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TNF-Alpha Represses Transcription of Human Bone Morphogenetic Protein-4 in Lung Epithelial Cells

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Abstract

Bone Morphogenetic Proteins are key signaling molecules in vertebrate development. Little is known about *Bmp* gene regulation in any organ. In *Drosophila*, the *Bmp* gene, *dpp* is regulated by Dorsal, the invertebrate homologue of Rel-NFκB. In this study we examined whether TNF-α, which activates NF-κB, can regulate *Bmp4* gene expression. TNF-α reduced *Bmp4* mRNA in lung adenocarcinoma A549 cells and repressed transcriptional activity of the human *Bmp4* promoter in a dose-dependent manner. Similar repression was observed when the *Bmp4* promoter was co-transfected with a p65 (RelA) expression vector in the absence of TNF-α treatment, suggesting that RelA mediates the effect of TNF-α. In support of this finding, the repressor effect of TNF-α on *Bmp4* was abrogated by a co-transfected dominant negative mutant of IκB (S32A/S36A). The human *Bmp4* promoter contains 3 putative consensus binding sites for NF-κB. Surprisingly, only one of the latter binding sites was capable of binding NFκB. Repressor effect of NFκB was not dependent on any of the three binding sites, but localized to a 122 bp fragment which bound both RelA and SP1. SP1 stimulated transcription, whereas increasing doses of RelA opposed this effect. In vivo, TNF-α inhibited branching morphogenesis and LacZ gene expression in *Bmp4-lacZ* transgenic lungs. These data support a model in which TNF-α-induced RelA interacts with SP1 to bring about transcriptional repression of *Bmp4* gene. These findings provide a mechanistic paradigm for interactions between mediators of inflammation and morphogenesis with relevant implications for normal lung development and pathogenesis of disease.

Keywords

NF-κB; Lung development; Inflammation; Branching morphogenesis

1. INTRODUCTION

BMP4 is a member of the Bone Morphogenetic Protein family of signaling molecules that constitute a subclass of the transforming growth factor-beta superfamily (Wozney et al.,

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1988;Hogan, 1996). In general, BMPs play key roles in a variety of processes in embryonic development, including bone formation and repair, organogenesis, pattern formation in the early embryo, and epithelial-mesenchymal interactions (Jones et al., 1991;Vainio et al., 1993;Fainsod et al., 1994;De Robertis and Sasai, 1996). On the basis of sequence similarities, members of the BMP subfamily can be further subdivided into three subgroups (BMP2 and BMP4 in group 1; BMP3/osteogenin in group 2; and BMP5, BMP6, BMP7 and BMP8 in group 3) (Hahn et al., 1992). *Bmp5* and *Bmp7* are expressed ubiquitously in the lung mesenchyme and the epithelium, respectively (King et al., 1994;Bellusci et al., 1996). *Bmp4* expression during lung development occurs with precise spatial and temporal specificity, being highly localized to select epithelial cells forming the branching tip of the airways. The operational connection between lung development and *Bmp4* was elegantly demonstrated by ectopic expression of *X-noggin*, an inhibitor of BMP4 or a dominant negative BMP4 receptor, Alk-6, both of which inhibited branching morphogenesis (Weaver et al., 1999).

Little functional data are currently available regarding the regulation of *Bmp4* gene expression in any organ. The genomes of both humans and mice contain a single *Bmp4* gene that generates multiple transcripts with 5'-end heterogeneity. Accordingly, two regions of promoter activity on the *Bmp4* gene were subsequently identified (Van den Wijngaard et al., 1999). In the mouse, only one of the two promoters has been functionally characterized (Kurihara et al., 1993;Feng et al., 1995). The 5' regulatory DNA of the human *Bmp4* gene has been cloned and sequenced (van den Wijngaard et al., 1996). As in the mouse, the two regions of the gene designated *hBmp4.1* (distal) and *hBmp4.2* (proximal) exhibit activity in cultured cells and generate distinct mRNAs (van den Wijngaard et al., 1996). Within each of the two human promoters, DNA sequence analysis has identified putative binding sites for a number of transcription factors. Amongst these, GATA-1 and Msx have been implicated in the regulation of the *hBmp4.1* promoter in osteosarcoma cell lines (Van den Wijngaard et al., 1999). Of interest to the current study, the DNA sequence of the *hBmp4.1* promoter includes putative consensus binding sites for members of the transcription factor family NF- κ B. The latter suggested that *Bmp4* may be a target of regulation by pro-inflammatory cytokines such as Tumor necrosis factor, TNF- α .

TNF- α is a non-glycosylated protein of 17 kDa with 157 amino acids that acts as a pleiotropic pro-inflammatory cytokine. TNF- α is produced primarily by activated macrophages, but also by a variety of other structural cell types including fibroblasts, epithelial and smooth muscle cells. Biological responses of TNF- α are mediated by specific binding either via a Type I or a Type II receptor (Tartaglia et al., 1991) expressed on the surface of many cell types. Binding of TNF- α to its receptors results in activation of the inflammatory response classically mediated by a wide variety of pro-inflammatory cytokines including interleukins, Interferon- γ and Transforming growth factor- β (TGF- β). In addition, intracellular signal transduction generated by TNF- α elicits a wide spectrum of other cellular responses, including modulation of differentiation and proliferation of a variety of cell types, and induction of apoptosis (Aggarwal, 2003). Lung-specific ectopic overexpression of *Tnf-alpha* (Miyazaki et al., 1995) and other inflammatory cytokines inhibits branching morphogenesis (DiCosmo et al., 1994;Ray et al., 1997).

Signal transduction in the TNF- α pathway occurs partly through the activity of the NF- κ B family of transcriptional factors. In unstimulated cells, NF- κ B exists in the cytoplasm as a homodimer or heterodimer of a family of structurally related proteins with a conserved Relhomology domain (Baldwin, 1996). To date, five proteins, RelA (p65), c-Rel, Rel-B, NF- κ B1 (p50/p105) and NF- κ B2 (p52/p100) have been identified (Baldwin, 1996). The classical NF- κ B dimer contains RelA and NF- κ B1 (May and Ghosh, 1998). NF- κ B is held in the cytoplasm by binding to inhibitory molecules known as I κ B (Baldwin, 1996). Cytokines such as TNF- α and IL-1 β are potent inducers of NF- κ B as are lipopolysaccharides. These

cytokines initiate a cascade of events that leads to degradation of I κ B by a ubiquitin-proteasome pathway that is triggered by phosphorylation. Degradation of I κ B leads to unmasking of the NF- κ B nuclear localization signal and its transport to the nucleus as an active transcription factor. It has been well documented that many signal transduction pathways in inflammation, cell proliferation and apoptosis can converge to activate NF- κ B (Mercurio and Manning, 1999).

