TRANSCRIPTIONAL REGULATION OF THE NOS3 GENE IN PULMONARY MYOFIBROBLAST DIFFERENTIATION AND IMPLICATIONS FOR THIS IN PULMONARY FIBROSIS.

A thesis submitted in partial fulfillment of requirements for Masters of Science Degree of the University of Nairobi (Comparative Mammalian Physiology).

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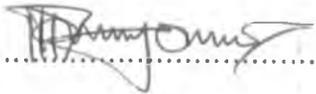
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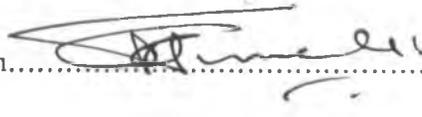
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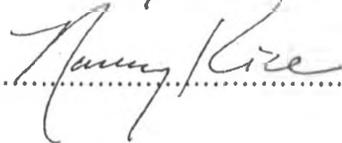
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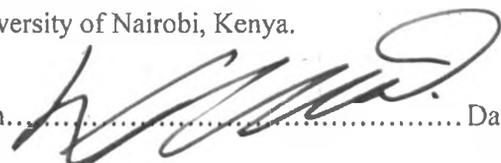
DEDICATION

This work is especially dedicated to all students from poor backgrounds especially Sub-Saharan Africa who strive to achieve their academic dreams, who understand the statement that "Anyone who has ever struggled with poverty knows how extremely expensive it is to be poor".

This work is also dedicated to my dear friend and companion Nelly Kanazi, and my family who have been a source of support and love. My beloved father Wilfred Ochwang'i and Mother Annastancia Serah Moraa, Brother Robert and Justus, Sisters Damarice, Linet, Lydia, Mercy, Diana and Purity.

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LIST OF ABBREVIATIONS

PBS: Phosphate buffered saline

PCR: Polymerase Chain Reaction

NO: Nitric oxide

ABSTRACT

Nitric oxide (NO) produced by endothelial cells via the catalytic action of nitric-oxide synthase (eNOS) represents an antifibrotic mechanism in the body. Previous studies suggest that nitric oxide (NO)-mediated signals regulates myofibroblast phenotypes and it is believed that a loss of this control may play an important role in development of pulmonary fibrosis. This work focused on the effect of specific regulators on NOS3 gene expression to elucidate the mechanisms by which nitric oxide levels are controlled in rat pulmonary myofibroblasts cells.

Rat NOS3 gene promoter was cloned in front of a luciferase reporter gene and transfection assays in rat pulmonary myofibroblasts were performed and cells were treated with a variety of potential regulators of NOS3. Promoter activity of NOS3 gene, were assayed using the Dual Luciferase reporter gene assay technique. The results showed that the rat NOS3 promoter was active in the rat pulmonary myofibroblasts with the human NOS3 promoter showing little or no activity. This study confirmed that TGFβ and LPS up regulates transcriptional activity while PMA decreases NOS3 transcription.NOS3 transcriptional activity decreased in cells treated with 23187, a calcium ionophore but increased when treated with EGTA suggesting that calcium concentrations could have a potential effect on regulating NO concentrations in the cell. Treatment with L-NAME (Nw-Nitro-L-arginine methyl ester), a known NOS3 selective inhibitor had no effect on the gene expression. S-NAP (S-nitroso-N-acetylpenicillamine), a known Nitric Oxide donor suppressed NOS3 transcriptional activity. From these results it can be concluded that high concentrations of NO inhibit NOS3 activity.NOS3 is regulated by several effectors in the cell that could be targets for pharmacological agents to help in protection against pulmonary fibrosis. This work initiated a

study to determine the functional elements involved in the transcriptional activity of the promoter by creation of deletion constructs, however this studies were not completed.

CHAPTER ONE

1.0   GENERAL INTRODUCTION

Myofibroblasts are mesenchymal cells that have the ultrastructural properties of both muscle and nonmuscle cells. Originally described as "modified fibroblasts" located in granulation tissue (Gabbiani *et al.,* 1971), they have subsequently been documented in a diverse array of tissues (reviewed in Powell *et al.,* 1999).There are numerous cell types that have been characterized as myofibroblasts, including stromal cells in organ loose connective tissue, pericytes that are found around capillaries, stellate cells in the nervous system, interstitial cells, mesangial cells in the kidneys, and granulation tissue fibroblasts. They are intriguing cells that have been described for decades, but their molecular, cellular and developmental properties have not been well elucidated. Myofibroblasts are highly plastic and diverse with their phenotypes depending on their tissue of origin and whether the tissue is normal or pathologic. Common features include expression of muscle, nonmuscle structural proteins, regulatory proteins and contractile proteins as well as secretion of extracellular matrix (ECM) (Forbes *et al.,* 1999).

During embryonic development the mesenchyme,a derivative of the mesoderm and ectodermal layers gives rise to mesenchymal stem cells(MSC) which then differentiate into bone, cartilage and other tissue derivatives (Caplan,2007). Similarly in the adult rare MSC cells grow, differentiate and die off and are replaced by other developing MSC cells thus, maintaining tissue integrity. Important MSC derivatived cells are adventitial fibroblast cells that are found in virtually all organs that are essential for normal development and tissue homeostasis.

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These adventitial fibroblasts that later differentiate to form myofibroblasts exhibit contractile properties thought to be essential in regulating blood flow (Forbes *et al.,* 1999). The origin and differentiation pathways of these myofibroblasts in different tissues have not been well characterized. Myofibroblasts are present during normal development and have been detected in developing kidney, brain, heart, lung, and brown adipose tissue. Their presence during development requires Platelet derived growth factor (PDGF), though the myofibroblasts of different tissues have different PDGF requirements, as demonstrated in mice (Lindahl *et al.,* 1998). It has also been reported that mice deficient for PDGF-A exhibit lung defects and die either during embryogenesis or just after birth. These defects are caused by lack of alveolar septation due to the absence of alveolar myofibroblasts (Bostrom *et al.,* 1996).

In addition to their normal cellular functions, myofibroblasts are also involved in wound repair. However, their persistence has implicated them in fibrosis in various tissues, such as liver, heart, and kidney (reviewed in Schurch *et al.,* 1998). During tissue injury resident adventitial fibroblasts transform into an activated state. This activated state participates in granulation tissue formation by exhibiting an increase in muscle protein gene expression which include alpha smooth muscle actin (ASMA) and skeletal-specific myosin heavy chain (MyHC) proteins; extracellular matrix secretion and contractility (Gabbiani,1992). This *in vivo* activation can also be reproduced in cell culture by treatment with numerous cytokines, notably endothelin 1(ET-1), angiotensin II (Ang II), (PDGF), and transforming growth factor β (TGF-β) (Desmouliere *et al.,* 1993). During normal wound healing myofibroblasts undergo apoptosis (Darby *et al.*, 1990; Clark 1993), but in certain circumstances, these cells persist and continue to secrete extracellular matrix. Persistent myofibroblasts have been implicated in interstitial fibrosis of the lung (Phan, 2002).

Although pulmonary fibrosis has diverse etiologies, there is a common feature characteristic of this process, namely, the abnormal deposition of extracellular matrix that effaces the normal lung tissue architecture. A key cellular source of this matrix is the mesenchymal cell population that occupies much of the fibrotic lesion during the active period of fibrosis. This population is heterogeneous with respect to a number of key phenotypes. One of these phenotypes is the myofibroblast, which are commonly identified by their expression of ASMA and by features that are intermediate between the bona fide smooth muscle cell and the fibroblast. The *de novo* appearance of myofibroblasts at sites of wound healing and tissue repair/fibrosis is associated with a period of active fibrosis which is considered to be involved in wound contraction. Furthermore, localization of myofibroblasts at sites undergoing active extracellular matrix deposition suggests an important role for these cells in the genesis of the fibrotic lesions. In recognition of the potential importance of these cells in fibrosis, and perhaps in its persistence or progression, previous studies have focused on the nature and precise role of these cells in the context of pulmonary fibrosis (Sem *et al.,* 2002). The presence of myofibroblasts in patients with pulmonary fibrosis is amply documented in both lung tissues taken from patients with pulmonary fibrosis as well as in those taken from animal models of the disease (Adler *et al.,* 1989, Mitchell *et al* 1989, Kuhn *et al* 1991, Pache *et al* 1998).There is however limited information regarding the mechanisms of this pathological fibroproliferation.

Several studies suggest that nitric oxide (NO)-mediated signals may be important in regulating myofibroblasts phenotypes which though heterogeneous in different tissues, share many common biochemical characteristics and are valid comparisons.

In the heart, long-term inhibition of NO synthesis leads to increased accumulation of ASMA positive myofibroblasts with associated collagen and fibronectin deposition in ischemic lesions causing hypertension and myocardial damage in rats (Pessanh. *et al.*, 2000). The myocardial healing process includes changes in extracellular matrix composition associated with the phenotypic modulation of fibroblasts. Early and later lesion areas showed a population of spindle-shaped fibroblast cells expressing ASMA content (Pessanh, 2000). NO has been shown to reduce myofibroblast accumulation and collagen deposition (Vernet. *et al.*, 2002).

In vivo, endothelial NO synthase (eNOS) gene knockout mice experience prolonged pulmonary fibrosis in response to the profibrotic agent bleomycin, suggesting that eNOS operates in down-regulating myofibroblast proliferation and/or apoptosis (Chung. *et al.*, 2003). These studies support the hypothesis that NO plays a role in the mechanism that down regulates myofibroblast phenotype expression. A cytokine thought to have significant effect on myofibroblast development is transforming growth factor-β (TGF-β). Through the action of its membrane-bound type I receptors, this cytokine elicits a wide range of cellular responses that regulate myofibroblast proliferation, differentiation and apoptosis (Zhang, 1999).

This study investigated the role of NO in pulmonary fibrosis at the NOS gene level by studying the transcriptional regulation of the eNOS gene promoter in pulmonary myofibroblasts. The study hypothesizes that NO regulates its own transcription.

1.1 OBJECTIVES

1. To study the role of NO via *NOS3* gene expression in pulmonary myofibroblasts under different growth conditions.

2. To study the role of various effector chemicals in regulation of *NOS3* gene in pulmonary myofibroblasts.

3. To map out the *NOS3* promoter gene elements important in myofibroblasts regulation.

1.2 HYPOTHESIS

It was hypothesized that NO regulates its own transcription.

CHAPTER TWO

2.0   LITERATURE REVIEW

2.1   Myofibroblast definition and morphology

Myofibroblasts are smooth muscle-like fibroblasts depending on tissue of occurrence. They variously had been referred to as smooth-muscle-like cells, activated smooth muscle cells, lipocytes or stellate cells (Moore et al., 1989). They are mesenchymal cells that possess both fibroblast, muscle-like and secretory characteristics and function in tissue development, remodeling and repair. Morphologically, myofibroblasts are stellate in all tissues; functionally, they are contractile and secrete various collagen isoforms and elastin (Serini, and Gabbiani, 1999).
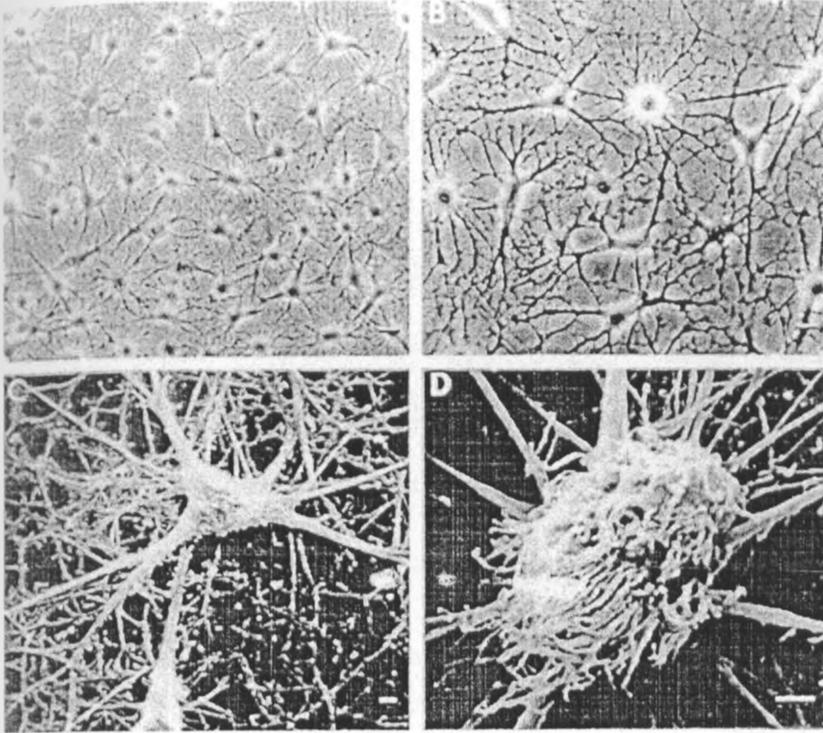
Fig 1: Phase-contrast micrographs (A and B) and scanning electron micrographs (C and D) of stellate myofibroblast cells.

The cells display a highly refractile cell body on phase-contrast microscopy and possesses a highly arborized array of cell processes with several orders of bifurcation. The cell processes are devoid of microvilli, whereas the cell body shows a dense array of long microvilli, giving it a shaggy appearance [From Valentich *et al.* (246)].

2.1.1 Myofibroblast function

It has been suggested that different organs contain fibroblasts with specific features and function (Sappino *et al* 1990b).They have been classified into subtypes depending on their main functions which include: fibrogenesis, tissue skeleton or barrier, intercellular communication system, contractile, endocrine and vitamin A storage (Kumuro, 1990). Other specific functions determined include production of growth factor and cytokine (Aggarwal and Pocsik, 1992; Bennet and Schultz, 1993), interaction with immune system (Phipps et al., 1990), and determination of epithelial differentiation (Cunha *et al.,* 1991; Hayashi et al., 1993).

2.2 Myofibroblast distribution in normal tissues

Typically, myofibroblasts are diverse and have been found in a variety of organs including the kidneys, the lungs, nerve tissue as well as blood vessels (Sappino *et al* 1990b). Immunohistochemical studies have shown that they express proteins typical of contractile cells such as desmin, skeletal specific myosin heavy chain and α smooth muscle actin (ASMA), suggesting that these cells participate in visceral contraction and/or organ remodeling. This view is supported by the observation that generally myofibroblasts are present in organs in which the capacities of remodeling are important (Schmitt-Graff *et al.,* 1994). It is not known whether the proportion of skeletal muscle cell markers (i.e., ASMA, desmin, and skeletal specific myosin heavy chain) in different fibroblast populations reflects precise functional activities or whether specific properties related to the expression of these different cytoskeletal proteins remain to be defined.

Functionally through mesenchymal-epithelial interactions, myofibroblasts are key components of organogenesis or morphogenesis, *i.e.*, the growth and differentiation of the tissue or organ

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(Simon-Assmann et al., 1995). Myofibroblasts function through the secretion of inflammatory and growth factors, expression of their receptors and secretion of interstitial matrix and/or basement membrane molecules (Fries et al., 1994). Myofibroblasts also play a fundamental role in many disease states, either through activation and proliferation or through apoptosis (Darby et al., 1990 and Sappino et al 1990) .They also play a central role in wound healing, presumably as an extension or accentuation of their role in normal growth and differentiation (Gabbiani and Rungger-Brandle 1981; Gabbiani et al 1971). They also appear to be involved in the formation and repair of the extracellular matrix (ECM) and proliferation and differentiation of epith...ial or parenchymal, vascular and neurogenic elements (D'Amore, 1992; Saunders and D'Amore 1992).

2.3   Cellular origin of myofibroblast cells

Previous studies suggest that granulation tissue fibroblasts arise from quiescent connective tissue cells. However, myofibroblasts can derive from at least three mesenchymal cell types: fibroblasts, pericytes and smooth muscle cells (MacDonald, 1959; Grillo, 1963; Ross et al., 1970). It seems likely that in a majority of situations, myofibroblasts derive from preexisting fibroblasts, but in some cases they may derive from pericytes and or/SM cells (Grimaud and Borojevic, 1977). Shum and McFarlane (1988) in their morphological studies showed that myofibroblasts can derive from vascular SM cells.

An intimate relationship between myofibroblasts and blood vessel wall has been described particularly during initial steps of granular tissue formation (Janssen, 1902; Fisher et al.; 1982).

In experimental granulation tissue, myofibroblasts derive from local fibroblasts; temporarily acquire markers of smooth muscle differentiation, such as ASMA, which disappear when the wound is closed (Darby et al., 1990). This study proposes that some local stimuli, probably distinct from those producing proliferation, induce SM differentiation markers in resident fibroblasts. Whether the distinct heterogeneity in the cytoskeletal phenotype of myofibroblasts is attributable to differentiation from a common cell type or from different cell types remain uncertain.
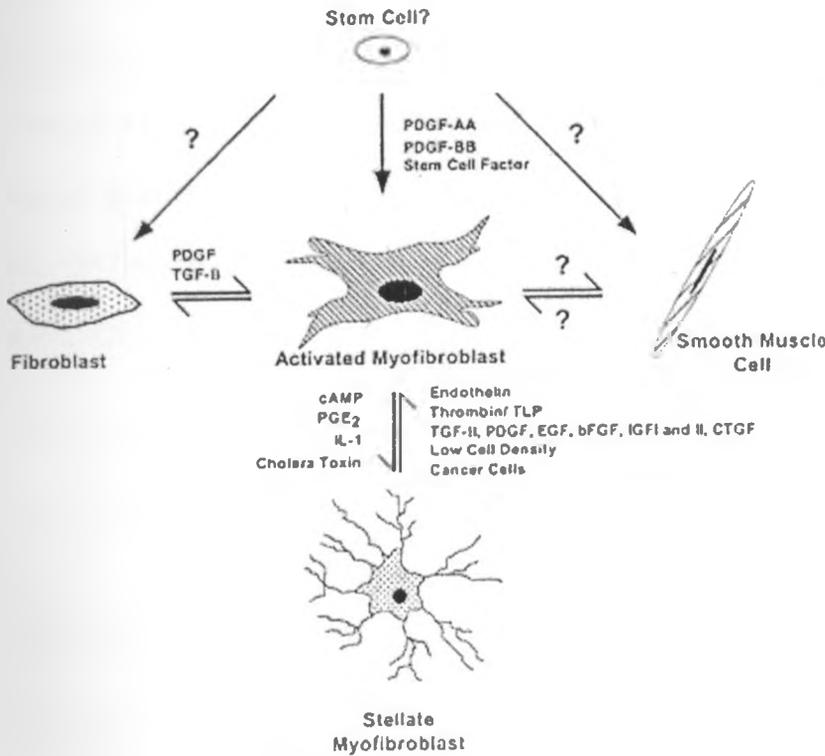
Fig.2. Proposed scheme depicting the origin, transdifferentiation, activation, and stellate transformation of myofibroblasts.

