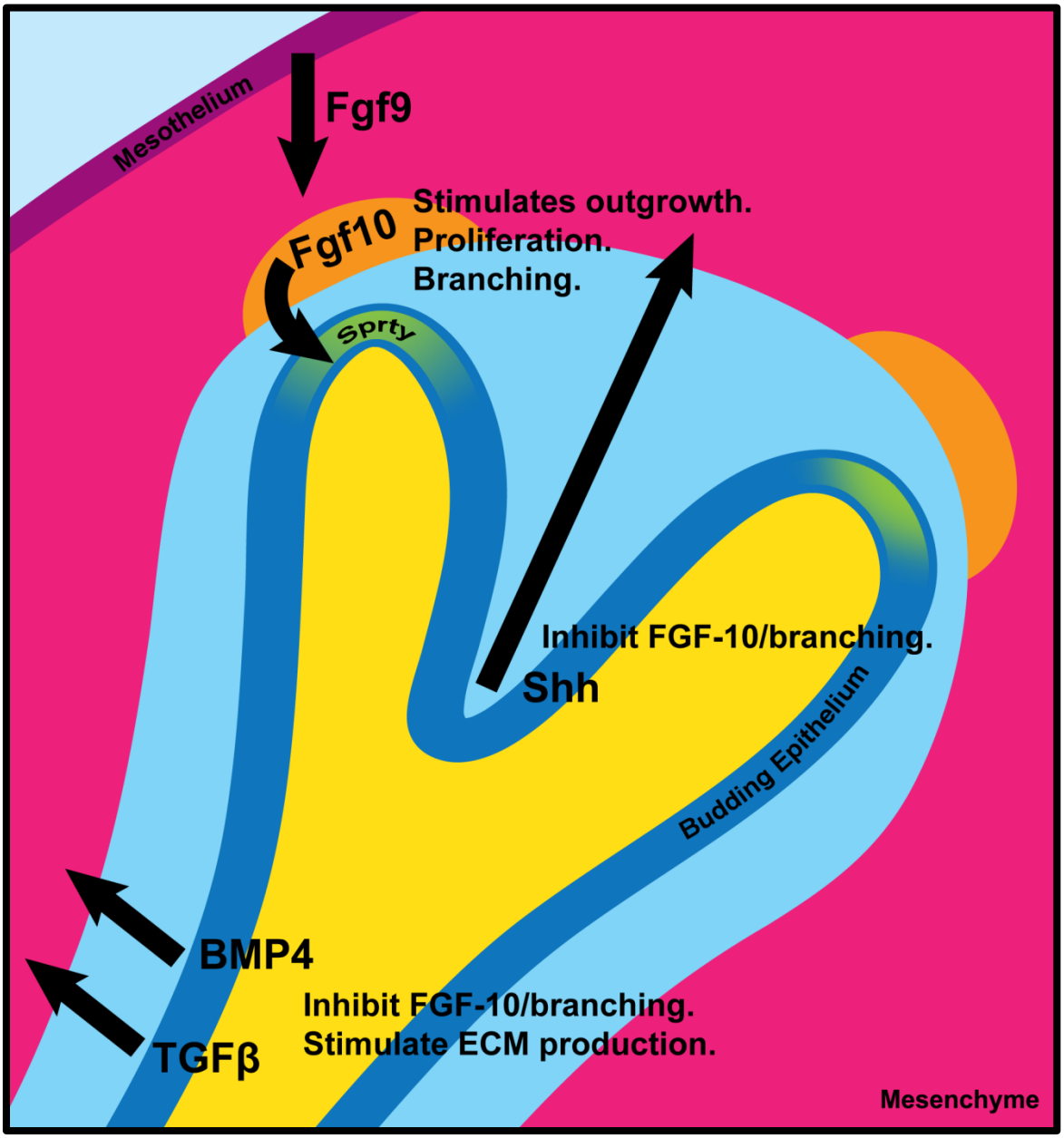

Mesothelium
Fgf9
Fgf10
Stimulates outgrowth.
Proliferation.
Branching.
Sprty
Inhibit FGF-10/branching.
Shh
Budding Epithelium
BMP4
TGFβ
Inhibit FGF-10/branching.
Stimulate ECM production.
Mesenchyme

Figure 1.1. Factors associated with lung branching morphogenesis. Signaling between the expanding epithelium and surrounding mesenchyme leads to growth and branching of the growing pulmonary tree. Pro-branching growth factors, including FGF-10, FGF-7, FGF-9, and Wnt2 and Wnt2b (not depicted) expressed by the mesenchyme stimulate outgrowth and proliferation in the epithelium. Anti-branching factors in the non-branching epithelial “trunk” such as SHH, BMP4 and TGFβ1/2 downregulate expression of pro-branching factors in the adjacent mesenchyme, restricting branching points to the terminal bifurcations and domain branching of the epithelium. Receptor tyrosine kinase (RTKs) activity also activates anti-RTK Sprouty 2 and Sprouty 4 in the epithelium, dampening RTK signaling, preventing excessive growth and proliferation.

Sp Family of Transcription Factors

The Sp family of transcription factors is comprised of at least nine separate genes in humans, designated Sp1 – Sp9. The Sp family is part of a larger superfamily of zinc finger transcription factors, the Sp/KLF family. The Sp/KLF family of transcription factors exist in various forms in *Drosophila sp.* (nine genes), *C. elegans* (six genes), and mammals (25 genes in humans) (Suske, Bruford et al. 2005). They are unified by their zinc finger DNA-binding domains, which are 81 amino acids in length and show considerable similarities in sequence binding. In Sp proteins this sequence motif is called a GC-box, and consists of a conserved sequence of GC-rich DNA (5'-(G/T)GGGCGG(G/A)(G/A)(C/T)-3') (Hagen, Muller et al. 1992; Sogawa, Kikuchi et al. 1993). Sp proteins are distinct from their Kruppel-like factor cousins because of their different binding sites, and presence of a ubiquitous but poorly-understood Sp box domain (Harrison, Houzelstein et al. 2000). These proteins have wide patterns of expression; some are ubiquitously expressed. Two of the most well studied members of this family, Sp1 and Sp3, are expressed in all cells and have been shown to be important in many normal developmental and physiological processes, as well as disease processes (Liu, Borras et al. 1997; Sapetschnig, Rischitor et al. 2002; Feng and Kan 2005).

The best characterized role of Sp1 is to recruit RNA polymerase to genes lacking TATA boxes (Faber, van Rooij et al. 1993; Wierstra 2008). In this capacity, Sp1 is responsible for activating transcription of a number of important genes (in the lung, for instance, FGF-10). Sp1 can potently potentiate

expression by binding to GC-boxes in the promoter, and recruiting RNA polymerase II. Sp1 possesses two activating domains, rich in glutamine residues, which activate transcription and recruit RNA polymerase II by binding components of the basal transcriptional machinery, such as components of the TATA-binding TFIID complex (Gill, Pascal et al. 1994). This recruitment appears to be further enhanced by synergistic homo-oligomerization of Sp1 at the promoter (Matsushita, Hagihara et al. 1998). Sp1 also possesses an N-terminal inhibitory domain, but the importance of this domain is not as well understood. The Sp box could function to downregulate some genes targeted by Sp1 through interactions with histone deacetylases (Kaczynski, Cook et al. 2003). Regulated proteolytic cleavage of the inhibitory domain has been suggested as a mechanism to regulate the ability of Sp1 to activate gene transcription.

Sp3 is strikingly similar in structure to Sp1 – the most obvious difference being the more C-terminal location of the inhibitory domain (Li, He et al. 2004). Perhaps unsurprisingly, Sp3 possesses many of the same properties as Sp1 and the two factors often synergistically affect gene expression (Zhang, Li et al. 2003) (Figure 1.2). Sp3 can bind to GC-boxes with equal affinity to Sp1, and can activate transcription with its similarly placed glutamine rich activating domains (Van Loo, Bouwman et al. 2003). In genes with single GC-boxes, Sp3 appears to activate transcription identically to Sp1. However, Sp3 is a potent inhibitory of gene transcription in genes with multiple GC-boxes. This antagonistic relationship between Sp1 and Sp3 is poorly understood, but could represent a model where Sp3 competitively displaces Sp1 from the promoter, or binds to Sp1

and prevents oligomerization (Hagen, Muller et al. 1994; Yu, Datta et al. 2003). Another possibility includes differing properties among the four described

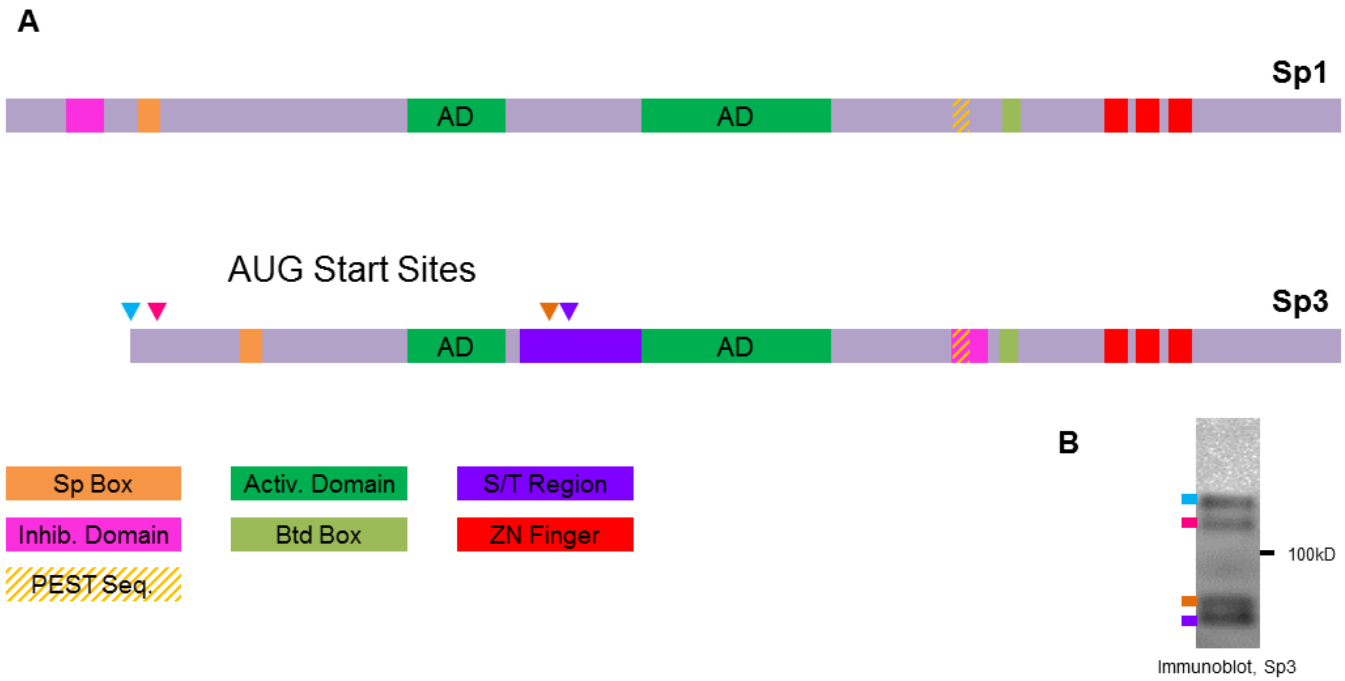


Figure 1.2. Overview of structural motifs in the Sp1 and Sp3 proteins. (A). Three zinc finger DNA-binding domains are the hallmark of transcription factors of the Sp family. Each member also contains a Buttonhead box (Btd) and Sp box domain, though the function of these motifs are not well understood. The inhibitory domain of Sp1 is located near the N-terminus, while in Sp3 the inhibitory domain is located closer to the zinc fingers nearer the C-terminus. The serine/threonine rich region is thought to play an important role in allowing for post-translational modifications in Sp3 (Suske 1999; Bouwman and Philipsen 2002). (B). Immunoblot for Sp3 from CHO cell nuclear lysate showing the four translationally-dependent isoforms.

translationally-dependent isoforms of Sp3. Four different AUG start sites exist in the single Sp3 mRNA, and each of these can serve as the initial amino acid in the protein – leading to four isoforms which appear in equal stoichiometry (Sapetschnig, Koch et al. 2004). The only described domain that is lost between the two longer and two shorter isoforms is the Sp box, the function of which remains unknown.

A series of post-translational modifications have also been implicated as potential mechanisms to modulate the activity of Sp1 and Sp3 in regards to transcriptional activation of target genes. Myriad modification processes, including phosphorylation, acetylation, and SUMOylation have been identified in Sp proteins (Braun, Koop et al. 2001; Ross, Best et al. 2002). SUMOylation in particular has been shown to vastly alter the activity of Sp3 in the nucleus, and could represent a master switch that shifts Sp3 from a transcriptional activator to a transcriptional repressor. In fact, inhibition of Sp3 SUMOylation by deletion of predicted SUMOylated lysine residues in the Sp3 protein altered the subnuclear distribution of Sp3 and prevented inhibitory regulation of target gene transcription (Ross, Best et al. 2002). SUMOylation sites have also been identified in the Sp1 protein, and it has been suggested recently that SUMOylation might prevent cleavage of the inhibitory domain from the N-terminus of Sp1. This has been predicted to damped the ability of Sp1 to increase transcription of target genes (Spengler and Brattain 2006).

It is interesting to note that mice null for Sp1 and Sp3 each die early in development. In the case of the Sp1 null mouse, loss of viability occurs at approximately E9.5. No single defect has been implicated in the death of these embryos, but they are much smaller and many organ tissues are highly disorganized.

This indicates that Sp1 is required for the proper development of a broad variety of tissues, and loss is incompatible with sustained normal development (Marin, Karis et al. 1997). Mice null for Sp3 survive embryonic development, but die perinatally, cyanotic and gasping for breath. Histological analysis reveals subtle differences in the microanatomy of the lung, including goblet cell dysplasia in the alveoli, thickened alveolar septa, and general epithelial disorganization. There are also defects in fetal dentition and ossification of long skeletal bones in Sp3 null mice (Bouwman, Gollner et al. 2000; Gollner, Dani et al. 2001). These results suggest that Sp3 is required for normal lung development, though no obvious physiological cause for the respiratory insufficiency has presently been identified beyond the subtly altered tissue structure. Perhaps most interesting is the fact while mice heterozygous for either Sp1 or Sp3 appear healthy and fertile, with no differences in viability or lung physiology, mice transheterozygous for Sp1 and Sp3 ($Sp1^{+/-}$; $Sp3^{+/-}$) die perinatally, again with lung dysmorphia and cyanosis (Kruger, Vollmer et al. 2007). This synthetic lethality implies a sophisticated interaction between Sp factors in the nascent lungs must be required for normal organogenesis of the lungs, and that loss of a single copy of each gene together yields deleterious results on the sophisticated developmental mechanisms employed in lung development.

Pathophysiology of Bronchopulmonary Dysplasia

Bronchopulmonary dysplasia is a disease that affects primarily premature children with a birth weight of less than 1000 grams. Many of these children are born

during a period of gestation corresponding to the saccular stage of lung development, 24 – 26 weeks. Approximately 10,000 new cases of BPD are diagnosed each year, with incidence of disease increased as birth rate decreases; “The incidence of BPD is 22% in infants with birth weights of less than 1500g. The incidence increases with decreased birth weight, so that 46% of infants with birth weight between 501-750g develop BPD (Fanaroff, Stoll et al. 2007; Bäckström 2010).” Because of evolving respiratory support protocols for premature neonates, the disease described as BPD has undergone a significant revision in pathology since its earliest description in the 1960s (Northway, Rosan et al. 1967; Northway and Rosan 1968). The BPD described in the 1960s and 1970s is typically referred to as “old BPD,” which is a disease associated with aggressive ventilator treatment and resulting fibrosis of the lung. This disease was frequently fatal, often from co-morbidities associated with pulmonary fibrosis such as pulmonary hypertension and cor pulmonale (Northway 1990). Antenatal steroids meant to hasten lung maturation, along with better ventilation management and administration of surfactants can ameliorate some of the respiratory symptoms associated with prematurity and has changed BPD into the more mild “new BPD,” which is frequently marked by sustained requirement for oxygen therapy, Often children perform well immediately after birth, but eventually require oxygen and ventilation assistance (Gould 2007). The pathophysiology of new BPD include unusually dilated alveoli, inflammation of the pulmonary epithelium, and abnormalities in the capillary bed (Jobe and Bancalari 2001; Coalson 2003).

There are several known risk factors associated with BPD. The most common and important risk factor for developing BPD is premature birth, with a strong inverse

correlation between birth weight and likelihood of developing BPD (Bancalari 2000). Ventilation has also been suggested to alter vascular and alveolar development of the neonatal lung (Albertine, Jones et al. 1999; Coalson, Winter et al. 1999; Bäckström 2010). Mechanical ventilation, though typically associated with the previous incarnation of BPD, persists as a risk factor in developing new BPD; this despite introduction of more sophisticated techniques to tightly control pressure in the lung thus lessening the risk of developing barotrauma. Other risk factors associated with BPD include hyperoxia, potentially patent ductus arteriosis, and complications of pregnancy including preeclampsia (Bonikos, Bensch et al. 1975; Warner, Stuart et al. 1998; Clyman, Cassady et al. 2009; O'Shea, Davis et al. 2012).

Perhaps the most intriguing risk factor for BPD is antenatal exposure to pro-inflammatory microbial immunogenic molecules during chorioamnionitis, inflammation of the fetal membranes. Chorioamnionitis has long been associated with preterm delivery and neonatal morbidity (Ramsey, Lieman et al. 2005; Galinsky, Polglase et al. 2013). Mammalian models of chorioamnionitis show a remarkable effect on inflammatory pathways in the lung, including alveolar hypoplasia and infiltration of immune cells (Bry, Hogmalm et al. 2010; Gantert, Jellema et al. 2012). Because the fetal lung epithelium is awash in amniotic fluid, bacterial endotoxin present in the amniotic fluid will necessarily affect those epithelial cells (Kallapur, Willet et al. 2001; Kramer, Ikegami et al. 2005). Previous work has shown that not only do epithelial cells respond to bacterial products in the amniotic sac, but that lung development is qualitatively altered by the presence of these toxins. Differences in pulmonary vasculature, including increased numbers of endothelial cells in the lung parenchyma occurred in the lungs of fetal mice treated with

intra-amniotic LPS injections (Miller, Benjamin et al. 2010). Similarly, exposure to inflammatory stimuli known to activate NF-κB increases differentiation of type II alveolar cells, suggesting a role for intracellular pathways activated by inflammatory signaling in the overall differentiation and maturation of the pulmonary epithelium (Prince, Okoh et al. 2004). Perhaps because of these differences in vasculature and airway cell differentiation, neonates with lungs exposed to NF-κB-activating stimuli actually perform better in the short time compared to stage-matched children without inflamed lungs (Watterberg, Demers et al. 1996; Newnham, Moss et al. 2002). Increased type II cells lead to earlier and increased production and secretion of surfactant proteins, ameliorating the effects of hyaline membrane disease and resulting respiratory distress syndrome (Watterberg, Demers et al. 1996). This temporary advantage is lost in children who go on develop BPD, whose demand for oxygen therapy and requirement for ventilation increase over time (Merritt, Cochrane et al. 1983; Ogden, Murphy et al. 1984; Groneck, Gotze-Speer et al. 1994; Murch, Costeloe et al. 1996).