In *Drosophila*, decapentaplegic, *dpp* is the invertebrate homologue of *Bmp4*. Decapentaplegic is repressed by Dorsal, which is the *Drosophila* homologue of Rel-NF- κ B family involved in the establishment of dorso-ventral axis during development (Nusslein-Volhard and Roth, 1989). Dorsal also activates *twist*, which is required for transcription of genes encoding FGF receptors. In the vertebrate limb, inhibition of Rel-NF- κ B activity represses *twist*, but stimulates *Bmp4* gene expression and results in arrest of outgrowth, suggesting negative control of *Bmp4* by NF- κ B in limb development (Bushdid et al., 1998; Kanegae et al., 1998). In the lung, studies by Muraoka et al (2000) showed that experimentally induced perturbations in *Nf- κ B* gene expression results in abnormal lung morphogenesis. As in the limb, inhibition of NF- κ B activity in the lung results in greatly increased *Bmp4* mRNA. Based on the *Drosophila* regulation of *dpp* by Dorsal, we examined the hypothesis that the human *Bmp4* may be a target of negative regulation by members of the Rel-NF- κ B family of transcription factors. In the current study, we show that TNF- α treatment of lung cells represses *Bmp4* gene transcription through the interaction of RelA and SP1 within a specific domain located around the transcriptional start site on the human *Bmp4* gene. Thus, the findings establish a functional relationship between mediators of inflammation (e.g. TNF- α) and key morphoregulatory molecules, such as *Bmp4*, that may have significant implications for both normal development as well as pathogenesis of disease.

2. EXPERIMENTAL PROCEDURES

2.1 Cell Culture

The human lung adenocarcinoma cell lines A549 and NCI H441 were obtained from ATCC and cultured in Ham's F12 and RPMI1640 medium (GIBCO-BRL, Gaithersburg, MD) respectively, supplemented with 10% fetal bovine serum, 2mM glutamine and 1% penicillin-streptomycin. Recombinant human TNF- α was purchased from R&D Systems, Inc. (Minneapolis, MN). Anti-NF- κ Bp65 antibody (sc-109x) and anti-SP-1 antibody (sc-59x) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

2.2 RNA isolation and Northern blotting

A549 cells were transfected with *pCMV4p65* plasmid for 48 h, and treated with 50ng/ml of TNF- α for 4 h before RNA isolation. Total RNA was isolated with TRIzol reagent (BRL Life Technologies, Inc), resolved on 1% agarose-formaldehyde gels and transferred to nylon membranes. The membranes were pre-hybridized at 42°C for 1 h in hybridization solution, then hybridized overnight in the same solution with [α -³²P] dCTP random labeled probes generated from a 1.5 kb *Bmp4* cDNA kindly provided by Dr. Brigid L. Hogan (Duke University, Durham, NC). After hybridization, the membranes were washed and exposed to X-ray film. The membranes were washed and re-probed with ³³P-end-labeled oligo probes to 18S rRNA for quantification.

2.3 Plasmid and deletion constructs

Plasmid constructs *pSLA4*, *pSLA4.1EX*, *pSLA4.1PX* and *pSLA4.1NX* were kindly provided by Dr. Wilma Steegenga at the University of Nijmegen, Netherlands. *pSLA4.1EX* contains a full length 1.3 kb EcoRI-XhoI *Bmp4* promoter 1 fragment (−1097/+242) on the vector *pSLA4*; *pSLA4.1PX* contains a 900 bp PstI-XhoI promoter 1 fragment (−633/+242); while

pSLA4.1NX bears a 500 bp NcoI-XhoI promoter 1 fragment (−253/+242) on *pSLA4* vector. *pSLA4.1/−42+242* contains a 284 bp fragment of *Bmp4* promoter 1 cloned into BamHI and XhoI sites of *pSLA4* vector. *pSLA4.1/−42+80* contains a 122 bp fragment of *Bmp4* promoter 1 cloned into BamHI and PstI sites of *pSLA4.1*. Sequences of nucleotides −253 to +20, −253 to −17, and −17 to +20 of *Bmp4* promoter were inserted upstream of the TK promoter of pCL74 and named *pTK/−17+20*, *pTK/−17+20*, *pTK/−17+20*, respectively. *pCL74* was made by inserting a PCR amplified 170bp (nucleotides −120 to +50) fragment of the Thymidine Kinase (TK) promoter into the BglII/HindIII of pGL2 basic vector.

Plasmid *pCMV-p50* (*Nf-kBp50*) and *pCMV-RelA* (*Nf-kBp65* or *RelA*) were the gifts from Dr. Warner C. Greene (J. David Gladstone Institutes, UC San Francisco, California). *pCMVp50* was constructed by cloning a HindIII/BglII fragment of *Nf-kBp50* cDNA into *pCMV4/kbfl* vector, while *pCMV-RelA* was constructed by cloning a HindIII/HindIII fragment of *Nf-kBp65* cDNA into the same vector. Plasmid *IκB* (*S32A/S36A*), a dominant negative human I-kappaB cloned in pUSEamp vector, was purchased from Upstate Biotechnology Inc (Waltham, MA). The S32A/S36A mutations in I-kappaB render the protein non-phosphorylatable, and consequently not degradable. The IL-6 constructs and its mutated form, IL-6mκB construct were kindly provided by Dr. Yamauchi-Takahara (Osaka University Medical School, Osaka, Japan). The IL-6 construct contains an IL-6 promoter fragment (−179 to +12) that is responsive to NF-κB. This fragment was inserted upstream of a *CMV* promoter that drives a luciferase reporter gene (Matsui et al., 1999). Plasmid *IL-6mκB* bears a mutation in IL-6 promoter fragment in which the NF-κB binding site was disrupted by converting GGGATTTTCC to ACATTGCACAATCT (Matsui et al., 1999).