PDGF, platelet-derived growth factor; TLP, tethered ligand protein; TGF-β, transforming growth factor-β; IL-1, interleukin-1; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; IGFI, insulin-like growth factor I; CTGF, connective tissue growth factor (reviewed in Powell *et al.*,1999).

2.4 Role of myofibroblasts in wound repair

During wound repair, fibroblasts participate in the formation of granulation tissue and modulate into myofibroblasts (Gabbiani et al., 1971; Darby et al., 1990). Myofibroblasts are poorly developed in early granulation tissue and are most numerous in the phase of wound contraction. At wound contraction they are organized in the architecture of the tissues in the form of several almost continuous layers parallel to the tissue surface, whereas small blood vessels are disposed perpendicularly to the fibroblastic layers and the wound surface (MacSween and Whaley, 1992). After healing, myofibroblasts and small vessels progressively disappear in the scar (Darby et al., 1990). It is conceivable that the myofibroblastic phenotype reverts to a quiescent form when the wound is closed, or myofibroblasts disappear selectively through apoptosis (Darby et al., 1990; Clark 1993; Gabbiani, 1992).). Multiple paracrine and autocrine mechanisms appear to be involved in regulation through a mechanism yet to be elucidated (reviewed in Powell *et al.*, 1999).

The process of wound healing involves release of proinflammatory cytokines, eicosanoids of the cyclooxygenase, lipoxygenase, and cytochrome P-450 family, NO, and a host of growth factors; the secretion of collagen and other matrix proteins; the elaboration of angiogenic, angiostatic, and nerve growth factors. If the wound is deep or open, the granulation tissue is converted to a scar (fibrosis) (Birchmeier and Birchmeier, 1993; Diehl and Rai, 1996). Myofibroblasts therefore appear to be to be key cells in events of wound healing and repair.

2.5 Myofibroblasts in idiopathic pulmonary fibrosis

Overally myofibroblasts respond to proinflammatory cytokines with elaboration of matrix proteins and additional growth factors and then postulated to undergo apoptosis following

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repair or scar formation which is a normal process (Desmouliere and Gabbiani, 1994). In some cases, however, following healing myofibroblasts are known to persist and this continuation of myofibroblasts and/or their activity, *i.e.* matrix deposition, in the absence of injury has implicated them in various pathological processes including pulmonary fibrosis and the molecular mechanism(s) that result in the sustained activation of these cells is still unclear.

Pathogenesis of pulmonary fibrosis remains incompletely understood. Studies of associated inflammation have led to the discovery of a number of cytokines and chemokines that are found to be important either directly or indirectly for the fibrotic process. However, the importance of inflammation in pulmonary fibrosis is unclear, and at the time of diagnosis the inflammatory component is variable and usually not responsive to anti-inflammatory therapeutic agents. Patients usually exhibit evidence of active fibrosis with increased numbers of activated fibroblasts, many of which have the phenotypic characteristics of myofibroblasts (Sem, 2002). At these sites, increased amounts of extracellular matrix deposition are evident with effacement of the normal alveolar architecture.

Animal model studies show the myofibroblast to be the primary source of type I collagen gene expression in active fibrotic sites. *In vitro* studies have shown differentiation of these cells from fibroblasts under the influence of certain cytokines as well as their susceptibility to NO apoptosis (Sem, 2002)

Although pulmonary fibrosis has diverse etiologies, there is a common feature characteristic of this process, namely, the abnormal deposition of extracellular matrix that affects the normal lung tissue architecture. A key cellular source of this matrix is the mesenchymal cell population that occupies much of the fibrotic lesion during the active period of fibrosis. This

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population is heterogeneous with respect to a number of key phenotypes. One of these phenotypes is the myofibroblast (Sem, 2002). In normal wound healing, the number of myofibroblasts gradually declines as the healing process is successfully completed (Darby and Gabbiani 1990 and Majno, 1971). Similarly, in a self-limiting model of pulmonary fibrosis, myofibroblasts gradually disappear as the active fibrotic phase is terminated (Zhang et al, 1994). In contrast, these cells persist and can be found in various stages of human pulmonary fibrosis where the disease is progressive (Zhang et al 1996). Therefore the mechanism of the myofibroblast disappearance is of potential interest since it can provide insight into the basis for its persistence and hence into the maintenance or progression of the fibrosis. Pulmonary fibrosis also known as idiopathic pulmonary fibrosis (IPF) is a progressive and largely fatal group of disorders that is quite prevalent worldwide.IPF affects up to 500,000 people in the United States alone (Selman, M., et al, 2004). There are five million people worldwide that are affected by this disease. As a consequence of misdiagnosis the actual numbers may be significantly higher. Of these more than 40,000 expire annually.

However, diagnoses have ranged from age seven to the eighties and typically when diagnosed they are in their forties and fifties although research indicates that many infants are afflicted by Pediatric Interstitial Lung Disease. At this time there is limited data on prevalence for this group   (Pulmonary Foundation, 2009)

The disease involves scarring of the lung with alveolar air sacs gradually replaced by fibrotic tissue. With this scarification, the tissue thickens causing an irreversible loss of the tissue's ability to transfer oxygen into the bloodstream. Most patients diagnosed with IPF are over 50 years of age and present with dyspnea and a nonproductive cough over months to years. Symptoms include: shortness of breath, particularly with exertion; chronic dry, hacking

cough; fatigue and weakness; discomfort in the chest; loss of appetite and rapid weight loss. Chest radiographs show bilateral peripheral based reticular opacities and honeycombing predominantly in lower lung regions. Prognosis is typically death two to five years from diagnosis. This disease can result from a number of different insults to the lung that can include toxic, autoimmune, drug-induced, traumatic injuries and also idiopathic causes (Thannickal, 2004). Myofibroblasts are the principal cells in the foci responsible for collagen matrix deposition, inflammation, and altered mechanical properties in the fibrotic lesions (Pardo and Selman, 2002). Therefore, a thorough understanding of the mechanisms involved in this foci formation is essential.

2.6 NO signaling in myofibroblast differentiation

Previous studies have shown that fibroblasts differentiate in response to multiple paracrine-mediated pathways and then undergo the phenotypic changes associated with myofibroblasts which include increased extracellular matrix deposition and increased contractility (Powell, 1999).

Multiple signaling pathways have been implicated in the process of this fibroblast differentiation into myofibroblasts. This work is interested is on one of these pathways, the NO signaling pathway. NO synthases (NOSs) enzymes convert L-arginine to L-citrulline leading to the formation of NO which is a free radical gas (Moncada and Higgs, 1993).

Three different forms of NOS have been identified based upon tissue of origin: neural NOS1 (nNOS), inducible NOS2 (iNOS), and endothelial NOS3 (eNOS) which are encoded by different genes, *NOS1*, *NOS2* and *NOS3*, respectively (Knowles and Moncada, 1994).

2.7 NOS3 promoter regulation

The present study focuses on the role of eNOS in myofibroblast function at the promoter level. Promoters such as the NOS3 promoter contain specific DNA sequences and response elements which provide a binding site for RNA polymerase and for transcription factors that recruit RNA polymerase. Promoters represent critical elements that can work in concert with other regulatory regions (enhancers, silencers, boundary elements/insulators) to direct the level of transcription of a given gene (Agullo, 2007). As promoters are typically immediately adjacent to the gene in question, positions in the promoter are designated relative to the transcriptional start site, where transcription of RNA begins for a particular gene (i.e., positions upstream are negative numbers counting back from -1, for example -100 is a position 100 base pairs upstream). To initiate transcription, a core promoter like NOS3 requires a minimal of Transcription Start Site (TSS) approximately -34 ,a binding site for RNA polymerase which encompass RNA polymerase I that transcribes genes encoding ribosomal RNA ;RNA polymerase II that transcribes genes encoding messenger RNA and certain small nuclear RNAs and RNA polymerase III: that transcribes genes encoding tRNAs and other small RNAs.NOS3 however requires polymerase I (Agullo, 2007).

Many eukaryotic promoters, between 10 and 20% of all genes, contain a TATA box (sequence TATAAA), which in turn binds a TATA binding protein which assists in the formation of the RNA polymerase transcriptional complex.

The TATA box typically lies very close to the transcriptional start site .However the eNOS promoter doesn't contain this element. (Agullo, 2007).

2.8 NO signaling pathway

The activity a gene promoter is regulated by various factors which include effector molecules which either activate or inactivate the gene expression and ultimately the cells. Many of the effectors tested on their effects on myofibroblast cells in this work regulate the NO signaling pathways through interactions (Fig 3). This represents a general model of regulation of NOS-III which is the same pathway that would occur in regulation of myofibroblast function (Agullo, 2007).
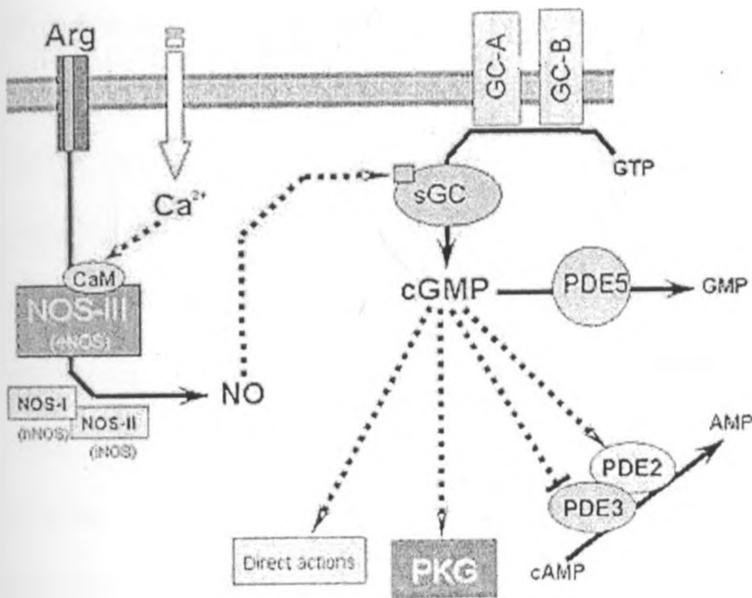
Fig.3. Cyclic GMP Transduction Pathway.

Continuous lines represent real chemical transformation of the compound. *Discontinuous* lines represent interaction with a target protein (Adopted from Blauplanet.com, 2003)

Cyclic GMP is seen to be synthesized by three different enzymes; guanylyl cyclase (sGC) and the particulate forms of guanylyl cyclase (GC-A and GC-B).Guanylyl cyclase is activated by nitric oxide (NO) by its interaction as a target protein. sGC catalyses conversion

of GTP into cGMP, cGMP-dependent protein kinase phosphorylates amino acids thus affecting the nature of the protein produced. There are two important events in the synthesis of nitric oxide which include: cytosolic calcium increase that activates constitutive nitric oxide synthases and uptake of L-arginine which is one of the substrates for the synthetic reaction.

2.9 Regulation of NO signaling by calcium

Calcimycin which is a calcium ionophore, also known as A23187 a mobile ion-carrier forms stable complexes with divalent cations (ions with a charge of +2) through an increase of intracellular Ca^{2+} levels in intact cells thus suppressing NOS3 promoter activity (Agullo, 2007). Calcimycin also uncouples oxidative phosphorylation, the process cells use to synthesize Adenosine triphosphate which they use for energy. In addition, it inhibits mitochondrial ATPase activity. All NO-synthases required for its activation to be bound to a calcium regulatory protein: calmodulin. iNOS tightly binds calmodulin even at resting calcium concentrations, and then it is active once it is synthetized. However, constitutive enzymes, eNOS and nNOS, only bind calmodulin when the intracellular calcium concentration increases up to a certain value. Agents that increase intracellular calcium concentration, either by allowing calcium entrance from the outside or by stimulating calcium mobilization from intracellular stores, can activate these constitutive enzymes.

It is now clear that eNOS is also regulated by pathways that are independent on changes in the intracellular calcium concentration: its activity is acutely dependent on intracellular localization and also dependent on phosphorylation at specific amino acids (Agullo, 2007).

Fig 4 presents the pathway used by other important factors in the regulation of transcription of the eNOS3 gene.
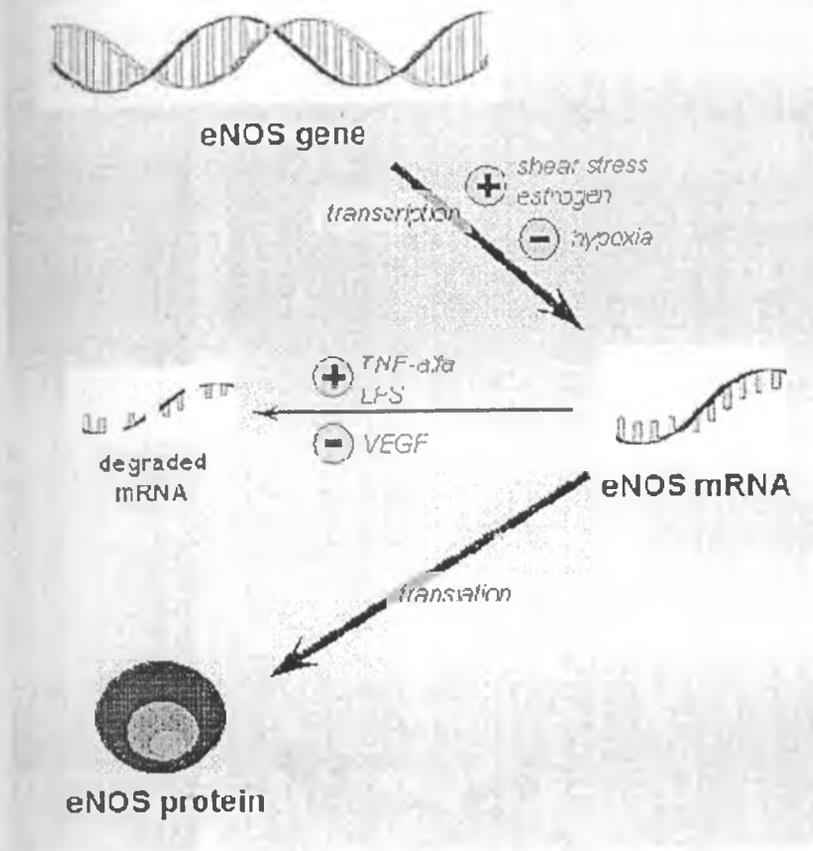
Fig. 4. eNOS regulation. [Adopted from Govers and Rabelink, Am J Physiol 2001, 280:F193]. Here, the expression of eNOS is clearly shown.

There are several factors that regulate the transcription of eNOS gene (shear stress, estrogen and hypoxia) and others that modulate the stability of its mRNA (tumor necrosis factor alfa or TNF-alfa, lipopolysacharide or LPS, and vascular endothelial group factor or VEGF).

Phorbol 12-myristate 13-acetate (PMA) is diester of phorbol and a potent tumor promoter often employed in biomedical research to activate the signal transduction enzyme protein kinase C (PKC). The effects of PMA on PKC result from its similarity to one of the natural activators of classic PKC isoforms, diacylglycerol (Agullo, 2007).Regulation of endothelial NOS (eNOS, NOS3 or NOS-III) has been extensively studied in recent years and found to

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involve multiple factors. It is complex and multiple regulatory pathways have been identified (Agullo, 2007). This study investigated transcriptional regulation of the NOS3 promoter and how some of these factors are linked to pulmonary myofibroblasts.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 NITRIC OXIDE SYTHASE 3 (NOS3) GENE PROMOTER CLONING AND EXTRACTION

Rat and human NOS3 gene promoter (gifts from Professor Li Jiang of University of

Pittsburgh, USA) were ligated unto pGL3-Basic vector plasmid DNA and pGL2-Basic vector

plasmid respectively (Promega Corporation).
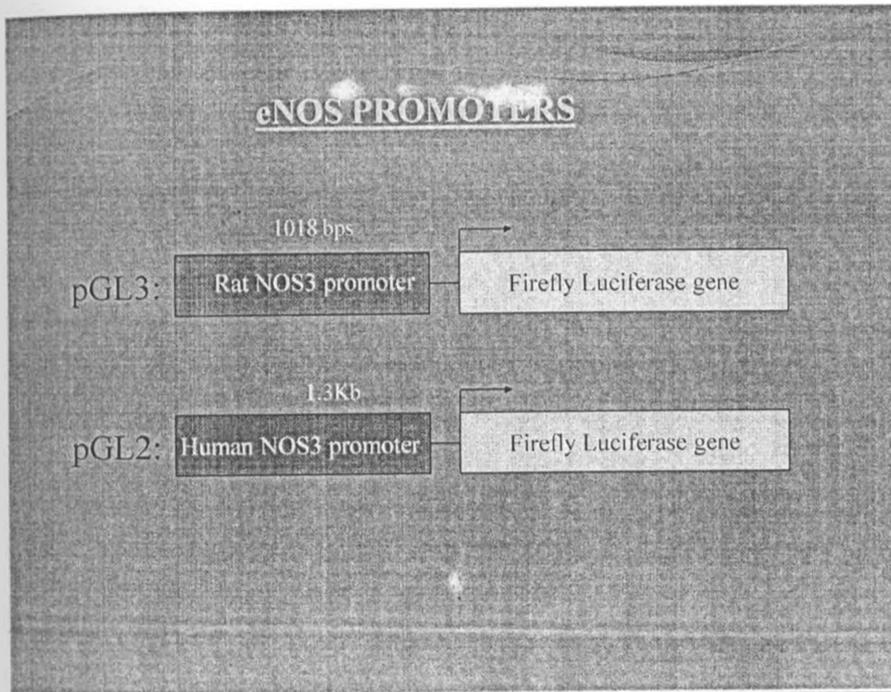
Fig 5: A schematic presentation of the eNOS promoter (Human and Rat)

These plasmid DNA molecules are autonomously replicating mini-chromosomes which are

double stranded. Most of them are circular and some can freely transfer between bacteria.

They perform this function through replication where the plasmid copies itself and

partitioning where each progeny cell receives a copy of the plasmid.

pGL3-Basic vector plasmid DNA is a typical genetically modified plasmid that has important components which include an origin of replication, a promoter ligated to the origin that is important in controlling the expression of the cloned genes, a polylinker (multiple cloning sites) used to clone DNA fragments to the backbone of the plasmid, a transcription terminator sequence to terminate the transcription of the cloned gene, and a selection marker used to isolate host cells taken up by the plasmid.

The promoter fragments and vector DNA were digested with restriction enzymes that generated compatible ends for cloning. The human promoter was inserted between KpnI and BglII restriction sites while the rat promoter was ligated between KpnI and XhoI restriction sites of the respective basic vectors.