Just as the etiology and nature of BPD has changed since it was first described in the 1960s, so too has the pathology. Classic BPD was marked by "severe airway epithelial lesions and smooth muscle hyperplasia, alternating sites of overinflation and atelectasis, extensive fibrosis, and severe vascular hypertensive lesions (Bonikos, Bensch et al. 1976; Coalson 2006)." In the newer incarnation of BPD, pathological findings are less severe. Epithelial and smooth muscle injuries are less obvious, and the lung vasculature appears healthier. While fibrosis is sometimes seen in new BPD, it is more sporadic and less severe than in classic BPD. The most consistent finding in new BPD are larger, dilated alveoli. These changes to alveolar structure allow for less

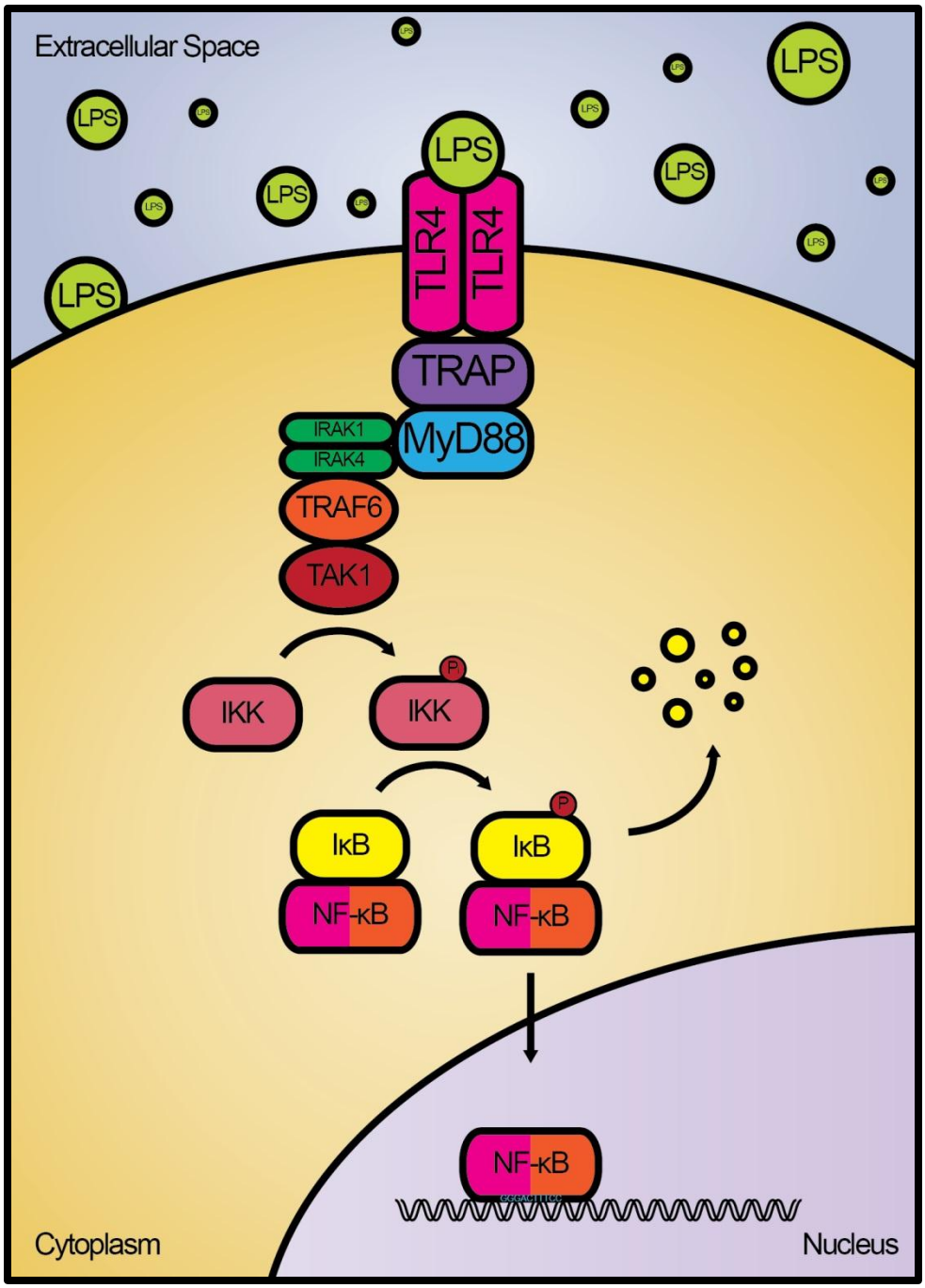
surface to exchange gasses, and could contribute to the nature of the disease (Bland and Coalson 2000). Based on pathological and molecular data, it has been suggested that BPD represents arrested development of lung maturation during the saccular stage of development (Hislop, Wigglesworth et al. 1987; Jobe 1999). This remains a fascinating question because understanding the basic physiological cause of the disease would inform better treatment strategies. Developmental processes occurring during the saccular stage could be compromised by factors contributing to BPD, disallowing further maturation of the lung (Benjamin, Smith et al. 2007; Benjamin, Carver et al. 2010).

NF-κB Signaling

The NF-κB signaling pathway is a cellular pathway that typically activates gene expression in cells exposed to pro-inflammatory stimuli. It consists of a heterodimeric transcription factor typically sequestered in the cytoplasm. Many different cell-surface receptors and intracellular signaling even can activate NF-κB. Receptors for cytokines, growth factors, and mediators of cell stress can activate NF-κB (Baeuerle and Baltimore 1996). One of the most well-known classes of receptors associated with NF-κB is the Toll-Like Receptor (TLR) family. TLRs bind to pathogen-associate molecular patterns (PAMPS), components of microbes that serve as hallmarks of infection (Kawai and Akira 2007). Once a TLR binds to its activating ligand, scaffolding proteins such as MyD88, IRAK1, IRAK4, TRAF6 and TAK1 are recruited to the cytoplasmic domain of the receptor (Richmond 2002). Under basal conditions, the NF-κB dimer is sequestered

in the cytoplasm by an inhibitory IκB. Upon activation of a relevant receptor, IKK complexes are phosphorylated and become active (Napetschnig and Wu 2013). Once phosphorylated, the IKK complex can then phorphoylate IκB, leading to protein degradation by the proteasome. Without IκB to mask the NF-κB heterodimer's nuclear localization signal, it is translocated into the nucleus (Beg, Ruben et al. 1992) (Figure 1.3).

In the nucleus, NF-κB typically acts as a transcriptional activator (Whitehouse, Khal et al. 2003). Conserved binding sequences located in the promoters of genes associated with immunological function allow cells to respond to a variety of immunogenic cues. Other functions transcriptionally


Extracellular Space
LPS
TLR4
TLR4
TRAP
IRAK1
IRAK4
MyD88
TRAF6
TAK1
IKK
IKK
P
IκB
NF-κB
IκB
NF-κB
P
NF-κB
Cytoplasm
Nucleus

F**igure 1.3. An overview of NF-κB signaling through TRL4 activation.** When TLRs bind their cognate PAMPS, LPS in the case of TLR4, downstream scaffolding proteins assemble on the cytoplasmic trail of the receptor (TRAP, MyD88, IRAK1/4, TAB2, TRAF6, TAB1 (Richmond 2002). Activation at the receptor level leads to phosphorylation and subsequent activation of the IKK complex. IKK then phosphorylates the inhibitory IκB, unmasking the nuclear localization sequence on NF-κB, allowing it to translocate into the nucleus and modulate transcriptional activity of target genes.

controlled by NF-κB include cell survival, cell differentiation, and cell proliferation; this diversity of target genes associated with NF-κB informs the pleiotropic effects of NF-κB activation (Narayanan, Higgins et al. 1993; Oeckinghaus and Ghosh 2009). Because NF-κB can play a central role in these cellular functions frequently associated with molecular developmental processes, it is unsurprising that NF-κB has been implicated in several developmental phenomena. Activated NF-κB can be found during fetal development of many structures in the mouse embryo, for instance the telencephalon, choroid plexus, cochlear canal, thymus, mammary glands, and lacrimal glands (Dickson, Bhakar et al. 2004). Components of the NF-κB pathways have also been shown to be required for some developmental processes. For instance, mice null for RelA die in utero due to massive apoptosis of the developing liver, while nice null for IKKβ develop defects in skin, skeleton, and hematopoietic system (Beg, Sha et al. 1995; Hu, Baud et al. 1999; Takeda, Takeuchi et al. 1999; Balkhi, Willette-Brown et al. 2012). An established role for NF-κB in normal embryogenesis implies that NF-κB is capable of associating with molecular signaling pathways and physiological mechanisms more typically associated with development to affect these processes. Activation of NF-κB is an important step in mediating cellular responses to environmental pro-inflammatory stimulation. Coordinated responses to microbial invasion and other stresses require cell to cell signaling via release of cytokines. These cytokines then influence paracrine (and also autocrine) behaviors geared towards the offending stimulus. These behaviors can include recruitment of pro-inflammatory cells to sites of inflammation, activating immunological cells to stimulate phagocytosis, and many other immunological functions (Blackwell, Hipps et al. 2011). Some cytokines known to be expressed by cells in which

NF-κB is active include IL-1β, , IL-6, IL-8, IFNγ, MIP-1α, RANTES, and TNFβ (Gupta, Sundaram et al. 2010).

In some circumstances, NF-κB acts as an inhibitor of target genes. NF-κB directly inhibits expression of intestinal trefoil factor (TFF3) (Baus-Loncar, Al-azzeh et al. 2004), E-cadherin (Chua, Bhat-Nakshatri et al. 2007) and Collagen1A1 (Rippe, Schrum et al. 1999). These genes have been implicated in developmental processes ranging from tissue migration during gastrulation to cell differentiation (Lesuffleur, Porchet et al. 1993; Shimizu, Yabe et al. 2005). This establishes a paradigm where NF-κB can direct concerted physiological responses by directly activating and inhibiting transcription of a diverse set of genes.

To further alter cell behavior when faced with pro-inflammatory stimuli, NF-κB has been described to interact with other transcription factors to modulate gene regulatory mechanisms. Several of these are associated with developmental pathways (Rual, Venkatesan et al. 2005), and some have been implicated in lung development specifically: retinoid X receptor, Notch1, and β-catenin (Mucenski, Wert et al. 2003; Garber, Zhang et al. 2006; Morimoto, Liu et al. 2010). NF-κB has also been shown to interact with Sp factors to co-regulate genes. Sp1 and NF-κB interact and situationally regulate target gene transcription. Much of this work has been discovered in viral transcription, especially in the biology of HIV infection. Interaction between NF-κB and Sp1 is required for HIV gene expression (Perkins, Edwards et al. 1993; Majello, De Luca et al. 1994). Sp3 and NF-κB have been proposed to interact to regulate the Collagen A1A gene, a gene also involved in the pulmonary basement membrane (Bigot, Beauchef et al. 2012). This effect appears distinct from the previously described cross-

interactions between Sp proteins with NF-κB binding sites, and NF-κB with GC boxes (Hirano, Tanaka et al. 1998; Liu, Hoffman et al. 2004). This interaction could suggest an intriguing mechanism to offer context to gene regulation by Sp1 and Sp3, transcription factors that appear to be express ubiquitously.

Research Plan Description

Several questions persist regarding the relationship between inflammation and subsequent developmental pathologies of the lung. Prior to this work, strong bodies of work regarding lung development and inflammatory effects on the adult lung existed. How inflammation affects lung development was less well understood. For instance, how does inflammation seemingly arrest lung development during the saccular stage? What are the relevant signaling molecules? What downstream pathways connect extracellular inflammatory signaling to intracellular effectors? What are the ultimate repercussions of developmental arrest? To answer these questions, we employed the following approaches:

NF-κB had previously been identified as a potential mediator of lung inflammation and subsequent pathology. Once we began questioning the role of NF-κB in regulating lung branching through FGF-10, we developed an FGF-10 reporter system using CHO cells and luciferase expression to more thoroughly interrogate the regulatory function. This system allowed for more precisely controlled NF-κB modulation through co-transfection with constitutively active IKKβ construct to activate NF-κB signaling, and a dominant negative IκB to totally blockade NF-κB. We similarly employed our reporter

system to determine the role that other transcription factors played on FGF-10 expression through co-transfection, most notably with Sp1 and Sp3. We chose CHO cells when developing this system because of their mesenchymal-like appearance, similar regulation of FGF-10 promoter activity to lung mesenchymal cells, and relative ease of transfection.

When NF-κB was determined to negatively regulate FGF-10, we sought to determine whether it did so directly or indirectly. We utilized two methods to test this: ChIP analysis, then *in silico* analysis and mutagenesis of predicted NF-κB binding sites in the FGF-10 promoter. Using primary lung mesenchymal cells, we used ChIP to determine whether NF-κB (then Sp1 and Sp3) occupied the FGF-10 promoter, and if LPS treatment altered enrichment of these factors. We then turned to *in silico* analysis to find NF-kB consensus sequences in the FGF-10 promoter. We used site-directed mutagenesis to remove these sites, and employed our reporter system to determine whether these sites were required for downregulation of FGF-10 by NF-κB. We extended these questions to determine how Sp1, Sp3 and NF-κB might interact to regulate FGF-10. We utilized an *in situ* proximity ligation assay to determine whether the proteins were within range to interact with one another.

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CHAPTER II

NF-κB Activation Limits Airway Branching through Inhibition of Sp1-Mediated Fibroblast Growth Factor-10 Expression

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Abstract

Bronchopulmonary dysplasia (BPD) is a frequent complication of preterm birth. This chronic lung disease results from arrested saccular airway development and is most common in infants exposed to inflammatory stimuli. In experimental models, inflammation inhibits expression of FGF-10 and impairs epithelial-mesenchymal interactions during lung development; however, the mechanisms connecting inflammatory signaling with reduced growth factor expression are not yet understood. Here we found that soluble inflammatory mediators present in tracheal fluid from preterm infants can prevent saccular airway branching. In addition, LPS treatment led to local production of mediators that inhibited airway branching and FGF-10 expression in LPS-resistant C.C3-Tlr4Lpsd/J fetal mouse lung explants. Both direct NF-κB activation

and inflammatory cytokines (IL-1β and TNFα) that activate NF-κB reduced FGF-10 expression, while chemokines that signal via other inflammatory pathways had no effect. Mutational analysis of the FGF-10 promoter failed to identify genetic elements required for direct NF-κB mediated FGF-10 inhibition. Instead, NF-κB activation appeared to interfere with the normal stimulation of FGF-10 expression by Sp1. Chromatin immunoprecipitation and nuclear co-immunoprecipitation studies demonstrated that the RelA subunit of NF-κB and Sp1 physically interact at the FGF-10 promoter. These findings indicate that inflammatory signaling through NF-κB disrupts the normal expression of FGF-10 in fetal lung mesenchyme by interfering with the transcriptional machinery critical for lung morphogenesis.

Introduction

Epithelial-mesenchymal interactions guide lung development. Cells that arise from the foregut endoderm line the airways. These epithelial airways are surrounded by mesenchyme, which drives airway extension and branching. The lung bronchial tree forms first, followed by expansion and branching of more distal saccular airways (Hogan and Yingling 1998; Perl and Whitsett 1999; Warburton, Schwarz et al. 2000). During branching morphogenesis, the fetal lung mesenchyme expresses multiple growth factors that act on airway epithelia. Fibroblast growth factor 10 (FGF-10) is among the key mesenchymal growth factors for lung development, promoting airway extension and branching (Weaver, Dunn et al. 2000; Weaver, Batts et al. 2003). During canalicular and

saccular airway formation (beginning at E14 in mice, 22 week gestation in humans), FGF-10 continues to stimulate branching of the terminal airways, which become alveolar ducts as the lung reaches maturity (Hokuto, Perl et al. 2003).

Abnormalities in lung branching morphogenesis can lead to human disease. In up to 50% of extremely preterm infants, saccular airway branching arrests, resulting in bronchopulmonary dysplasia (BPD; (Christou and Brodsky 2005; McDorman 2005)). Arrested airway branching in BPD results in fewer alveolar units available for gas exchange and smaller overall lung volumes (May, Prendergast et al. 2009). While many factors likely contribute to BPD pathogenesis, exposure to inflammation clearly increases BPD risk. Infants exposed prenatally to chorioamnionitis (infection or inflammation of the amniotic membranes, uterus, or placenta) or postnatally to bacteremia and sepsis are more likely to develop BPD (Watterberg, Demers et al. 1996; Klinger, Levy et al. 2010). These clinical observations suggest inflammatory mediators transmitted to the lungs might prevent normal canalicular and saccular stage lung development. The process by which inflammatory signaling disrupts normal developmental processes is not clear, but recent findings have provided clues to the mechanisms linking inflammation and BPD pathogenesis.

Activation of the innate immune system in fetal mice inhibits saccular stage lung development. Injecting Gram-negative bacterial lipopolysaccharide (LPS), a classic activator of innate immunity, into the amniotic fluid of mice at E15 causes cystic dilation of saccular airways and inhibits airway branching. LPS also acts in a Toll Like Receptor 4 (TLR4) dependent manner to inhibit saccular airway branching in lung explants (Prince, Dieperink et al. 2005). In preventing normal morphogenesis, LPS inhibits

expression of FGF-10, a key mesenchymal growth factor required for airway branching (Benjamin, Smith et al. 2007). Because the fetal lung mesenchyme is not normally in contact with the external environment, LPS and other bacterial constituents may not directly contact mesenchymal cells to inhibit FGF-10 expression and disrupt branching. Instead, innate immune activation may lead to local production of soluble inflammatory mediators that affect the lung mesenchyme. While innate immune signaling appears to alter branching morphogenesis in part through reduced FGF-10 expression, several important questions remain unanswered, including: 1) what are the critical inflammatory mediators in the airways of premature infants at risk for BPD?, 2) what are the important down-stream signaling pathways?, and 3) how do these pathways lead to reduced expression of FGF-10 (and possibly other mediators) and impaired mesenchymal-epithelial interactions that block normal lung development? Identifying the connections between inflammatory signaling and lung morphogenesis is critical for understanding the disease mechanisms leading BPD.

Because preterm infants are commonly exposed to an inflammatory environment immediately before birth, we tested whether the inflammatory milieu in the airways of newborn preterm infants contains substances that could inhibit lung development. We then measured the ability of soluble inflammatory mediators to alter FGF-10 expression in fetal lung mesenchyme and found that mediators that activate NF-κB signaling can inhibit FGF-10 expression. Investigation in to the mechanism of FGF-10 inhibition by NF-κB revealed that NF-κB blocks FGF-10 expression by interfering with the ability of Sp1 to drive FGF-10 transcription. Interactions between NF-κB and Sp1 may provide a

potential target for preserving FGF-10 expression during lung development with implications for novel approaches for preventing or treating BPD in preterm infants.

Materials and Methods

Reagents and Cell Culture

Phenol-extracted, gel purified *E. coli* LPS (O55:B5) was purchased from Sigma. Recombinant IL-1β, TNFα, MIP1α, and MCP-1 were purchased from R&D Systems. Parthenolide, the IKKβ inhibitor BMS345541 and the MAPK inhibitors PD98059, SB203580, U0126, FPTIII, and ZM336372 were purchased form EMD Biosciences. Antibodies against RelA and Sp1 were from Santa Cruz. Rat anti-E-Cadherin was purchased from Zymed. Alexa-conjugated secondary antibodies were purchased from Invitrogen.

Animals and Saccular Explant Culture

BALB/cJ and C.C3-Tlr4Lpsd/J mice were obtained from Jackson Laboratories. For timed matings, the morning of vaginal plug discovery was defined at Embryonal day 0 (E0). Our procedure for culturing saccular stage fetal lung explants has been described (Prince, Okoh et al. 2004; Dieperink, Blackwell et al. 2006; Benjamin, Smith et al. 2007). Briefly, E16 mice were euthanized and the fetal mouse lungs dissected free of surrounding structures. The lung tissue was minced into 0.5-1 mm^3 cubes and

cultured on an air-liquid interface using permeable supports (Costar Transwell) and serum-free DMEM. Explants were cultured at 37˚C in 95% air/ 5% CO_2 for up to 72 hours. To isolate conditioned media from BALB/cJ explants, LPS was included in culture media at a concentration of 250 ng/ml. After 72 h of culture, the media was removed and stored at -80˚C. Conditioned media from control and LPS-treated BALB/cJ explants were then added to freshly isolated C.C3-Tlr4Lpsd/J explants.

Immunostaining, Imaging, and Analysis

To quantify saccular airway branching in cultured lung explants, brightfield images of explants were acquired following 24 h and 72 h of culture. The explant area and number of saccular airways along the periphery of each explant were measured with the assistance of the Image Processing Toolkit (Reindeer Graphics) within Photoshop (Adobe). Airway branching was then expressed as the number of new branches/mm2 that formed between 24 h and 72 h of culture in each explant. For immunostaining of Sp1 and E-cadherin in fetal mouse lungs, E16 BALB/cJ mouse lungs were dissected, rinsed in PBS, and processed in increasing sucrose concentrations. Lungs were then frozen in OCT and stored at -80˚C. Cryostat sections (8 μm) were fixed with 4% paraformaldehyde, permeabilized with 0.1 % Triton X-100, and stained with antibodies against Sp1 and E-cadherin. Alexa-conjugated secondary antibodies were used for fluorescent detection and nuclei were labeled with DAPI. Images were acquired using an Olympus FV1000 laser scanning confocal microscope.