2.4 Transient transfection, Luciferase Assay and β-galactosidase assay

Transient transfection of H441 cells and A549 cells was performed with SuperFect reagent as described by the manufacturer (Qiagen Inc, Valencia, CA). In brief, cells were cultured in 12 well culture dishes overnight to reach 70-75% confluence. In each well, 1 ug of test construct, 1 ug of transactivator plasmids and 1 ug of pSV-β gal (Promega Corp, Madison, WI) were mixed with 15 ml of SuperFect reagent in serum free and antibiotic free medium. The mixture of plasmid and SuperFect was added to the cells after incubation for 30 min at room temperature. Transfected cells were continuously cultured for an additional 6 hours. The mixtures were then replaced with regular medium containing serum and antibiotic. Approximately 48 h after transfection, cells were washed with phosphate-buffered saline and harvested with cell lysis buffer (Promega Corp, Madison, WI). Both luciferase and β-galactosidase levels were tested by Promega Luciferase assay kit and β-galactosidase assay kit (Promega Corp, Madison, WI). For the luciferase assay, 25ul of cell lysate was added in 100ul luciferase reagent and immediately measured in a luminometer. For the β-galactosidase assay, 25 ul of cell lysate was mixed with 50 ul of β-galactosidase reagent, and then incubated at 37° C until color was developed. The O.D 420 was measured on a spectrophotometer. The values of β-galactosidase were used to normalize the luciferase activity. Each experiment was conducted in triplicate and repeated between 2 and 3 times. Data are presented as means ± SE. Student *t*-test was used for statistical analysis of the data.

2.5 Oligonucleotides for EMSA

Annealed double stranded oligonucleotides used for probes and cold competitors in electrophoretic mobility shift assays were purified by gel electrophoresis on 3% low melting agarose (Promega, Madison, WI), excised and then eluted using QIAEXII (Qiagen Inc, Valencia, CA). Two picomoles of annealed oligonucleotides were end-labeled with T4 polynucleotide kinase and [γ -³³P] ATP as described (Amersham Pharmacia Biotech, Piscataway, NJ). The labeled probes were purified further from unincorporated [γ -³³P] ATP using G-25 Sephadex column (Boehringer Mannheim, Indianapolis, IN). The DNA sequences

of oligos used for probes are as follows: Oligo1 (BMP4.1/-1097to-1057): GAATTCGGATCTGGGCAAGTCCCTTTAACCTGGTAGTCC; Oligo2 (BMP4.1/-590 to-556): GAGCTCATTTACTGGGGTCTACCTCAGGGTCATCA; Oligo3 (BMP4.1/-531to-493): CTTTAAGAATGTTTTGGAAGGAAATTCACCTTTTAACA; OligoA (BMP4.1/-45to +9): GGCTCCCATCCCCAGAAAGGGAGGCGAGCGAGGGAG; OligoB (BMP4.1/-17to+20): GAGGGAGGAGGGAAGGAGGGAGGGGCCGCCGGGGA; OligoC (BMP4.1/+14to+49): CGGGGAAGAGGAGGAGGAAGGAAAGAAAGAAAGCG and OligoD(BMP4.1/+43 to +80): AAAGCGAGGGAGGGAAGAGGAGGAAGGAAGATGCGA

2.6 Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed as described (Li et al., 2002). In brief, nuclear extracts were prepared using a mini-extraction procedure (Bohinski et al., 1994). Five microgram of the nuclear extract was incubated with [γ -³²P]ATP end-labeled oligonucleotide probe with or without cold competitor in 12.5 mM Tris-HCl (pH7.5), 62.5 mM NaCl, 0.62 mM DTT, 10% Glycerol, 0.05% NP-40, 0.05 g/l Poly(dI-dC) in a total volume of 20 l at 4°C for 20 minutes. The antibodies were added and incubated at 4°C for 20 minutes before adding oligonucleotides. Bound and free probes were separated by gel electrophoresis on a 4.5% non-denaturing polyacrylamide gel. The gels were dried and exposed to x-ray film.

2.7 Embryonic lung explant culture

For embryonic lung explant culture, whole embryonic lungs were isolated from E11 embryos of normal and transgenic *Bmp4-lacZ* mice. These lungs were placed on 0.8 um nitrocellulose membranes (Millipore, Bedford, MA) atop stainless steel grids. The grids with the lung tissues were placed on the top of the BGJB medium supplemented with 50 units of penicillin and streptomycin. For TNF α treated lungs, 30 ng/ml of recombinant human TNF- α was added in the culture medium. After 48 hours of culture in 5% CO₂ at 37°C, lung tissues were removed from membranes and washed with PBS following 5% PFA fixing. The lung tissues from *Bmp4-lacZ* transgenic mice were stained with X-gal solution overnight according to standard protocols.

3. RESULTS

3.1 TNF-alpha reduces steady-state levels of Bmp4 mRNA in human lung A549 epithelial cells

To determine whether the endogenous human *Bmp4* gene is responsive to TNF-alpha, sub-confluent A549 cells were treated with or without 50 ng/ml of TNF-alpha for 48 hours. Total RNA was isolated from these cells and fractionated by gel electrophoresis on agarose gels and Northern blotted onto Nytran membranes. The membrane was probed with a ³²P-labeled 1.5 kb *Bmp4* cDNA (A generous gift from Dr. B. Hogan, Duke University, Durham, NC). As shown in Figure 1, *Bmp4* is expressed constitutively in cultured A549 cells. Levels of *Bmp4* mRNA in H441 cells were somewhat lower. In A549 cells TNF-alpha repressed the steady state level of *Bmp4* mRNA (Figure1). Densitometric analysis of the Northern blots showed a nearly 30 percent reduction ($p < 0.05$, t -test) in *Bmp4* mRNA levels in response to TNF-alpha. TNF-alpha had little if any impact on H441 cells. These data demonstrate that the steady-state level of the endogenous *Bmp4* mRNA is repressed by TNF-alpha in lung A549 cells.

3.2 Regulation of Bmp4 promoter/enhancer by TNF-alpha

To determine whether the impact of TNF-alpha on *Bmp4* mRNA occurs at the transcriptional level, transient transfection studies were performed using the two human *Bmp4.1* and *Bmp4.2* promoters, linked to luciferase. The *Bmp4.2-luciferase* showed no response to TNF-alpha (data not shown). The *Bmp4.1* promoter/enhancer, *pSLA4.1 EX-Lux* showed constitutive

activity in both H441 and A549 cells (Figure 2). Subsequent treatment of transfected cells with increasing doses of TNF- α ligand for 24 hours showed significant repression of *hBmp4.1-luciferase* promoter activity (Figure 2A). Although there was variability in the response, maximum repression of *hBmp4.1-luciferase* activity was observed at 100 ng/ml of TNF- α in A549 cells. In H441 cells, in contrast to the unresponsiveness of the endogenous *Bmp4* mRNA (Figure 1), 5 ng/ml of TNF- α was sufficient for maximum repression of the transfected *Bmp4.1-luciferase* plasmid (Figure 2A). In parallel control experiments, we showed positive response of an IL-6 promoter/enhancer-luciferase construct to both 5 and 10 ng/ml of TNF- α in A549 cells (Figure 2B). Higher concentrations of TNF- α also stimulated the IL-6 promoter, but were not as effective as 5-10 ng/ml doses.