Competent bacterium *Escherichia coli* (DH5α) that were sourced from Takara Bio Incorporation were transformed by heat shock to take up the plasmid DNA. These are cells that have been chemically transformed by growing to mid-log phase, harvested and treated with CaCl$_2$. Cells treated in such a way are said to be competent. The competent cells were mixed with the DNA on ice, followed by a brief incubation at 42 ^{0}C (heat shock).Transformed bacteria were then grown in liquid LB (Miller's) growth media from Invitrogen Corporation with 200μl ampicillin (50mg/ml).The plasmids had lactamase gene that confers resistance to ampicillin. The transformed bacteria that take up this plasmid can grow in LB medium containing ampicillin. Ampicillin inhibits cells wall synthesis by interfering with peptioglycan cross linking. Inoculated media flasks were then put in a shaking incubator at 37°C (230 rpm for 16 hours).Upon transformation, the *E.coli* cells were grown in LB medium for 45-60 minutes, to allow expression of the antibiotic resistance gene.

Bacterial cells were harvested by centrifugation at 6000 gravity using a sorvall centrifuge-RC 5C Plus. The pelleted bacteria contained the plasmid DNA.

3.2 NITRIC OXIDE SYTHASE 3 (NOS3) GENE PROMOTER PLASMID DNA PURIFICATION AND QUANTIFICATION.

The plasmid DNA was purified using QIA filter midi-prep plasmid purification kit per manufacturer's protocol from Qiagen Company. This process entailed resuspending the pellet in a suspension buffer containing Tris/EDTA and RNaseA which will suspend the pellet and digest the RNA.The bacteria was then lysed with lysis buffer that contains NaOH/SDS; SDS denatures protein while NaOH denatures DNA.The lysate was neutralized with neutralization buffer containing potassium acetate that causes the covalently closed plasmid DNA to reanneal (supernatant) and protein and bacterial DNA to form a complex with potassium (precipitate).The supernatant was then applied to the spin column and the DNA eluted from the column.Eluted DNA was quantified using a Nano drop spectrophotometer at an absorbance of 260nm using 10mM Tris as a blank..

3.3 RESTRICTION DI3ESTION

The eluted DNA contained either the rat or the human NOS3 gene promoter, ligated into pGL3-Basic vector plasmid DNA or PGL2-Basic vector plasmid respectively. The rat-NOS3 promoter was cleaved from the plasmid vector using restriction enzyme that cut DNA fragments cutting at specific sequences, usually four to twelve base-pairs in length and can produce blunt or overhanging (sticky) ends.

3.3.1 Sequential restriction digestion

Sequential restriction digestion was done to digest the NOS3 promoter fragment. In the present study we used KpnI, HindIII and XhoI restriction endonucleases (Promega).Because of the various salt concentrations the restriction digestion was done starting with buffer J for KpnI and then buffer D for XhoI. For HindIII restriction enzyme, reaction buffer E containing 100mM NaCl was used while for KpnI restriction enzyme buffer J containing 0% NaCl was used. It should be noted that at first we choose restriction sites upstream and downstream of the promoter and therefore used KpnI and HindIII. To specifically cut the Rat-NOS3 promoter from the plasmid construct, a restriction digestion using KpnI and XhoI restriction endonucleases was done. A sequential restriction digestion starting with buffer J (0%Nacl) for KpnI and buffer D (150mM Nacl) for XhoI was done. Agarose gel electrophoresis was then done using 0.7% Agarose gel TBE to visualize the DNA bands.
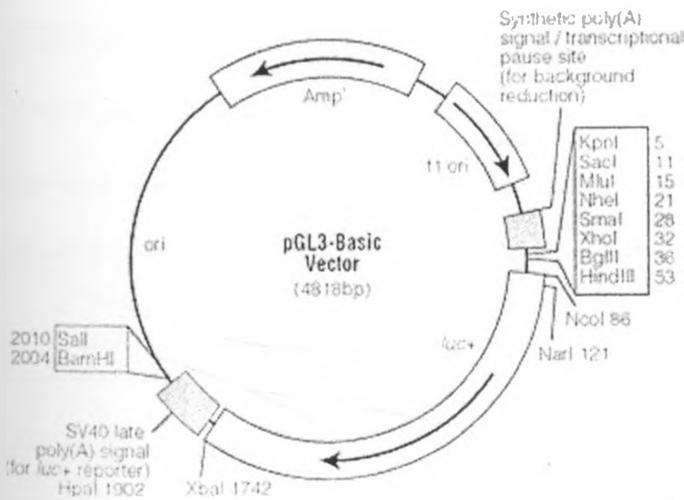
Fig 6: pGL3-Basic vector where the rat-NOS3 promoter is inserted

(Adopted from Promega Corporation).This is a negative control that lacks eukaryotic promoter and enhancer sequences.

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3.4   pGL3-NOS3 DNA PROMOTER SEQUENCING

The pGL3-NOS3 plasmid was sequenced by chain termination method using the Big Dye Terminator v3.1 Cycle sequencer to determine the exact sequence of the promoter as per the manufacturer's protocol (appliedbiosystems; appendix 9). Sequencing by chain-termination method involves the synthesis of a DNA strand by a DNA polymerase I using a single stranded template with a forward and reverse primer.Sythesis is initiated at the site where an oligonucleotide primer anneals to the template. The synthesis reaction is terminated by the incorporation of a nucleotide analog (ddNTP) that terminates elongation. When proper mixtures of dNTPS and one of the four ddNTPs are used, polymerization will be terminated randomly at each possible site. Once the sequence information of the promoter and plasmid was known, the function of the DNA sequence was known using National centre for biotechnology information (NCBI) (U.S.A), a public biological sequence database. The database sequence search was done by doing a search in GenBank of NCBI with Basic Local Alignment Search Tool (BLAST) tool. In the present study, the rat NOS3 was confirmed to be cloned into the KpnI and XhoI site of pGL3-basic by the sequencing.

3.5   CELL CULTURE

3.5.1 GROWING AND PASSAGING OF PULMONARY MYOFIBROBLASTS CELLS.

To determine the transcriptional regulation of the NOS3 gene promoter activity in relation to pulmonary fibrosis, rat myofibroblast cells were grown and passaged in growth and differentiating conditions and then used in transfection with the promoters. Gene expression

was assayed using the Dual Luciferase reporter gene assay technique (Promega).Passaging and transfection procedure was conducted inside a laminar flow hood under sterile conditions. Myofibroblast cells were cultured from liquid nitrogen onto petri dish plates .The cells were grown in normal growth media containing DMEM (Dulbecco's Modified Eagles Medium from Gibco 11960), 10% FBS (Fetal Bovine Serum), Penicillin streptomycin, glutamate and 1.0M HEPES pH 7.4 for 48 hours until they were 80% confluent All the media used was prewarmed at 37°C in a water bath. This was to ensure that physiological conditions for growth of cells were maintained. The plates were labeled appropriately. The myofibroblast cells in plates were washed with 0.15M phosphate buffered saline (PBS) pH 7.2 to remove any dead cells and debri. The cells were harvested by detaching from the plates using 1X trypsin that cleaves the extracellular matrix connections cells have made to adhere to the plastic and placed for 2-3 minutes in 37 $^{\circ}$C incubator. The high temperature allows trypsin to work more efficiently. After incubation, cells were viewed using an inverted microscope to check for"balling" to determine if they have lost contact with the culture plates. The plates were whacked on counter very hard to further release the cells from the plastic. Most of the cells were harvested in media containing 1% FBS and then used for ransfection.

The cells that were to be used for the next passage were diluted 1:3 or 1:4 typically, with normal growth media and pipetted onto new, labeled plates and placed back into the incubator to grow ready for the next passage. Preparation of the various percentages of media is shown in the appendix.

For the transfection experiments, cells were counted using a hemacytometer and seeded to 24 well plates at a density of 4×10^5 cells/well. They were incubated for 16 hours in 37°C prior to transfection.

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3.5.2 TRANSFECTION OF PULMONARY MYOFIBROBLASTS AND PROMOTER ASSAY

Pulmonary myofibroblasts cells of 80% confluence between passages 4-5 were used. These cells had a density of $4-5 \times 10^5$ cells/well which is the optimal density for an effective transfection and were cotransfected with SV 40 Luciferase which is a positive control that contains SV40 promoter and enhancer sequences resulting in strong expression of *luc+* in many types of cells including myofibroblasts. pGL3 basic which is the negative control, lacks the eukaryotic promoter and enhancer sequences. Rat and Human NOS3 promoters in pGL3-basic plasmid DNA were cotransfected with a Renilla Luciferase construct downstream of the Thymidine kinase promoter (RLTK) to normalize activity levels and control for variability in transfection efficiency. The plasmid DNA was transfected using cationic lipid based product, Lipofectamine 2000 from Invitrogen using the transfection protocol (appendix 7). Cells were allowed to grow for 36-48 hours in a 5% Carbon dioxide-water jacketed incubator following transfection, at which time, they were harvested and assayed for Luciferase activity using the Dual Luciferase assay reporter gene technique. This procedure was used to assay both human and rat NOS3 promoters for relative activity using a 20/20 Turner Luminometer which records the luminescence of Firefly and Renilla Luciferase activities.

Cells grown under normal growth conditions i.e. 10% Fetal Bovine Serum (FBS) or differentiating conditions i.e. 0%FBS, were assayed as described above. Firefly Luciferase activity was normalized to that of Renilla Luciferase activity.

3.5.3 PROMOTER ACTIVITY ASSAY IN PULMONARY MYOFIBROBLAST CELLS IN THE PRESENCE OF EFFECTORS: PMA, TGFβ, LPS, A23187, S-NAP, L-NAME and EGTA.

The activity of a promoter is regulated by various factors which include enhancers, inhibitors and effectors. In the present study, effectors known to affect myofibroblast activity were tested on how they alter NOS3 gene expression in pulmonary myofibroblast cells. This could either down regulate or up regulate the transcription of the promoter through different pathways. Various concentrations were used in order to make comparisons of their regulation. Concentrations were chosen based upon the 1/2 life of each molecule as well physiologically relevant concentration that elicits an effect .The effectors included PMA (Phorbor-12-myristate-13-acetate) from Calbiochem for which 20μM and 2μM concentrations were tested; TGFβ (Transforming growth factor β)(Calbiochem) for which 10ng/ml and 5ng/ml concentrations were tested; LPS (Lipopolysaccharide) supplied from Sigma which was tested at 10ng/ml concentration; L-NAME (Nω-nitro-L-arginine methyl ester) a potent eNOS3 selective inhibitor from Sigma-Aldrich for which 1mM and 10mM concentrations were tested; S-NAP (S-nitroso-N-acetylpenicillamine) a nitric oxide donor supplied from Calbiochem for which 0.1mM and 1 mM concentrations were tested ; EGTA (Ethyl glycol tetra acetate) a calcium chealator for which 1mM and 1 μM concentrations were tested ;and A23187 a calcium ionophore from Calbiochem at 1μM concentration. All the above effectors were tested for changes in rat-NOS3 and human-NOS3 promoter activity in pulmonary myofibroblasts as described above (3.5.2) by performing the dual Luciferase reporter gene assay.

3.6 DELETION ANALYSIS OF NOS3 PROMOTER ELEMENTS.

The locations of functional elements within a DNA fragment are often determined by making a set of unidirectional deletions and then assaying for changes in biological activity. The deletion series is to map out the specific regions of the promoter that are important in transcriptional regulation of NOS3 gene regulation in pulmonary myofibroblast cells.

Any difference seen in treatments using the same effectors will be ascribed to that region in the promoter. This will then provide information regarding these actors in the promoter that are responsible for transcriptional regulation of the NOS3 gene. These factors can subsequently be manipulated in the design of a rational therapeutic design for pulmonary fibrosis. The different binding sites of the rat eNOS promoter were obtained from vector NTI programme and positions of the deletion series identified as shown in figure 9. The deletion constructions were made by inverse PCR using different sets of primers; one forward primer and one reverse primer for each construct, this would then amplify the entire plasmid in opposite directions, the region to be deleted is not amplified and the construct produced is ligated. The polymerase chain reaction (PCR) serves to copy DNA. It uses repeated cycles, each of which consists of three steps. The reaction solution containing DNA molecules (to be copied), polymerases (which copy the DNA), primers (which serve as starting DNA) and nucleotides (which are attached to the primers) is heated to 95°C. This causes the two complementary strands to separate, a process known as denaturing or melting. Lowering the temperature to 55°C causes the primers to bind to the DNA, a process known as hybridization or annealing. The resulting bonds are stable only if the primer and DNA segment are complementary, i.e. if the base pairs of the primer and DNA segment match. The polymerases then begin to attach additional complementary nucleotides at these sites, thus strengthening the bonding between the primers and the DNA.

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The temperature is again increased, this time to 72°C. This is the ideal working temperature for the polymerases used, which add further nucleotides to the developing DNA strand. At the same time, any loose bonds that have formed between the primers and DNA segments that are not fully complementary are broken. Each time these three steps are repeated the number of copied DNA molecules doubles. After 20 cycles about a million molecules are cloned from a single segment of double stranded DNA. The temperatures and duration of the individual steps described above refer to the most commonly used protocol. A number of modifications have been introduced that give better results to meet specific requirements. (Mullis, 1987)
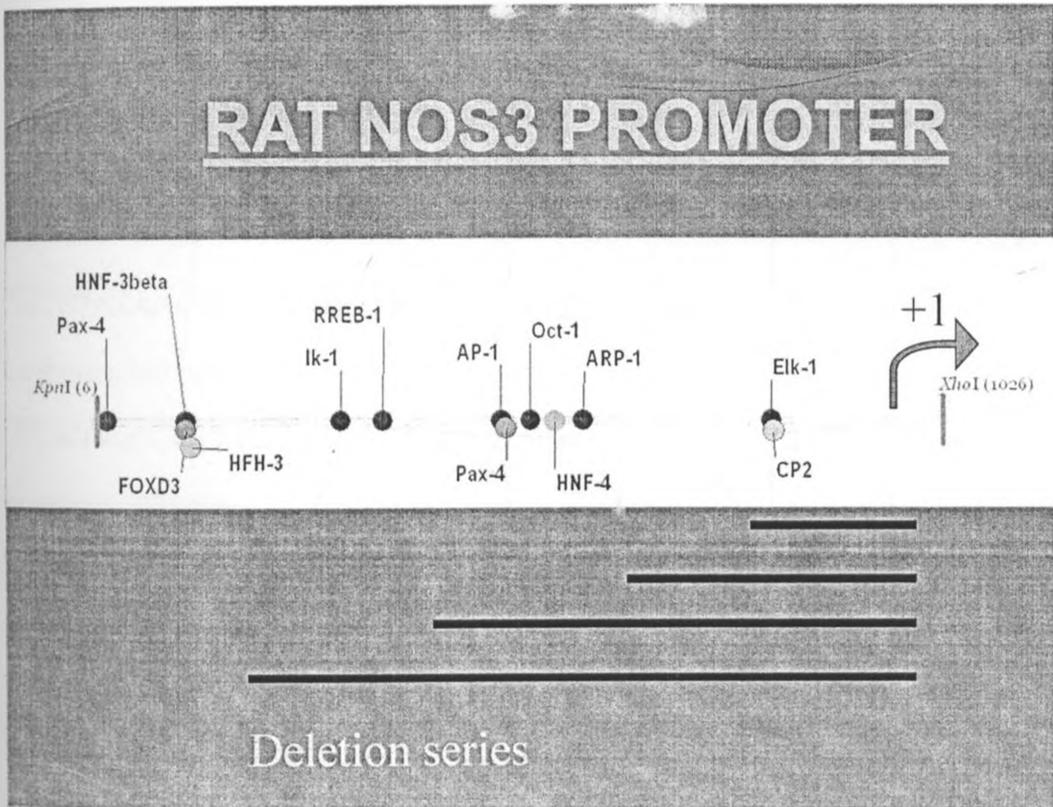
Fig 7: Schematic presentation of the various transcriptional factor binding sites of the Rat eNOS promoter and positions of the deletion series.

The sizes of the constructs produced are 5.6 Kb, 5.4Kb, 5.2Kb and 5.0Kb respectively.

The four deletion constructs of the NOS3 promoter were made by inverse PCR by designing One forward and four reverse primers. All primers were ordered from Integrated DNA . Technologies (IDT).

Forward Primer: 5' CTGCGATCTAAGTAACGTTGGC 3' with 22 base pairs,CG content of 50% and melting point of 60.53 degrees celcius.This would then amplify the entire plasmid running forward starting from the region immediately after XhoI restriction site at position 1030.

Table 1: Characteristics of various reverse primers

Reverse Primers	Melting temperature(Celsius)	Number of bps	GC content %	Primer binding position on the promoter
5'GCTGATAAGTGAGAACCCAGGT3' For approx.200 bps.	59.64	22	50	172
5'TCAGAGTCCTTTGGAAGCTTG3' For approx .400 bps.	59.6	21	47.62	407
5'ATGACGCATGTTTCCCTGG3' For approx.600 bps.	61.89	19	52.63	596
5'GGAGCTAGGTATTTGGGTGTACAG3' For approx .800bps.	60.3	24	50	779

The first reverse primer amplifies the entire plasmid in the opposite direction deleting 200 base pairs (6-172), the second reverse primer deletes 400 base pairs (6-407), the third primer deletes 600 base pairs (6-596) and the fourth primer deletes 800 base pairs (6-779).

Before performing the definitive inverse PCR experiment, a series of PCR experiments were done using different polymerase enzymes to optimize annealing temperatures in which the primers will effectively work in amplification. Firstly, Pfu Hotstart PCR master mix (from Stratagene) was used which is 2X formulation of Pfu Turbo hot start DNA polymerase, PCR reaction buffer, magnesium and dNTPS. Pfu Turbo hot start PCR master mix is formulated with heat labile monoclonal antibodies that, at room temperature effectively neutralize DNA polymerase and 3'-5'exonuclease activity.

Full enzyme activity is regained upon denaturation of the antibody during the initial denaturation step. It retains high fidelity, sensitivity and yield. An experimental set up was made with both the reverse and forward primer and the PCR run at 52 ^{0}c.Since no amplification was observed following gel electrophoresis another polymerase enzyme was used. 5'master mix DNA polymerase from Fisher Scientific Company, was then used and run on a PCR gradient of annealing temperature of 50-55°C, conditions were then adjusted to a PCR gradient of 53 °C -58 °C. No amplification was observed following agarose gel electrophoresis and therefore another polymerase enzyme; Taq polymerase from New England Biolabs was then used at an annealing temperature of 50.3 °C. This annealing temperature was found to be optimal. After optimizing of PCR experimental conditions, Pfu ultra high fidelity DNA polymerase was then used at this annealing temperature of 50.3 degrees Celsius for 30 seconds for 21 cycles. The success of the PCR experiment was checked on 0.7% gel. The constructs were purified by excising the gel using QIAEXII

Agarose Gel Extraction designed for the extraction of 40bps to 50 bps DNA fragments from 0.3-2% standard or low melt Agarose gels in TAE or TBE buffers.