Tracheal Aspirate Collection and Cytokine Measurement

Tracheal aspirate samples were obtained from intubated preterm infants. All protocols were reviewed and approved by the Institutional Review Board at the University of Alabama at Birmingham. Eligible patients were delivered between 23-28 wk gestation and intubated on the first day of life. Tracheal aspirates were collected prior to 24 h of age. Endotracheal suctioning was performed using an enclosed inline suction catheter. One ml of sterile saline was instilled into the endotracheal tube and fluid was aspirated into an enclosed specimen trap. Following centrifugation at 2000 x g at 4˚C for 5 minutes, the supernatant was filtered through a 0.45 μm low protein binding syringe filter (Millipore), aliquoted, and stored at -80˚C. Urea concentration in each aspirate was measured using the Quantichrom Urea Assay kit (Bioassay Systems). Samples were diluted to a final urea concentration of 0.1 mg/dl before use. Patient identifiers were not obtained, but presence or absence of maternal chorioamnionitis was recorded. All cases of chorioamnionitis were confirmed by placental pathology. IL-1β, TNFα, and IL-8 concentrations in each sample were measured by SearchLight Assay (Pierce Endogen). Each sample was measured in triplicate over multiple dilutions.

RNA Isolation, Reverse Transcription, and Real-Time PCR

Total RNA was isolated from fetal lung explants and cultured mesenchyme using Trizol reagent and standard protocols. Three explants were pooled for each sample. First strand cDNA was synthesized using oligo-dT primers and MMLV reverse transciptase (Superscript II, Invitrogen). PCR primers designed using Beacon Designer

software (BioRad) were validated by performing electrophoresis and melting temperature analysis of the PCR product. Standard concentration curves were done for each primer pair used. Two-step real time PCR was performed with a BioRad MyiQ thermocycler and SYBR green detection (BioRad). We normalized gene expression to GAPDH in each sample. The 2-ΔΔCT method was used to compare gene expression levels between samples (Arocho et al., 2006). Independent experiments were performed at least three separate times. Data between groups were compared by ANOVA to test for significant differences.

Plasmids

The FGF-10 luciferase reporter plasmid in the pXPI vector was generously supplied by Benoit Bruneau (Agarwal, Wylie et al. 2003). This construct contains an approximately 6 kb BamHI fragment from the murine FGF-10 gene placed immediately upstream of the luciferase coding sequence. Serial truncations of the FGF-10 promoter were generated by PCR mutagenesis. Deletions were confirmed by restriction digest and sequencing. The predicted NF-κB binding site in the FGF-10 promoter located 555 bp upstream of the start site was deleted from the 0.9 kb FGF-10-D truncation using site-directed mutagenesis. Primers compatible with the QuikChange II mutagenesis system (Agilent) were synthesized (5’ - CTT CCC CCC TCC CTG TTC CCA GCA GCT T - 3’; 5’ - AAG CTG CTG GGA ACA GGG AGG GGG GAA G - 3’). Positive transformants were sequenced to ensure deletion. FGF-10 luciferase reporter constructs were co-transfected into CHO cells using SuperFect lipofection reagent

(Qiagen). pSV-β-Gal was included to control for transfection efficiency. After 48 hours, cells were lysed and luciferase activity was measured with Steady-Glo reagent (Pierce) and a microtiter plate luminometer. Light units were normalized to β-galactosidase levels.

Constitutively active IKKβ and dominant-negative IκB cDNA constructs (17) were cloned into pCDNA3.1/CT-GFP for expression in CHO cells. Murine Sp1 was cloned from E15 fetal mouse lung RNA by reverse transcription and PCR using the following primers: 5' - GCC ACC ATG AGC GAC CAA GAT CAC TCC A - 3'; 5' - GGA AAC CAT TGC CAC TGA TAT T - 3'. The full-length Sp1 sequence was first subcloned into pCR2.0, sequenced, excised by restriction digestion, and ligated into pCDNA3.1/CT-GFP for expression.

Co-Immunoprecipitation

For co-immunoprecipitation of Sp1 and p65, CHO cells were lysed with ice-cold hypotonic lysis buffer (10 mM HEPES pH 7.6, 1 mM EDTA, 60 mM KCl, 0.5% NP-40, 1 mM DTT and protease inhibitors (Halts, Pierce)). Lysates were centrifuged for 10 min at 16,000 x g (4˚C) for separation of nuclear pellet from cytoplasmic supernant. The nuclear fraction was resuspended in 250 mM Tris-HCl pH 7.8, 1 mM DTT, and protease inhibitors and frozen at -80˚C. Samples were then thawed and diluted with nuclear dilution buffer (0.01% SDS, 1.1% Triton-X100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.0, and 167 mM NaCl). Nuclear and cytoplasmic fractions were pre-cleared with Protein G agarose beads (Sigma) and immunoprecipitated overnight. After washing,

immunoprecipitates were boiled in Laemmli sample buffer, separated by SDS/PAGE, and transferred to PVDF membranes. Blots were blocked with 5% BSA, probed with anti-RelA antibodies, and developed by chemiluminescence.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed using primary fetal mouse lung mesenchymal cells. Control and LPS-treated cells were fixed with 1% formaldehyde and lysed. DNA was sheared by sonication, phenol:chloroform extracted, and precipitated with ethanol. Samples were then incubated with anti-Sp1 and anti-p65 antibodies for immunoprecipitation. Following stringent washing, DNA-protein linkages were disrupted and released DNA fragments were phenol:chloroform extracted and precipitated with ethanol. The 320 bp region of the FGF-10 promoter (upstream of and including the transcriptional start site) was detected by PCR. Products were analyzed by agarose gel electrophoresis.

Results

Inflammatory mediators in newborn lungs inhibit airway branching

To investigate whether the inflammatory milieu in the airways of newborn preterm infants contains substances that could inhibit lung development, we tested patient samples using an experimental model of saccular lung development. We isolated

tracheal aspirate fluid from 22 newborn extremely preterm infants divided into three distinct groups. Nine infants were exposed to maternal chorioamnionitis, seven infants were born preterm due to maternal pre-eclampsia, and six infants were delivered preterm without an identifiable cause and without clinical or pathological evidence of chorioamnionitis. Each tracheal aspirate sample was separately added to E15 BALB/cJ fetal mouse lung explants to measure effects on airway branching and FGF-10 expression. As shown in Figure 2.1, samples from infants exposed to chorioamnionitis significantly inhibited the formation of new saccular airway branches in explants compared to pre-eclampsia and control preterm labor samples. Representative images in Figure 2.1A-D show that tracheal aspirate fluid from infants exposed to chorioamnionitis caused formation of more cystic, poorly branched airways (Figure 2.1B,D). In contrast, tracheal aspirate fluid from infants delivered preterm due to maternal pre-eclampsia had no effect on airway branching or formation (Figure 2.1A,C). New airway branches were significantly reduced in explants exposed to aspirates from the chorioamnionitis samples compared to control explants grown in media alone or with tracheal aspirates from the other patient groups (Figure 2.1E). Tracheal aspirates from patients exposed to chorioamnionitis also contained elevated levels of inflammatory cytokines compared to preterm infants born due to maternal pre-eclampsia or undiagnosed causes (Figure 2.1H, Table 2.1). LPS was not detected in any of the tracheal aspirate samples tested (< 0.1 EU/ml). Together, these data suggest that soluble inflammatory mediators in the airway of infants exposed to chorioamnionitis can inhibit saccular airway morphogenesis.

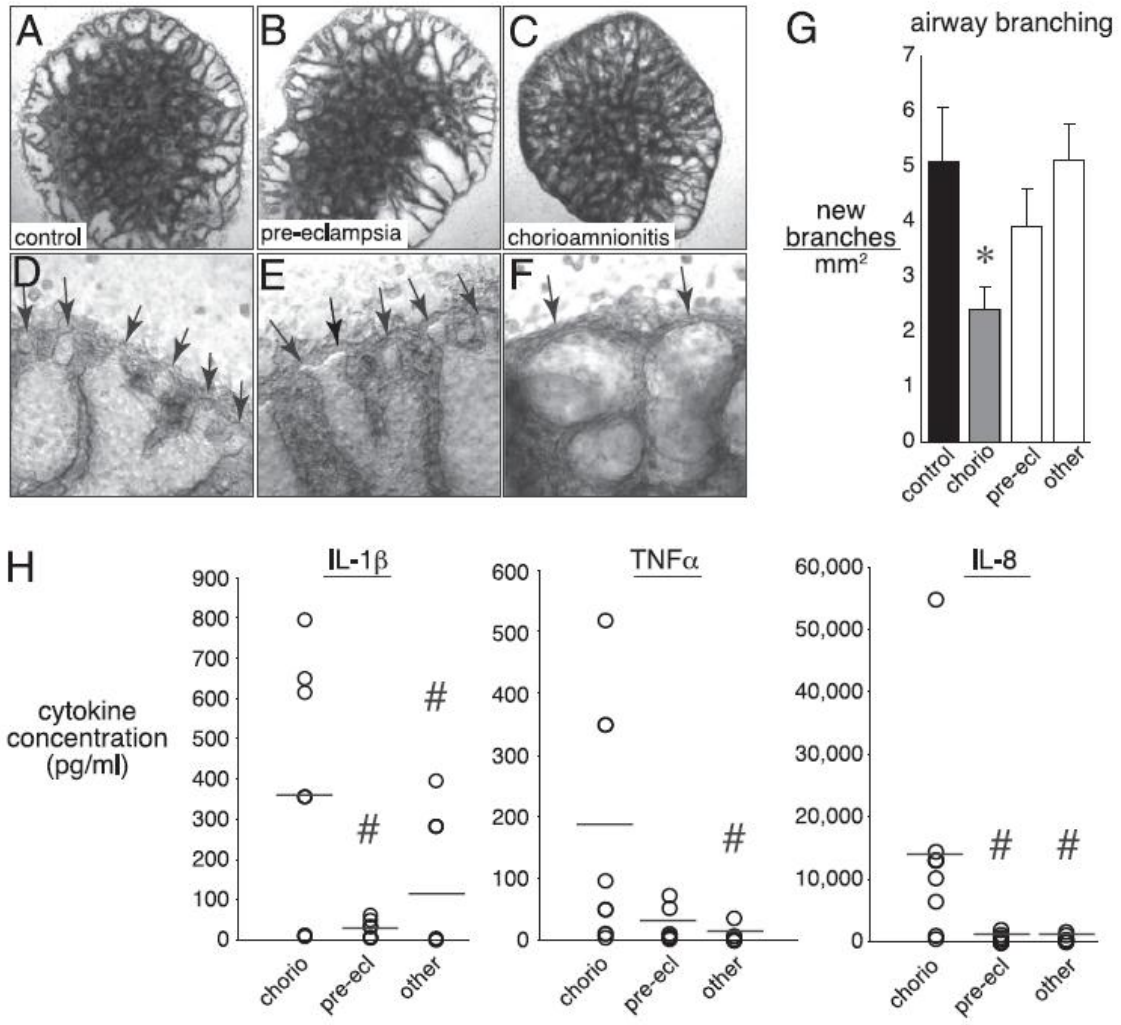

Figure 2.1. Tracheal aspirate fluid from newborn patients exposed to chorioamnionitis inhibits airway branching. Tracheal fluid was aspirated from intubated extremely preterm infants following delivery and added to saccular stage fetal mouse lung explants. (A-D). Brightfield images of mouse lung explants cultured with tracheal aspirate fluid from a patient born preterm due to maternal pre-eclampsia (A,C) or maternal chorioamnionitis (B,D). Higher magnification images are shown in (C,D). (E). The number of new saccular airway branches that formed during the culture period was lower when tracheal fluid from chorioamnionitis patients was added to the media (* $P < 0.05$, 9 chorio samples, 7 pre-ecl samples, 6 others). (F). Concentrations of IL-1β, TNFα, and IL-8 were measured in each patient sample. Elevated cytokine levels were detected in chorioamnionitis samples. (# $P < 0.05$ compared to chorio, n = 9 chorio samples, 7 pre-ecl samples, 6 others).

Cause of preterm labor	IL-6 (pg/ml)	IL-10 (pg/ml)	IP-10 (pg/ml)	RANTES (pg/ml)
Chorioamnionitis	114.2 (± 138.2)	9.9 (± 28.3)	139.2 (± 726.7)	76.4 (± 212.8)
Pre-eclampsia	22.5 (± 29.8)	6.1 (± 4.5)	46.9 (± 24.3)	24.3 (± 11.5)
Other	14.9 (± 14.9)	1.8 (± 2.2)	22.3 (± 22.8)	8.8 (±132.8)

Table 2.1. Inflammatory mediator concentrations in tracheal aspirate fluid obtained from preterm infants. Samples were obtained via endotracheal suctioning during the first 24 h of life and assayed using a SearchLight Assay platfor (Pierce Endogen). Samples were grouped based on the clinical and pathological cause of preterm birth. Values are reported as median (± standard deviation) for 9 chorioamnionitis samples, 7 pre-eclampsia samples, and 6 other samples.

Since data from patient samples suggested that soluble mediators could inhibit airway branching, we asked whether these mediators are produced locally in fetal lungs. To address this issue, we collected LPS-conditioned media from BALB/cJ fetal mouse lung explants. LPS stimulated the release of multiple inflammatory mediators into the media (Figure 2.2). We then added the LPS-conditioned media to explants from C.C3-Tlr4Lpsd/J mice, which are resistant to the direct effects of LPS due to a loss of function mutation in TLR4. LPS-conditioned media from BALB/cJ explants substantially inhibited saccular airway branching in C.C3-Tlr4Lpsd/J explants (Figure 2.3A-G) and reduced FGF-10 expression (Figure 2.3H). These effects were reduced when the NF-κB inhibitor parthenolide was included in the media (Figure 2.3C,F). Together, these data indicate that soluble inflammatory mediators produced in the lungs can inhibit FGF-10 expression and saccular airway branching, possibly through NF-κB signaling.

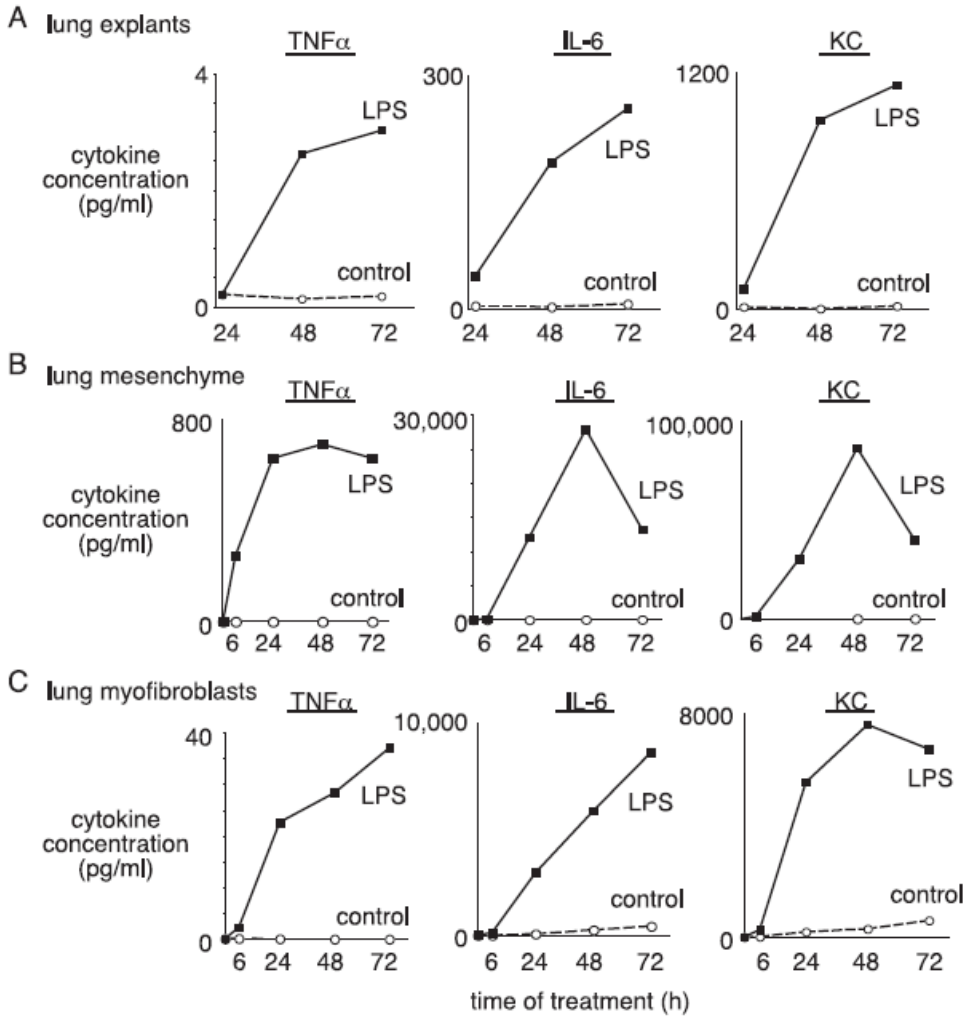

Figure 2.2. Inflammatory mediators produced by fetal mouse lung explants, mesenchymal cells, and myofibroblasts. To verify that cells within the fetal mouse lung were responsive to innate immune activation, 250 ng/ml of E. coli LPS was added to E15 fetal mouse lung explants (A), primary cultures of fetal lung mesenchyme (B), or cultured fetal mouse lung myofibroblasts (C). Myofibroblast cultures were obtained by first isolating fetal mouse lung mesenchymal cells, and passaging over 10 times to obtain a homogeneous population of cells that contained alpha-smooth muscle actin-positive stress fibers (not shown). For all experiments, LPS was added in serum-free DMEM. At indicated time points, the media was harvested and inflammatory mediator concentrations were measured using a luminex assay. All time points and conditions were repeated in triplicate, and mean value is shown for each data point.

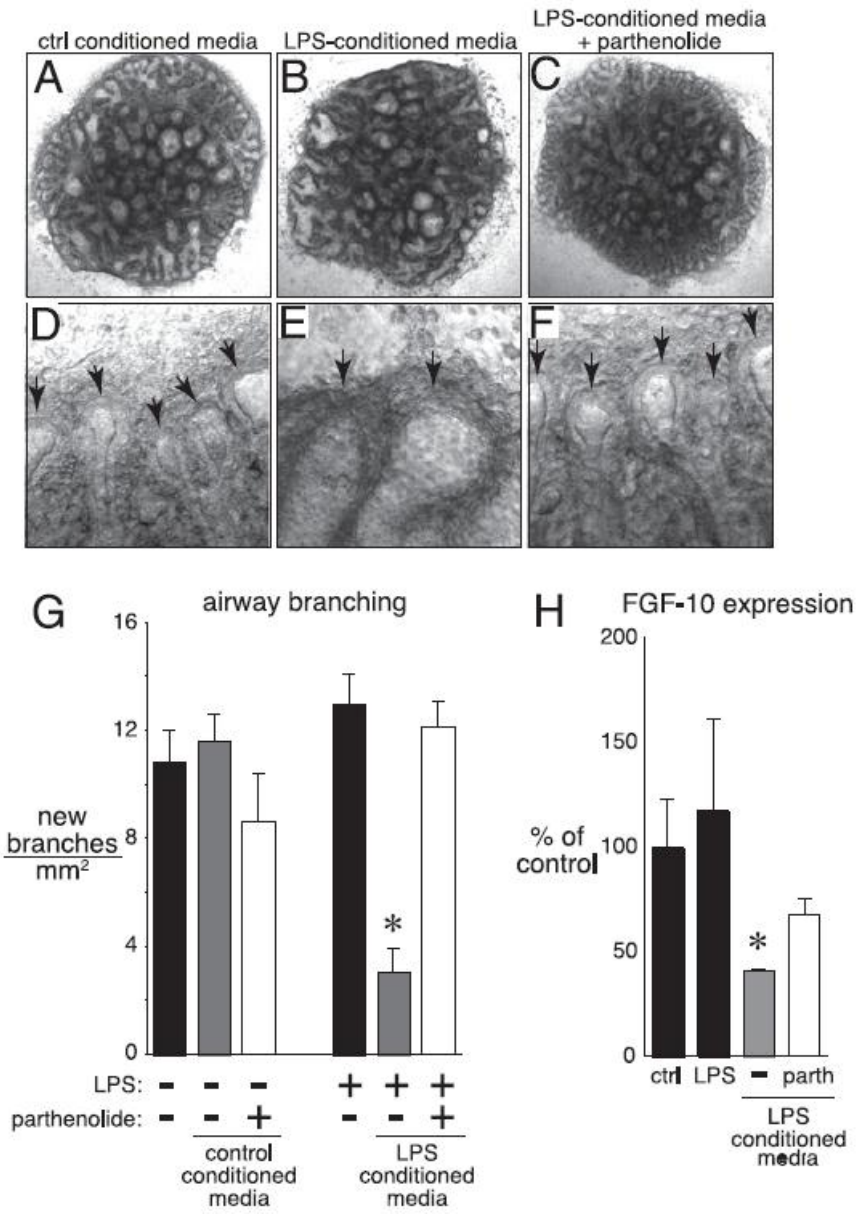

Figure 2.3. LPS-conditioned media disrupts saccular airway branching in TLR4 mutant explants. Media from control and LPS-treated BALB/cJ explants was added to LPS-resistant C.C3-Tlr4Lpsd/J explants. (A-F) Brightfield images of C.C3-Tlr4Lpsd/J explants cultured with control conditioned media (A,D), LPS-conditioned media (B,E), or LPS-conditioned media with the NF-κB inhibitor parthenolide (1 μM; C,F). Higher magnification images shown in (D-F). (G) LPS-conditioned media inhibited formation of new saccular airways in C.C3-Tlr4Lpsd/J explants (* $P < 0.001$, $n = 7$). (H) LPS-conditioned media inhibited FGF-10 expression in C.C3-Tlr4Lpsd/J explants as measured by real-time PCR (* $P < 0.05$, $n = 9$).