3.3 TNF- α -induced repression of hBmp4.1 promoter is mediated by RelA

Many biological effects of TNF- α at the nuclear level are mediated by members of the NF- κ B family of transcriptional factors. In unstimulated cells NF- κ B proteins are complexed with inhibitory molecules known as I κ B which retain them in an inactive form in the cytoplasm (Baldwin, 1996). Activation of TNF- α (and other inflammatory cytokines) signal transduction pathway leads to phosphorylation of I κ B, which results in degradation of I κ B and unmasking of NF- κ B nuclear localization signal. As a result, NF- κ B is then transported to the nucleus where it mediates the regulation of target gene expression (For a review see Baldwin, AS 1996). We conducted experiments with a dominant negative mutant of I κ B, I κ B(S32A/S36A) that fails to undergo phosphorylation, thereby abrogating NF- κ B mediated, TNF- α -induced gene regulation. As shown in the previous experiments (Figure 2A) TNF- α repressed transcriptional activity of the *hBmp4.1-luciferase* construct by 60% in A549 cells (Figure 3, compare 1 to 2). In contrast, whereas I κ B(S32A/S36A) alone had little or no effect on basal promoter activity (Figure 3, compare 1 to 3) it entirely abrogated the repressor activity of 100 ng/ml of TNF- α ligand (Figure 3, compare 2 to 4), demonstrating that the effect of TNF- α on the *Bmp4* promoter is mediated through NF- κ B.

To date, five distinct NF- κ B protein members with a conserved Rel-homology domain have been identified (Baldwin, 1996). The classical NF- κ B dimer consists of p65, otherwise known as RelA, and NF- κ B1, also referred to as p50/105 (May and Ghosh, 1998). To determine which of the two specific NF- κ B member protein(s) is involved in TNF- α repression of the *hBmp4* promoter, we examined the effects of RelA and p50 on transcriptional activity of the full length *hBmp4* promoter construct, *pSLA4.1 EX-Lux* in A549 and H441 cells. Co-transfection of *pSLA4.1 EX-Lux* with *pCMV4p50* resulted in a nearly three-fold increase in its activity in A549 cells (Figure 4A). No increased activity was observed in H441 cells. Co-transfection with *pCMV4-RelA* alone or in combination with *pCMV4p50* repressed *pSLA4.1 EX-Lux* activity by approximately 75 percent in both H441 and A549 cells (Figure 4A). In parallel studies and as positive control, we also used the IL-6 promoter/enhancer-Lux construct and its mutant derivatives (Figure 4B). The IL-6 promoter was stimulated by NF- κ B RelA protein. Also, as expected, the mutant construct, *IL6-mkB*, which carries a point mutation that disrupts the NF- κ B binding (Motomura et al., 1998) showed no response to RelA. These data strongly suggest that the repressor effect of TNF- α may be mediated through the activity of RelA.

3.4 Only one of the three putative NF- κ B binding sites on the hBmp4.1 promoter binds RelA

DNA sequence analysis of the 1.3 kb *pSLA4.1EX* has revealed three putative consensus binding sites for NF- κ B, located at -1085, -577 and -514 upstream of the transcriptional start site (+1) (Figure 6). To identify whether these sites can bind NF- κ B RelA protein, we synthesized three oligonucleotide probes, designated NF- κ B-Oligo1, NF- κ B-Oligo2, and NF- κ B-Oligo3 which represent each of the putative NF- κ B binding sites respectively. The binding of nuclear proteins to the latter oligos was then analyzed by Electrophoretic Mobility Shift Assays (EMSA) (Figure

5). Nuclear extract was isolated from A549 cells transfected with the RelA expression construct and treated with 50ng/ml of TNF-alpha to activate nuclear localization of RelA protein. The results show that a number of nucleoprotein complexes are formed when any of the three oligos was incubated with the A549-derived nuclear extract (Lanes A, D and G). Binding specificity of the nucleoproteins to each of the oligonucleotide probes was examined by inclusion of excess cold competitor oligonucleotides used for each probe (Lanes B, E, and H). Inclusion of anti-RelA antibodies in the binding reaction resulted in appearance of a supershifted complex (Arrow) only in reactions using NF-kB-Oligo3 (Lane I). These data suggest that demonstrable binding of RelA to the *hBmp4* promoter can be shown only for the third NF-kB binding site, represented by NF-kB-Oligo3.

3.5 None of the putative NF-kB binding sites mediates RelA-induced repression of *hBmp4.1* promoter

To determine whether any of the putative binding sites functions in TNF-alpha-induced, RelA-mediated repression of *hBmp4* transcription, we examined a number of serial deletion constructs of the full length *hBmp4.1* promoter. Deletion of 464 nucleotides from the 5' end of *pSLA4.1EX* removes the most distal putative NFkB binding site located at -1085. However, the resulting construct, *pSLA4.1PX* showed both the positive and the negative response to *pCMV-p50*, and *pCMV-RelA*, respectively (Figure 6, B). To our surprise, further deletion of the other two, more proximal NFkB putative binding sites, at -577 and -514, also had little impact on basal promoter activity, *pCMV-p50* positive response, and *pCMV-RelA*-induced repression of the *pSLA4.1NX* construct (Figure 6, C). In fact, the construct *pSLA-42+80*, which includes merely 42 nucleotides of the promoter sequences upstream of the transcriptional start site, exhibited similar activities, albeit at much lower magnitude compared to the full length *pSLA4.1EX* plasmid (Figure 6, E).

Thus, the construct *pSLA4.1/-42+80* represents the minimum sequences that confer both basal promoter activity and NF-kB responsiveness to the *hBmp4.1* promoter. DNA sequence analysis of this region revealed no discernible RelA binding sites, but included putative binding sites for SP1. Since interactions between SP1 and NF-kB have been previously reported in HeLa cells (Hirano et al., 1998), we examined functional interactions between the two transcription factors on the *pSLA4.1/-42+80* construct by transient co-transfection studies (Figure 7). As expected, *pCMV-RelA* decreased *pSLA4.1/-42+80* promoter activity (Figure 7A). In contrast, transfection with a plasmid expressing SP1, increased *pSLA4.1/-42+80* activity. However, induction of *pSLA4.1/-42+80* promoter activity was strongly repressed by increasing amounts of RelA expression plasmid, suggesting that the inhibitory effect of RelA is dominant over the stimulatory effect of SP1. In contrast to these results, interactions between SP1 and NFkBp50 were found to be synergistic in activating the *pSLA4.1/-42+80* promoter construct (Figure 7B).