The DNA band was excised from the agarose gel with a sharp clean scalpel and weighed in a colorless tube. Buffer QX1 was added to solubilize the sample. It was then resuspended in buffer QIAEXII to solubilize the agarose and bind the DNA.The sample was then centrifuged and supernatant was discarded. The pellet was then resuspended in buffer QX1 to remove residual agarose contaminants. It was washed twice with buffer PE to remove residual salt contaminants, the pellet was air-dried because vacuum drying will cause over drying and decrease elution efficiency. The DNA eluted in 10mM Tris at pH of 8.5. the maximum elution efficiency is achieved between pH 7.0 and 8.5.The elutant contained the purified DNA.After confirming presence of a strong band using a sample of the elute, a phosphorylation reaction was set up and then reaction product cleaned using Wizard^{R}SV Gel and PCR clean up system (Promega) resuspending in 10μl.This is designed to extract and purify DNA fragments of 100bp to 10 kb from standard or low-melt Agarose gels or to purify PCR products directly from a PCR amplification.PCR products are commonly purified to remove excess nucleotides and primers. This membrane bound system can bind up to 40 microgram's DNA and allow recovery of isolated DNA fragments or PCR products. It is based on the ability of DNA to bind to silica membranes in the presence of chaotropic salts. After amplification an aliquot of PCR reaction was added to the membrane Binding solution and directly purified.DNA was isolated using microcentrifugation to force the dissolved gel slice or PCR reaction through the membrane while simultaneously binding the DNA on the surface of the silica. After washing the isolated DNA fragment or PCR product, the DNA was eluted in water. The product was then used for the ligation reaction using DNA Ligase on

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10X buffer to ligate the ends of the deletion constructs and incubated for 16 °C overnight. This plasmid constructs were transformed into competent *E.coli* cells.

Upon transformation, the *E.coli* cells were grown in LB liquid medium for 45-60 minutes, to allow expression of the antibiotic resistance gene. They were the plated on selective LB agar plates. Those cells that took up the plasmid grew and were isolated, purified and contained the deletion constructs as described in 3.1 above. This will was used to test the effect of each deletion on NOS3 gene expression in pulmonary myofibroblasts by performing the Luciferase assay as described above (3.5.2).

3.7   STATISTICAL ANALYSIS

All values were expressed as mean± standard error of mean (SEM) using Microsoft Excel 2007 and all values were normalized to the control. Difference in means among different concentrations used was analyzed by one way ANOVA .P-values <0.05 were considered significant.

CHAPTER FOUR

4.0   RESULTS

4.1   GENERATION OF A LUCIFERASE REPORTER GENE CONTAINING THE PROMOTER REGION OF THE RAT AND HUMAN NOS3 GENE.

To study how the NOS3 gene promoter is transcriptionally regulated, it was important to generate a clone of the luciferase reporter gene and the promoter. Competent bacterium *Escherichia coli* (DH5α) that had been transformed by taking up the plasmid DNA and grown in liquid LB growth media was harvested by centrifugation after 16 hours of incubation. Plasmid DNA containing the NOS3 promoter was isolated using mid: prep from gen. Eluted DNA was quantified using a Nano-drop spectrophotometer at an absorbance of 280nm and yielded 1095.9ng/μl.

Restriction digestion was done to digest the NOS3 promoter fragment with KpnI and XhoI restriction endonucleases and was expected to give a 1018 base pairs fragment containing the promoter sequence. Agarose gel electrophoresis of sequential restriction digestion using KpnI restriction enzyme in buffer J and the XhoI restriction enzyme in buffer D for 4 hours at 37^0C showed clearly the insert of around 1018 base pairs which translates to the NOS3 rat promoter insert as shown in figure 9.
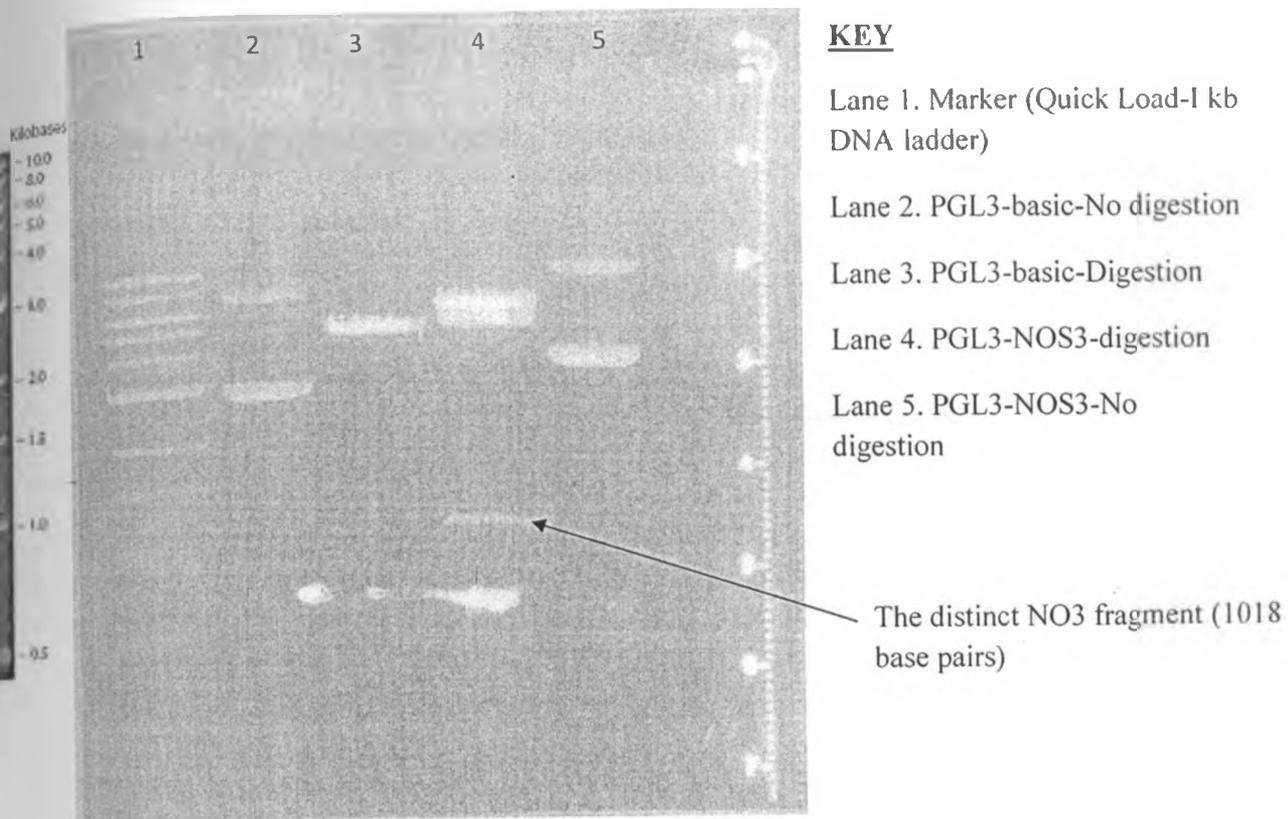
Fig 8: 0.7% Agarose gel electrophoresis of sequential restriction digestion with KpnI in buffer J four hours and the XhoI for four hours.

There is an insert as shown by the band in well 4 and this is 1018 base pairs which translate to the distinct NOS3 rat promoter insert.

4.2   PGL3-NOS3 DNA PROMOTER SEQUENCE

The NOS3 gene fragment was sequenced to ascertain its properties and to help in primer design. There were 1018 base pairs between KpnI and XhoI restriction sites corresponding to the rat NOS3 promoter construct in PGL3.This was consistent with the results from the gene bank of vector NTI gene analysis programme. The sequencing was to help in the construction of primers for inverse PCR and to ascertain the exact sequence.

GTGGGGGGCCAGAACATTTCTCTATCGATA**GGTACC**GAGCTCTTACGCGTAGTCCA
GCCAACACAAATCCAAGATGGTTTGTCTGCCTGTCTGCCTCTCCCAGTCTTGCCTC
TCCCTGTCTCTTAAGTTTCTGGGGGGTTTTGTTTTGTTTGTTTTTCCTTATAGTTTCTT
TCTTGGGCCTCTGAGGTCTCAAACTCCCACTCCTGTACACCCAAATACCTAGCTCC
TAGCTTTCTATCAGATGTAGGTAGTATAGAACTACAAACTCCAACATGCATGTCT
GCCTAAGGTGCCCAAAATGTGCTGGTATGTATCTCCCTGCATTCTGGGAATTGTA
GTTTTGCCTAGCCCACACTCAGTGTCCACTCCCCCACCCCCAAACTCTCCCCTGTA
GGCCATCTGCCTCTGCCCTGGTGGCTAGGTCCACTGACCTGCTGCCCCAGGGAAA
CATGCGTCATTTGACAGGATTGGAGGTGGAGGCCTTGGATGGCAGCTTCCTGCCC
CTTTGTATCCCCCCACTTGAGTCATGGGGTGTGGGGGTTTCAGGAAATTGAGATG
AGAATGGGAAAATGCCCTAATACCAGGCAAAAGGACAAAATGTCACTGCATCCT
TGCTGAACCTGTGTCCCCAAGCTTCCAAAGGACTCTGAGATAGAAGAGAGCAAG
GGGTCCAGGATTAACCTAGAGATCTCTGTGGTCACAGGAATATGATATTCCATTG
CTCTGGTACTGGCCCAGTGCACAAGGCCCTCCTACTGTGGCCCAACACCACCAAA
GCATCTTTCCCGCCCTGCAGTAGCCCTCTAATGGACACCTGGGTTCTCACTTATCA
GCTCTAGCCCTCATGGCGGAACCCAGGCGTCCGGCCCCCCACCCTCTGGGTCAGT
GGGCATGAAGCCGAGGTTTTAGAGCCTCCCTGCCGGCCTTGTTCCTGTCCCATTGT
GTGTGGGACAGGGGCGGGGCGAGGGCCAGCATCTGAGAGCCCCCTCCCACTACC
CCCTCCCTGCTTCCTAAAGGAAAAGGCCAGGACTCTTGTTGAGCAGTCAGCAGAG
TGG**CTCGAG**ATCTGCGATCTAGTAAGCTTGGCATTCCGGTACTGTGGGTAAAGCC

ACCATGGAGACCGCCCAAAAACATAAAGAAAGCCCGGCGCATTCTATCCGCTGA
GATGAACGCTTGGAAGAGCAACTGCATTAAGGGCTTATTAG

Fig 9: The NOS3 promoter sequence obtained by chain termination method using the Big Dye

Terminator v3.1 showing the promoter sequence between KpnI and XhoI restriction sites

(underlined and bold).

4.3 LUMINESCENT ACTIVITY OF RAT- NOS3 PROMOTER IN PULMONARY MYOFIBROBLASTS (BASELINE)

The purpose of this experiment was to determine whether the rat and human NOS3 promoters were active in rat pulmonary myofibroblast cells and therefore form a baseline for the other experiments. The relative firefly activity/renilla activity of the negative control, pGL3 basic that lacks eukaryotic promoter and enhancer sequences mean value was 0.253±0.0624;the mean relative activity value for the positive control, SV 40 Luciferase which contains SV40 promoter and enhancer sequences resulting in strong expression of *luc+* was 7.728±1.3758;the mean relative activity value for the Rat-NOS3 promoter with a Renilla Luciferase construct uuwnstream of the Thymidine kinase promoter (RLTK was 2.358±0.6744 and the mean relative activity value of Human-NOS3 promoter with a Renilla Luciferase construct downstream of the Thymidine kinase promoter (RLTK was 0.0878±0.0240. This experiment shows that rat NOS3 promoter has activity in rat myofibroblast cells while the human NOS3 promoter does not (Fig 10). Therefore, the human NOS3 promoter was not used in any additional studies.
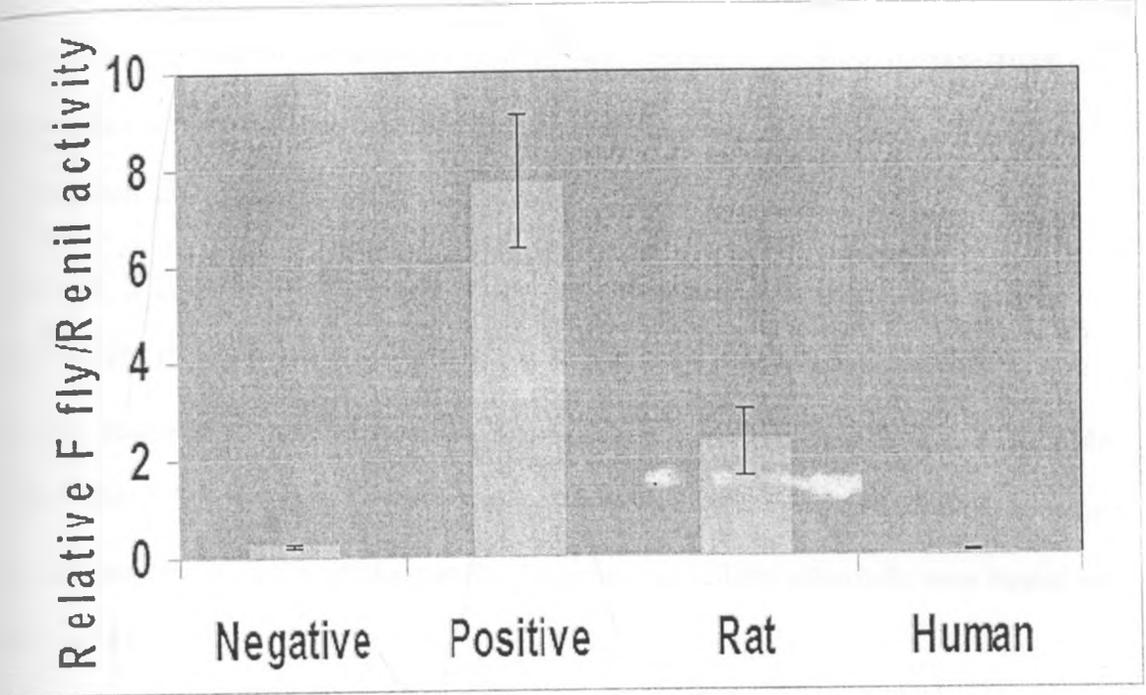
Fig 10: Base line data of the relative Fire fly activity/Renilla activity of PGL3 basic (Negative control), SV 40 Luciferase (Positive control), the Rat-NOS3 promoter and Human NOS3 promoter in rat pulmonary myofibroblast cells with error bars shown (P-value <0.05).

4.4 LUMINESCENT ACTIVITY OF RAT- NOS3 PROMOTER IN PULMONARY MYOFIBROBLASTS IN THE PRESENCE OF EFFECTORS: PMA, TGFβ and LPS.

Luminescent activity of the rat-NOS3 promoter was assayed in presence of PMA, TGFβ and LPS whose effect would be significant in provision of a better understanding on how nitric oxide levels are regulated in pulmonary myofibroblast cells.

For PMA, a diester of phorbol known to activate the signal transduction enzyme protein kinase C (PKC), the relative Firefly activity/Renilla activity of the negative control; rat without treatment mean value was 1.0 ± 0.00, the relative activity mean value for 20 μM PMA was 0.50 ± 0.2751; the relative activity mean value for 2μM PMA was 1.03 ± 0.1319.From the results, there was a significant decrease in transcriptional activity when cells were treated with 20μM PMA (P value 0.01) (Fig 11).

LPS acts to up regulate the transcription of eNOS gene by modulating the stability of its mRNA while TGFβ acting through the SMAD pathway which acts as transcription factors that regulate the expression of certain genes. The relative Firefly activity/Renilla activity of the negative control; rat without treatment mean value was 1.0 ± 0.00; the relative activity mean value for 10ng TGFβ was 1.52 ± 0.4895 and this results show an increase in transcriptional activity; the relative activity mean value for 5ng TGFβ was 1.12 ± 0.08651 and the relative activity mean value for 10μg LPS was 1.39 ± 0.4351 showing a slight increase in transcriptional activity (Fig 12).