Inflammatory mediators down-regulate FGF-10 expression through the NF-κB pathway

To better understand which cytokines and pathways might regulate FGF-10 expression, we treated primary fetal mouse lung mesenchyme with LPS or individual cytokines and chemokines. IL-1β and TNFα, which activate NF-κB, inhibited FGF-10 expression, while the CC chemokines MCP-1 and MIP1α had no effect (Figure 2.4A). LPS, IL-1β, and TNFα each inhibited FGF-10 expression within 4 h of treatment, and this reduction in gene expression persisted for up to 48 hours (Figure 2.4B).

Since several lines of investigation suggested that NF-κB signaling was involved in the reduction of airway branching and FGF-10 expression by inflammatory mediators, we asked whether the NF-κB pathway might directly inhibit FGF-10 expression. For these studies, we utilized an FGF-10-luciferase reporter construct in transfected CHO cells. As shown in Figure 2.4C, both IL-1β and TNFα inhibited FGF-10 promoter activity as measured by luciferase activity. In these cultured cells, parthenolide could not completely block the effects of IL-1β or TNFα, but the more specific IKKβ inhibitor BMS345541 did prevent the decrease in FGF-10 luciferase expression. We next used a molecular approach to further test if direct activation or inhibition of NF-κB, in the absence of inflammatory mediators, could alter FGF-10 expression. Co-transfection of CHO cells with a constitutively active IKKβ mutant (caIKK, (Cheng, Han et al. 2007)) with the FGF-10-luciferase reporter construct inhibited FGF-10 promoter activity (Figure 2.4D). We then used a dominant-negative IκB mutant to block NF-κB

activity (Cheng, Han et al. 2007). Co-transfection of the dnIκB mutant increased FGF-10 luciferase expression, indicating baseline regulation of FGF-10 by NF-κB in absence of inflammatory stimuli. To verify that the effects we measured were not due to MAPK activation, we used chemical inhibitors of various MAPK targets to test if they affected FGF-10 expression. As seen in Figure 2.4E, IL-1β inhibited FGF-10 luciferase activity in the presence of each inhibitor, suggesting MAPK activity was not required to inhibit FGF-10 expression.

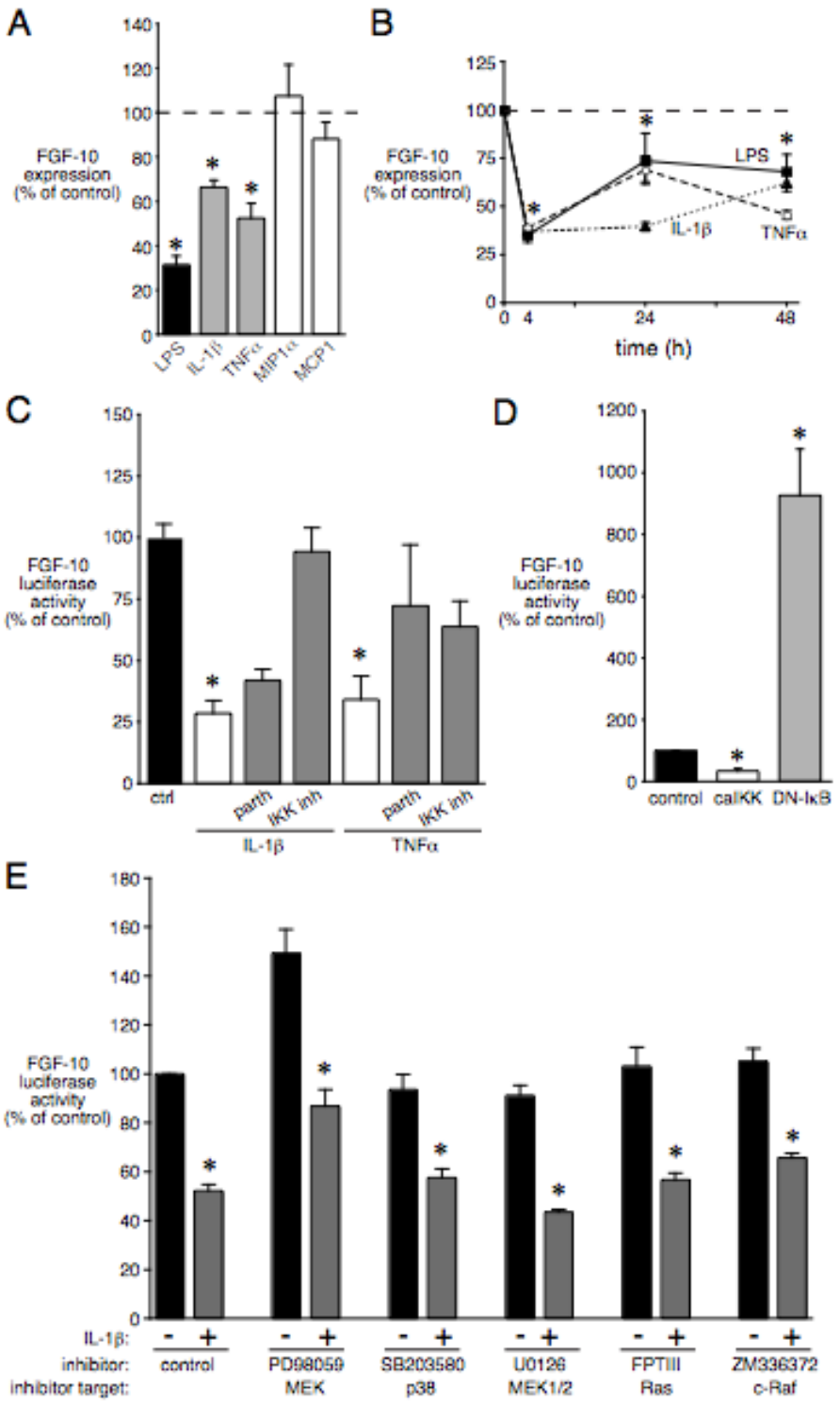
A
FGF-10 expression (% of control)
LPS
IL-1β
TNFα
MIP1α
MCP1
B
FGF-10 expression (% of control)
LPS
IL-1β
TNFα
time (h)
C
FGF-10 luciferase activity (% of control)
ctrl
parth
IKK inh
parth
IKK inh
IL-1β
TNFα
D
FGF-10 luciferase activity (% of control)
control
calKK
DN-IκB
E
FGF-10 luciferase activity (% of control)
IL-1β:
inhibitor:
inhibitor target:
control
PD98059
MEK
SB203580
p38
U0126
MEK1/2
FPTIII
Ras
ZM336372
c-Raf

Figure 2.4. NF-κB activation inhibits FGF-10 gene expression. (A). Primary fetal mouse lung mesenchymal cells were treated with LPS (250 ng/ml), IL-1β (10 ng/ml), TNFα (10 ng/ml), MIP1α (100 ng/ml), or MCP-1 (100 ng/ml). Following 4 h of treatment, RNA was isolated and FGF-10 expression measured by real-time PCR. (* $P < 0.05$, $n = 12$). (B). Time course of changes in FGF-10 gene expression following treatment of primary fetal mouse lung mesenchymal cells with LPS (solid line), TNFα (dashed line), or IL-1β (dotted line). (* $P < 0.05$, $n = 4$ for IL-1β and TNFα, $n = 30$ for LPS). (C). IL-1β and TNFα inhibit FGF-10 reporter activity. CHO cells transfected with FGF-10 luciferase were treated with IL-1β or TNFα in the absence (white bars) or presence (grey bars) of parthenolide (parth; 10 μm) or the IKKβ inhibitor BMS-345541 (IKK inh; 1 μM). (* $P < 0.05$, $n = 6$). (D). Overexpression of a constitutively active IKKβ mutant (caIKK) inhibited FGF-10 reporter activity (white bar). Expressing a dominant-negative isoform of IκB (DN-IκB) increased FGF-10 reporter activity (grey bar). (* $P < 0.05$, $n = 6$). (E) Chemical MAPK inhibitors did not prevent IL-1β-mediated inhibition of FGF-10 reporter activity (* $P < 0.05$, $n = 6$).

Sp1 and NF-κB interactions regulate FGF-10 transcription

Analysis of the FGF-10 promoter sequence revealed multiple predicted NF-κB binding sites within 5.9 kb of the transcriptional start site (Figure 2.5A). To whether these sites are required for inhibition of FGF-10 by NF-κB, serial truncations of the FGF-10 promoter were expressed in CHO cells. As seen in Figure 2.5B,C, IL-1β and caIKKβ inhibited activity of all of the truncated promoters, even the shortest construct (FGF-10-D) containing 350 bp upstream of the start site and only a single predicted NF-κB binding site. Deletion of this predicted NF-κB binding site did not prevent inhibition by caIKKβ (Figure 2.5D), suggesting that NF-κB may inhibit FGF-10 expression via interaction with other factors. The FGF-10 promoter region contains multiple GC boxes, which are predicted to bind Sp1 family transcription factors (Briggs, Kadonaga et al. 1986; Wierstra 2008). Interestingly, Sp1 expression was heterogeneous in developing fetal mouse lung, with mesenchymal cells adjacent to branching airways having variable levels of Sp1 expression (Figure 2.6B). Transfecting Sp1 increased FGF-10 reporter activity in a concentration-dependent manner (Figure 2.6C). These data, along with the multiple GC boxes in the FGF-10 promoter, suggest that Sp1 may be an important stimulator of FGF-10 expression in the fetal lung mesenchyme.

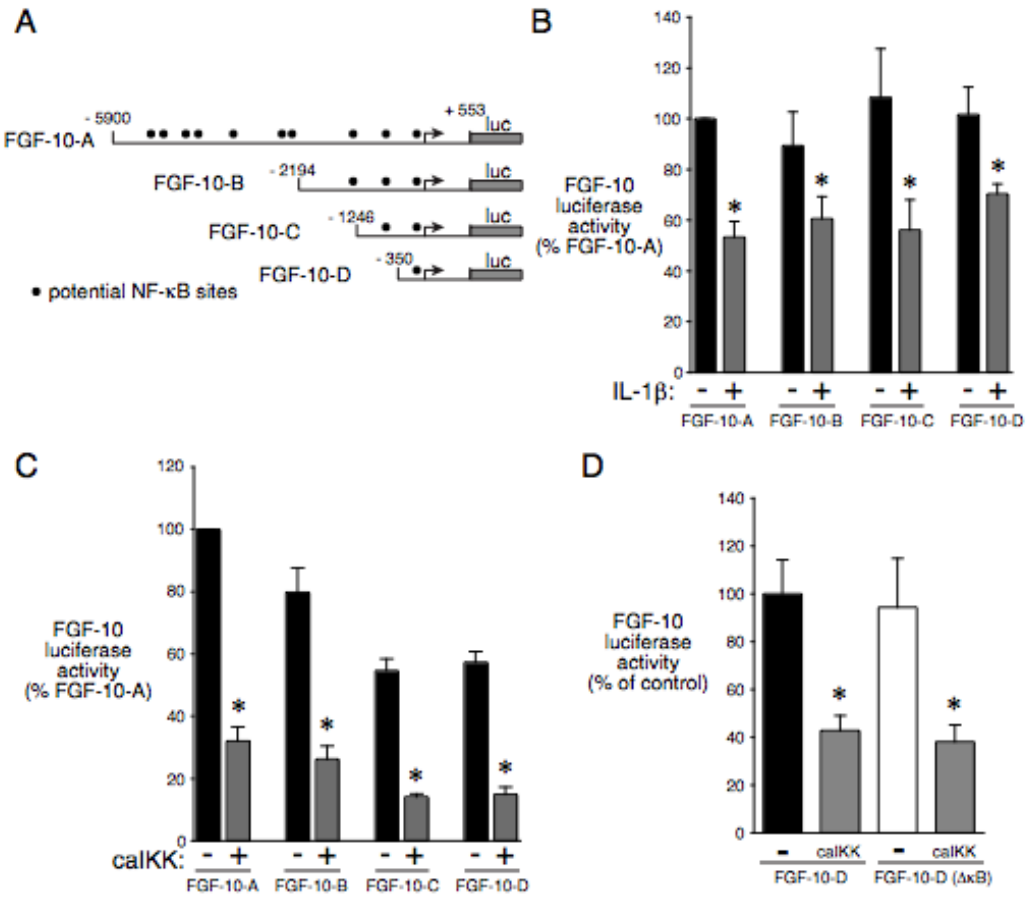

Figure 2.5. NF-κB inhibits FGF-10 promoter activity via a non-canonical interaction near the FGF-10 transcriptional start site. (A). Schematic diagram showing predicted NF-κB binding sites (•) along the FGF-10 promoter. Serial truncations of the FGF-10 promoter were generated as indicated. The FGF-10-D construct contained only a single predicted NF-κB binding site. (B,C). Deletion of all but a 350 kb upstream region of the FGF-10 promoter retained sequences required for inhibition by IL-1β (B) or calKK (C). (* $P < 0.05$, $n = 4$). (D). Deletion of the predicted NF-κB binding site in the FGF-10-D construct (ΔκB) did not prevent inhibition by calKK. (* $P < 0.05$, $n = 5$).

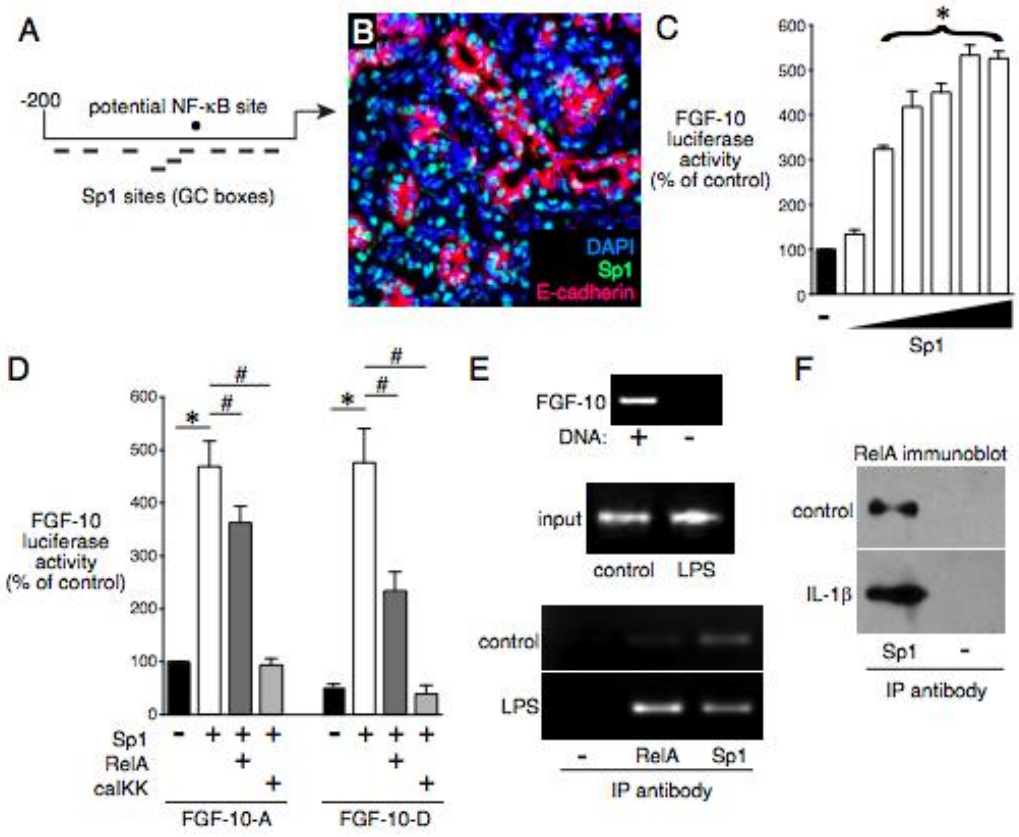

Figure 2.6. NF-κB activation interferes with Sp1-mediated FGF-10 expression. (A). Schematic diagram showing location of GC boxes predicted to bind Sp1 (black boxes) in relation to the predicted NF-κB binding site (•) and transcriptional start site in the FGF-10 promoter. (B). Sp1 immunolocalization in fetal mouse lung. Cells within E16 fetal lung mesenchyme showed variable expression of Sp1 (green). Airway epithelia indicated by E-cadherin staining (red). Nuclei labeled with DAPI (blue). (C). Sp1 increased FGF-10 reporter activity. Increasing amounts of Sp1 cDNA were co-transfected into CHO cells along with the FGF-10 luciferase reporter. (* $P < 0.05$, $n = 4$). (D). NF-κB prevents Sp1-mediated FGF-10 expression. CHO cells were co-transfected with FGF-10 luciferase, Sp1 (white bars), and either RelA (dark grey bars) or calKK (light grey bars). Experiments were repeated with the FGF-10-D truncated promoter with similar results (right hand side of graph). (* $P < 0.05$, $n = 6$). (E). LPS increases recruitment of RelA to the FGF-10 promoter. Primary fetal mouse lung mesenchymal cells were treated with LPS and transcription factor binding to the FGF-10 promoter was measured by chromatin immunoprecipitation. A small amount of RelA-FGF-10 interaction was detected in control cells, with increased signal in LPS treated cells. Sp1 appears to bind the FGF-10 promoter both in control and LPS-treated samples. (E). Sp1 and RelA interact by co-immunoprecipitation. Sp1 was immunoprecipitated from control and IL-1β-treated CHO cell nuclear lysates. Samples were immunoblotted with antibodies against RelA, demonstrating presence of RelA in Sp1 immunoprecipitates.

As Sp1 was expressed in fetal lung mesenchyme and stimulated FGF-10 expression, we wondered whether NF-κB activation might interfere with Sp1-mediated FGF-10 transcription. Co-transfection of either the RelA component of NF-κB or caIKKβ inhibited the ability of Sp1 to drive FGF-10 expression, suggesting a negative interaction between Sp1 and NF-κB on the FGF-10 promoter (Figure 2.6D). We further investigated potential Sp1 and RelA interaction using chromatin immunoprecipitation. Primary fetal lung mesenchymal cells were isolated, cultured, and treated with LPS. Crosslinked DNA was sheared into 300-500 bp fragments and protein-DNA complexes were immunoprecipitated using antibodies against Sp1 and RelA. We measured binding of Sp1 and RelA to the FGF-10 promoter using PCR primers flanking a 320 bp region immediately upstream of the transcriptional start site. In control cells, FGF-10 DNA was detected in Sp1 immunoprecipitates, but was barely detectable in RelA samples (Figure 2.6E). LPS treatment stimulated interaction between the FGF-10 promoter and both Sp1 and RelA. In addition to this ChIP data, RelA and Sp1 were co-immunoprecipitated from CHO cells, further demonstrating protein-protein interaction (Figure 2.6F). Collectively, these data indicate that RelA and Sp1 interact at the FGF-10 promoter, and that NF-κB may prevent Sp1-mediated FGF-10 expression.