Because the transient transfection assays suggest that the inhibitory effect of RelA may be based on its interactions with stimulatory factors such as SP1, we further characterized their interactions by EMSA. Accordingly, we synthesized four partially overlapping oligonucleotides designated OligoA, OligoB, OligoC and OligoD (Figure 8A) that span the entire 122 bp of *hBmp4.1* DNA in *pSLA4.1/-42+80* plasmid. Using the latter oligonucleotides as probe, we examined by EMSA the ability of each to bind to either SP1 or RelA. Figure 8B shows a representative set of the results. Nucleoprotein complexes that contain both SP1 and RelA proteins, supershifted by either anti-RelA or anti-SP1 antibodies (asterisks), were exclusively found when OligoB was used as probe (Figure 8B, Lanes 5-8). Since OligoB contains sequences between -16 and +19 nucleotides, the region that includes NFkB responsiveness may span the transcriptional start site. To verify that the DNA fragment contained between nucleotides -16 and +19 is necessary for regulation of *hBmp4.1* promoter

by NF- κ B, we made constructs from which this stretch of DNA was deleted. Since deletion of the DNA sequences between -17 and $+20$ removes at least part of the *hBmp4.1* basal promoter, we cloned all fragments upstream of a thymidine kinase minimal promoter element. Thus, the parent construct was *pTK/-253+20* (Figure 9) which includes 273 nucleotides from the *hBmp4.1* promoter (Please see Experimental Procedures). Using the latter construct, we made a 3' deleted construct, *pTK/-253-17* from which the putative NF κ B-responsive site located between -17 and $+20$ nucleotides was deleted. Reciprocally, we also generated a construct that includes only the sequences between -17 and $+20$ of the *hBmp4.1* promoter, designated as *pTK/-17+20*. Transient co-transfection with *pCMV-RelA* was conducted in A549 cells and the results are shown in Figure 9. As expected, deletion of the 37 nucleotide fragment between -17 and $+20$ in *pTK/-253-17* entirely abolished RelA-responsiveness (Figure 9). Reciprocally, when placed upstream of the minimal TK promoter this fragment was sufficient to reconstitute RelA-responsiveness.

3.6 TNF-alpha Represses Bmp4 Gene Activity in Lung Explants

An important question relates to whether the in vitro findings described in the current study bear relevance to the regulation of *Bmp4* gene expression in vivo. To address this question, embryonic day 11.5 (E11.5) mouse lungs were excised and placed in an explant culture using serum-free media as previously described. Under normal conditions, the embryonic lungs undergo a process of growth and development that closely resembles branching morphogenesis (Bellusci et al., 1996). In the control samples, branching morphogenesis occurred within the 48 hour period of incubation as shown in Figure 10, Panels A and C. In contrast, branching morphogenesis of lung explants treated with either 10ng/ml or 30ng/ml of TNF-alpha was slowed resulting in lungs that were smaller in size and less branched (Figure 10, Panels B and D). To determine the impact of TNF-alpha on *Bmp4* gene regulation, we explanted E11.5 embryonic lungs from mice that carry a knock-in LacZ gene in the *Bmp4* locus. In these mice, expression of LacZ faithfully mimics *Bmp4* gene expression (Weaver et al., 1999). When explanted in culture and treated under normal conditions (control), *Bmp4* gene activity as evidenced by LacZ expression is localized to the tip of the branching lung epithelium (Figure 10, Panel C Arrows). Treatment of the *Bmp4-lacZ* lung explants with 30 ng/ml of TNF-alpha resulted in smaller, less branched lungs concomitant with profound repression of LacZ activity (Figure 10, Panel D, arrows). These data clearly demonstrate that as in the in vitro conditions, TNF-alpha in embryonic lung explants represses the activity of the resident *Bmp4* gene.

4. DISCUSSION

Although *Bmp4* is a critical regulator of morphogenesis in many organs, regulation of its gene expression has not been adequately studied. Based on temporal and spatial specificity of its expression pattern during embryonic development it is likely that *Bmp4* regulation is complex and precisely controlled by various transcription factors and inter- and intra-cellular signaling pathways. In the present study we tested the ability of the pro-inflammatory cytokine, TNF-alpha to regulate the *hBmp4.1* promoter activity in lung epithelial carcinoma cell lines. The rationale for the studies was based on a number of observations. First, during establishment of dorso-ventral axis in *Drosophila* development, *Dorsal* the homologue of Rel-NF- κ B represses *dpp*, the invertebrate homologue of *Bmp* (Nusslein-Volhard and Roth, 1989). Second, both in the vertebrate limb and in the lungs, experimentally-induced inhibition of NF- κ B leads to increased *Bmp4* mRNA levels (Muraoka et al., 2000). Thirdly, the 5' region of the human *Bmp4* gene (i.e. *hBmp4.1* promoter) contains consensus binding sites for the TNF-alpha activated transcription factor NF- κ B.

TNF-alpha treatment of H441 and A549 cells decreased the steady-state level of mRNA from the endogenous resident *Bmp4* gene only in the latter cell line (Figure 1). The reason for the

lack of a measurable response in H441 cell is unknown, but may reflect differences, such as mRNA stability in *Bmp4* gene regulation. TNF- α also reduced the activity of a construct containing the *hBmp4.1* promoter cloned upstream of luciferase reporter (Figure 2), suggesting that the effect of TNF- α was at the transcriptional level. To assess whether the latter observation was mediated through NF- κ B, we conducted transient co-transfection studies in which the activity of NF- κ B was inhibited through over-expression of a dominant negative I κ B, which is not phosphorylated and hence blocks the entry of NF- κ B into the nucleus. The results of these studies confirmed that the repressor effect of TNF- α on the *hBmp4.1* promoter is mediated through the activity of NF- κ B (Figure 3). Additional transient co-transfection experiments revealed that the NF- κ B protein p50 is an inducer of *hBmp4.1* activity whereas RelA or p65 acts as a repressor (Figure 4).