The data presented had been normalized to the control which is the rat no treatment. The raw values are presented in the appendix.
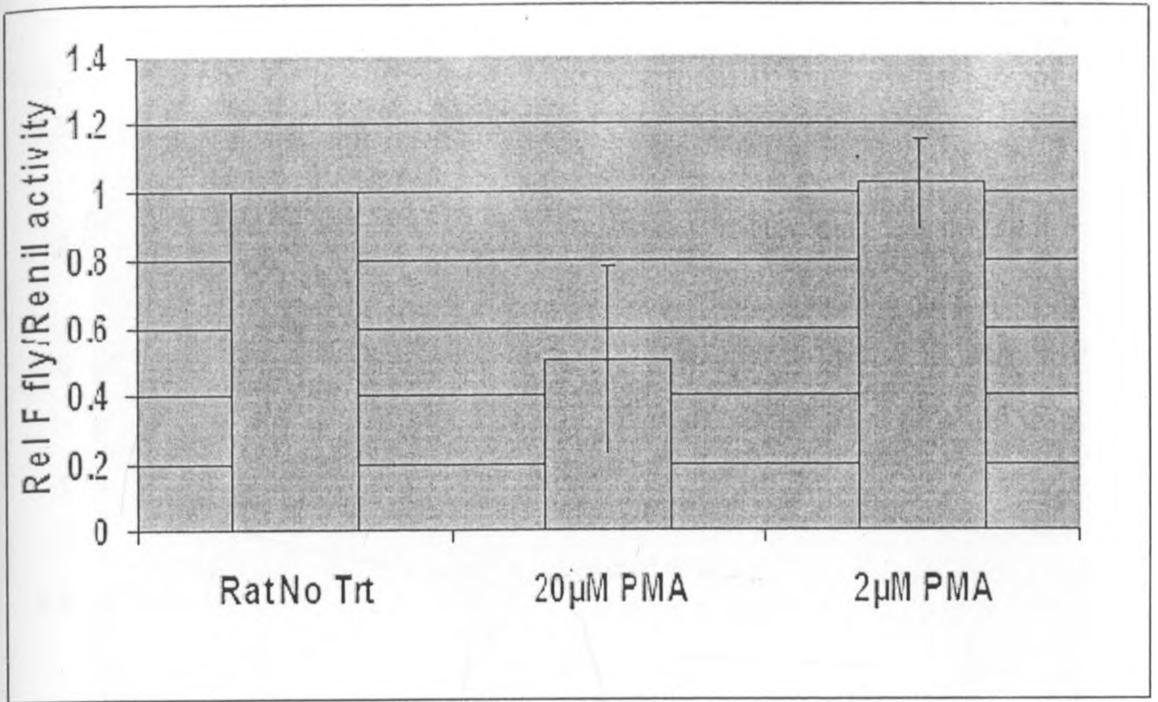
Fig 11: The relative fire fly activity/Renilla activity of Rat promoter without treatment, 20µM PMA and 2 µM PMA concentrations in rat pulmonary myofibroblast cells (Mean values were normalized to the control).
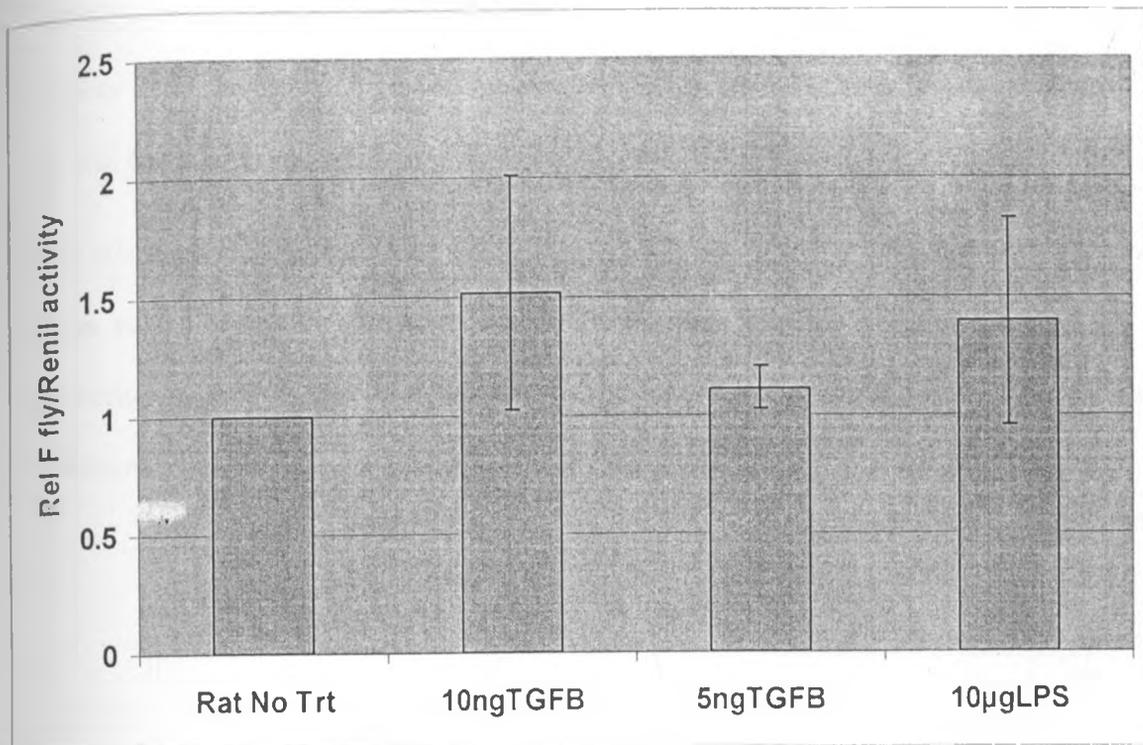
Fig 12: The relative fire fly activity/Renilla activity of Rat promoter without treatment, 10ng/μl TGFβ, 5ng/μl TGFβ and 10μg/μl LPS concentrations in rat pulmonary myofibroblast cells (Mean values were normalized to the control).

4.5   LUMINESCENT ACTIVITY OF NOS3 PROMOTER IN PULMONARY MYOFIBROBLASTS IN THE PRESENCE OF EFFECTORS: SNAP(S-nitroso-N-acetylpenicillamine) and L-NAME (Nw-Nitro-L-arginine methyl ester).

S-NAP and L-NAME are effectors known to specifically regulate NOS3 activity; S-NAP increases NO levels while L-NAME is a specific inhibitor of NOS3.The relative Firefly

activity/Renilla activity of the negative control; rat without treatment mean value was 1.0±0.00, the relative activity mean value for 1mM L-NAME was 1.11±0.1072, the relative activity mean value for 10 mM L-NAME was 1.06±0.16866, these results show that L-NAME doesn't have any effect on transcriptional activity (Fig 13).

The relative activity mean value for 0.1mM S-NAP was 0.99±0.1477, the relative activity mean value for 1mM S-NAP was 0.29±0.0470 which showed a significant decrease in transcriptional activity.The data presented had been normalized to the control which is Rat no treatment (Fig 14).
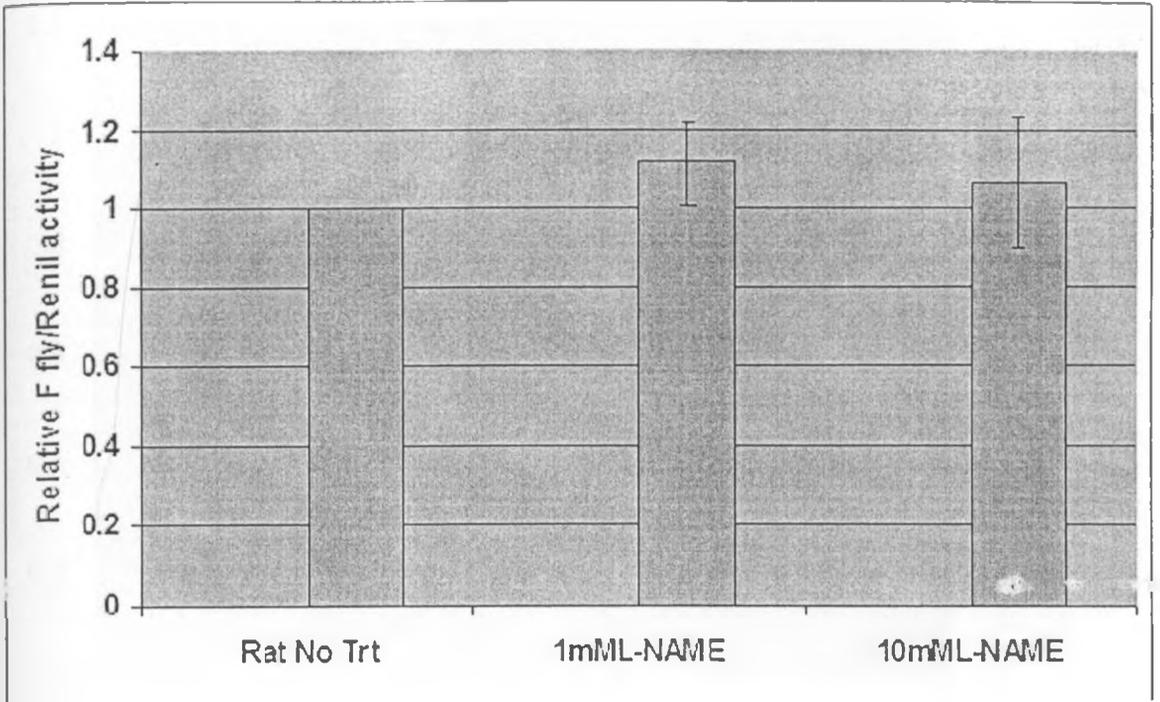
Fig 13: The relative Firefly activity/Renilla activity of the negative control; rat without treatment, 1mM L-NAME,10 mM L-NAME concentrations in rat pulmonary myofibroblast cells (Mean values were normalized to the control).
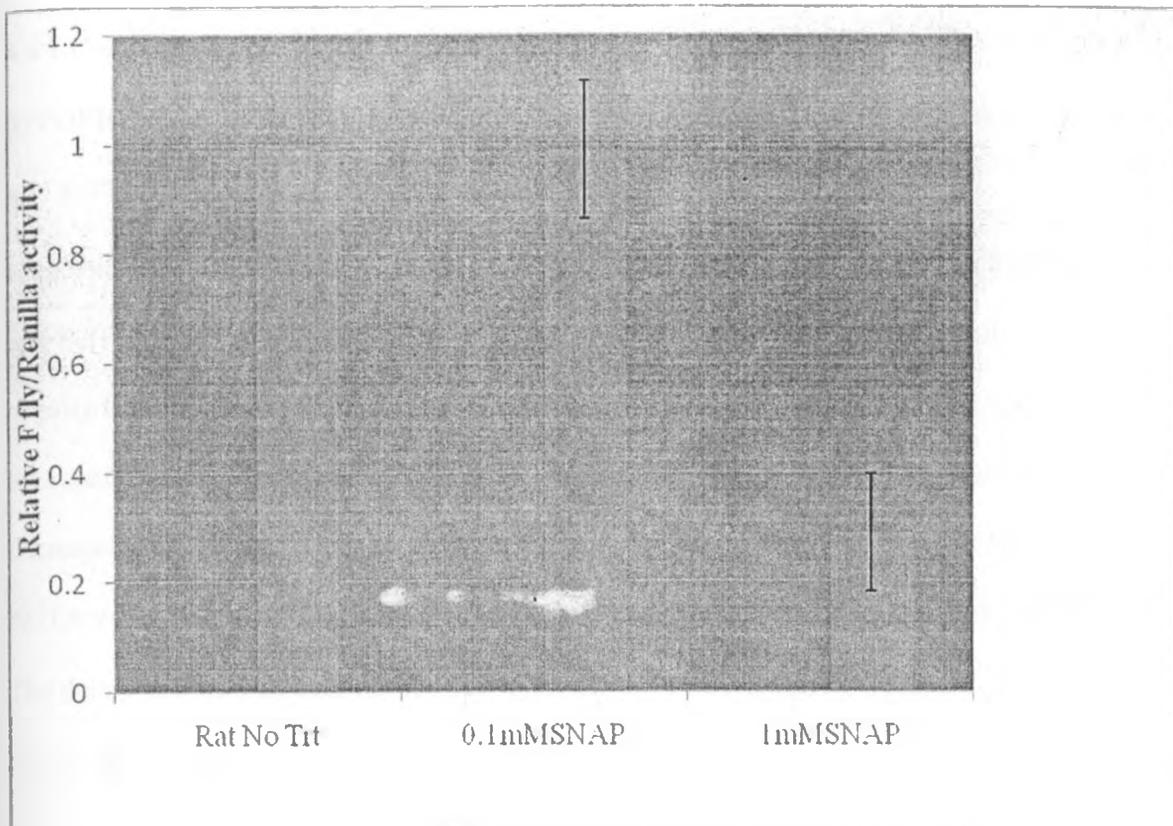
Fig 14: The relative Firefly activity/Renilla activity of the negative control; rat without treatment, 0.1mM SNAP and 1mM SNAP concentrations in rat pulmonary myofibroblast cells.(Mean values were normalized to the control).

4.6 LUMINESCENT ACTIVITY OF NOS3 PROMOTER IN PULMONARY MYOFIBROBLASTS IN THE PRESENCE OF EFFECTORS: 23187 (Calcium ionophore) and EGTA (Ethyl glycol tetra acetate).

Intracellular calcium concentrations also regulate NOS3 activity and 23187,a calcium ionophore and EGTA,a calcium chelator was used to determine this effect. The relative Firefly activity/Renilla activity of the negative control; rat without treatment mean value was 1.0 ± 0.00, the relative activity mean value for $1\mu M$ 23187 was 0.51 ± 0.1256.This results show that 23187 decreased NOS3 transriptional activity (Fig 15) .The relative activity mean value for $1mM$ EGTA was 0.94 ± 0.19814 and the relative activity mean value of $1\mu M$ EGTA was $1.23\pm0.205.74$. The data presented had been normalized to the control which is Rat no treatment. The raw values are presented in the appendix
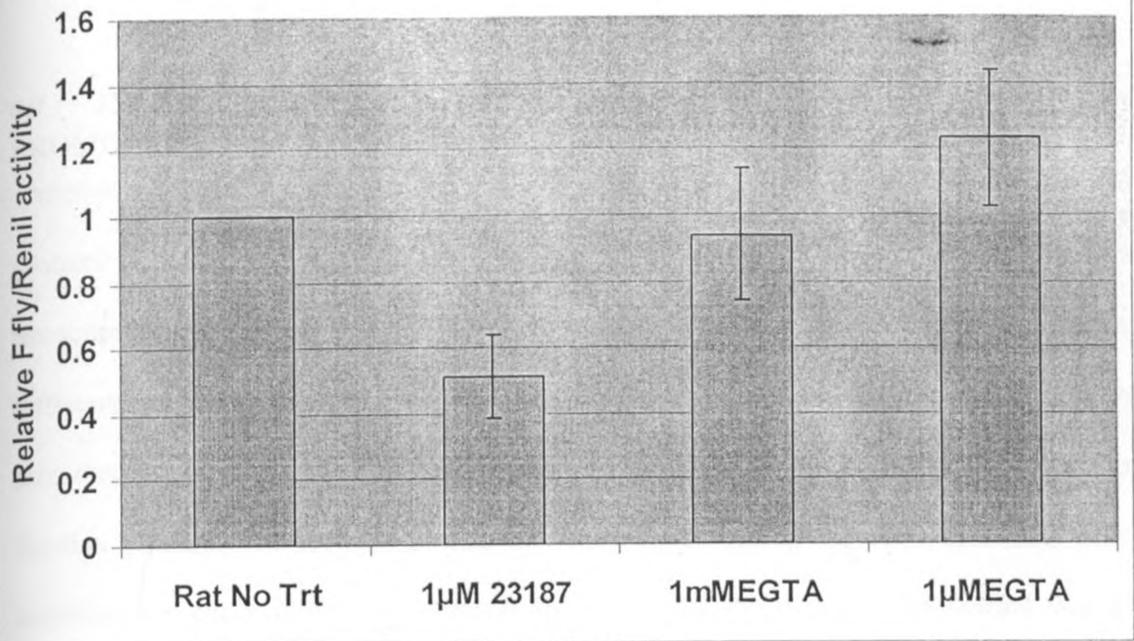
Fig 15: The relative Firefly activity/Renilla activity of the negative control; rat without

treatment, $1\mu M$ 23187, 1mM EGTA and $1\mu M$ EGTA concentrations in pulmonary

myofibroblast cells (Mean values were normalized to the control).

4.7 INVERSE PCR RESULTS OF THE DELETION ANALYSIS OF NOS3 PROMOTER.

Deletion synthesis involves making a set of unidirectional deletions and then assaying for changes in biological activity to map out the specific regions of the promoter important in transcriptional regulation of NOS3 gene regulation in pulmonary myofibroblast cells. Any difference seen in treatments using the same effectors will be ascribed to that region in the promoter. Agarose gel electrophoresis results showing the deletion constructions made by deletion primers; one forward primer and one reverse primer for each construct, which amplified the entire plasmid in opposite directions and the region to be deleted was not amplified and the construct produced ligated. The first reverse prime. amplifies the entire plasmid in the opposite direction deleting 200 base pairs (6-172), the second reverse primer deletes 400 base pairs (6-407), the third primer deletes 600 base pairs (6-596) and the fourth primer deletes 800 base pairs (6-779). Agarose gel electrophoresis results showed non-specific amplification in a gradient of annealing temperatures of 50-55^{0}C showing non distinct deletion constructs (Fig 17). Agarose gel electrophoresis results of an amplification of a gradient annealing temperature of 53-58°C showed stronger amplified fragments (Fig 18). Fig 19 shows an amplification of Reverse primer 3 at an annealing temperature of 51^{0}C, the other primers however seemed not to amplify at this temperature. Agarose gel electrophoresis results showed that all the reverse and the forward primer worked well in an annealing temperature of 50.3^{0}C with both Taq DNA polymerase and Pfu ultra high fidelity DNA polymerase both yielding strong bands showing the deletion constructs (Fig 20 and Fig. 21). The bands were then cut out from the gel using the QIAEX II Gel extraction kit and quantified using Nano drop ND 1000 spectrophotometer yielding 14.98ng/μl for reverse 1 deletion construct, 6.70ng/μl for reverse 2 deletion construct, 5.95ng/μl for reverse 3 deletion

construct and 6.53ng/µl for reverse primer 4 deletion construct. The low yields from the gel were expected. Future directions would be that the deletion constructs will be used to test the cells and map out the exact transcriptional factors regulating promoter activity.

The amplified deletion constructs fragments

Fig 16: 0.7% Agarose gel electrophoresis results of Inverse PCR using 5'prime master mix on a gradient of annealing temperatures 50-55oC (50.4 oC, 52.2 oC, 54.3 oC and 55.3 oC). The controls didn't not contain the plasmid.The whole plasmid with cloned promoter was 5.8Kb, the expected MW for R1 was 5.6Kb, the expected MW for R2 was 5.4Kb, the expected MW for R3 was 5.2 Kb and the expected MW for R4 was 5.0Kb.