Discussion

In preterm infants, inflammation arrests saccular airway formation, leading to BPD (Jobe 1999; May, Prendergast et al. 2009). Identifying how inflammation alters the epithelial-mesenchymal interactions critical for lung development is important for understanding BPD pathogenesis. We show here that NF-κB activation inhibits expression of FGF-10, a key growth factor for lung development. Both LPS and soluble inflammatory mediators that signal through the NF-κB pathway can decrease FGF-10 in the fetal lung mesenchyme and in heterologous reporter systems. Interestingly, these effects do not appear to require direct DNA binding of NF-κB to a canonical NF-κB response element in the FGF-10 promoter. Instead, our data suggest that NF-κB interacts with Sp1 at the FGF-10 promoter, inhibiting the ability of Sp1 to drive FGF-10 expression. The ability of NF-κB to disrupt normal Sp-1 mediated expression during development may link inflammation and altered lung morphogenesis at the molecular level.

A variety of microbial pathogens can cause chorioamnionitis and neonatal sepsis (Garland, Ni Chuileannain et al. 2002; Klinger, Levy et al. 2009). The risk of developing BPD following exposure to infection or inflammation is not exclusive to a particular pathogen. Many different microorganisms and microbial products activate NF-κB (Li and Verma 2002), making this a common mechanism by which infection and inflammation can inhibit normal lung

development. The cellular site of NF-κB activation may also play an important role in BPD pathogenesis. Our data show that inflammation and NF-κB activation can alter the expression of genes in the fetal lung mesenchyme in disease. As these cells are critical for airway development, they may be key targets of inflammation. In gaining access to and targeting the fetal lung mesenchyme, microbial products and TLR agonists must breach the barriers provided by the airway epithelia or pulmonary vascular endothelia to directly activate NF-κB and inhibit FGF-10 in the interstitially located mesenchymal cells. Alternatively, the initial cellular site of inflammatory response may be the airway epithelia or lung macrophage. These activated cells may then release soluble inflammatory mediators including TNFα and IL-1β that then activate NF-κB in fetal lung mesenchyme, inhibiting FGF-10 and preventing airway branching. By either path, NF-κB activation in fetal lung mesenchymal cells appears to be a key mechanism in arresting lung morphogenesis.

During the innate immune response, NF-κB stimulates expression of many pro-inflammatory genes. This pathway linking microbial products and cytokines to increased gene expression has been well studied (Ozato, Tsujimura et al. 2002). In the absence of inflammatory stimuli, NF-κB subunits are bound to IκB in the cell cytoplasm where they remain quiescent. Activation of innate immune signaling via microbial substances or cytokines increases IKK activity, leading to phosphorylation and degradation of IκB, and releasing NF-κB to traffic into the nucleus. The termination of NF-κB activation can occur by displacing the NF-κB complex from genomic DNA or by targeted degradation of nuclear NF-κB

proteins. The mechanisms linking NF-κB to suppression of gene expression, however, are not as well understood. Many of the genes activated by NF-κB are pro-inflammatory cytokines or contribute to the cellular inflammatory response. Genes downregulated by NF-κB are less numerous, are involved in more diverse cellular roles, and do not have obvious connections to the inflammatory response (Sitcheran, Cogswell et al. 2003; Campbell, Rocha et al. 2004). Because of NF-κB's transactivation function when bound to DNA, inhibition of gene expression may involve protein-protein interactions where NF-κB subunits prevent binding or activation of other stimulatory transcription factors, resulting in decreased gene expression. Interestingly, NF-κB appears to interfere with Sp1-mediated transcription in several genes, including BMP4 (Zhu, Li et al. 2007), TGFBRII (Bauge, Beauchef et al. 2008), and the collagen genes Col1A2 (Verrecchia, Wagner et al. 2002) and Col2A1 (Chadjichristos, Ghayor et al. 2003). While genes inhibited by Sp1-NF-κB interaction may plan similar roles in tissue morphogenesis and wound healing, a more genome wide assessment is required before determining the significance of this mechanism.

Our data suggest that NF-κB inhibits the normal stimulatory function of Sp1 on FGF-10 expression. Sp1 strongly activates FGF-10 expression, likely through binding the multiple GC boxes present in the FGF-10 promoter. Increasing Sp1 concentrations often amplify the expression of genes like FGF-10 with arrays of GC boxes. Amplification appears to involve both Sp1-DNA binding and the formation of large tertiary Sp1-Sp1 complexes that function to recruit components of the basal transcriptional apparatus (Courey, Holtzman et al.

1989). NF-κB and Sp1 interactions can both promote and inhibit transcriptional activation. NF-κB and Sp1 cooperate to increase HIV-1 transcription by binding distinct but immediately adjacent DNA sequences (Perkins, Agranoff et al. 1994). Sp1 binds consensus NF-κB sequences in the P-selectin (Hirano, Tanaka et al. 1998), NR1 (Liu, Hoffman et al. 2004), and IL-6 promoters (Kang, Brown et al. 1996), competing with RelA for DNA binding. Sp1 and RelA also interact at the protein-protein level (Kuang, Berk et al. 2002), as we also demonstrated in CHO cells. We speculate that RelA may prevent Sp1 mediated amplification and tertiary Sp1-Sp1 complex formation on the FGF-10 promoter. NF-κB activation may also recruit additional inhibitory transcription factors to the FGF-10 promoter, reducing gene expression. The Sp family member Sp3 can act as an inhibitor of Sp1-mediated gene amplification, binding GC boxes but preventing Sp1-Sp1 complex formation (Majello, De Luca et al. 1997; Yu, Datta et al. 2003). Our future studies will further test these potential mechanisms for regulating FGF-10 expression. The findings presented here also raise the possibility that NF-κB activation could cause a change in mesenchymal cell differentiation. Instead of expressing critical growth factors or other genes important for fetal lung morphogenesis, mesenchymal cells exposed to inflammation may adopt a more mature fibroblast-like phenotype. While these cells would normally be found in fully developed tissues, they could lack the ability to drive airway morphogenesis in an immature lung.

Identifying how components of the inflammatory signaling cascade interact with normal morphogenic pathways will help us understand the molecular

mechanisms of developmental disorders. Our data show that soluble inflammatory mediators produced locally in the fetal or neonatal lung are capable of inhibiting lung morphogenesis. Production of inflammatory cytokines that activate the IKKβ/NF-κB pathway inhibits mesenchymal expression of FGF-10. By determining if these changes are reversible and then discovering interventions to restart developmental processes following inflammatory exposures, we may find new therapeutic targets for preventing or treating morbidities associated with preterm birth.

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CHAPTER III

Interactions between NF-κB and SP3 Connect Inflammatory Signaling with Reduced FGF-10 Expression

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Abstract

Inflammation inhibits normal lung morphogenesis in preterm infants. Soluble inflammatory mediators present in the lungs of patients developing bronchopulmonary dysplasia disrupt expression of multiple genes critical for development. However, the mechanisms linking innate immune signaling and developmental programs are not clear. NF-κB activation inhibits expression of the critical morphogen fibroblast growth factor-10 (FGF-10). Here we show that interactions between the RelA subunit of NF-κB and Sp3 suppress Sp1-mediated FGF-10 expression. Sp3 co-expression reduced Sp1-mediated FGF-10 promoter activity, suggesting antagonistic interactions between Sp1 and Sp3. Chromatin

immunoprecipitation of LPS-treated primary mouse fetal lung mesenchymal cells detected increased interactions between Sp3, RelA, and the FGF-10 promoter. Expression of a constitutively active IKKβ mutant not only decreased FGF-10 promoter activity, but also increased RelA-Sp3 nuclear interactions. Expression of a dominant-negative IκB, which blocks NF-κB nuclear translocation, prevented inhibition of FGF-10 by Sp3. The inhibitory functions of Sp3 required sequences located in the N-terminal region of the protein. These data suggested that inhibition of FGF-10 by inflammatory signaling involves the NF-κB-dependent interactions between RelA, Sp3, and the FGF-10 promoter. NF-κB activation may therefore lead to reduced gene expression by recruiting inhibitory factors to specific gene promoters following exposure to inflammatory stimuli.

Introduction

During fetal lung development, spatially restricted expression of mesenchymal growth factors stimulates cell proliferation, elongation of epithelial tubes, and expansion of newly formed airways (Morrisey and Hogan 2010). Fibroblast growth factor-10 (FGF-10) is expressed in the lung mesenchyme from the earliest stages of development and is critical for lung formation. By activating its receptor FGFR2b on adjacent epithelial cells, FGF-10 stimulates both proliferation and airway branching (Arman, Haffner-Krausz et al. 1999; Ohuchi, Hori et al. 2000). Mice lacking either FGF-10 or FGFR2b develop only

rudimentary lung structures containing tracheas but lacking bronchial airways (Min, Danilenko et al. 1998; Arman, Haffner-Krausz et al. 1999). During later stages of lung development, transgenic expression of an FGF-10 antagonist or addition of inhibitory antibodies disrupts normal airway elongation and branching (Peters, Werner et al. 1994; Celli, LaRochelle et al. 1998; Hokuto, Perl et al. 2003). While many genes play important roles in lung development, data clearly implicate FGF-10 as a major regulator of lung morphogenesis.

Defects in FGF-10 expression contribute to lung disease in both children and adults. Preterm infants with severe BPD have reduced saccular airway and alveolar duct formation and have lower FGF-10 expression in their lungs (Christou and Brodsky 2005; Benjamin, Carver et al. 2010). In adult patients, FGF-10 haploinsufficiency can lead to abnormal pulmonary function and chronic obstructive pulmonary disease (COPD) (Klar, Blomstrand et al. 2011). In both COPD and BPD, lung inflammation plays a key role in disease pathogenesis, and also inhibits FGF-10 expression (Baraldi and Filippone 2007; Benjamin, Smith et al. 2007; Bhandari and Bhandari 2009). Inflammatory signaling may therefore interfere with the mechanisms regulating FGF-10 expression and formation and maintenance of normal lung architecture.

Microbial products and inflammatory mediators stimulate lung inflammation by binding pattern recognition receptors on the surface of cells (Beutler, Hoebe et al. 2003; Newton and Dixit 2012). While the expression of multiple receptors provides a diverse detection repertoire at the cell surface, many of these receptors signal through overlapping intracellular pathways that

activate the transcription factor NF-κB (Klar, Blomstrand et al. 2011; Oeckinghaus, Hayden et al. 2011). In quiescent cells, NF-κB resides in the cytoplasm bound to IκB. When cell surface receptors detect inflammatory stimuli, IKKβ phosphorylates IκB, displacing it from NF-κB and leading to IκB degradation (Huxford, Huang et al. 1998; Birbach, Gold et al. 2002). NF-κB is then free to traffic into the nucleus, where it regulates gene transcription. In addition to driving the acute innate immune response, the NF-κB signaling pathway also influences wound repair, tumor formation, and tissue morphogenesis (Guttridge, Albanese et al. 1999; Cao and Karin 2003; Adams, Pankow et al. 2007; Ben-Neriah and Karin 2011; Chen, Meng et al. 2011).

Because inflammation plays a major role in the pathogenesis of BPD, we previously investigated how innate immunity and NF-κB activation in the fetal lung affects expression of genes important for normal development. NF-κB activation by microbial products or inflammatory mediators inhibits FGF-10 expression in the fetal mouse lung, leading to alterations in normal airway formation (Benjamin, Smith et al. 2007).

NF-κB most commonly acts as a transcriptional activator; the mechanisms by which NF-κB can reduce gene transcription are less well characterized. Inhibition of FGF-10 does not involve direct interaction between NF-κB and canonical DNA binding elements in the FGF-10 promoter (Blackwell, Hipps et al. 2011). We hypothesized that NF-κB might therefore inhibit FGF-10 expression by regulating the activity of other transcription factors.

The FGF-10 promoter lacks a TATA box sequence but contains multiple conserved GC rich regions predicted to bind Sp proteins. Sp1 is a potent activator of TATA-less gene transcription. The related Sp family member Sp3 can act as both a transcriptional activator and repressor, depending on cellular context (Suske 1999). We previously showed that Sp1 drives FGF-10 transcription and that this effect can be inhibited by NF-κB activation (Benjamin, Carver et al. 2010). However, it was not clear how NF-κB might inhibit Sp1-mediated transcription in the absence of conserved predicted NF-κB binding sites. Here we show that NF-κB activation recruits Sp3 to the FGF-10 promoter, where it functions as a transcription inhibitor. This novel mechanism may provide new insight into how inflammation can alter expression of developmentally important genes.

Materials and Methods

Reagents

Gel purified *Escherichia coli* LPS (O55:B5) was purchased from Sigma-Aldrich (St. Louis, MO). Recombinant IL-1β was purchased from R&D Systems. The IκB kinase β (IKKβ) inhibitor BMS-345541 was purchased from EMD Biosciences (San Diego, CA). Abs against RelA, Sp1, and Sp3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-FLAG was purchased from Sigma (St. Louis, MO). Rabbit anti-GFP was purchased from

Millipore (Bedford, MA). Rat anti-E-cadherin and Alexa Fluor-conjugated secondary Abs were procured from Invitrogen (Carlsbad, CA) CHO-K1 cells were obtained from ATCC and cultured as directed in Ham's F-12 medium supplements with 10% fetal bovine serum from Atlanta Biologicals (Atlanta, GA).

Plasmids

The FGF-10 luciferase reporter in the pXPI vector was generously supplied by Benoit Bruneau (Gladstone Institute of Cardiovascular Disease, San Francisco, CA). This construct contains ~6-kb BamHI fragment from the murine FGF-10 gene placed immediately upstream of the luciferase coding sequence. Serial truncations were made using PCR mutagenesis. Deletions were confirmed by restriction digest and sequencing. The Sp1 and Sp3 expression plasmids were generated by inserting the murine cDNA for these genes into the pcDNA3.1/CT-GFP vector (Invitrogen, Carlsbad, CA). Mutations made within the Sp3 coding sequence were produced using the QuickChange II XL mutagenesis system (Agilent, Palo Alto, CA), and were confirmed by sequencing. FGF-10 luciferase reporter constructs were co-transfected into CHO cells using SuperFect (Qiagen, Velencia, CA). pSV-β-Gal was used to control for transfection efficiency. Cells were lysed after 48 h, and luciferase activity measured by Steady-Glo system (Promega, Madison, WI) on a microtiter plate with a luminometer. Arbitrary light units were normalized to β-galactosidase activity.

Immunostaining, imaging, and analysis

To quantify mean fluorescent intensity in explanted fetal mouse lung, optical sections of explants immunostained for Sp1 and Sp3 were collected on an Olympus FV1000 laser scanning confocal microscope (Olympus, Melville, NY). The images were then imported into SlideBook (Intelligent Imaging Innovations Inc, Ringsby, CT). Individual nuclei were selected and their mean fluorescent intensity for both channels was quantified.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed using primary fetal mouse lung mesenchymal cells (Yamamoto, Baldwin et al. 2011). Control and LPS-treated cells were fixed with 1% formaldehyde and lysed. DNA was sheared by sonication, phenol:chloroform extracted, and precipitated with ethanol. Samples were then incubated with anti-Sp1, anti-Sp3, and anti-RelA Abs for immunoprecipitation. Following stringent washing, DNA–protein linkages were disrupted and released DNA fragments were phenol:chloroform extracted and precipitated with ethanol. The 320-bp region of the FGF-10 promoter (upstream of and including the transcriptional start site) was detected by PCR. Products were analyzed by agarose gel electrophoresis.

DNA-based ELISA

Quantitative protein assay for Sp1 and Sp3 was performed using TransAm transcription factor ELISA (Active Motif, Carlsbad, CA). Control and LPS treated 3T3 cells were grown in culture to 90% confluency, harvested, and lysed by hypotonic buffer and 0.5% NP-40. Nuclei were pelleted and equal amounts of nuclear proteins were applied to wells coated with oligonucleotide containing consensus binding sequences for Sp1 or Sp3. After incubation with primary antibodies that recognize Sp1 and Sp3 only in their DNA-bound states, an HRP-conjugated secondary antibody provided quantitative detection of Sp1 and Sp3 by spectrophotometry. 5 µg of nuclear extract from MCF-7 cells, supplied by the manufacturer, was used as a positive control. Wells without nuclear extract were used as negative controls.

In situ proximity ligation assay

CHO cells were transfected with plasmids expressing FLAG-tagged RelA and cIKKβ, dnIκB, or empty vector. Cells were fixed 24 hours after transfected in 4% PFA before being permeabilized with 0.1% Triton X-100 (Pierce, Rockford, IL) and blocked with normal donkey serum (Sigma). Interactions between FLAG-RelA and Sp3 were measured using the Red Duolink II In situ PLA kit (OLink Bioscience, Uppsala, Sweden). Cells were incubated with anti-FLAG and anti-Sp3 primary antibodies overnight. After washing with PBS, the cells were incubated with PLA anti-Mouse MINUS and PLA anti-Rabbit PLUS for 1 hour at 37°C in a humidified chamber. After washing, the ligation, polymerization, and

hybridization steps of the Duolink II protocol were carried out as instructed by the manufacturer. Slides were mounted in medium containing DAPI. Control cells were not incubated with the anti-FLAG antibody. Mounted cells were imaged using an inverted Olympus BX-81 fluorescence microscope, and analyzed using SlideBook software.

