Plosa, Erin J.

Regulation of FGF-10 Expression by Inflammatory Signals, Erin J. Plosa, M.D.

I. SPECIFIC AIMS

Bronchopulmonary dysplasia (BPD) causes significant morbidity in preterm infants. Lung morphogenesis in infants with BPD arrests during the saccular stage of lung development, when terminal airways branch and expand into alveolar ducts. In a mouse model of chorioamnionitis, developed in the Prince laboratory *E. coli* lipopolysaccharide (LPS) injected into the amniotic fluid resulted in decreased saccular branching and large, simplified airways [1]. These experimental findings are consistent with clinical observations correlating inflammation with the development of BPD. The Prince laboratory has previously shown that LPS inhibits Fibroblast growth factor-10 (FGF-10) expression in fetal mouse lungs and in primary fetal lung mesenchymal cells. Human patients with BPD also have reduced FGF-10 expression in their lungs compared to controls [2]. FGF-10 may therefore be a target of inflammation in BPD pathogenesis.

Bacterial products such as LPS activate the innate immune system through binding cell surface Toll-Like Receptors [3]. Toll-like receptor signaling results in the activation of the transcription factor NF-κB, stimulating the expression of pro-inflammatory genes [4]. Our preliminary data suggest that LPS and inflammatory cytokines that activate NF-κB can inhibit FGF-10 expression. *The overall goal of the project is to identify the molecular mechanisms linking inflammation and reduced FGF-10 expression in the fetal lung.* ***This proposal will test the specific hypothesis that NF-κB activation directly inhibits FGF-10 transcription.*** We will use biochemical and molecular approaches to test this hypothesis in the following 3 Aims:

Aim 1. Test if NF-κB activation inhibits FGF-10 expression. Bacterial products inhibit FGF-10 expression in fetal mouse lungs and in cultured fetal lung mesenchymal cells. Because many inflammatory stimuli can activate NF-κB, we will test if NF-κB activation specifically reduces FGF-10 expression.

a. Using primary fetal lung mesenchymal cells, we will test if the NF-κB activators LPS, IL-1β, and TNFα inhibit FGF-10 expression as measured by real time PCR.

b. CHO cells transfected with a FGF-10-luciferase reporter plasmid will be co-transfected with p65 or a constitutively active IKK2 mutant. Each of these cDNAs will increase NF-κB activation, allowing us to test if they also inhibit FGF-10 promoter activity.

Aim 2. Determine if blocking NF-κB protects FGF-10 from the effects of inflammatory mediators. If NF-κB activation inhibits FGF-10, then specifically blocking NF-κB should protect FGF-10 expression from the effects of inflammation. We will use both biochemical and genetic approaches to interfere with NF-κB activation in primary NGL fetal lung mesenchyme and FGF-10-luciferase expressing CHO cells.

a. We will use the specific IKK2 inhibitor BMS-345541 to inhibit NF-κB activation in NGL fetal lung mesenchymal cells and transfected CHO cells. We will then test if this inhibitor prevents LPS, IL-1β, and TNFα from inhibiting FGF-10 expression.

b. FGF-10 luciferase expressing CHO cells will be co-transfected with a plasmid expressing a dominant-negative mutant IκB, which prevents NF-κB activation. We will then treat these cells with IL-1β and TNFα to test if NF-κB activation is required for these inflammatory cytokines to inhibit FGF-10 expression.

Aim 3. Identify the sites of NF-κB interaction with the FGF-10 promoter. The FGF-10 promoter region is poorly defined. Our preliminary experiments identified a 350 bp region of the FGF-10 gene that retains inhibition by IL-1β. This region contains at least one consensus NF-κB binding site. We will use molecular approaches to test if this region can mediate NF-κB inhibition of FGF-10.

a. We will perform further truncations of the FGF-10 promoter to test if sequential deletion prevents the effects of IL-1β on promoter activity as measured using the luciferase reporter in transfected CHO cells

b. We will remove the predicted NF-κB binding site just upstream of the FGF-10 start site by both deletion and point mutation to test if this sequence is required for NF-κB inhibition of FGF-10-luciferase activity. We will use both IL-1β and co-transfection with p65 and constitutively active IKK2 to activate NF-κB.

c. Once we identify a region of the FGF-10 promoter that is required for NF-κB inhibition, we will insert this sequence into a control luciferase plasmid to test if it is sufficient to provide NF-κB responsiveness.