The amplified fragment
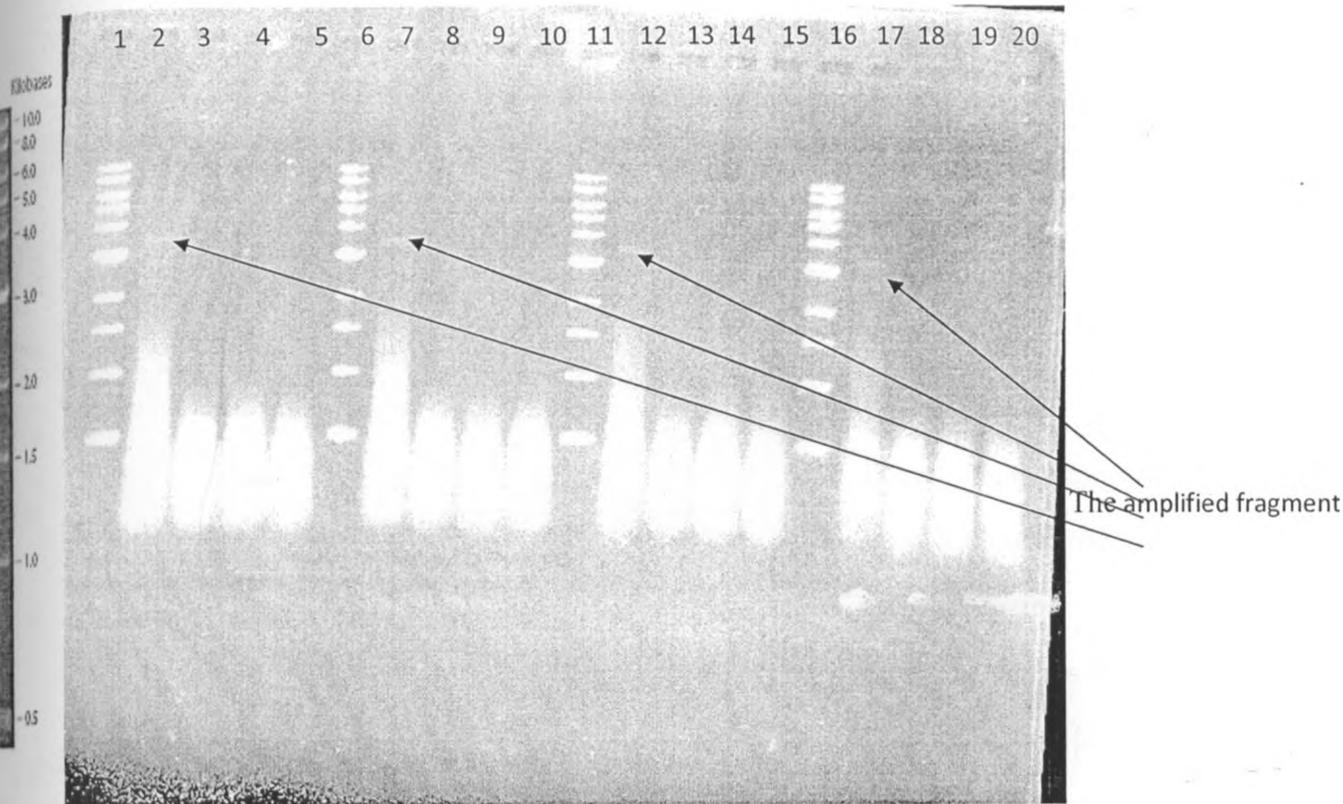
KEY: Lane **1.** DNA marker Lane **2.** Plasmid F+R1 (53.4) Lane 3-5. Controls Lane **6.** DNA marker Lane **7.** Plasmid F+R1 (55.2) Lane 8-10 Controls Lane **11.** DNA marker Lane **12.** Plasmid F+R1 (56.7) Lane 13-15 Controls Lane **16.**DNA marker Lane **17.**Plasmid F+R1 (58.5) Lane 18-20 Controls

Fig 17: 0.7% Agarose gel electrophoresis results of Inverse PCR using 5'prime master mix(2.5X) on a gradient of 53-58oC(53.4 oC,55.2 oC,56.7 oC and 58.5 oC) on the thermo cycler.

The controls didn't not contain the plasmid. The expected MW for R1 was 5.6Kb, the expected MW for R2 was 5.4Kb, the expected MW for R3 was 5.2 Kb and the expected MW for R4 was 5.0Kb.

KEY

Lane 1. DNA ladder

Lane 2. Plasmid+RI

Lane 3. Control

Lane 4. Blank plasmid

Lane 5. Plasmid+R2

Lane 6. Control

Lane 7. Blank plasmid

Lane 8. Plasmid+R3

Lane 9. Control

Lane 10. Blank plasmid

Lane 11. Plasmid+R4

Lane 12. Control
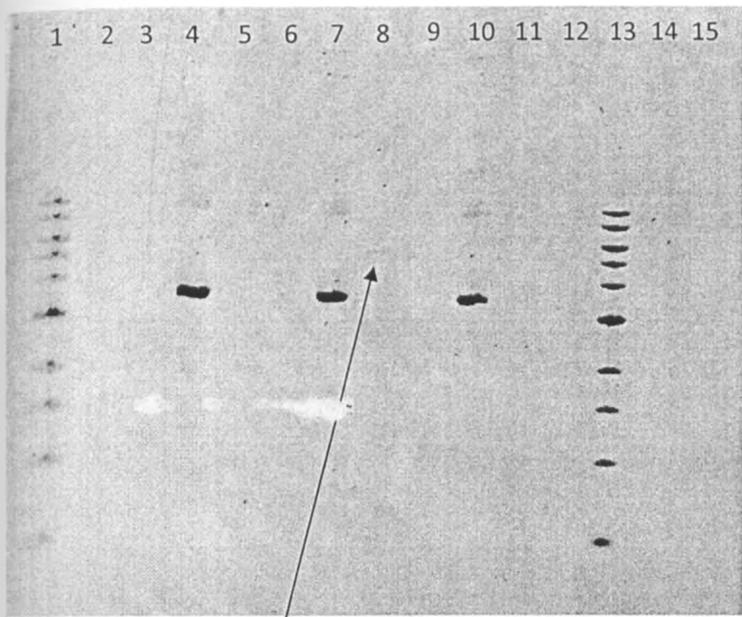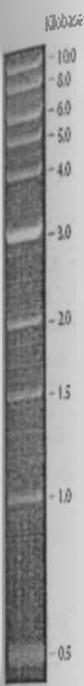
Lane 13. DNA ladder .

The fragment amplified by R3

Fig 18: 0.7% Agarose gel electrophoresis results of Inverse PCR using 5'prime master mix (2.5X) at an annealing temperature of 51oC.

The controls didn't not contain the plasmid. The expected MW for R1 was 5.6Kb, the expected MW for R2 was 5.4Kb, the expected MW for R3 was 5.2 Kb and the expected MW for R4 was 5.0Kb.

Strong amplified deletion constructs
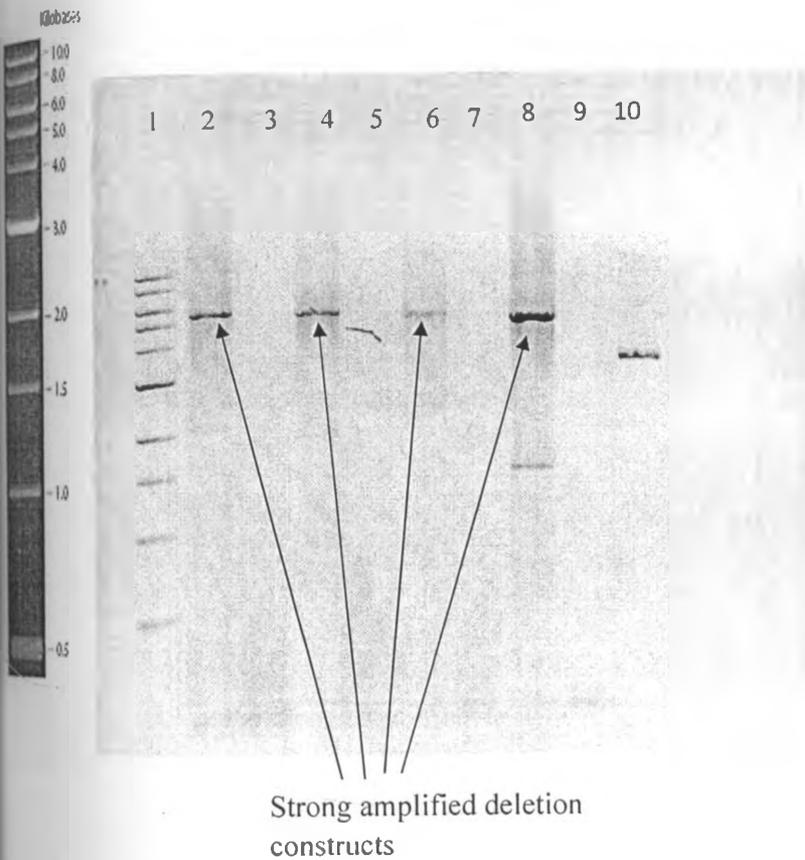
Fig 19: 0.7% Agarose gel electrophoresis results of inverse PCR using Taq DNA polymerase

from New England Biolabs annealing temperature of 50.3oC.

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The controls didn't not contain the plasmid .The expected MW for R1 was 5.6Kb, the expected MW for R2 was 5.4Kb, the expected MW for R3 was 5.2 Kb and the expected MW for R4 was 5.0Kb.

KEY

1. Plasmid control

2. DNA ladder

3. F+R1

4. Control

5. F+R2

6. Control

7. F+R3

8. Control

9. F+R4

10. Control

Strong amplified deletion constructs
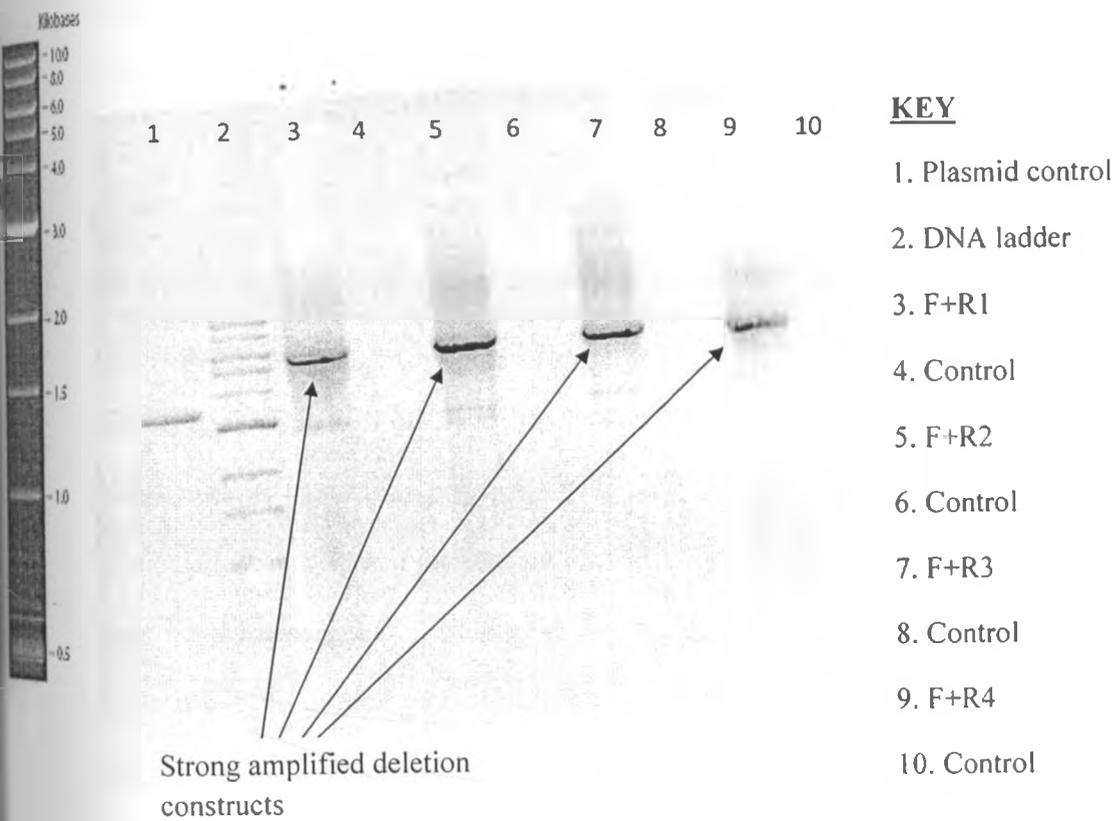
Fig 20: 0.7% Agarose gel electrophoresis results of inverse PCR using Pfu ultra high fidelity DNA polymerase at an annealing temperature of 50.30C .

The expected MW for R1 was 5.6Kb, the expected MW for R2 was 5.4Kb, the expected MW for R3 was 5.2 Kb and the expected MW for R4 was 5.0Kb.

CHAPTER FIVE

5.0   DISCUSSION AND CONCLUSION

Regulation of endothelial NO synthase (NOS) has been extensively studied, and its complex and multiple regulatory pathways elaborated (Agullo, 2007). However the effect of the rat eNOS promoter regulation on pulmonary myofibroblasts is not fully elucidated. This study examined the transcriptional regulation of the NOS3 gene in pulmonary myofibroblast cells and how this can be useful as a step in finding a therapeutic design for pulmonary fibrosis. Studies show this disorder affects over five million people worldwide and as a consequence of misdiagnosis, the actual numbers may be significantly higher (Pulmonary Foundation, 2009). Myofibroblasts are the principal cells responsible for collagen matrix deposition, inflammation, and altered mechanical properties in the fibrotic lesions of pulmonary fibrosis (Pardo and Selman, 2002). Studies show that NO produced by endothelial cells via eNOS conversion of L-arginine to L-citrulline has a role in myofibroblast differentiation and death thus representing a potential antifibrotic mechanism in the body. Several studies suggest that NO-mediated signals do this through regulating myofibroblast phenotypes. Studies suggest that inhibition of NO production leads to increased accumulation of myofibroblasts (Vernet *et al*, 2002). Hence an enhanced expression of eNOS in response to pharmacological interventions could provide protection against fibrosis, specifically interstitial pulmonary

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fibrosis (IPF) emanating from altered characteristics of myofibroblasts. Studies on the rat eNOS promoter can be extrapolated to humans due to genomic similarity of the two species.

To determine the effects of various compounds in positively or negatively regulating promoter activity of myofibroblast, different treatments of cells was performed. The results demonstrated that treatment of rat pulmonary myofibroblast cells with Phorbor-12-myristate-13-acetate (PMA) regulates NOS3 gene transcription in a dose dependent manner, whereas high concentrations of transforming growth factor beta (TGFβ) up-regulates the expression of NOS3 gene. Results further showed when NOS3 activity is inhibited by Nw-Nitro-L-arginine methyl ester (L-NAME), an eNOS3 selective inhibitor; there is no significant NOS3 gene expression. Furthermore, increased production of NO by S-nitroso-N-acetylpenicillamine (S-NAP), which is a NO donor, suppresses eNOS promoter activity which therefore shows that increased production of Nitric oxide regulated its own transcription confirming our hypothesis. This study demonstrated that increase in entry of calcium into the cells suppresses eNOS expression by the effect seen by Calcium ionophore (23187). This study therefore demonstrates that NOS3 promoter is transcriptionally regulated. Recent studies suggest that the expression of the eNOS gene may be activated via a transcriptional mechanism (Nishida *et al*, 1992).

Prior to the studies on gene expression in the cultured cells, the promoter was cloned to pGL3-Basic plasmid had been grown, purified, cleaved by restriction endonucleases and sequenced. Results confirmed the 1018 base pairs of rat eNOS promoter when Blast analysis was performed in Genebank. As a point of reference, the human eNOS promoter was also

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initially used together with rat eNOS promoter in transfection of the pulmonary myofibroblast cells. However, the human eNOS promoter was not active in the rat pulmonary myofibroblast cells and this was not used after the first set of experiments. This inactivity may have been due to the species difference between the cells and promoter.

There are several factors that regulate the transcription of eNOS gene such as shear stress (Davis *et al*, 2004), estrogen and hypoxia. Other factors modulate the stability of its mRNA which includes tumor necrosis factor alpha (TNF-α), lipopolysacharide (LPS) and vascular endothelial growth factor (VEGF).This study investigated the effect of PMA a diester of phorbol on transcriptional regulation of eNOS3 gene.. MA is known to activate the signal transduction enzyme protein kinase C (PKC) and its effects on this molecule result from its similarity to one of the natural activators of classic PKC isoforms, diacylglycerol (Agullo,2007). PKC is a family of enzymes that are involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on these proteins. PKC enzymes in turn are activated by signals such as increases in the concentration of diacylglycerol or Ca^{2+}. Hence PKC enzymes play important roles in several signal transduction cascades (Mellor and Parker, 1998). The regulatory domain or the amino-terminus of the PKCs contains several shared sub regions. The C1 domain, present in all of the isoforms of PKC has a binding site for diacylglycerol (DAG) as well as non-hydrolysable, non-physiological analogues called phorbol esters such as PMA. This domain is functional and capable of binding DAG in both conventional and novel isoforms; however, the C1 domain in atypical PKCs is incapable of binding to DAG or phorbol esters. The C2 domain acts as a Ca^{2+} sensor and is present in both conventional and novel isoforms, but functional as a Ca^{2+} sensor only in the conventional. The pseudo

substrate region, which is present in all three classes of PKC, is a small sequence of amino acids that mimic a substrate and bind the substrate-binding cavity in the catalytic domain, lack crital serine, threonine phosphoacceptor residues, keeping the enzyme inactive.

When Ca^{2+} and DAG are present in sufficient concentrations, they bind to the C2 and C1 domain, respectively, and recruit PKC to the membrane. This interaction with the membrane results in release of the pseudo substrate from the catalytic site and activation of the enzyme (Mellor and Parker, 1998).Upon activation; protein kinase C enzymes are translocated to the plasma membrane by RACK proteins (membrane-bound receptor for activated protein kinase C proteins). The protein kinase C enzymes are known for their long-term activation: They remain activated after the original activation signal or the Ca^{2+}-wave is gone. This is presumably achieved by the production of diacylglycerol from phosphatidylinositol by a Phospholipase; fatty acids may also play a role in long-term activation (Mellor and Parker, 1998).PMA may be acting through this PKC signal transduction pathway to regulate the transcription of the eNOS3 gene.

A multiplicity of functions has been ascribed to PKC. Recurring themes are that PKC is involved in receptor desensitization, in modulating membrane structure events, in mediating immune responses, in regulating cell growth, and in learning and memory and in regulating transcription which is the focus of this study (Mellor and Parker, 1998). These functions are achieved by PKC mediated phosphorylation of other proteins and the substrate proteins present for phosphorylation are variable, since protein expression is different between different kinds of cells. Thus, effects of PKC are cell-type specific. The results of this study demonstrated that high concentrations of PMA (20µM) increased NOS3 gene expression and the fact that PMA resembles one of the natural activators of classic PKC isoforms,

diacylglycerol, we can therefore postulate that it acts in the same cascade through the PKC pathway to down regulate the transcription of NOS3 gene in pulmonary myofibroblasts (Agullo, 2007). This finding can therefore be an important tool in pharmacological interventions investigations.

Moreover, this study also demonstrated that increasing calcium in the cell decreases eNOS3 promoter activity in rat pulmonary myofibroblast cells by the effects seen by Calcimycin (A23187) while chelation of calcium by Ethylene glycol tetra acetic acid (EGTA) enhances expression of NOS3.

EGTA is a polyamino carboxylic acid, a chelating agent that is related to the better known EDTA, but with a much higher affinity for calcium than for magnesium ions (Bett *et al* 2002). Calcimycin, which is a calcium ionophore, is a mobile ion-carrier that forms stable complexes with divalent cations (ions with a charge of +2) through the increase of intracellular Ca^{2+} levels in intact cells thus suppressing NOS3 promoter activity (Agullo,2007). Calcimycin also uncouples oxidative phosphorylation, the process cells use to synthesize ATP which they use for energy and also inhibits mitochondrial ATPase activity (Agullo, 2007). All NO-synthases required for its activation to be bound to a calcium regulatory protein: calmodulin. iNOS tightly binds calmodulin even at resting calcium concentrations, and then it is active once it is synthesized. However, constitutive enzymes, eNOS and nNOS, only bind calmodulin when the intracellular calcium concentration increases up to a certain value. Agents that increase intracellular calcium concentration like Calcimycin, either by allowing calcium entrance from the outside or by stimulating calcium mobilization from intracellular stores, can activate these constitutive enzymes. It is now clear that eNOS is also regulated by pathways that are independent of changes in the intracellular

calcium concentration and that eNOS activity is largely dependent on intracellular localization and on phosphorylation at specific amino acids (Agullo, 2007).