Results

Sp3 is expressed throughout development in the fetal lung

We hypothesized that NF-κBinhibits FGF-10 transcription by interfering with other transcriptional activators. Because Sp1 interacts with GC-rich sequences, such as those found in the FGF-10 promoter (Fig. 3.1C) and stimulates FGF-10 promoter activity, we first tested if Sp1 and Sp3 might both regulate FGF-10. Sp3 binds similar GC-rich regions as Sp1, but can function as a transcriptional inhibitor. Like Sp1, Sp3 was expressed throughout fetal mouse lung development in both epithelial and mesenchymal cell populations (Fig. 3.1A). Expression appeared heterogenous, with higher levels of nuclear staining in some individual cells compared to others. Immunoblotting confirmed expression in the fetal mouse lung with the four previously identified Sp3

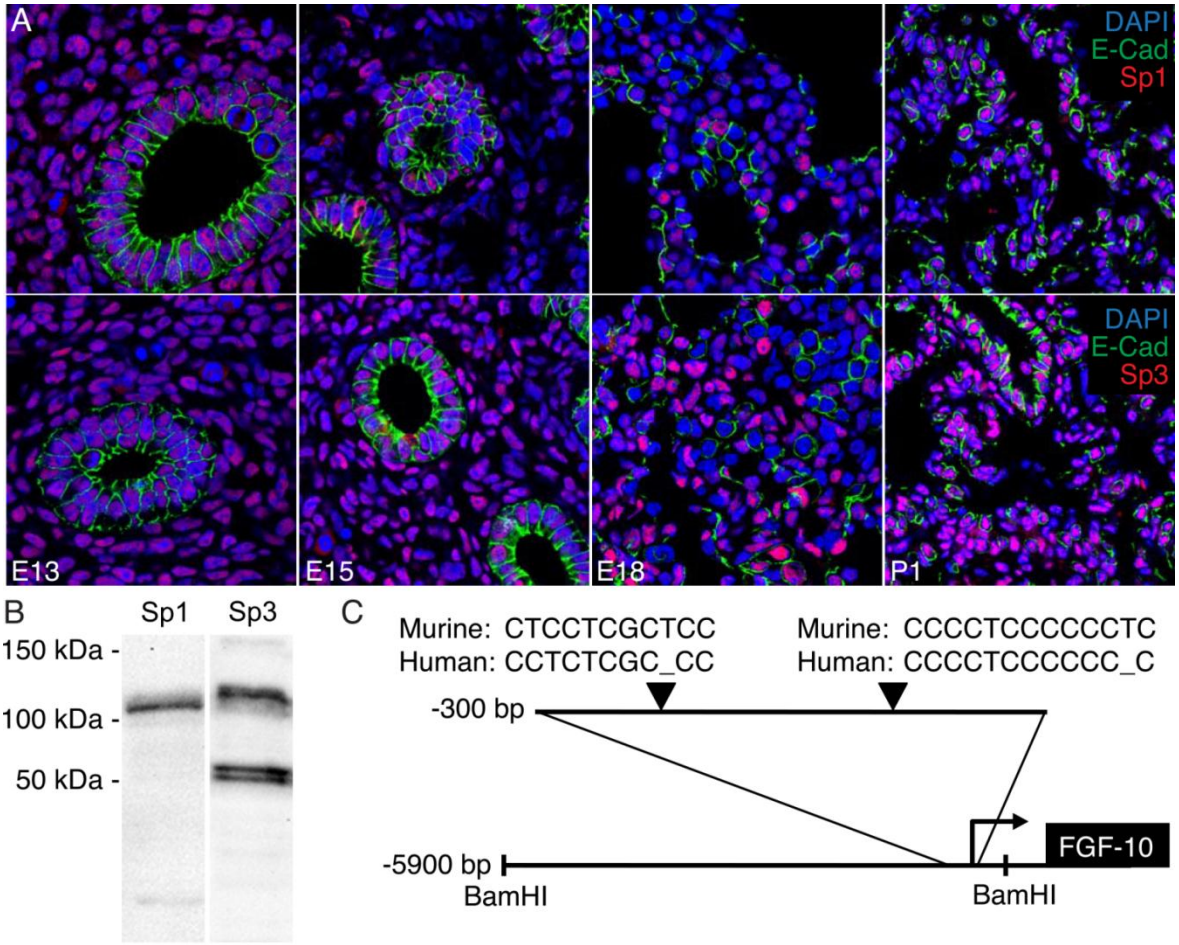
A
DAPI
E-Cad
Sp1
DAPI
E-Cad
Sp3
E13
E15
E18
P1
B
Sp1
Sp3
150 kDa -
100 kDa -
50 kDa -
C
Murine: CTCCTCGCTCC
Human: CCTCTCGC_CC
Murine: CCCCTCCCCCCTC
Human: CCCCTCCCCCC_C
-300 bp
-5900 bp
BamHI
BamHI
FGF-10

Figure 3.1. Sp1 and Sp3 expression in the fetal mouse lung. A, Immunostaining of lung sections from embryonal day 13 (E13) through postnatal day 1 (P1) mice using antibodies against Sp1 (top panels) or Sp3 (bottom panels). Airway epithelial cells were immunostained using an antibody against E-cadherin (E-Cad), and nuclei were labeled with DAPI. B, Immunoblot of total fetal mouse lung homogenate using antibodies against Sp1 (left) and Sp3 (right). C, Sequence comparison of murine and human fgf-10 promoter regions, showing conserved GC-rich regions upstream of the transcriptional start site.

isoforms present in fetal lung homogenate and in primary fetal lung mesenchymal cell lysates (Fig. 3.1B).

Sp3 decreases Sp1-mediated FGF-10 promoter activity

To test if Sp3 could inhibit FGF-10 transcription, we employed an FGF-10 luciferase reporter plasmid containing a 6 kb region of the mouse Fgf10 gene that included the transcriptional start site and 5' UTR. In transfected CHO cells, expression of increasing amounts of mouse Sp3 cDNA did not change basal FGF-10 luciferase activity (Fig. 3.2A). As we demonstrated previously, Sp1 increased FGF-10 luciferase expression, but co-expression with increasing Sp3 inhibited this activation in a concentration dependent manner, returning reporter expression to basal levels (Fig. 3.2A). These data suggested that Sp1 and Sp3 have opposing functions on FGF-10 transcription.

NF-κB activation recruits Sp3 to the FGF-10 promoter

Because LPS and inflammatory mediators that signal through NF-κB reduce FGF-10 expression, we tested if NF-κB activation might increase the amount of Sp3 present at the FGF-10 promoter. We treated primary fetal lung mesenchymal cells with LPS and performed ChIP analysis using antibodies against Sp1, Sp3 and the NF-κB subunit RelA (Fig. 3.2B). In control cells, we detected Sp1-FGF-10 interactions; faint bands were also detected in samples precipitated with antibodies against Sp3 and RelA. However, in LPS-treated

cells, interactions between all three proteins and the FGF-10 promoter region were strongly detected. We obtained similar results using a molecular approach to increase NF-κB activation. Expressing a constitutively active IKKβ mutant (cIKKβ, in which serines 177/181 are replaced with negatively charged glutamate residues) (Mercurio, Murray et al. 1999) increased Sp3-FGF-10 interactions in CHO cells (Fig. 3.2C), further supporting the possible connection between Sp3 and NF-κB in regulating the FGF-10 promoter. We next tested the functional effects of both Sp3 and increased IKKβ activity. Sp3 and cIKKβ separately inhibited Sp1-mediated FGF-10 promoter activity and co-expressing Sp3 and cIKKβ appeared to have an additive inhibitory effect (Fig. 3.2C). Collectively, the ChIP and co-expression data suggested that Sp3 might play a role in NF-κB dependent FGF-10 inhibition.

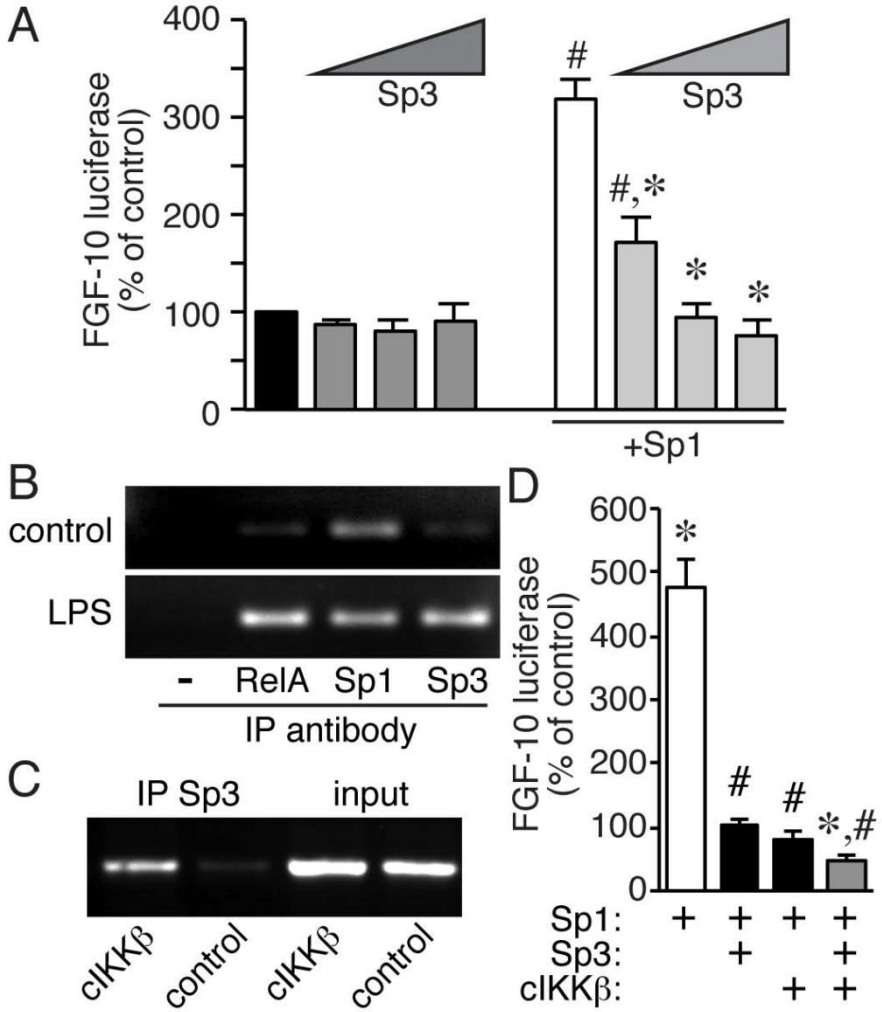

Figure 3.2. Sp1 and Sp3 antagonistically regulate FGF-10 promoter activity. A, CHO cells were transfected with FGF-10 luciferase and increasing concentrations of Sp3 cDNA in the absence or presence of Sp1 cDNA. Sp1 expression increased FGF-10 luciferase activity, and this effect was inhibited by Sp3. #P < 0.01 compared to control. *P < 0.01 compared to Sp1 only (n = 6). B, ChIP analysis of the FGF-10 promoter in primary fetal mouse lung mesenchymal cells. Cells were cultured in the absence or presence of LPS (250 ng/ml) for 4 h. DNA:protein complexes were immunoprecipitated with antibodies against RelA, Sp1, Sp3 or rabbit IgG. The immunopreciptated DNA was amplified by PCR using primers flanking a 350 bp of the FGF-10 promoter immediately upstream of the transcriptional start site. C, Chromatin immunoprecipitation from CHO cells expressing either constitutively active mutant IKKβ (caIKKβ) or GFP control plasmid. Anti-Sp3 antibodies used as in B. Input DNA included on right. D, Expression of either Sp3 or caIKKβ (black bars) inhibited Sp1-activated FGF-10 luciferase activity. Co-expression of both Sp3 and caIKKβ (grey bar) had an additive inhibitory effect. #P < 0.01 compared to Sp1 only, *P < 0.01 compared to control (n = 4).

Sp1 and Sp3 levels are not altered by NF-κB activation

LPS and NF-κB activation could reduce FGF-10 transcription by changing the relative levels of Sp1 and Sp3. As seen in Figure 3.3, activation of NF-κB by treating fetal lung mesenchyme with LPS (Fig. 3.3A) or CHO cells with rIL-1β (Fig. 3.3B) did not change the amount of Sp3 in nuclear extracts based on immunoblotting. Importantly, we also did not observe any differences in the relative abundance of each of the four naturally occurring Sp3 isoforms. Treating CHO cells with rIL-1β also did not cause changes in Sp1 or Sp3 protein concentration in nuclear extracts as measured by DNA-based ELISA (Fig. 3.3C). We next tested this hypothesis by immunostaining control and LPS-treated lung explants from E15 fetal mice. In figure 3.3D, Sp1 and Sp3 expression appear similar in control and LPS-treated explants. Quantification of nuclear fluorescence intensity in cells located in the mesenchyme showed that LPS caused small changes in both Sp1 and Sp3 (Fig. 3.3E,F) but did not appear to change the relative Sp1:Sp3 ratio. In addition, LPS and the IKKβ inhibitor BMS345541 also did not change the relative expression or subcellular localization of Sp3 in primary fetal mouse lung mesenchymal cells (Fig. 3.3G). These data suggest the effects of NF-κB on Sp-mediated gene expression in the fetal lung mesenchyme did not involve dramatic changes in protein levels or subcellular localization.

Modification by SUMO is not required for Sp3 to regulate FGF-10 – The inhibitory effects of Sp3 on the FGF-10 promoter activity could involve post-translational modifications. SUMOylation has been reported to switch Sp3 from a

transcriptional activator to a repressor (Ross, Best et al. 2002). We therefore mutated the lysine residues in Sp3 shown to be SUMO modified and tested Sp3 repressor function (Fig. 3.4A,B). Mutant Sp3 lacking either one or both SUMO sites was still able to inhibit FGF-10 promoter activity, suggesting that SUMOylation does not play a role in the ability of Sp3 to repress FGF-10 expression (Fig. 3.4C).

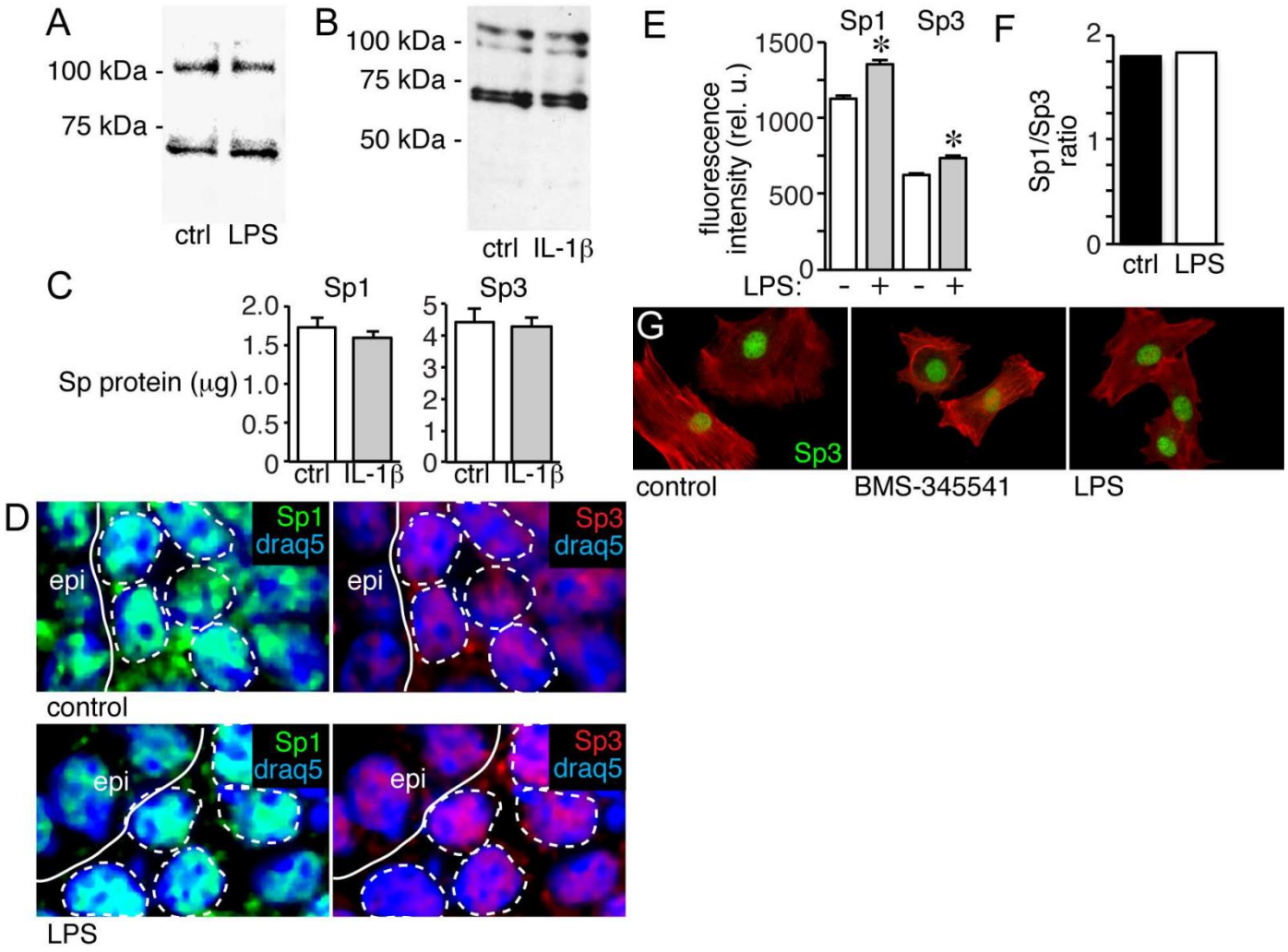

Figure 3.3. NF-κB activation does not alter relative Sp1 and Sp3 expression levels. A,B, Representative Sp3 immunoblot from control and LPS-treated mouse fetal lung mesenchymal cells (A) and control and IL-1β treated CHO cells (B), showing no differences in Sp3 expression level or relative isoform abundance. C, The protein levels of Sp1 (left) and Sp3 (right) in control and IL-1β treated CHO cell nuclei were measured using DNA-binding ELISA. D-F, Control and LPS-treated E15 fetal mouse lung explants were immunolabeled with antibodies against Sp1 and Sp3. Cell nuclei within the mesenchyme were identified by confocal microscopy. Sum fluorescence intensity in approximately 700 cell nuclei was quantified in control and LPS-treated samples. LPS increased both Sp1 and Sp3 expression similarly (B, *P < 0.01), having no net effect on the Sp1/Sp3 ratio (C). G, NF-κB activation did not alter Sp3 subcellular localization. Primary fetal mouse lung mesenchymal cells were immunolabeled with antibodies against endogenous Sp3 following treatment with the IKKβ inhibitor BMS-345541 or LPS. Cells were counterstained with phalloidin (red) and DAPI (blue).

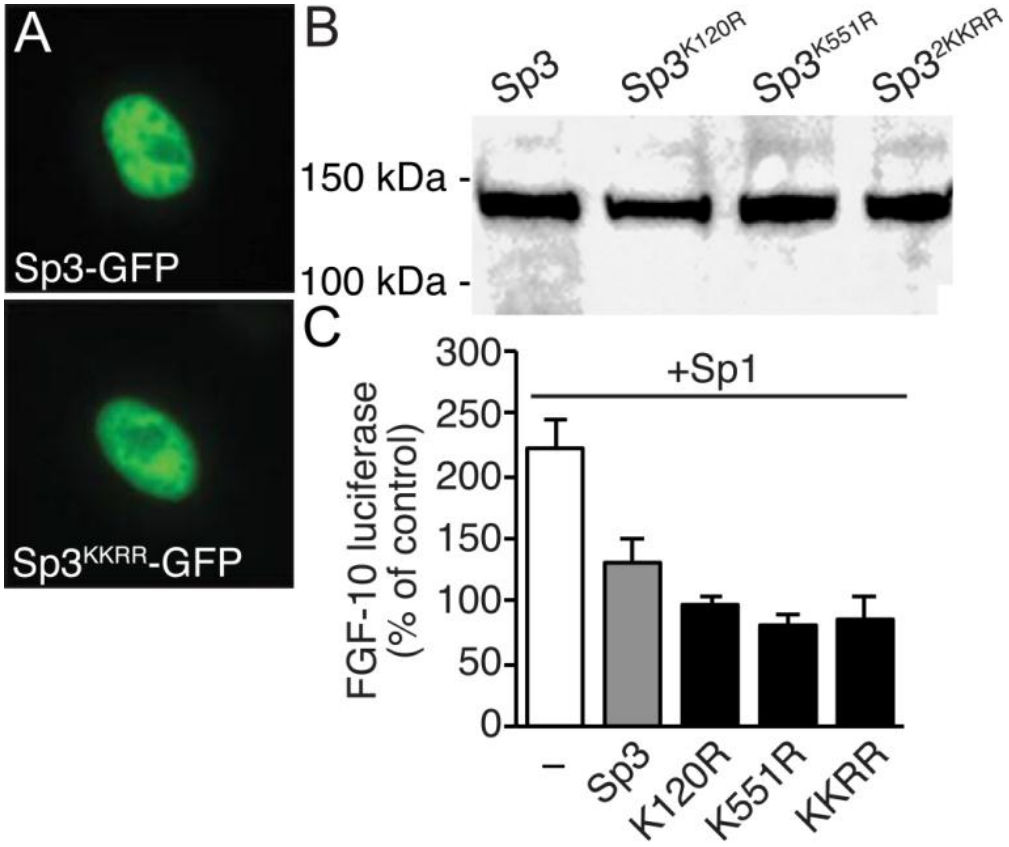

Figure 3.4. Mutation of Sp3 SUMOylation sites does not alter ability of Sp3 to inhibit FGF-10 promoter activity. CHO cells were transfected with Sp3 mutants containing single amino acid mutations (K120R and K551R) or mutation of both SUMOylation sites (KKRR). A, Sp3-GFP and Sp3KKRR-GFP localized to the cell nucleus when expressed in CHO cells. B, Immunoblot of transfected CHO cells showed that each Sp3 SUMOylation mutant was expressed in CHO cells at the predicted MW. C, Mutation of SUMOylated lysine residues in Sp3 did not affect the ability of Sp3 to inhibit Sp1-mediated FGF-10 promoter activity (n = 8).