Three NF- κ B consensus binding sites were identified by DNA sequence analysis at positions -1085, -577 and -514 within the 5' region of the *hBmp4.1* promoter. Direct examination of these sites by EMSA revealed that only one site, located at -514 was capable of binding NF- κ B protein (Figure 5). This is not surprising since identification of the NF- κ B binding sites was merely based on presence or absence of consensus DNA sequences. Further functional examination, using deletional analysis of the *hBmp4.1* promoter revealed no relationship between the three consensus putative binding sites and the repressor activity of RelA, nor stimulatory activity of p50 on the *hBmp4.1* promoter. Thus, we have concluded that the binding revealed by EMSA to the site located at -514 has little or no functional consequences on the regulatory role of NF- κ B on the *hBmp4.1* promoter.

The sites of cis-active DNA elements responsive to both stimulatory activity of NF- κ B p50 and repressor activity of RelA were localized by deletional analysis to a fragment of 122 base pairs. This fragment was further divided into three regions and the sequences located within -17 and +20 were shown to bind nucleoprotein complexes that contained both NF- κ B RelA and SP1 proteins by EMSA (Figure 8B). The ubiquitous transcription factor Sp1 is composed of four transactivation domains and three zinc-finger DNA binding domains that form complexes on GC-rich DNA motifs (Courey and Tjian, 1988). We found a GC-rich motif that fits the sequence requirements as a consensus binding site for Sp1 within the DNA sequences of fragment between -17 to +20 of *hBmp4.1* promoter. Furthermore, when cloned upstream of luciferase, an oligonucleotide spanning the region of -42 and +80 (nucleotides) conferred not only basal promoter activity, but also responsiveness to both SP1 and NF- κ B proteins. In transient co-transfection studies the promoter activity of the construct containing oligo -42 to +80 increased in response to Sp1 (Figure 7). Importantly, RelA also interacted with the same fragment, but repressed its transcriptional activity. Stimulatory effect of Sp1 was overcome in a dose dependent manner by increasing amounts of the RelA p65 expression plasmid, suggesting that RelA can inhibit the positive impact of Sp1 on *pSLA 4.1/-42/+80* (Figure 7). Thus, it appears that at least some of the responsive cis elements are located around the site of transcriptional start site. Presence of Sp1 sites around the transcriptional start site and in coding sequences has been reported for other genes. For example CCCTC-binding factor binds to the region that includes the first two exons of the hTERT gene which encodes the catalytic subunit of Telomerase, and represses its transcription (Renaud et al., 2005). Antagonistic and synergistic interactions between NF κ B and other promoter-bound transcription factors have been previously described. Functional interference of Sp1 and NF- κ B through the same DNA binding site has been reported (Hirano et al., 1998). In addition, direct protein~protein interactions between Sp1 and NF- κ B transcription factors have also been observed (Perkins et al., 1993; Hirano et al., 1998). The data presented in the current report are consistent with the latter model and may explain the molecular basis of NF κ B-induced repression of the *hBmp4.1* promoter.

To demonstrate the *in vivo* relevance of the *in vitro* transient co-transfection experiments, we examined the response of explanted genetically modified embryonic lungs to TNF- α . The impact of TNF- α on lung branching morphogenesis has not been studied in detail. Early reports suggested that TNF- α enhanced embryonic lung branching morphogenesis (Jaskoll et al., 1994). However, numerous studies in both human and animal models have shown a correlation between TNF- α and inhibition of lung growth and development. For example, endotoxin which is a potent inducer of TNF- α administered to fetal sheep *in utero* inhibits lung development and causes respiratory distress in postnatal life (Kallapur et al., 2004). Also, the effect of TNF- α on lung gene expression, such as that of the pulmonary surfactant protein B has been documented to involve repression (Berhane et al., 2000). In our study we used genetically modified mice that carry a knock-in LacZ gene in the *Bmp4* locus. Morphogenesis of E11.5 *Bmp4-lacZ* lungs in culture was inhibited by 10 ng/ml of TNF- α (Figure 10). Inhibition was accompanied by repression of *Bmp4* gene expression as evidenced by reduced or absent LacZ staining in the branching epithelium (Figure 10). In previously published studies, repression of *Bmp4* either through over-expression of Noggin, an inhibitor, or dominant negative receptor in transgenic mice resulted in inhibition of lung morphogenesis (Weaver et al., 1999). Thus, it is likely that TNF- α -induced inhibition of branching observed in studies described in Figure 10 may be caused by its repressor effect on *Bmp4* gene expression.

The physiological implications of the findings reported here can be important both in the context of normal development as well as in injury and pathogenesis of lung disease. As stated above, *Bmp4* is a crucial component of the program that directs embryonic lung morphogenesis and may also be critical for maintenance of pulmonary homeostasis in adult organisms. Muraoka et al. (2000) showed that NF- κ B is expressed both in the epithelial and mesenchymal compartments of the lung and experimentally-induced changes in its level lead to changes in *Bmp4* mRNA and perturbation of embryonic lung morphogenesis. Also, even though Sp1 is regarded as a ubiquitous transcription factor, its expression is highly regulated during embryonic development and cellular differentiation (Saffer et al., 1991). Thus, it is possible that the activity of NF- κ B and its interactions with Sp1, may be a part of a program that controls precise expression pattern of *Bmp4* in the lung and other tissue. Furthermore, pro-inflammatory cytokines and TNF- α in particular are well recognized as mediators of injury in a variety of disease settings including neonatal chronic lung disease known as bronchopulmonary dysplasia. It is currently thought that mediators of inflammation may interfere with the morphogenetic mediators and derail the normal processes of repair and development, leading to a pathology described as alveolar hypoplasia. The linkage between inflammation and morphogenesis, however, has been elusive. Repressor activity of TNF- α -induced NF- κ B on *Bmp4* gene expression provides an intriguing paradigm for the mechanisms by which inflammation may derail normal morphogenetic processes.

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Abbreviations

NF- κ B, Nuclear Factor κ B; bp, base pairs; dpp, decapentaplegic; I κ B, Inhibitor of κ B; kDa, kiloDaltons; TNF, Tumor Necrosis Factor; cDNA, DNA complementary to RNA; rRNA, ribosomal RNA; TK, thymidine kinase.