This study aimed at finding out the effect of chealation of calcium ions or the availability of the calcium ions to the cells in relation to transcriptional regulation of the eNOS3 promoter. The study confirmed that increase of calcium concentration by Calcimycin suppresses eNOS3 promoter activity in rat pulmonary myofibroblast cells and therefore postulates that this may be due to uncoupling of oxidative phosphorylation and inhibition of mitochondrial ATPase activity in these cells by calcium. Removal of calcium by the action of EGTA up regulates the expression of NOS3 gene, this study postulates that this may be acting through the pathway independent of calcium concentration but dependent on eNOS intracellular localization and on its phosphorylation at specific amino acids.

The results also demonstrated that blocking NO production has no effect on the expression of NOS3 gene in pulmonary myofibroblast cells through the effect seen by N^w-Nitro-L-arginine methyl ester (L-NAME) which is a potent eNOS selective inhibitor (Rotzinger *et al*, 1995). These results therefore suggest that L-NAME does not act through transcriptional mechanism but posttranscriptional through the inhibition of eNOS protein production by suppression of transduction. Moreover this study has demonstrated that high concentrations of the NO down regulates eNOS expression and therefore we can confirm the hypothesis that NO acts to regulate its own transcription. S-nitroso-N-acetylpenicillamine (S-NAP) is a NO donor and liberates NO spontaneously without any requirement for enzyme degradation. Recent studies have shown that S-NAP has a retaining ability to produce cyclic-guanosine monophosphate (Shaffer *et al*, 1992). S-NAP is formed by addition of a nitroso group to a sulfur atom of an

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amino acid residue a process known as S-nitrosation or S-nitrosylation which is a reversible process and a major form of posttranslational modification of protein (Yang *et al* 2007).

S-nitrosylated proteins (SNO's) serve to transmit nitric oxide (NO) bioactivity and to regulate protein function through mechanisms analogous to phosphorylation. NO donors target specific amino acids motifs; leading to changes in protein activity, protein interactions, or subcellular location of target proteins. NOS activity leads directly to SNO formation. NOSs are hemoproteins that combine reductase and oxygenase catalytic domains in one monomer to synthesize NO from the terminal nitrogen atom of L-arginine in the presence of NADPH and oxygen. NOSs target specific Cys residues for S-nitrosylation (Gaston *et al*, 2003). Thiol S-nitrosylation and NO transfer reactions (transnitrosation reactions) are involved in virtually all classes of cell signaling, ranging from regulation of ion channels and G-protein coupled reactions to receptor stimulation and activation of nuclear regulatory protein (Gaston *et al*, 2003). We postulate that S-NAP may be operating through this pathway possibly targeting the eNOS protein.

This work also focused on the effects of TGF-β which is a protein that controls proliferation, cellular differentiation, and other functions in most cells and plays a role in immunity, cancer, heart disease, diabetes, and Marfan syndrome. TGF-β acts as an antiproliferative factor in normal epithelial cells and at early stages of oncogenesis. Some cells secrete TGF-β, and also have receptors for TGF-β. Cancerous cells increase their production of TGF-β, which also acts on surrounding cells (Khalil, 1999). TGF-β induces apoptosis in numerous cell types and · postulated to act through the SMAD pathway (Khalil, 1999). SMADs are proteins that modulate the activity of transforming growth factor beta and often in complex with other SMADs/CoSMAD, act as transcription factors that regulate the expression of certain genes.

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TGF-β may therefore be acting through the SMAD pathway to upregulate the expression of eNOS in pulmonary myofibroblast cells.

The SMAD pathway is the canonical signaling pathway that TGF-β family members signal through. In this pathway, TGF-β dimers bind to a type II receptor which recruits and phosphorylates a type I receptor. The type I receptor then recruits and phosphorylates a receptor regulated SMAD (R-SMAD). SMAD3, an R-SMAD, has been implicated in inducing apoptosis.

The R-SMAD then binds to the common SMAD (coSMAD) SMAD4 and forms a heterodimeric complex. This complex then enters the cell nucleus where it acts as a transcription factor for various genes, including those that activate the mitogen-activated protein kinase 8 pathway, which triggers apoptosis (Khalil, 1999). Transcription factors perform this function alone or with other proteins in a complex, by promoting (as an activator), or blocking (as a repressor) the recruitment of RNA polymerase (the enzyme that performs the transcription of genetic information from DNA to RNA) to specific gene.

A defining feature of transcription factors is that they contain one or more DNA-binding domains (DBDs), which attach to specific sequences of DNA adjacent to the genes that they regulate. (Roeder, 1996). This study demonstrated that Lipopolysaccharide (LPS) upregulate the transcription of eNOS gene by modulating the stability of its mRNA (Fig 4) (Govers and Rabelink, 2001).

This study desired to gain insight into the mechanisms of endothelial nitric oxide synthase (eNOS) gene expression by doing a deletion synthesis to map regions of the promoter important for basal transcription in rat pulmonary myofibroblast cells. This method is consistent with relevant work that was done by Zhang, 1995.

Future aims of this work will be to use the constructs to transfect the rat pulmonary myofibroblast cells for determination of the various elements driving eNOS promoter activity. It will also be interesting to see the expression of NOS3 under the effect of the various compounds used in this study and see the difference with the deletion constructs. The factors and elements can therefore be manipulated to bring the effects desired in the investigation of a rational therapeutic design especially TGF-β may be of intervention importance.

This study therefor overally concludes that the rat eNOS promoter is active in pulmonary myofibroblasts and the activity of the eNOS promoter appears to be regulated by various mechanisms including NO itself. The suppression of the eNOS promoter by high concentrations of NO suggests that possibly during the inflammatory process of IPF, NO is produced by iNOS resulting in suppression of eNOS activity.

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APPENDICES

APPENDIX 1: PREPARATION OF MEDIA
Preparation of 10% media

From the DMEM (Dulbecco's Modified Eagles Medium from Gibco11960) - bottle remove 70 mls of fluid to make space for the other contents to be added. To the bottle add:

- 50 mls FBS((Fetal Bovine Serum)-in the freezer -20 degrees Celsius

- 5 mls Pen strep(Penicillin-streptomycin) in the freezer -20 degrees Celsius

- 5 mls L-glutamate in the freezer(-20 degrees Celsius)

- 10 mls HEPES(Buffer free acid) always refrigerated(+4 degrees Celsius)

Mix the contents to make 10% media. All this procedure is done in the hood and all sterile conditions should be maintained.

Preparation of 0% media

This contains no FBS and no antibiotics.

- ❖ From DMEM remove 15 ml

- ❖ Add 5 ml L-glutamate

- ❖ Add 10ml HEPES

Gently mix and refrigerate. Do all this under sterile conditions under the hood.

Preparation of 1% FBS (Fetal bovine serum)

- ❖ Take 49.5ml 0% media

- ❖ Add 0.5ml FBS

- ❖ Mix and refrigerate.

APPENDIX 2: RAT AND HUMAN PROMOTERS RAW DATA

Experiment 1. Passage P+2 1:3

Samples			Fire fly #1	Renilla #2	Ratio	
	1	r 1	5.511	63.21	0.087	
		r 2	2.461	27.8	0.089	
		r 3	4.384	46.33	0.095	
Avg		=	4.119	45.78	0.09	Negative control
%CV		=	37.44	38.68	4.394	
Std Dev		=	1.542	17.71	0.004	
	2	r 1	484.5	91.7	5.284	
		r 2	693.7	126.9	5.467	
		r 3	291.6	54.11	5.39	
Avg		=	489.9	90.9	5.38	Positive control
%CV		=	41.05	40.04	1.711	
Std Dev		=	201.1	36.4	0.092	
	3	r 1	206.3	119.1	1.733	
		r 2	362.9	199.2	1.822	
		r 3	129.7	82.66	1.569	
Avg		=	233	133.6	1.708	Rat promoter
%CV		=	51.03	44.61	7.521	
Std Dev		=	118.9	59.61	0.128	
	4	r 1	2.242	86.83	0.026	
		r 2	2.395	96.42	0.025	
		r 3	1.948	54.44	0.036	
Avg		=	2.195	79.23	0.029	Human promoter
%CV		=	10.35	27.76	21.02	
Std Dev		=	0.227	22	0.006	

Experiment 2: P+4 1:3

Samples			Fire fly #1	Renilla #2		Ratio		
	1	r 1	0.556	1.424		0.39		
		r 2	0.514	1.293		0.397		
		r 3	0.598	1.034		0.579		
Avg		=	0.556	1.25	NaN			Negative control
%CV		=	7.636	15.88	NaN			
Std Dev		=	0.042	0.199	NaN			
	2	r 1	3.94	68.92		0.057		
		r 2	7.057	54.95		0.128		
		r 3	7.588	0.878		8.642		
Avg		=	6.195	41.59		2.943		Positive control
%CV		=	31.81	86.42		167.7		
Std Dev		=	1.971	35.94		4.936		
	3	r 1	6.064	1.311		4.625		
		r 2	8.239	0.94		8.764		
		r 3	7.257	30.84		0.235		
Avg		=	7.187	11.03		4.541		Human promoter
%CV		=	15.15	155.5		93.91		
Std Dev		=	1.089	17.16		4.265		
	4	r 1	11.21	38.43		0.292		
		r 2	14.07	47.36		0.297		
		r 3	19.9	8.945		2.225		
Avg		=	15.06	31.58		0.938		Rat promoter
%CV		=	29.4	63.66		118.8		
Std Dev		=	4.429	20.1		1.115		

Experiment 3: P+4 1:3 Repeated

			Firefly	Renilla		
10		r 1	0.226	1.172	0.193	
		r 2	0.175	1.231	0.143	
		r 3	0.22	1.363	0.161	
Avg		=	0.207	1.256	0.166	Negative control
%CV		=	13.35	7.784	15.42	
Std		=	0.028	0.098	0.026	

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Dev

			Firefly	Renilla		
	11	r 1	51.88	6.557	7.912	
		r 2	0.027	0.015	1.847	
		r 3	69.45	6.652	10.44	
Avg		=	40.45	4.408	6.733	Positive control
%CV		=	89.23	86.32	65.6	
Std Dev		=	36.1	3.805	4.417	
	12	r 1	0.978	5.272	0.186	
		r 2	1.359	7.276	0.187	
		r 3	1.084	6.18	0.175	
Avg		=	1.14	6.243	0.183	Human promoter
%CV		=	17.23	16.07	3.401	
Std Dev		=	0.196	1.003	0.006	
	13	r 1	33.99	10.19	3.336	
		r 2	46.05	13.02	3.538	
		r 3	51.35	17.56	2.925	
Avg		=	43.8	13.59	3.266	Rat promoter
%CV		=	20.31	27.36	9.568	
Std Dev		=	8.896	3.718	0.312	

Experiment 4: P+2 1:3

			Firefly	Renilla		
	5		0.881	8.919	0.099	Negative control
	6		412.2	35.22	11.7	Positive control
	8	r 1	3.148	56.49	0.056	
		r 2	2.486	50.08	0.05	
Avg		=	2.817	53.28	0.053	Human Promoter
%CV		=	16.61	8.513	8.159	
Std Dev		=	0.468	4.536	0.004	
	9	r 1	18.22	33.09	0.55	
		r 2	2.041	4.806	0.425	
Avg		=	10.13	18.95	0.488	Rat Promoter
%CV		=	112.9	105.6	18.23	
Std Dev		=	11.44	20	0.089	

Experiment 5: P+4 1:3

Samples		Firefly #1	Renilla #2	Ratio	
1	r 1	1.048	3.047	0.344	
	r 2	1.3	3.256·	0.399	
	r 3	0.792	2.409	0.329	
Avg	=	1.047	2.904	0.357	Negative
%CV	=	24.25	15.19	10.37	
Std Dev	=	0.254	0.441	0.037	
2	r 1	119.4	13.69	8.723	
	r 2	70.87	7.921	8.946	
	r 3	103.8	12.86	8.072	
Avg	=	98.04	11.49	8.58	Positive
%CV	=	25.29	27.15	5.295	
Std Dev	=	24.8	3.12	0.454	
3	r 1	0.891	8.512	0.105	
	r 2	1.652	18.47	0.089	
	r 3	1.517	17.23	0.088	
Avg	=	1.353	14.74	0.094	Human
%CV	=	29.98	36.83	9.843	
Std Dev	=	0.406	5.427	0.009	
4	r 1	69.17	24.89	2.779	
	r 2	65.79	25.21	2.609	
	r 3	77.9	25.8	3.019	
Avg	=	70.95	25.3	2.803	Rat
%CV	=	8.81	1.827	7.347	
Std Dev	=	6.251	0.462	0.206	

Fig 26: Graphic Dual Luciferase Assay one point data.

	Exp.1	Exp.2	Exp.3(R PT)	Exp.4(6 w)	Exp.5	Exp.6
▫ Negative	0.09	0.445	0.166	0.099	0.357	0.36
▣ Positive	5.38	2.943	6.733	11.7	8.58	11.03
▫ Rat	1.708	0.938	3.266	0.488	2.803	4.945
▫ Human	0.029	4.541	0.183	0.053	0.094	0.08
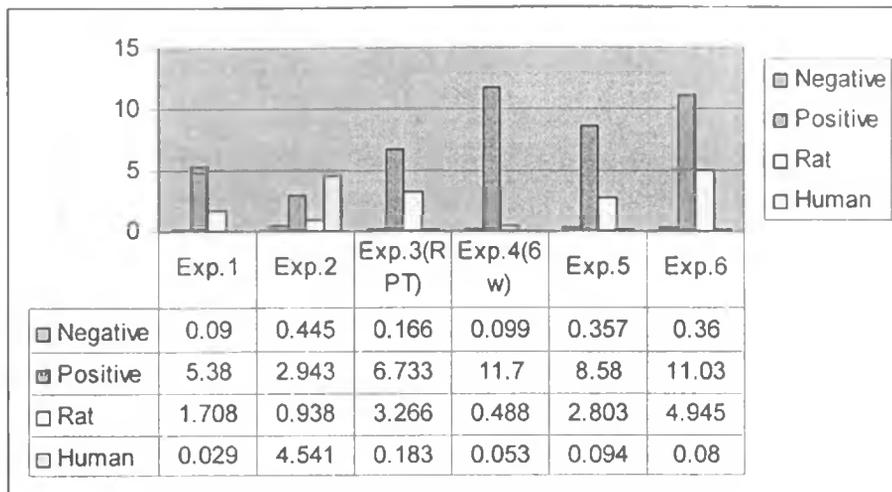
ANALYSIS OF RESULTS

	EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	STDV	STDERR
Negative	0.09	0.445	0.166	0.099	0.357	0.36	0.152959	0.062432
Positive	5.38	2.943	6.733	11.7	8.58	11.03	3.370616	1.375762
Rat	1.708	0.938	3.266	0.488	2.803	4.945	1.652323	0.674418
Human	0.029		0.183	0.053	0.094	0.08	0.058802	0.024001

	AVERAGE
Negative	0.252833
Positive	7.727667
Rat	2.358
Human	0.0878

APPENDIX 3: PMA, TGFβ, AND LPS RAW DATA.

Experiment 1

Samples		Firefly #1	Renilla #2	Ratio		
1	r 1	49.1	8.324	5.898		
	r 2	0.062	0.145	0.424		
	r 3	41.93	1.564	26.82		
Avg	=	30.36	3.344	11.05	Positive control	
%CV	=	87.23	130.7	126.1		
Std Dev	=	26.48	4.37	13.93		
2	r 1	0.009	0.136	0.067		
	r 2	0	0.161	0		
	r 3	0	0.133	0		
Avg	=	0.003	0.143	0.022	Negative control	0.022
%CV	=	173.2	10.55	173.2		
Std Dev	=	0.005	0.015	0.039		
3	r 1	61.25	9.286	6.595		
	r 2	0.047	0.151	0.315		
	r 3	43.48	1.938	22.44		
Avg	=	34.93	3.792	9.783	Repeated + control	9.783
%CV	=	90.15	127.7	116.5		
Std Dev	=	31.48	4.842	11.4		
4	r 1	0.131	0.14	0.936		
	r 2	0	0.141	0		
	r 3	0.011	0.163	0.07		
Avg	=	0.047	0.148	0.335	Rat control no treatment	0.335
%CV	=	152.8	8.719	155.4		
Std Dev	=	0.073	0.013	0.521		
5	r 1	1.71	1.724	0.992		
	r 2	0.453	0.372	1.218		
	r 3	0.264	0.337	0.784		
Avg	=	0.809	0.811	0.998	20 micro molar PMA	0.998
%CV	=	97.19	97.58	21.77		
Std Dev	=	0.786	0.791	0.217		
6	r 1	1.263	1.044	1.21		
	r 2	81.94	37.19	2.203		
	r 3	3.85	2.102	1.832		
Avg	=	29.02	13.44	1.748	2 micro molar	1.748

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			PMA		

					PMA	
%CV	=	158	153	28.72		
Std Dev	=	45.85	20.57	0.502		
7	r 1	5.297	2.283	2.32		
	r 2	0.027	0.201	**0.133**		2.53
	r 3	11.51	4.216	2.731		
					10 nanogram/ml	
Avg	=	5.613	2.233	1.728	TGF	
%CV	=	102.5	89.91	80.82		
Std Dev	=	5.75	2.008	1.397		
8	r 1	0.214	0.234	0.917		
	r 2	71.9	32.52	2.211		1.195
	r 3	0.108	0.236	0.459		
					5 nanogram/ml	
Avg	=	24.07	11	1.195	TGF	
%CV	=	172	169.5	76		
Std Dev	=	41.42	18.64	0.909		
9	r 1	5.435	5.597	0.971		
	r 2	0.011	0.115	**0.098**		0.753
Avg	=	2.723	2.856	0.535	10 microgram/ml LPS	
%CV	=	140.8	135.7	115.4		
Std Dev	=	3.835	3.877	0.617		