Sp3 interacts with the RelA subunit of NF-κB

We next tested if activation and nuclear import of NF-κB subunits could recruit Sp3 to the FGF-10 promoter and inhibit transcription. Using an in situ proximity ligation assay that detects colocalization of two proteins within 40 nm, we tested if RelA and Sp3 colocalize within cell nuclei (Fig. 3.5). RelA-Sp3 colocalization was detected in control cells by quantifying fluorescent intensity in individual cell nuclei (Fig. 3.5A). Transfection with cIKKβ increased sum fluorescence approximately 3-fold (Fig. 3.5B). We next tested the effect of expressing a dominant-negative IκB which cannot be phosphorylated by IKK and therefore prevents NF-κB nuclear import. In cells transfected with dnIκB, we did not detect RelA-Sp3 colocalization above background levels (Fig. 3.5C-E). Spatial interaction between RelA and Sp3 therefore correlates with NF-κB activation.

To determine whether Sp3 requires NF-κB to downregulate FGF-10, we co-transfected Sp3 and dnIκB into CHO cells with an FGF-10 luciferase reporter. When NF-κB activation was inhibited with dnIκB expression, Sp3 did not decrease FGF-10 promoter activity, but instead activated FGF-10 expression (Fig. 3.5F). These data suggest that nuclear import of activated of RelA recruits Sp3 to the FGF-10 promoter where it then inhibits FGF-10.

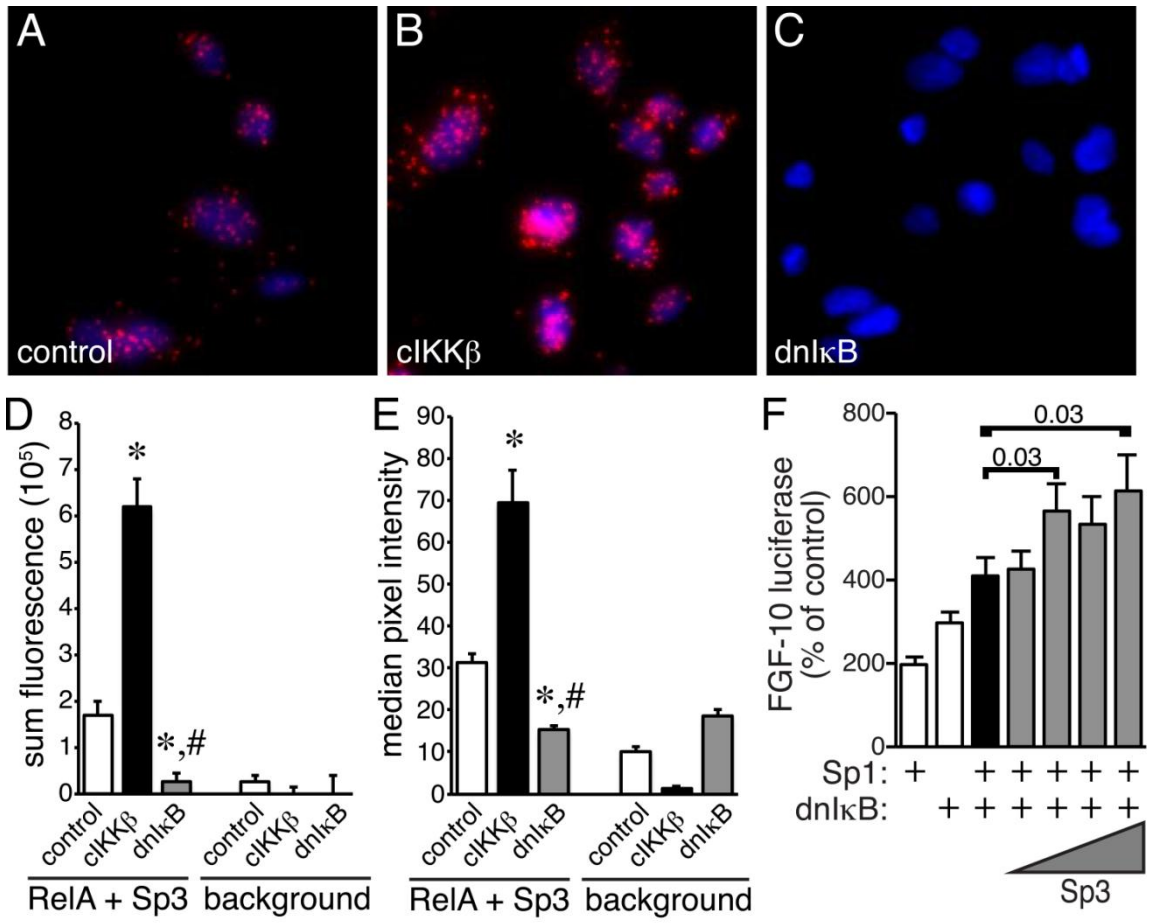

Figure 3.5. Interactions between Sp3 and nuclear RelA regulate FGF-10 promoter activity. A-C, Evidence for Sp3-RelA interactions using an in situ proximity ligation assay (PLA). CHO cells were transfected with FLAG-tagged RelA and either control empty vector (A), a constitutively active IKKβ mutant (cIKKβ) (B), or a dominant-negative IκB mutant that prevents nuclear RelA translocation (C). Following fixation and processing, red fluorescence was visualized at sites where Sp3 and RelA-FLAG localized to within 40 nm of each other. Nuclei were labeled with DAPI. Fluorescence microscopy images were acquired from 60-70 cells within each group. Expression of cIKKβ increased both the sum fluorescence intensity (D) and median pixel intensity (E), consistent with increased RelA-Sp3 colocalization (*P < 0.01). Expression of dnIκB reduced in situ PLA signal to background levels. F, Increasing levels of Sp3 (0.1, 0.2, 0.4 or 0.8 µg) did not inhibit Sp1-mediated FGF-10 promoter activity when CHO cells were co-transfected with dnIκB (n = 6).

The N-terminal region of Sp3 is required for FGF-10 inhibition

We next determined if each of the four translation-dependent isoforms of Sp3 were equally able to inhibit FGF-10 expression. The ATG start sites in Sp3 were mutated so that each plasmid expressed only a single Sp3 isoform. Each isoform had similar nuclear localization and was expressed at the appropriate molecular weight (Fig. 3.6A,B). While expression of the longest isoform reduced FGF-10 promoter activity, the shortest Sp3 isoform, which lacks the N-terminal region had no effect even at the highest cDNA concentration (Fig. 3.6C). We next tested two additional N-terminal Sp3 mutants. Sp3 lacking the N-terminal 149 amino acids ($Sp3^{2.5}$), and Sp3 lacking the conserved Sp region located between amino acids 44-90 ($Sp3^{\Delta SP}$) localized to the cell nucleus. However, both $Sp3^{2.5}$ and $Sp3^{\Delta SPBox}$ had reduced ability to inhibit Sp1-mediated FGF-10 promoter activity (Fig. 3.6D,E). These data suggest that the inhibitory function of Sp3 requires structural elements located at the N-terminus of the protein.

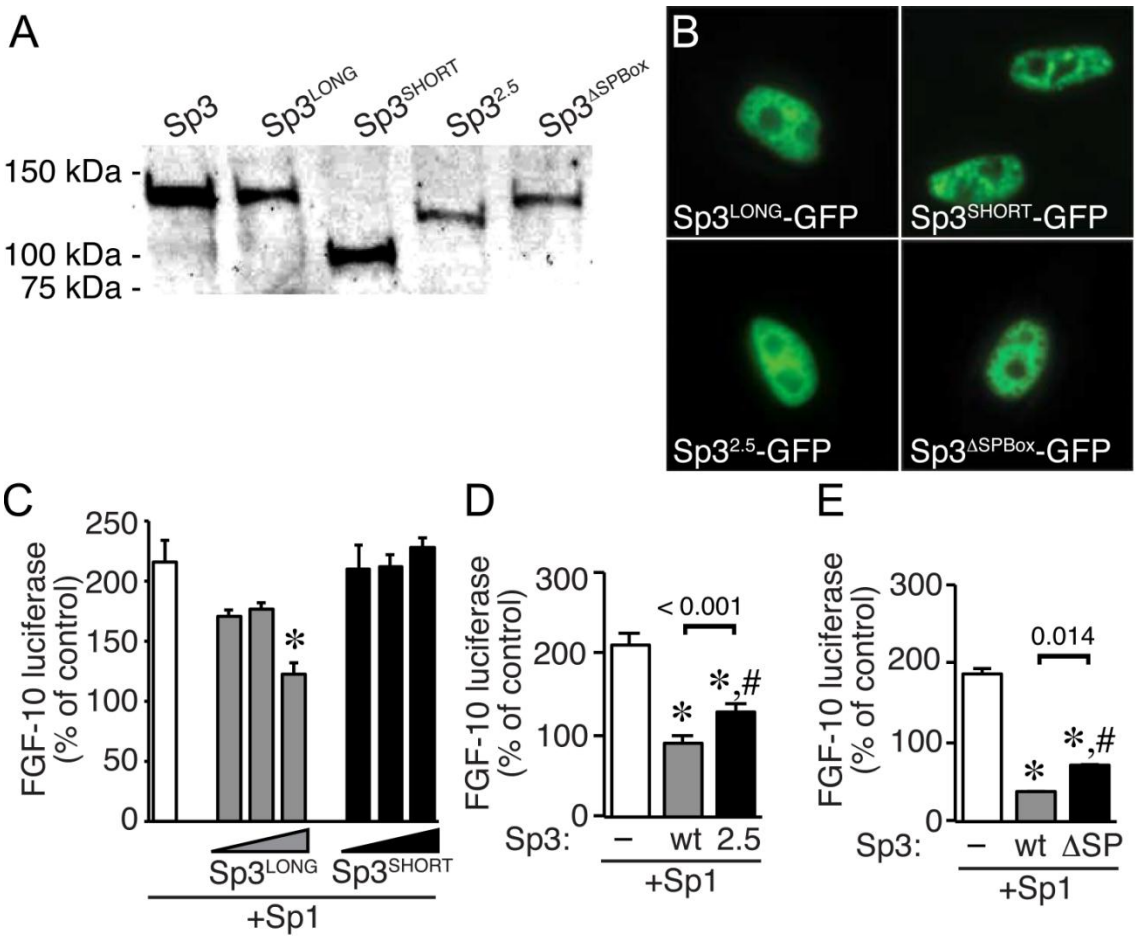

Figure 3.6. The N-terminus of Sp3 is required for inhibition of Sp1-mediated FGF-10 expression. A, Sp3-GFP fusion proteins containing N-terminal mutations were expressed in CHO cells. $Sp3^{LONG}$ and $Sp3^{SHORT}$ correspond to the longest and shortest Sp3 isoforms that are expressed from the full length Sp3 mRNA. $Sp3^{2.5}$ lacks the 149 N-terminal amino acids. $Sp3^{\Delta SPBox}$ lacks the Sp Box domain located between amino acids 44-90. Immunoblotting for GFP confirms that each mutant Sp3 construct was expressed at the expected MW. B, Sp3-GFP mutant isoforms localized to the cell nucleus when expressed in CHO cells. C, Increasing amounts (0.2, 0.4, 0.8 µg) of full-length Sp3 ($Sp3^{LONG}$, grey bars) inhibited Sp1-mediated FGF-10 promoter activity (* $P < 0.05$ compared to Sp1 only; n = 3). The shortest Sp3 isoform ($Sp3^{SHORT}$, black bars) failed to inhibit Sp1-mediated FGF-10 promoter activity. D,E, Sp3 mutants lacking either the 149 N-terminal amino acids (D) or the Sp box (E) were less effective at inhibiting Sp1-mediated FGF-10 promoter activity than wild type Sp3 (* P compared to Sp1 alone, # P compared to wild type Sp3; n = 6).

Discussion

Our data show that NF-κB activation decreases FGF-10 expression by altering Sp protein-mediated transcription. We determined that NF-κB activation increases interactions between the transcription factor Sp3 and the FGF-10 promoter, decreasing FGF-10 expression. Sp3 is incapable of downregulating FGF-10 without NF-κB nuclear translocation, and the N-terminus of the Sp3 protein is required for this effect. These findings identify an important molecular mechanism regulating FGF-10 expression.

In extremely preterm infants that develop BPD, inflammation disrupts the normal developmental programs that control lung morphogenesis. While the connections between inflammatory signals and altered development have long been suggested, the mechanisms linking immunity and development have been less clear. Within the developing lung mesenchyme, microbial products and cytokines activate NF-κB and inhibit FGF-10 expression, and prevent normal epithelial-mesenchymal interactions during airway formation (Benjamin, Smith et al. 2007; Benjamin, Carver et al. 2010). Our results here reveal a novel mechanism linking inflammation-mediated NF-κB activation and abnormal transcriptional regulation of FGF-10.

The NF-κB subunit RelA interacts with Sp3 to inhibit FGF-10 transcription. This inhibitory function could occur in one of several ways. RelA could act as a molecular chaperone within the nuclear microenvironment, recruiting Sp3 to the

FGF-10 locus. In cells expressing FGF-10 in the absence of inflammatory stimuli, RelA is mostly cytoplasmic. Interaction of Sp3 with the FGF-10 promoter is minimal and FGF-10 expression is high due to the stimulatory function of Sp1. However, when cells are exposed to inflammatory signals, the nuclear transport of RelA increases Sp3 levels at the FGF-10 promoter, inhibiting Sp1- mediated transcription. As part of this model, RelA-Sp3 complexes could specifically interact with the FGF-10 promoter (as well as other Sp-regulated genes also inhibited by NF-κB).

Alternatively, Sp3-RelA interactions could cause functional switching. Sp3 appeared to activate FGF-10 transcription when NF-κB was inhibited. Therefore interacting with RelA may turn Sp3 into a transcriptional repressor. Additionally, NF-κB activation and binding of RelA to the FGF-10 gene could change the structure or modification of the FGF-10 promoter-enhancer region, leading to increased Sp3 binding and perhaps preventing Sp1 from promoting transcription. These types of interactions may help explain how transcription factors like NF-κB can function as both activators and repressors given different genetic contexts.

Examples of transcription factors functioning both as activators and repressors are found throughout molecular biology (Smale 2011). The retinoic acid receptor family of RAR/RXR complexes normally function as co-repressors, inhibiting transcription. In the presence of ligand, additional co-activators are recruited and promote gene expression. In this example, the RAR/RXR acts somewhat as a molecular scaffold, allowing ligand-dependent recruitment of additional factors (Delescluse, Cavey et al. 1991; Benko, Love et al. 2003). For

genes regulated by Wnt and β-catenin signaling, activated β-catenin can switch TCF family transcription factors from repressors to activators (Bienz 1998; Roose and Clevers 1999). β-catenin also promotes exchange of repressor TCF proteins for activating family members (Daniels and Weis 2005). This exchange of activating and inhibiting transcription factors has similarities to the one we propose here. Activated RelA increases the localization of the repressor Sp3 at the FGF-10 promoter that is occupied primarily by Sp1 in the absence of inflammatory stimuli. As many genes contain GC boxes in their promoter regions, transcriptional repression by RelA-Sp3 complexes could target more genes than just FGF-10.

The inhibitory function of Sp3 requires the N-terminal peptide region of the protein. Expression of Sp3 isoforms lacking the amino-terminal 302 amino acids did not inhibit FGF-10 promoter activity, and a smaller truncation of the 149 N-terminal region resulted in an intermediate inhibitory ability of Sp3. Within the Sp3 N-terminal domain, deletion of the conserved Sp box reduced, but did not completely abolish inhibitory function. The differential function of the various Sp3 isoforms is particularly interesting. Expressed from a single, full-length mRNA, internal ATG start sites produce peptides of 60, 62, 100, and 102 kDa (Sapetschnig, Koch et al. 2004). As each isoform appears to be expressed in all cell types examined to date, the differential roles of short and long Sp3 peptides are unknown (Kennett, Udvadia et al. 1997; Kennett, Moorefield et al. 2002). As Sp box domains are described in all Sp family members, the potentially unique properties of the Sp3 Sp box are not clear (Suske, Bruford et al. 2005). Future

experiments will better define this region and how it might regulate interactions between Sp3 and other transcription factors, including RelA and Sp1.

NF-κB can activate and inhibit gene expression by distinct mechanisms. For activation of innate immunity genes, NF-κB directly binds to the gene promoter via a consensus NF-κB binding sequence (Kunsch, Ruben et al. 1992). In addition, members of the NF-κB family possess a transactivating domain that can recruit additional transcription factors to discrete regulatory sites (Fujita, Nolan et al. 1992). NF-κB also represses the expression of a smaller subset of genes, many of which are involved in development and repair (Rippe, Schrum et al. 1999; Zhang and Kone 2002; Chua, Bhat-Nakshatri et al. 2007). The mechanisms responsible for suppression by NF-κB are less well understood. NF-κB can stimulate the expression of transcriptional regulators that then inhibit downstream target genes. For instance, NF-κB interacts with histone deacetylases to guide these enzymes to targets of NF-κB suppression (Ashburner, Westerheide et al. 2001). This particular mechanism is thought to transcriptionally silence specific genes, such as the pro-apoptotic gene BNIP3 in cardiomyocytes (Shaw, Zhang et al. 2006), as well as tempering activation of NF-κB targets, including TNFα and IL-8 (Ashburner, Westerheide et al. 2001). NF-κB directly inhibits expression of intestinal trefoil factor (TFF3) (Baus-Loncar, Al-azzeh et al. 2004), E-cadherin (Chua, Bhat-Nakshatri et al. 2007) and Collagen1A1 (Rippe, Schrum et al. 1999). These inhibitory functions of NF-κB suggest a regulatory role in development.

Many components of the NF-κB signaling pathway are required for normal fetal development and tissue morphogenesis. Mice deficient for IKKβ die shortly after birth with profound developmental defects in skin, skeleton, and hematopoietic system (Hu, Baud et al. 1999; Takeda, Takeuchi et al. 1999; Balkhi, Willette-Brown et al. 2012). In addition to its role in lymphocyte survival, NF-κB prevents apoptosis during development. Mice null for RelA die in utero with massive liver apoptosis (Beg, Sha et al. 1995). An emerging body of literature also implicates NF-κB in regulating neural repair and neural synapse integrity. NF-κB influences neuronal excitability in the amygdala, protecting neurons in this critical region of the brain from excitoxicity while simultaneously promoting long term potentiation (Albensi and Mattson 2000; Avital, Goshen et al. 2003). NF-κB has also been implicated in promoting neural regeneration and neural synapse pruning in the hippocampus (Guenard, Dinarello et al. 1991; Lynch 1998; Avital, Goshen et al. 2003; Li, Liu et al. 2003). NF-κB signaling therefore regulates developmental processes in addition to the innate immune response. By interacting with other developmental transcription factors, NF-κB provides an additional level of expression control. These molecular mechanisms may provide unique insight into the connections between inflammation, immunity, and the pathogenesis of developmental diseases.

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CHAPTER IV

Conclusions

Prior to this work, the primary questions remaining with regard to how growth factor expression is altered in bronchopulmonary dysplasia focused almost entirely on the mechanism of FGF-10 regulation. With the foreknowledge that FGF-10 is crucial for lung branching and maturation, and that FGF-10 expression is reduced in the lungs of children suffering from BDP, a mechanistic approach could be determined that analyzes the FGF-10 promoter and attempts to discovered the relevant factors responsible for expression under normal circumstances. This informed how different factors might alter expression under pathological conditions. Our approach here was three-fold: Determine the signaling pathway responsible for FGF-10 suppression, determine how that pathway effects FGF-10 promoter activity, finally elucidating a mechanism for the effects.

What is the relevant signaling pathway linking extracellular pro-inflammatory stimuli to changes in FGF-10 gene regulation in mesenchymal cells?

To confirm NF-κ as the relevant pathway for pro-inflammatory signaling in the lung, we assayed FGF-10 expression by RT-PCR after exposure to LPS and several other cytokines that signal through NF-κB or their own cognate receptors.

We found that only those activating NF-κB downregulated FGF-10. We further tested this by activating or blocking NF-κB with a constitutively active IKKβ construct or a dominant negative IκB construct, respectively. Blocking NF-κB strongly increased, while activating it decreased, FGF-10 promoter activity. These experiments suggest that NF-κB activation strongly downregulates FGF-10 promoter activity and subsequent expression.

Are the effects of NF-κB on FGF-10 direct or indirect?