These experiments will test our novel hypothesis that the inflammatory transcription factor NF-κB can directly inhibit transcription of the developmentally critical FGF-10 gene. Not only will this work be important in better understanding the pathogenesis of BPD, but will also provide critical insight into the basic biological mechanisms regulating both innate immunity and mammalian organ development.

II. RESEARCH STRATEGY

1. Significance

Up to 50% of infants born before 27 weeks will require oxygen for more than 28 days and continue to require respiratory support at 36 weeks gestation [5]. Patients with BPD are more likely to be rehospitalized for respiratory illnesses and often have impaired pulmonary function and exercise tolerance at school age [6, 7]. Our understanding of BPD pathogenesis is evolving. Our understanding of BPD pathogenesis is evolving. Lung morphogenesis in infants with BPD arrests during the saccular stage of lung development, when terminal airways branch and expand into alveolar ducts. In infants with BPD, these terminal airways remain dilated with fewer branches and subsequently lower numbers of alveoli. This results in a reduced surface area available for gas exchange and potentially an immature alveolar capillary bed [8, 9].

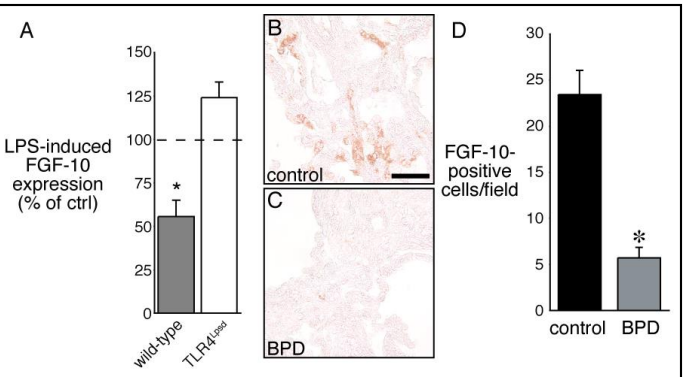

Figure 1. Reduced FGF-10 in lungs exposed to inflammation and in BPD. **(A).** *E. coli* LPS inhibits FGF-10 gene expression in wild-type fetal mouse lungs, but not in lungs from TLR4 mutant mice. **(B-D).** Decreased FGF-10 expression in immunostained lung sections from infants that died with BPD compared to age-matched controls. (*$P < 0.05$, n = 6).

Chorioamnionitis, defined as inflammation or infection of the uterine wall, placenta, and amniotic membranes, is a frequent cause of preterm delivery and increases the risk of BPD [10, 11]. In animal models of chorioamnionitis, bacterial products and inflammation arrests normal lung development. In sheep, prenatal exposure to inflammatory stimuli lead to large, simplified alveoli [12]. In a mouse model of chorioamnionitis by injecting LPS into the amniotic fluid of pregnant mice resulted in decreased saccular branching and large, simplified airways in the offspring [1]. Detailed analysis of the LPS-exposed fetal lungs revealed reduced saccular airway elongation and branching [1]. FGF-10 is a mesenchymal growth factor critical for normal lung development that promotes airway elongation and branching [13]. The Prince laboratory has previously showed that LPS inhibits FGF-10 expression in fetal mouse lungs and in primary fetal lung mesenchymal cells. Human BPD patients have reduced FGF-10 expression in their lungs compared to controls (**Fig. 1**), [2]. Overall BPD remains a common pediatric disease. The incidence of BPD in preterm infants has not significantly improved during recent years. Current therapies are largely empiric, as the mechanisms leading to BPD are unknown. This proposal builds on clinical observations, animal studies, and more recent experimental data to directly examine the transcriptional regulation of growth factor expression in the face of inflammatory stimuli. This would provide a route to alter the arrest of lung development seen in BPD. Specific knowledge regarding the pathogenesis of BPD could lead to novel therapies to prevent or treat disease. This study will test the mechanisms linking inflammation and inhibition of FGF-10 gene expression. The mechanistic experiments proposed will be performed in controlled, rigorous, in vitro cell culture models.

2. Innovation

This proposal will use novel tools to ask critical questions about BPD pathogenesis, an important disease in children. By using primary fetal lung mesenchymal cells from NGL reporter mice and from conditionally immortalized SV40 mice, we will be able to perform rigorous experiments into the molecular signaling in the fetal lung mesenchyme that were not previously possible.