5. REFERENCES

- Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol* 2003;3:745–56. [PubMed: 12949498]
- Baldwin AS Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* 1996;14:649–83. [PubMed: 8717528]
- Bellusci S, Henderson R, Winnier G, Oikawa T, Hogan BL. Evidence from normal expression and targeted misexpression that bone morphogenetic protein (Bmp-4) plays a role in mouse embryonic lung morphogenesis. *Development* 1996;122:1693–702. [PubMed: 8674409]
- Berhane K, Margana RK, Boggaram V. Characterization of rabbit SP-B promoter region responsive to downregulation by tumor necrosis factor-alpha. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L806–14. [PubMed: 11053014]
- Bohinski RJ, Di Lauro R, Whitsett JA. The lung-specific surfactant protein B gene promoter is a target for thyroid transcription factor 1 and hepatocyte nuclear factor 3, indicating common factors for organ-specific gene expression along the foregut axis. *Mol Cell Biol* 1994;14:5671–81. [PubMed: 8065304]
- Bushdid PB, Brantley DM, Yull FE, Blaeuer GL, Hoffman LH, Niswander L, Kerr LD. Inhibition of NF-kappaB activity results in disruption of the apical ectodermal ridge and aberrant limb morphogenesis. *Nature* 1998;392:615–8. [PubMed: 9560159]
- Courey AJ, Tjian R. Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. *Cell* 1988;55:887–98. [PubMed: 3142690]
- De Robertis EM, Sasai Y. A common plan for dorsoventral patterning in Bilateria. *Nature* 1996;380:37–40. [PubMed: 8598900]
- DiCosmo BF, Geba GP, Picarella D, Elias JA, Rankin JA, Stripp BR, Whitsett JA, Flavell RA. Airway epithelial cell expression of interleukin-6 in transgenic mice. Uncoupling of airway inflammation and bronchial hyperreactivity. *J Clin Invest* 1994;94:2028–35. [PubMed: 7962549]
- Fainsod A, Steinbeisser H, De Robertis EM. On the function of BMP-4 in patterning the marginal zone of the *Xenopus* embryo. *Embo J* 1994;13:5015–25. [PubMed: 7957067]
- Feng JQ, Chen D, Cooney AJ, Tsai MJ, Harris MA, Tsai SY, Feng M, Mundy GR, Harris SE. The mouse bone morphogenetic protein-4 gene. Analysis of promoter utilization in fetal rat calvarial osteoblasts and regulation by COUP-TFI orphan receptor. *J Biol Chem* 1995;270:28364–73. [PubMed: 7499338]
- Hahn GV, Cohen RB, Wozney JM, Levitz CL, Shore EM, Zasloff MA, Kaplan FS. A bone morphogenetic protein subfamily: chromosomal localization of human genes for BMP5, BMP6, and BMP7. *Genomics* 1992;14:759–62. [PubMed: 1427904]
- Hirano F, Tanaka H, Hirano Y, Hiramoto M, Handa H, Makino I, Scheidereit C. Functional interference of Sp1 and NF-kappaB through the same DNA binding site. *Mol Cell Biol* 1998;18:1266–74. [PubMed: 9488441]
- Hogan BL. Bone morphogenetic proteins in development. *Curr Opin Genet Dev* 1996;6:432–8. [PubMed: 8791534]
- Jaskoll T, Boyer PD, Melnick M. Tumor necrosis factor-alpha and embryonic mouse lung morphogenesis. *Dev Dyn* 1994;201:137–50. [PubMed: 7873786]
- Jones CM, Lyons KM, Hogan BL. Involvement of Bone Morphogenetic Protein-4 (BMP-4) and Vgr-1 in morphogenesis and neurogenesis in the mouse. *Development* 1991;111:531–42. [PubMed: 1893873]
- Kallapur SG, Bachurski CJ, Le Cras TD, Joshi SN, Ikegami M, Jobe AH. Vascular changes after intra-amniotic endotoxin in preterm lamb lungs. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L1178–85. [PubMed: 15321788]
- Kanegae Y, Tavares AT, Izpisua Belmonte JC, Verma IM. Role of Rel/NF-kappaB transcription factors during the outgrowth of the vertebrate limb. *Nature* 1998;392:611–4. [PubMed: 9560158]
- King JA, Marker PC, Seung KJ, Kingsley DM. BMP5 and the molecular, skeletal, and soft-tissue alterations in short ear mice. *Dev Biol* 1994;166:112–22. [PubMed: 7958439]
- Kurihara T, Kitamura K, Takaoka K, Nakazato H. Murine bone morphogenetic protein-4 gene: existence of multiple promoters and exons for the 5'-untranslated region. *Biochem Biophys Res Commun* 1993;192:1049–56. [PubMed: 8507180]