Because NF-κB is typically understood to activate gene expression rather than suppress gene expression, we were eager to understand the mechanism of the regulatory role that NF-κB was exacting on FGF-10. By identifying a minimal FGF-10 promoter fragment capable of responding to NF-κB activity, then deleting the sole NF-κB consensus binding sequence in that fragment, we were able to test whether the effect was direct or indirect. To our surprise, removing the NF-κB binding element did not abrogate the ability of NF-κB to downregulate FGF-10 promoter activity. These data indicate that NF-κB negatively regulates FGF-10 expression in an indirect way.

What is the mechanism of NF-κB-mediated FGF-10 gene suppression?

If NF-κB did not bind to the FGF-10 promoter at the predicted site and directly downregulate FGF-10, then how did it accomplish such a strong downregulation of expression? We used in silico analysis to determine other factors binding in the region ChIP experiments suggested NF-κB might occupy on the FGF-10 promoter. We found several conserved GC boxes in the region.

We then tested the effects of GC box-binding Sp1 and Sp3 on FGF-10 expression. We determined that Sp1 increases FGF-10 expression, and that Sp3 decreases that Sp1-mediated response. We also determined that Sp1 constitutively occupies the FGF-10 promoter by ChIP analysis, but that Sp3 and NF-κB are enriched at the promoter after LPS treatment. We further tested whether Sp3 and NF-κB require one another for this response, and found that Sp3 requires NF-κB to downregulate Sp1-mediated FGF-10 expression. In situ proximity ligation experiments confirmed that Sp3 and NF-κB interact after NF-κB activation. This suggests that Sp1 is a potent activator of FGF-10 expression under normal circumstances, but that during times of physiological stress through inflammation, Sp3 and NF-κB cooperatively downregulate normal FGF-10 expression.

This new knowledge could lead to novel treatments for diseases such as BPD in which lung development is halted as a result of decreased FGF-10 signaling. By understanding the regulatory mechanisms that regulate FGF-10 during injury and inflammation, better therapies can be devised to restore FGF-10 to appropriate levels by directly targeting Sp1, Sp3 and NF-κB and the rich regulatory networks associated with these genes. These therapies might also be applied to scenarios in which alveolar cells are acutely injured, since FGF-10 is known to play a role in stimulating differentiation of alveolar cells. Emerging research continues to reveal that developmental pathways are crucial for lung recovery and regeneration after insult; expanding our knowledge of the factors

that govern these pathways will continue to allow us to more elegantly treat diseases of the lungs.

To continue to understand this regulatory mechanism, several more questions need to be addressed. First, while the overall function of Sp1, Sp3, and NF-κB has been determined experimentally in this work, the precise mechanism of Sp3/NF-κB downregulation of FGF-10 has not been clearly defined. This knowledge is key to exploiting potential therapeutic targets. Second, it has not yet been established whether suppressing Sp3 activity leads to protection from inflammation and epithelial injury. Finally, this regulatory mechanism has only been described to regulate FGF-10 in the branching lung; other genes in other locations could potentially be regulated in the same way.

Future Directions

Examining the Role of Sp1/Sp3/NF-κB in Regulating a Potential Pro-Branching Morphogeneis Gene Regulatory Network

Determining the role that Sp factors play in lung development is significantly hampered by the lethal nature of null alleles for Sp1 and Sp3 (Marin, Karis et al. 1997; Bouwman, Gollner et al. 2000; Kruger, Vollmer et al. 2007). While mice possessing null alleles for each gene are available, to date no conditionally null allele has been made available. For Sp1 this totally precludes

studying the effects of Sp1 deficiency on lung development beyond the earliest patterning of the presumptive lung because Sp1 null animal die just as the lungs are specified. A mouse with conditionally null Sp1 alleles flanked by LoxP sites crossed to mice expressing Cre recombinase from the Surfactant Protein C promoter would allow for analysis of lungs deficient for Sp1 in the epithelium (Okubo and Hogan 2004). Similar crosses to mice expressing Cre recombinase from Dermo1 promoter would allow for analysis of lungs deficient for Sp1 in the mesenchyme (Sosic, Richardson et al. 2003). Because FGF-10 is expressed in the mesenchyme, the Dermo1-Cre;$Sp1^{Flox/Flox}$ mice might provide better insight into the role that Sp1 plays there.

Because Sp3 embryos live until birth, in utero lung development can be studied in the global absence of Sp3. Early experimental evidence suggests that fetal lung mesenchymal cells derived from mice heterozygous for Sp3 are capable of responding to LPS treatment, but that response could be blunted relative to wild type cells. Interestingly, FGF-10 expression in $Sp3^{+/-}$ cells was higher in non-treated cells, and remained higher after LPS treatment. This supports the assertion that Sp3 negatively regulates FGF-10, and also suggests that the negative regulation is constitutive (Figure 4.1).

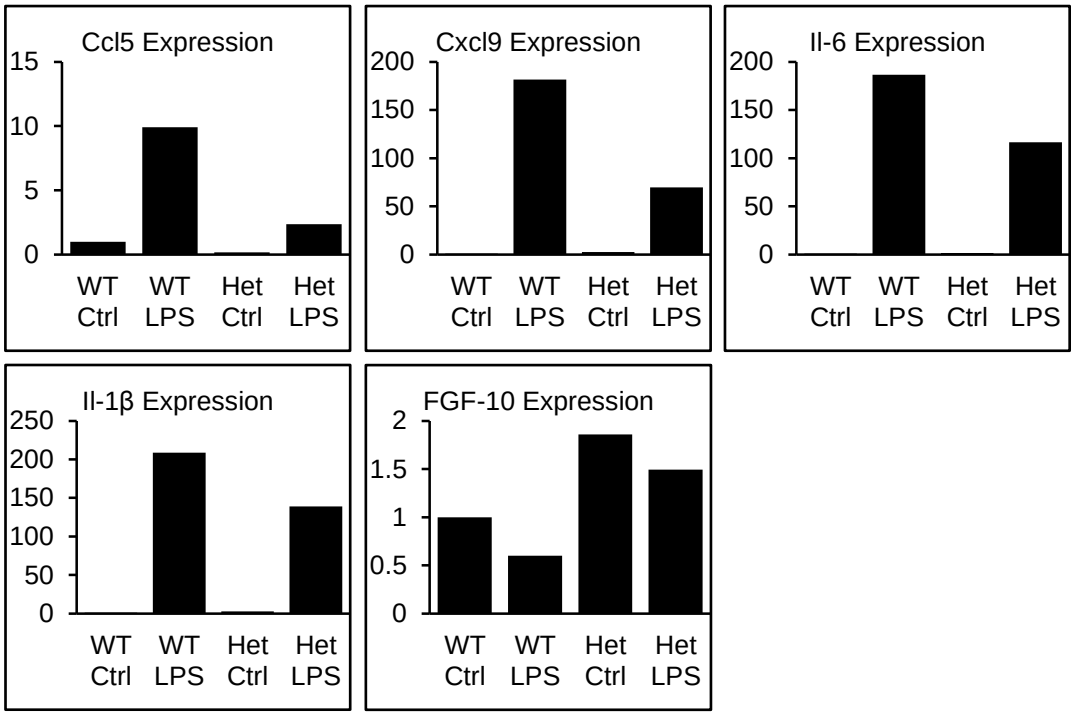

Figure 4.1. Gene expression data from primary lung mesenchymal cells harvested from mice heterozygous for Sp3. Lung mesenchymal cells were derived from e15 fetuses and split into two dishes: one control and one treated for four hours with LPS. Quantitative RT-PCR for genes associated with an inflammatory response revealed that $Sp3^{+/-}$ cells mount a potentially weaker response to LPS-treatment, and express more FGF-10 with and without LPS than wild type cells.

The paradigm presented here for controlling FGF-10 expression basally by Sp1 and under inflammatory conditions by Sp3 and NF-κB suggests a novel mechanism for utilizing ubiquitously expressed transcription factors to direct highly tissue-specific events (i.e. branching morphogenesis). That this mechanism is specific to a single gene in a single organ for a single process seems unlikely given that FGF-10 is used for similar purposes in other developmental systems such as the salivary and lacrimal glands (Jaskoll, Abichaker et al. 2005; Qu, Carbe et al. 2011). Similarly, branching morphogenesis of the lung requires the coordinated regulation of myriad genes. Likelier, then, is a scenario in which Sp1/Sp3/NF-κB regulate any number of genes required for branching morphogenesis. Preliminary gene expression data suggests that a subset of genes is downregulated similarly to FGF-10 in lung mesenchymal cells treated with LPS (downregulated at least two-fold, p-value <0.01). These genes, including Gata4, Fzd2, IGF-1, and FGF-18 are known to play important roles in patterning and development of the lung. *In silico* analysis of their promoters reveals similarities to the FGF-10 promoter, with each possessing at least two conserved GC boxes and several possess a conserved NF-κB binding site (Figure 4.2A). Early luciferase reporter assays of the genes indicate that they could respond in the same way as FGF-10 to Sp1 and NF-κB, though less dramatically (Figure 4.2B).

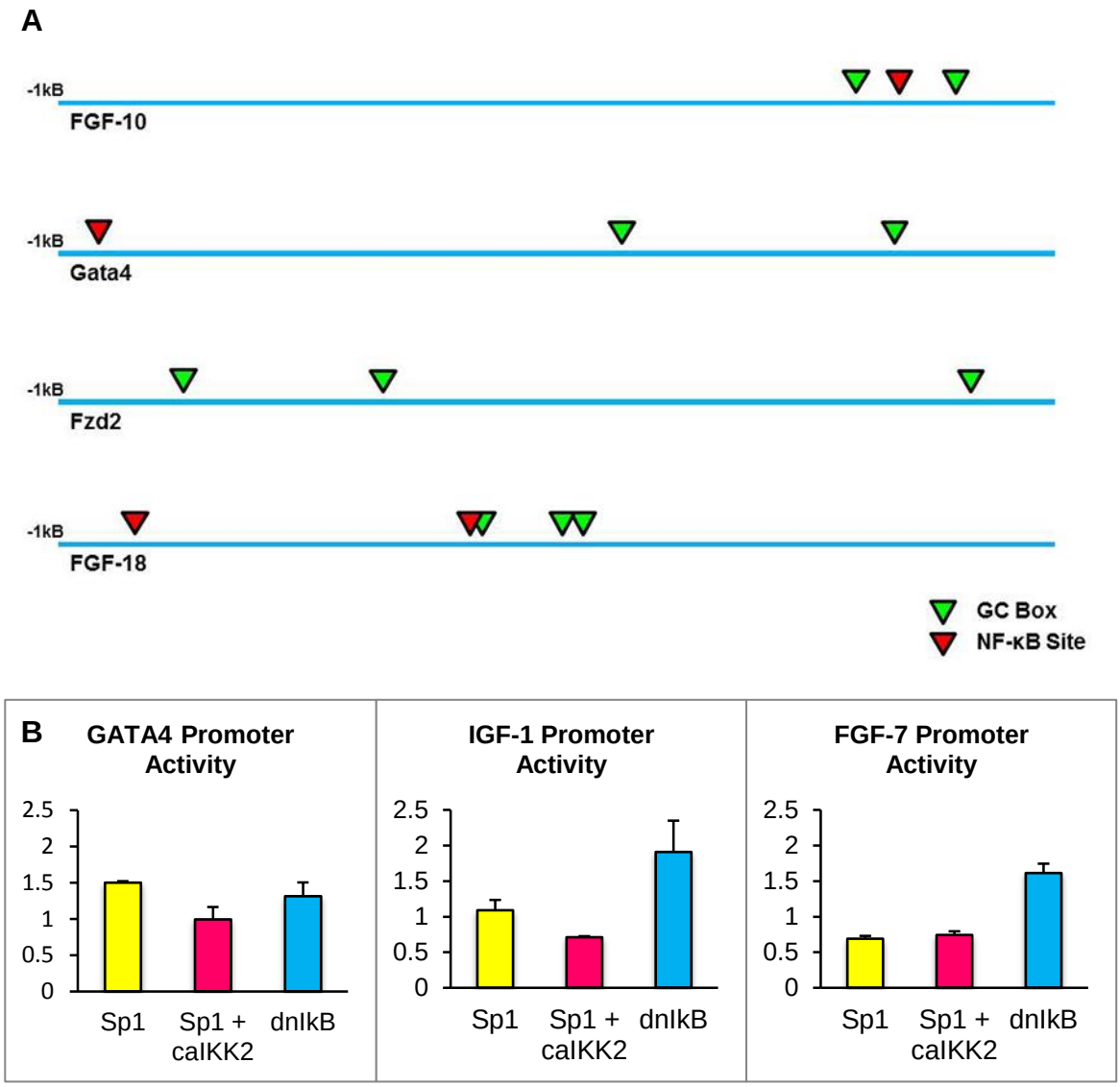

Figure 4.2. Promoter map of three factors downregulated in lung mesenchymal cells by LPS treatment. Conserved consensus sequences associated with binding Sp proteins and NF-κB appear in many of the promoters, a trait shared with FGF-10. Luciferase reporter assays show responses similar to FGF-10, in contrast to FGF-7 which lacks predicted GC boxes or NF-κB consensus binding sites.

Extending that assertion, then, perhaps this trio of transcription factors could regulate developmental processes in other systems. This has been illustrated for other genes on a limited basis, but without convincing evidence of interaction between the three factors.

In mice mentioned above, or cells/tissues derived from those mutant mice, many genes can be examined by genomic approaches. Because FGF-10 is a mesenchymally-derived growth factor, candidate genes for initial analysis might include other genes expressed by lung mesenchymal cells during lung development. Such genes include FGF-7, BMPR1, TGFβR3, HIP, and genes associated with mesenchymal identity such as smooth muscle actin and vimentin.

Determining Whether Proper Branching Morphogenesis is Restored in Lungs Overexpressing Sp1 or Deficient for Sp3

Children with chorioamnionitis are more likely to suffer from BPD, and their lungs have less FGF-10 expression (Benjamin, Smith et al. 2007). Results presented here indicate that Sp1 positively regulates FGF-10 relative to Sp3/NF-κB; so the question remains whether additional Sp1 could overcome the blockade of FGF-10 in these lungs? One way to test such a theory would be to overexpress Sp1 in explanted saccular stage mouse lung treated with LPS or pro-inflammatory cytokines. This could be accomplished genetically with an allele overexpressing Sp1, or with an adenovirus expressing Sp1. Branch counts

and morphometry could reveal whether these lungs branch more under basal conditions with additional Sp1, and whether they are protected from inflammatory injury. Presumably they would recapitulate *in vitro* data presented here indicating that heightened Sp1 increases FGF-10 expression. Hypothesizing the overall results of this experiment is complicated by the robust feedback inhibition innate in the developing lung towards excessive FGF signaling.

Because Sp3 appears to be important for downregulating FGF-10 in the face of active NF-κB, it would also be interesting to determine whether Sp3 deficiency protects against NF-κB-mediated lung dysmorphia. A model of chorioamnionitis where LPS is injected directly into the amniotic sac could be employed on these animals, and differences in severity of lung disease assayed (Prince, Okoh et al. 2004). If mice overexpressing Sp1 become available, they could also be utilized in this experiment. The hypothesis based on present data predict that Sp1 overexpression and Sp3 deficiency would protect lungs against the effects of inflammation.

Ellucidating a Mechanisms by which Sp3 and NF-κB Dampen Sp1-Mediated FGF-10 Expression

The specific mechanism by which Sp3 and NF-κB collaboratively suppress Sp1-mediated FGF-10 expression remains unknown. Several different possibilities exist. Sp3 and NF-κB could displace Sp1 from the FGF-10 promoter, or they could somehow deactivate its ability to activate transcription.

One intriguing possibility lies in the ability of Sp1 to oligomerize and synergistically activate gene transcription (Courey, Holtzman et al. 1989). It is possible that Sp3 and NF-κB bind to Sp1 and prevent this oligomerization, thus dampening its ability to activate FGF-10 expression (Figure 4.1). Quantitative ChIP studies could be used to determine how much Sp1 remains at the FGF-10 promoter after NF-κB activation. This would indicate whether displacement is the primary mechanism of suppression. On the other hand, determining oligomerization could be more difficult. FRET of a mixed population of fluorescently tagged Sp1 could potentially be used, which decrease FRET signal after NF-κB activation indicating a loss of oligomerization (Biener, Charlier et al. 2005). Similarly, glutaraldehyde crosslinking of mixed epitope tagged variants of Sp1 might be used, and visualization on an acrylamide gel could determine whether lower order oligomers/monomers becames more prevalent after NF-κB activation (Fadouloglou, Kokkinidis et al. 2008).

The Sp3 protein could also be used to help further elucidate a mechanism for FGF-10 regulation. Several important domains in the Sp3 protein could be further analyzed by deletion or mutation to determine their importance. While SUMOylation does not appear to be required for FGF-10 regulation, several other sites of post-translational modification (and thus potential regulation of Sp3 function) are known to exist in the N-terminus of the protein. Scanning mutagenesis of phosphorylation sites, for instance, could reveal a residue crucial to activating the repressive nature of Sp3 with regard to FGF-10. Similarly, as the DNA-binding domain of Sp1 has been suggested to mediate interaction

between Sp1 and the RelA subunit of NF-κB (Suske, Bruford et al. 2005), the extremely similar DNA-binding domain of Sp3 could be key in mediating a regulatory interaction between Sp3 and the NF-κB dimer. An interaction with the DNA-binding domain of Sp3 could also explain the presence of NF-κB but apparent unimportance of NF-κB consensus binding sequences in the FGF-10 promoter – it could bind using Sp3 as an "adapter."

An alternative approach for answering this question might lie in genetic analysis and sequencing of children who perform better than expected when faced with prematurity and other risk factors for BPD. If unique mutations in the Sp3 alleles of these children are discovered, they could similarly determine the important regulatory machinery in the Sp3 protein for downregulating FGF-10. These experiments could lead to a new paradigm for understanding how ubiquitously expressed transcription factors can regulate highly context specific genes.

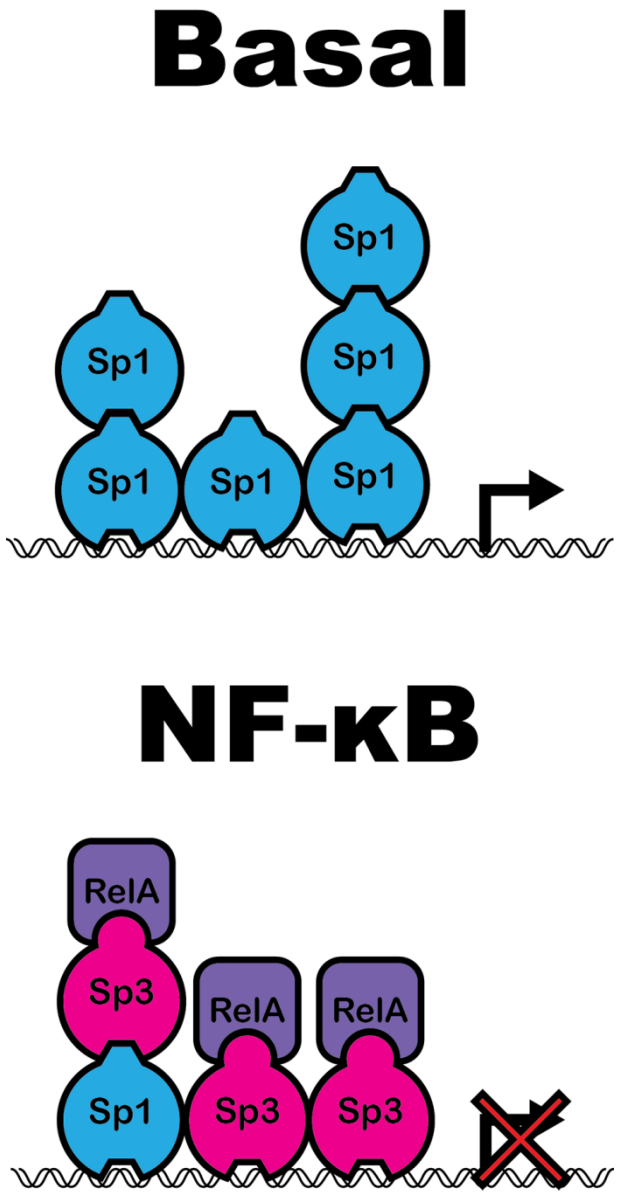

Figure 4.3. Potential models depcting how Sp3 and NF-κB might inhibit Sp1-mediated FGF-10 expression.

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