3. Rationale

Recent studies have shown that inflammation can disrupt normal human development in extremely preterm infants. At the molecular level, this likely represents interference between the inflammatory signaling pathways and normal developmental mechanisms. This proposal will specifically test if the pro-inflammatory transcription factor NF-κB inhibits FGF-10 promoter activity. These experiments would represent a key early step in understanding at the molecular level how inflammation might disrupt fetal development. If we can identify how inflammation inhibits FGF-10, then we can concentrate our efforts on designing more specific therapies.

Instead of trying to globally block inflammation, a more specific approach might be to protect FGF-10 expression, and therefore normal lung development, in the face of ongoing inflammation. Similar approaches may be used in studying development of the brain and gastrointestinal system.

4. Study Design

We have isolated primary mesenchymal cells from the fetal lungs of temperature-sensitive SV-40 transgenic mice [14]. These cells can be maintained in an immortalized state at 33 ˚C, with return to primary cell features when cultured at 37 ˚C. In preliminary experiments, these cells behaved similarly to primary BALB/cJ mesenchymal cells. We have also isolated fetal lung mesenchymal cells from NGL reporter mice, which express both GFP and luciferase downstream of a NF-κB response element. We will use mutant isoforms of IKK2 and IκB to manipulate the NF-κB signaling pathway within transfected CHO cells. These tools will specifically test if NF-κB activation can inhibit the activity of the FGF-10 promoter, thereby reducing FGF-10 expression. The tools and reagents necessary for these studies are currently available and in use by the Prince lab.

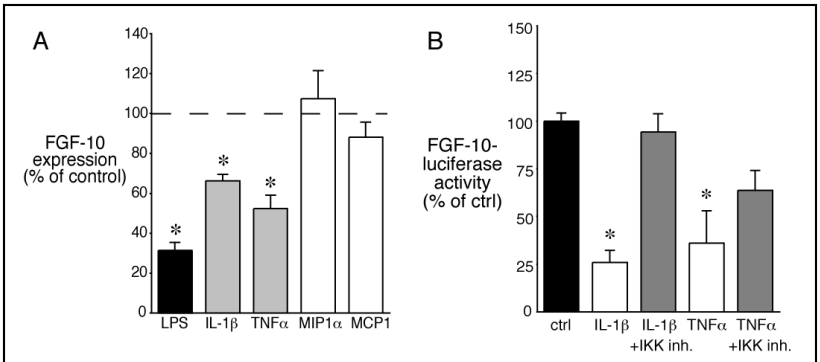

Figure 2. Cytokines that activate NF-κB inhibit FGF-10 expression. **(A).** LPS, IL-1β, and TNFα inhibit FGF-10 gene expression in fetal mouse lung mesenchymal cells. (**P* < 0.05, n = 9). **(B).** IL-1β and TNFα inhibit FGF-10-luciferase activity in CHO cells. The IKK inhibitor BMS345541 blocked this effect. (**P* < 0.05, n=6).

Aim 1: Test if NF-κB activation inhibits FGF-10 expression. We will first verify that activated NF-κB can inhibit FGF-10 expression. We will treat primary mouse fetal lung mesenchymal cells with LPS, IL-1β, and TNFα, each well-described NF-κB activators. Preliminary data suggest that the NF-κB agonists LPS, IL-1β, and TNFα can each inhibit FGF-10 expression (**Fig. 2A**). We will perform dose-response experiments with each agent using NGL mesenchymal cells, measuring luciferase activity to quantify NF-κB activation. Cells will then be treated for 1 h, 4 h, 12 h, and 24 h with each agent. We will then isolate RNA from control and treated cells, measuring FGF-10 gene expression by Real-Time PCR. Gene expression levels will be normalized to GAPDH, which is not affected by inflammatory stimuli (previous data from Prince lab). The entire set of experiments will be repeated at least 3 times. As a positive control, we will measure the expression of MIP1 α, which is robustly induced by NF-κB. For negative controls, we will treat cells with MCP-1, an inflammatory chemokine that does not activate NF-κB.