- Li C, Zhu NL, Tan RC, Ballard PL, Derynck R, Minoo P. Transforming growth factor-beta inhibits pulmonary surfactant protein B gene transcription through SMAD3 interactions with NKX2.1 and HNF-3 transcription factors. *J Biol Chem* 2002;277:38399–408. [PubMed: 12161428]
- Matsui H, Ihara Y, Fujio Y, Kunisada K, Akira S, Kishimoto T, Yamauchi-Takahara K. Induction of interleukin (IL)-6 by hypoxia is mediated by nuclear factor (NF)-kappa B and NF-IL6 in cardiac myocytes. *Cardiovasc Res* 1999;42:104–12. [PubMed: 10435001]
- May MJ, Ghosh S. Signal transduction through NF-kappa B. *Immunol Today* 1998;19:80–8. [PubMed: 9509763]
- Mercurio F, Manning AM. NF-kappaB as a primary regulator of the stress response. *Oncogene* 1999;18:6163–71. [PubMed: 10557108]
- Miyazaki Y, Araki K, Vesin C, Garcia I, Kapanci Y, Whitsett JA, Piguet PF, Vassalli P. Expression of a tumor necrosis factor-alpha transgene in murine lung causes lymphocytic and fibrosing alveolitis. A mouse model of progressive pulmonary fibrosis. *J Clin Invest* 1995;96:250–9. [PubMed: 7542280]
- Motomura T, Kasayama S, Takagi M, Kurebayashi S, Matsui H, Hirose T, Miyashita Y, Yamauchi-Takahara K, Yamamoto T, Okada S, Kishimoto T. Increased interleukin-6 production in mouse osteoblastic MC3T3-E1 cells expressing activating mutant of the stimulatory G protein. *J Bone Miner Res* 1998;13:1084–91. [PubMed: 9661072]
- Muraoka RS, Bushdid PB, Brantley DM, Yull FE, Kerr LD. Mesenchymal expression of nuclear factor-kappaB inhibits epithelial growth and branching in the embryonic chick lung. *Dev Biol* 2000;225:322–38. [PubMed: 10985853]
- Nusslein-Volhard C, Roth S. Axis determination in insect embryos. *Ciba Found Symp* 1989;144:37–55. [PubMed: 2673683]discussion 55–64, 92–8
- Perkins ND, Edwards NL, Duckett CS, Agranoff AB, Schmid RM, Nabel GJ. A cooperative interaction between NF-kappa B and Sp1 is required for HIV-1 enhancer activation. *Embo J* 1993;12:3551–8. [PubMed: 8253080]
- Ray P, Yang L, Zhang DH, Ghosh SK, Ray A. Selective up-regulation of cytokine-induced RANTES gene expression in lung epithelial cells by overexpression of IkappaBR. *J Biol Chem* 1997;272:20191–7. [PubMed: 9242696]
- Renaud S, Loukinov D, Bosman FT, Lobanekov V, Benhattar J. CTCF binds the proximal exonic region of hTERT and inhibits its transcription. *Nucleic Acids Res* 2005;33:6850–60. [PubMed: 16326864]
- Saffer JD, Jackson SP, Annarella MB. Developmental expression of Sp1 in the mouse. *Mol Cell Biol* 1991;11:2189–99. [PubMed: 2005904]
- Tartaglia LA, Weber RF, Figari IS, Reynolds C, Palladino MA Jr. Goeddel DV. The two different receptors for tumor necrosis factor mediate distinct cellular responses. *Proc Natl Acad Sci U S A* 1991;88:9292–6. [PubMed: 1718003]
- Vainio S, Karavanova I, Jowett A, Thesleff I. Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. *Cell* 1993;75:45–58. [PubMed: 8104708]
- Van den Wijngaard A, Pijpers MA, Joosten PH, Roelofs JM, Van zoelen EJ, Olijve W. Functional characterization of two promoters in the human bone morphogenetic protein-4 gene. *J Bone Miner Res* 1999;14:1432–41. [PubMed: 10457277]
- Van den Wijngaard A, van Kraay M, van Zoelen EJ, Olijve W, Boersma CJ. Genomic organization of the human bone morphogenetic protein-4 gene: molecular basis for multiple transcripts. *Biochem Biophys Res Commun* 1996;219:789–94. [PubMed: 8645259]
- Weaver M, Yingling JM, Dunn NR, Bellusci S, Hogan BL. Bmp signaling regulates proximal-distal differentiation of endoderm in mouse lung development. *Development* 1999;126:4005–15. [PubMed: 10457010]
- Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA. Novel regulators of bone formation: molecular clones and activities. *Science* 1988;242:1528–34. [PubMed: 3201241]

Figure 1.

The effect of TNF-alpha treatment on endogenous *Bmp4* gene expression in lung adenocarcinoma, A549 and H441 cell lines. Upper Panel, Northern blot of steady state levels of mRNA in the two cell lines treated with and without TNF-alpha. Lower Panel, quantification of mRNA levels from the above Northern blot by densitometry.

Figure 2.

TNF-alpha represses the activity of a human *Bmp4* promoter-luciferase construct, transfected into A549 and H441 cells. Panel A: The construct *pSLA4.1EX* was transfected into the latter cell lines and then treated with various doses of TNF-alpha as indicated. Luciferase values, subsequent to normalization, were plotted against doses of TNF-alpha. Panel B. As a control, an *IL-6* promoter-luciferase construct was transfected into A549 cells and the cells were treated with doses of TNF-alpha as indicated. Luciferase values were normalized and plotted against TNF-alpha doses used.

Figure 3.

The repressor effect of TNF- α is mediated through NF κ B. The full length human *Bmp4* promoter-luciferase construct, *pSLA4.1EX* was co-transfected with either Ikb (Bars 3 and 4) or a control plasmid (Bars 1 and 2). The cells were subsequently treated with either TNF- α (bars 2 and 4) or control carrier (Bars 1 and 3).

Figure 4.

RelA mediates the inhibitory effect of TNF-alpha on transcriptional activity of the human *Bmp4* promoter. Panel A. A549 or H441 cells were co-transfected with *pSLA4.1EX* with and without expression plasmids for RelA, or p50. Normalized luciferase values are plotted as shown. Panel B. As a control for Panel A, wild type or IL6 mutant promoter-luciferase constructs were co-transfected with and without RelA expression plasmid in A549 cells and the normalized luciferase values plotted as shown.

Figure 5.

Only one of the 3 putative NFkB binding sites on the human *Bmp4* promoter binds RelA. Electrophoretic mobility shift assay performed with nuclear extract from A549 cells. NFkB-oligo1, NFkB -oligo2 and NFkB -oligo3 sequences are shown in Materials & Methods. Only the binding site in NFkB-oligo3 showed supershifted band with the anti-RelA antibody, indicating binding of RelA to the DNA sequences on this probe (Lane I).

Figure 6. Co-transfection of serial deletion constructs of the human *Bmp4* promoter, *pSLA4.1EX* with expression vectors for RelA and P50 in A549 cells. Normalized luciferase measurements were plotted as shown.

Figure 7.

Co-transfection of the minimal responsive *Bmp4* promoter fragment with expression plasmids for either RelA (Panel A) or P50 (Panel B), combined with an Sp1 expression plasmid. In Lanes 3 to 6, increasing quantities of either RelA or P50 were used. Normalized luciferase values were plotted as shown.

Figure 8.

Panel A. Nucleotide sequence of the 96 bp minimal DNA fragment within the human *Bmp4* promoter and the oligonucleotides derived from it that were used in EMSA. Panel B. EMSA with nuclear extracts from A549 cells. The probes are the oligonucleotides shown in Panel A. Supershifted complexes with anti-RelA and anti-SP1 are pointed out by the arrowhead on the right and the asterisks (SS = supershifted).

Figure 9. A 37 bp fragment (nucleotides -17 to +20) of *Bmp4* promoter mediates the response to RelA, or p50. pTK/-253+20, pTK/-253-17, or pTK/-17+20 was cotransfected with NFkB p65, NFkB p50, or control vector in A549 cells. Normalized luciferase values were plotted as shown.

Figure 10.

Expression of the endogenous *Bmp4* gene is repressed in lung explants by TNF-alpha. Wild type and *Bmp4-lacz* embryonic E11 lungs were cultured as explants and treated without (A and C) or with 30 ng/mL of TNF-alpha (B and D) for two days according to the procedures described in Materials & Methods. Arrows show the sites of LacZ staining in untreated lung. Arrowheads show extinguished LacZ staining in the lung in response to TNF-alpha.