We will test the effects of NF-κB on the activity of a FGF-10-luciferase reporter construct in transfected CHO cells. Preliminary data shows that this reporter has strong basal activity in CHO cells, and was inhibited by IL-1β (**Fig. 2B**; of note, CHO cells are resistant to LPS). We will treat FGF-10-luciferase expressing CHO cells with increasing concentrations of IL-1β and TNFα to test if these activators of NF-κB can inhibit FGF-10 promoter activity in a concentration-dependent manner. In addition, we will co-transfect CHO cells with expression plasmids containing p65, the major active subunit of NF-κB or a constitutively active mutant form of IKK2 (cIKK2) [15]. Overexpression of either construct increases net NF-κB activity. We will test if p65 or cIKK2 overexpression inhibits FGF-10 promoter activity. Our preliminary data suggests that p65 overexpression may inhibit FGF-10-luciferase activity (**Fig. 3**). As positive control, parallel experiments will be performed in CHO cells transfected with a NF-κB-luciferase reporter that contains a consensus NF-κB response element driving luciferase expression [16]. For a negative control, we will transfect CHO cells with a PAI1-luciferase construct, which is very sensitive to TGFβ signaling, but not affected by NF-κB activation. All of these reagents are available in the Prince lab.

Aim 2: Determine if blocking NF-κB protects FGF-10 from the effects of inflammatory mediators. If NF-κB is an essential negative regulator of FGF-10 expression, blocking NF-κB should protect FGF-10 from the effects of inflammatory mediators. We will first test this using the specific inhibitor BMS345541, which by

blocking IKK2 activity prevents NF-κB activation [17]. To determine the concentration required for inhibition, we will treat NGL fetal lung mesenchymal cells with a range of BMS345541 concentrations (100 pM – 10 μM), and then treat the cells with IL-1β. Luciferase activity will quantify NF-κB activation. Once we have established an inhibitor concentration that can inhibit NF-κB but not cause visible cell toxicity, we will pretreat mesenchymal cells with BMS345541, then add LPS, IL-1β, or TNFα for 1 h, 4 h, 12 h, and 24 h. FGF-10 gene expression will be measured by Real-Time PCR. Untreated cells and cells treated only with inhibitor will be used as controls.

We will also use BMS345541 to inhibit NF-κB activation in transfected CHO cells. As CHO cells may have different inhibitor sensitivity, the dose-response will be repeated in CHO cells transfected with a NF-κB luciferase reporter construct. We will then pretreat FGF-10-luciferase expressing CHO cells with BMS345541, followed by treatment with IL-1β and TNFα. Measuring luciferase activity will determine if this IKK inhibitor can block the effects of inflammatory cytokines on FGF-10 promoter activity. Other inhibitors of NF-κB have been reported, but are less specific for the NF-κB pathway and have significant cell toxicity in our experience.

Transfection of CHO cells with a dominant-negative mutant form of IκB (DNIκB) will provide another approach for testing the role of NF-κB. CHO cells will be transfected with both FGF-10-luciferase and a mammalian expression vector containing the DNIκB coding sequence. This mutant protein prevents activation and nuclear trafficking of NF-κB subunits into the nucleus [18]. After transfection, we will treat cells with IL-1β and TNFα, then measuring FGF-10-luciferase activity. Preliminary data suggests this approach may be feasible, as DNIκB expression increased basal FGF-10-luciferase activity (**Fig. 3**). Parallel experiments using the NF-κB luciferase reporter will be used as a control to ensure that DNIκB expression inhibits NF-κB activation.

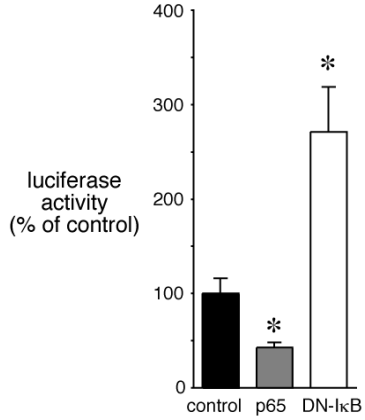

Figure 3. Components of NF-κB signaling regulate the FGF-10 promoter. CHO cells expressing FGF-10-luciferase were co-transfected with the NF-κB subunit p65 or a mutant IκB (*$P < 0.05$, n = 4).

Aim 3: Identify the sites of NF-κB interaction with the FGF-10 promoter. Our preliminary data identified a region of the FGF-10 promoter within 350 bp of the transcriptional start site that retained responsiveness to IL-1β (**Fig. 4**). We plan to use molecular approaches to identify the sequences within this region required and sufficient for NF-κB inhibition of FGF-10. Using a PCR mutagenesis strategy, we will perform smaller truncations of this 350 bp region, testing each mutant for both baseline FGF-10 promoter activity and responsiveness to IL-1β. Cotransfection with p65 and cIKK2 will also test the effects of NF-κB activation.

In silico analysis of this region has identified at least one potential NF-κB binding site (**Fig. 4C**). We plan to mutate this site by both deletion and site directed mutagenesis (to a scrambled sequence), again measuring basal FGF-10 promoter activity and IL-1β response. If we identify a minimal region required for the IL-1β response, we will insert this region of the FGF-10 promoter into a control luciferase reporter plasmid to test if this sequence is sufficient to instill NF-κB inhibition to another gene. We will use luciferase

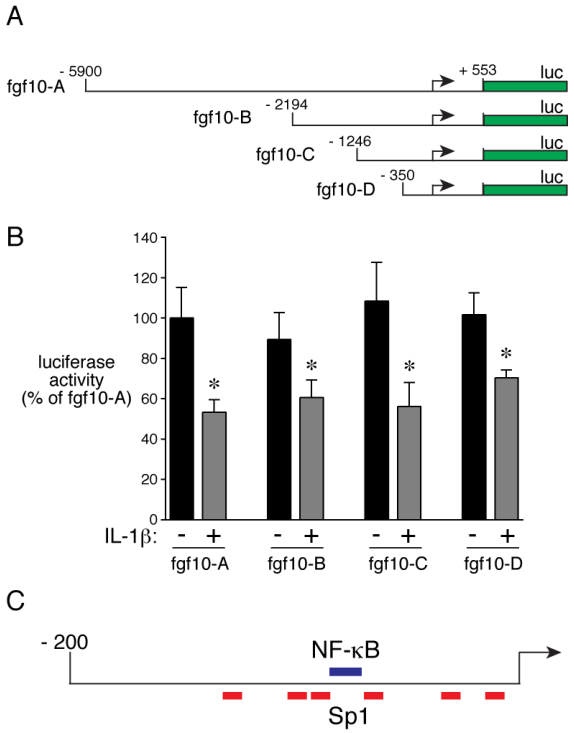

Figure 4. Analysis of the FGF-10 promoter. **(A).** Schematic of deletion mutants generated by truncating the 5' end of the FGF-10 promoter. **(B).** Each FGF-10-luciferase construct was transfected into CHO cells. IL-1β inhibited promoter activity of all FGF-10 promoter mutants tested. **(C).** Predicted binding NF-κB and Sp1 binding sites in the FGF-10 promoter region within 200 bp of the start site.

plasmids without any promoter sequence and with a SV40 promoter for these experiments. Positive experiments will be repeated with TNFα to ensure that other NF-κB activators give similar results.

Mutation of the FGF-10 promoter could result in disruption of basal activity, making it difficult to determine if NF-κB is still inhibitory. Other members of the Prince laboratory are currently performing chromatin immunoprecipitation to test if p65 or p50 directly interact with the FGF-10 promoter. NF-κB could interact with another transcription factor (such as Sp1) and regulate FGF-10 promoter activity without directly binding a specific promoter sequence. Such an interaction has been reported in the literature and could be tested using the tools available [18].

Detailed methodology:

Cell culture and transfection. Primary fetal lung mesenchymal cells have been isolated from embryonal day 16 lungs of both tsSV40 and NGL transgenic mice. Cells are maintained in DMEM with 10% FBS. Immortalized tsSV40 cells are cultured at 33 ˚C with γ-interferon. CHO cells are maintained in Ham's-F12 with 10% FBS. For transfection, CHO cells plated at 50% confluency are incubated in serum free media with cDNA/lipofectamine complex for 4h. Complete media is the added and the cells are studied 24-72 h later. All luciferase transfections include a β-galactosidase expression vector (pSport), enabling correction for differences in transfection efficiency.

Real-Time PCR. Total RNA is isolated from cultured cells using Trizol reagent. cDNA is synthesized by reverse-transcription and an oligo-dT primer. Real-Time PCR is then performed using an iQ5 optical thermocycler (Bio-Rad) and gene-specific TaqMan probes (ABI). GAPDH is used as a control gene. Threshold values (C_T) are compared between groups by ANOVA and gene expression values are represented as percentage of control using the $2^{\Delta\Delta CT}$ method.

Luciferase measurement. Transfected CHO and NGL cells are lysed in Reporter Lysis Buffer (Promega), centrifuged to remove any cellular debris, and measured using Steady-Glo luciferase reagent (Promega) and a 96-well luminometer. Beta-galactosidase activity is measured on aliquots of each sample, and used to normalize luciferase activity each sample.

III. REFERENCES

1. Prince, L.S., et al., *Toll-like receptor signaling inhibits structural development of the distal fetal mouse lung.* Dev Dyn, 2005. **233**(2): p. 553-61.
2. Benjamin, J.T., et al., *FGF-10 is decreased in bronchopulmonary dysplasia and suppressed by Toll-like receptor activation.* Am J Physiol Lung Cell Mol Physiol, 2007. **292**(2): p. L550-8.
3. Beutler, B., et al., *How we detect microbes and respond to them: the Toll-like receptors and their transducers.* J Leukoc Biol, 2003. **74**(4): p. 479-85.
4. Ghosh, S., M.J. May, and E.B. Kopp, *NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses.* Annu Rev Immunol, 1998. **16**: p. 225-60.
5. Bancalari, E. and N. Claure, *Definitions and diagnostic criteria for bronchopulmonary dysplasia.* Semin Perinatol, 2006. **30**(4): p. 164-70.
6. Korhonen, P., et al., *Respiratory outcome in school-aged, very-low-birth-weight children in the surfactant era.* Acta Paediatr, 2004. **93**(3): p. 316-21.
7. Smith, L.J., et al., *Reduced exercise capacity in children born very preterm.* Pediatrics, 2008. **122**(2): p. e287-93.
8. Jobe, A.J., *The new BPD: an arrest of lung development.* Pediatr Res, 1999. **46**(6): p. 641-3.
9. Husain, A.N., N.H. Siddiqui, and J.T. Stocker, *Pathology of arrested acinar development in postsurfactant bronchopulmonary dysplasia.* Hum Pathol, 1998. **29**(7): p. 710-7.
10. Newton, E.R., *Preterm labor, preterm premature rupture of membranes, and chorioamnionitis.* Clin Perinatol, 2005. **32**(3): p. 571-600.
11. Gibbs, R.S., *The relationship between infections and adverse pregnancy outcomes: an overview.* Ann Periodontol, 2001. **6**(1): p. 153-63.

12. Moss, T.J., et al., *Early gestational intra-amniotic endotoxin: lung function, surfactant, and morphometry.* Am J Respir Crit Care Med, 2002. **165**(6): p. 805-11.
13. Hogan, B.L. and J.M. Yingling, *Epithelial/mesenchymal interactions and branching morphogenesis of the lung.* Curr Opin Genet Dev, 1998. **8**(4): p. 481-6.
14. Jat, P.S., et al., *Direct derivation of conditionally immortal cell lines from an H-2Kb-tsA58 transgenic mouse.* Proc Natl Acad Sci U S A, 1991. **88**(12): p. 5096-100.
15. Sadikot, R.T., et al., *Selective I kappa B kinase expression in airway epithelium generates neutrophilic lung inflammation.* J Immunol, 2003. **170**(2): p. 1091-8.
16. Yull, F.E., et al., *Bioluminescent detection of endotoxin effects on HIV-1 LTR-driven transcription in vivo.* J Histochem Cytochem, 2003. **51**(6): p. 741-9.
17. Burke, J.R., et al., *BMS-345541 is a highly selective inhibitor of I kappa B kinase that binds at an allosteric site of the enzyme and blocks NF-kappa B-dependent transcription in mice.* J Biol Chem, 2003. **278**(3): p. 1450-6.
18. Zhu, N.L., et al., *TNF-alpha represses transcription of human Bone Morphogenetic Protein-4 in lung epithelial cells.* Gene, 2007. **393**(1-2): p. 70-80.

BUDGET

Cell culture media and supplies	$1500
Luciferase Assay supplies	$800
Real Time PCR core lab and supplies	$1700
Biochemical and molecular reagents	$1000

TIMELINE

	Months 1-3	Months 4-6	Months 7-9	Months 10-12
Aim 1	Real time PCR, CHO cell transfection	p65, cIKK2 transfection		
Aim 2	IKK2 inhibition	DNIκB transfection		
Aim 3		FGF-10 promoter deletions	FGF-10 promoter deletions, point mutations	FGF-10 promoter insertion