Experiment 2

		Firefly	Renilla			
Samples		#1	#2	Ratio		
1	r 1	1.78	5.885	0.302		
	r 2	1.732	6.412	0.27		
	r 3	1.286	5.063	0.254		
Avg	=	1.599	5.787	0.276	Negative C	0.276
%CV	=	17.02	11.75	8.939		
Std Dev	=	0.272	0.68	0.025		
2	r 1	111.1	11.03	10.08		
	r 2	133.1	12.46	10.69		
	r 3	170.9	18.66	9.159		
					Positive	
Avg	=	138.4	14.05	9.975	C	9.975
%CV	=	21.86	28.89	7.723		
Std Dev	=	30.25	4.059	0.77		

					Rat No	
3	r 1	70.76	27.83	2.543		
	r 2	54.09	24.41	2.216		
	r 3	55.45	22.58	2.456		
Avg	=	60.1	24.94	2.405	T	2.405
%CV	=	15.41	10.68	7.039		
Std Dev	=	9.26	2.663	0.169		
4	r 1	1.656	1.511	1.096		
	r 2	2.55	2.313	1.102		
	r 3	1.996	1.773	1.126		
Avg	=	2.067	1.866	1.108	20 micro PMA	1.108
%CV	=	21.81	21.9	1.402		
Std Dev	=	0.451	0.409	0.016		
5	r 1	64.73	23.79	2.721		
	r 2	76.92	28.47	2.701		
	r 3	57.06	21.3	2.6/9		
Avg	=	66.24	24.52	2.7	2 micro PMA	2.7
%CV	=	15.12	14.86	0.766		
Std Dev	=	10.01	3.644	0.021		
6	r 1	92.59	35.61	2.6		
	r 2	63	29.26	2.153		
	r 3	71.01	28.61	2.482		
Avg	=	75.54	31.16	2.412	10 ng TGF	2.412
%CV	=	20.26	12.41	9.616		
Std Dev	=	15.31	3.867	0.232		
7	r 1	90.99	29	3.138		
	r 2	64.87	23.16	2.801		
	r 3	74.9	26.81	2.794		
					5 ng	
Avg	=	76.92	26.32	2.911	TGF	2.911
%CV	=	17.13	11.21	6.752		
Std Dev	=	13.18	2.95	0.197		
8	r 1	48.56	19.69	2.466		
	r 2	64.49	22.64	2.848		
	r 3	48.12	17.58	2.738		
Avg	=	53.72	19.97	2.684	10 ng LPS	2.684
%CV	=	17.36	12.75	7.322		
Std Dev	=	9.326	2.545	0.197		

Experiment 3

Samples		Firefly #1	Renilla #2	Ratio		
1	r 1	1.208	3.596	0.336		
	r 2	1.61	5.138	0.313		
	r 3	1.198	4.62	0.259		
Avg	=	1.339	4.451	0.303	Negative control	0.303
%CV	=	17.56	17.63	13.03		
Std Dev	=	0.235	0.785	0.039		
2	r 1	80.05	5.491	14.58		
	r 2	106.2	6.165	17.22		
	r 3	131.2	8.161	16.08		
Avg	=	105.8	6.606	15.96	Positive control	15.96
%CV	=	24.17	21.02	8.307		
Std Dev	=	25.57	1.388	1.326		
3	r 1	83.87	14.97	5.602		
	r 2	119.5	19.55	6.111		
	r 3	96.31	12.94	7.445		
					Rat No	
Avg	=	99.89	15.82	6.386	Trt	6.386
%CV	=	18.11	21.42	14.9		
Std Dev	=	18.09	3.389	0.952		
4	r 1	0.125	0.35	0.359		
	r 2	1.07	0.301	3.554		
	r 3	0.068	0.246	0.279		
Avg	=	0.421	0.299	1.397	20 micro PMA	0.319
%CV	=	133.5	17.4	133.7		
Std Dev	=	0.562	0.052	1.869		
5	r 1	79.96	11.38	7.025		
	r 2	86.09	10.51	8.194		
	r 3	43.6	8.81	4.949		
Avg	=	69.88	10.23	6.723	2 micro PMA	7.61
%CV	=	32.86	12.78	24.45		
Std Dev	=	22.96	1.308	1.643		
6	r 1	85.3	16.58	5.143		
	r 2	95.2	19.07	4.991		
	r 3	75.15	19.83	3.79		
Avg	=	85.22	18.5	4.642	10 ng TGF	4.642
%CV	=	11.76	9.175	15.97		
Std Dev	=	10.02	1.697	0.741		

7	r 1	115.7	20	5.784			
	r 2	81.7	14.63	5.584			
	r 3	110.6	16.48	6.708			
					5ng		
Avg	=	102.7	17.04	6.025	TGF		6.025
%CV	=	17.85	16.01	9.946			
Std Dev	=	18.33	2.728	0.599			
8	r 1	56.68	10.24	5.537			
	r 2	51.36	10.76	4.772			
	r 3	50.92	9.434	5.398			
Avg	=	52.98	10.14	5.235	10 micro LPS		5.235
%CV	=	6.055	6.599	7.785			
Std Dev	=	3.208	0.669	0.408			

Experiment 4

			Firefly	Renilla	Ratio		
	3	r 1	0.023	0.081	0.289		
		r 2	0.006	0.034	0.163		
		r 3	0.004	0.035	0.118		
Avg		=	0.011	0.05	0.19	Negative C	0.1405
%CV		=	97.28	53.5	46.67		
Std Dev		=	0.011	0.027	0.089		
	4	r 1	0.038	0.028	1.346		
		r 2	0.1	0.045	2.221		
		r 3	0.401	0.225	1.778		
Avg		=	0.18	0.1	1.782	Positive C	1.782
%CV		=	108	109.7	24.53		
Std Dev		=	0.194	0.109	0.437		
	5	r 1	0.819	2.14	0.383		
		r 2	0.607	2.393	0.253		
		r 3	0.254	1.019	0.249		
Avg		=	0.56	1.851	0.295	Rat NO Trt	0.295
%CV		=	51.04	39.52	25.79		
Std Dev		=	0.286	0.731	0.076		
	6	r 1	0.364	2.278	0.16		

	r 2	0.428	1.464	0.292		
	r 3	0.438	0.354	1.239		
					20mM	
Avg	=	0.41	1.365	0.564	PMA	0.226
%CV	=	9.77	70.75	104.4		
Std						
Dev	=	0.04	0.966	0.588		
7	r 1	0.815	2.055	0.397		
	r 2	1.125	2.6	0.433		
	r 3	0.886	0.279	3.174		
Avg	=	0.942	1.645	1.335	2mM PMA	0.415
%CV	=	17.24	73.78	119.4		
Std						
Dev	=	0.162	1.213	1.593		
8	r 1	1.012	0.641	1.579		
	r 2	0.369	0.483	0.764		
	r 3	0.948	0.847	1.12		
Avg	=	0.776	0.657	1.154	10ng TGF	1.154
%CV	=	45.6	27.74	35.37		
Std						
Dev	=	0.354	0.182	0.408		
9	r 1	0.508	0.336	1.514		
	r 2	0.398	0.282	1.408		
	r 3	0.349	0.17	2.055		
Avg	=	0.418	0.263	1.659	5ng TGF	1.461
%CV	=	19.48	32.22	20.93		
Std						
Dev	=	0.081	0.085	0.347		
10	r 1	1.098	0.062	17.64		
	r 2	0.412	0.102	4.04		
	r 3	0.288	0.112	2.572		
Avg	=	0.599	0.092	8.082	10ug LPS	3.306
%CV	=	72.88	28.49	102.8		
Std						
Dev	=	0.437	0.026	8.306		

Experiment 5

Samples		Firefly #1	Renilla #2	Ratio	
1	r 1	· 0.07	0.192	0.364	
	r 2	0.061	0.164	0.37	
	r 3	0.03	0.117	0.251	
Avg	=	0.053	0.158	0.328	Negative C
%CV	=	39.68	23.95	20.37	
Std Dev	=	0.021	0.038	0.067	
2	r 1	1.709	0.246	6.957	
	r 2	0.688	0.132	5.227	
	r 3	2.369	0.366	6.475	
					Positive
Avg	=	1.589	0.248	6.22	C
%CV	=	53.32	47.3	14.35	
Std Dev	=	0.847	0.117	0.893	
3	r 1	5.463	2.396	2.28	
	r 2	4.389	2.596	1.691	
	r 3	1.898	1.097	1.73	
					Rat No
Avg	=	3.917	2.03	1.9	Trt
%CV	=	46.69	40.09	17.34	
Std Dev	=	1.829	0.814	0.329	
4	r 1	2.39	2.234	1.07	
	r 2	1.968	1.985	0.991	
	r 3	1.022	0.465	2.197	
Avg	=	1.793	1.561	1.419	20 uM PMA
%CV	=	39.05	61.31	47.52	
Std Dev	=	0.7	0.957	0.674	
5	r 1	3.023	2.115	1.429	
	r 2	4.759	2.552	1.865	
	r 3	2.558	1.906	1.343	
					2uM
Avg	=	3.447	2.191	1.545	PMA
%CV	=	33.65	15.06	18.1	
Std Dev	=	1.16	0.33	0.28	
6	r 1	4.508	2.458	1.834	
	r 2	1.882	1.938	0.971	
	r 3	2.847	2.946	0.966	
Avg	=	3.079	2.448	1.257	10 ng TGF

%CV	=	43.14	20.6	39.74	
Std Dev	=	1.328	0.504	0.5	
7	r 1	0.862	0.373	2.313	
	r 2	0.867	0.347	2.499	
	r 3	1.183	0.403	2.932	
					5ng
Avg	=	0.971	0.374	2.581	TGF
%CV	=	18.9	7.519	12.31	
Std Dev	=	0.184	0.028	0.318	
8	r 1	0.167	0.162	1.032	
	r 2	0.702	0.272	2.583	
	r 3	0.532	0.238	2.233	
					10ug
Avg	=	0.467	0.224	1.949	LPS
%CV	=	58.58	25.21	41.73	
Std Dev	=	0.274	0.056	0.814	

ANALYSIS OF RESULTS

	EXP1 11-26-08	EXP2 11-29-08	EXP3 12-02-08	EXP4 12-14-08
Positive	9.783	9.975	15.96	6.22
Negative Rat No	0.335	0.276	0.303	0.328
Trt 20µM	0.998	2.405	6.386	1.9
PMA 2µM	1.748	1.108	1.397	1.419
PMA	1.728	2.7	6.723	1.545
10ngTGF☐	2.53	2.412	4.642	1.257

5ngTGF⬚	0.688	2.911	6.025	2.581
10µgLPS	0.535	2.684	5.235	1.949

Dual Luciferase Results Analysis treatments(Normalized values)

	Exp1	Exp2	Exp3	Exp4	AVERAGE	STDV	STDERR
Rat No Trt	1	1	1	1	1	0	0
20µM PMA	2.979104	0.460707	0.049953	1	0.503553	0.476471	0.275099
2µM PMA	5.21791	1.122661	1.191669	0.766102	1.026811	0.228402	0.131872
10ngTGF⬚	7.552239	2.412	0.726903	1.40678	1.515227	0.847767	0.489473
5ngTGF⬚	1.195	1.210395	0.94347	4.952542	1.116288	0.149863	0.086526
10µgLPS	2.247761	1.116008	0.⬚⬚9762	11.20678	1.39451	0.753637	0.435125

APPENDIX 4:A23187, SNAP, L-NAME AND EGTA RAW DATA.

Experiment 1

Samples		Firefly #1	Renilla #2	Ratio	
	r 1	0.62	2.215	0.28	
	r 2	0.984	3.457	0.285	
	r 3	0.772	3.541	0.218	
Avg	=	0.792	3.071	0.261	Negative C
%CV	=	23.1	24.18	14.23	
Std Dev	=	0.183	0.743	0.037	
2	r 1	72.24	6.713	10.76	
	r 2	105.8	8.585	12.32	
	r 3	74.25	7.745	9.586	
					Positive
Avg	=	84.09	7.681	10.89	C
%CV	=	22.37	12.21	12.6	
Std Dev	=	18.81	0.938	1.372	
3	r 1	17.2	9.731	1.767	
	r 2	32.29	21.44	1.506	
	r 3	21.3	16.3	1.307	

Avg	=	23.6	15.83	1.527	Rat No TRT
%CV	=	33.07	37.09	15.13	
Std Dev	=	7.803	5.87	0.231	
4	r 1	3.997	2.975	1.343	
	r 2	4.246	4.294	0.989	
	r 3	4.61	3.333	1.383	
Avg	=	4.284	3.534	1.238	1 uM 23187
%CV	=	7.2	19.29	17.53	
Std Dev	=	0.308	0.682	0.217	
5	r 1	25.19	19.25	1.309	
	r 2	27.8	20.7	1.343	
	r 3	19.32	17.44	1.108	
Avg	=	24.1	19.13	1.253	1 mM L-NAME
%CV	=	18.02	8.538	10.15	
Std Dev	=	4.343	1.633	0.127	
6	r 1	17.71	13.65	1.298	
	r 2	16.89	14.94	1.13	
	r 3	27.16	17.77	1.528	
Avg	=	20.59	15.46	1.319	10 mM L-NAME
%CV	=	27.71	13.66	15.13	
Std Dev	=	5.705	2.111	0.2	
7	r 1	10.32	3.434	3.005	
	r 2	16.54	5.673	2.916	
	r 3	16.9	7.685	2.199	
Avg	=	14.59	5.597	2.707	0.1mM S-NAP
%CV	=	25.36	37.99	16.33	
Std Dev	=	3.699	2.126	0.442	
8	r 1	0.865	4.668	0.185	
	r 2	0.666	2.08	0.32	
	r 3	0.853	2.642	0.323	
Avg	=	0.795	3.13	0.276	1 mM S-NAP
%CV	=	14.01	43.49	28.52	
Std Dev	=	0.111	1.361	0.079	
10	r 1	27.48	18.64	1.475	
	r 2	39.48	32.56	1.212	
	r 3	34.3	32.07	1.07	
Avg	=	33.76	27.76	1.252	EGTA 1mM
%CV	=	17.83	28.47	16.4	
Std Dev	=	6.019	7.902	0.205	
11	r 1	41.2	33.32	1.236	

		Firefly #1	Renilla #2	Ratio		
	r 2	38.19	30.15	1.267		
	r 3	40.32	38.5	1.047		
Avg	=	39.9	33.99	1.183	EGTA 1mM	
%CV	=	3.875	12.4	10.05		
Std Dev	=	1.546	4.216	0.119		

Experiment 2

Samples		Firefly #1	Renilla #2	Ratio		
1	r 1	0.162	0.555	0.292		
	r 2	0.13	0.506	0.257		
	r 3	0.114	0.434	0.264		
Avg	=	0.136	0.498	0.271	Negative C	0.271
%CV	=	18.04	12.2	6.961		
Std Dev	=	0.024	0.061	0.019		
2	r 1	15.57	0.444	35.09		
	r 2	17.81	0.546	32.61		
	r 3	11.95	0.495	24.13		
					Positive	
Avg	=	15.11	0.495	30.61	C	30.61
%CV	=	19.56	10.36	18.78		
Std Dev	=	2.955	0.051	5.749		
4	r 1	1.299	0.785	1.655		
	r 2	2.551	1.988	1.283		
	r 3	1.266	0.93	1.362		
					Rat NO	
Avg	=	1.705	1.234	1.433	trt	1.433
%CV	=	42.94	53.21	13.68		
Std Dev	=	0.732	0.657	0.196		
5	r 1	0.087	0.227	0.385		
	r 2	0.162	0.24	0.676		
	r 3	0.176	0.272	0.645		
Avg	=	0.142	0.246	0.569	1uM A2387	0.569
%CV	=	33.6	9.52	28.08		
Std Dev	=	0.048	0.023	0.16		
6	r 1	1.845	1.29	1.43		
	r 2	2.015	1.283	1.571		
	r 3	1.801	1.012	1.781		

Avg	=	1.887	1.195	1.594	1 mM L-NAME	1.594
%CV	=	5.973	13.28	11.08		
Std Dev	=	0.113	0.159	0.177		
7	r 1	1.08	0.797	1.355		
	r 2	1.92	0.605	3.172		
	r 3	2.274	1.029	2.21		
Avg	=	1.758	0.81	2.246	10 mM L-NAME	2.246
%CV	=	34.89	26.17	40.47		
Std Dev	=	0.613	0.212	0.909		
8	r 1	1.38	1.321	1.044		
	r 2	1.7	1.307	1.3		
	r 3	1.065	0.447	2.384		1.172
Avg	=	1.382	1.025	1.576	0.1mM SNAP	
%CV	=	22.97	48.87	45.13		
Std Dev	=	0.317	0.501	0.711		
9	r 1	0.369	0.828	0.446		
	r 2	0.463	0.75	0.618		
	r 3	0.931	1.33	0.7		
Avg	=	0.588	0.969	0.588	1 mM SNAP	0.558
%CV	=	51.23	32.52	22.05		
Std Dev	=	0.301	0.315	0.13		
10	r 1	2.489	1.4	1.778		
	r 2	1.805	1.275	1.416		
	r 3	2.478	1.291	1.919		
Avg	=	2.257	1.322	1.704	1 mM EGTA	1.704
%CV	=	17.35	5.153	15.24		
Std Dev	=	0.392	0.068	0.26		
11	r 1	2.856	1.255	2.275		
	r 2	3.039	1.176	2.583		
	r 3	2.419	0.985	2.455		
Avg	=	2.771	1.139	2.438	1 uM EGTA	2.438
%CV	=	11.49	12.18	6.343		
Std Dev	=	0.318	0.139	0.155		

Experiment 3.

Samples		Firefly #1	Renilla #2	Ratio		
1	r 1	0.052	0.51	0.102		
	r 2	0.126	0.457	0.276		
	r 3	0.062	0.311	0.199		
Avg	=	0.08	0.426	0.192	Negative C	0.192
%CV	=	50.46	24.16	45.49		
Std Dev	=	0.04	0.103	0.087		
2	r 1	14.52	0.563	25.78		
	r 2	5.47	0.424	12.89		
	r 3	7.529	0.424	17.75		
					Positive	
Avg	=	⌐ 171	0.47	18.81	C	18.81
%CV	=	51.7	17.06	34.59		
Std Dev	=	4.742	0.08	6.505		
3	r 1	6.463	1.799	3.592		
	r 2	29.02	6.645	4.368		
	r 3	57.69	9.984	5.778		
Avg	=	31.06	6.143	4.579	Rat NO TRT	4.579
%CV	=	82.67	67	24.21		
Std Dev	=	25.67	4.115	1.109		
4	r 1	2.635	1.316	2.001		
	r 2	0.01	0.091	0.111		
	r 3	0.342	0.32	1.068		
Avg	=	0.996	0.576	1.06	1 uM A23187	1.061
%CV	=	143.5	113.1	89.12		
Std Dev	=	1.429	0.651	0.945		
5	r 1	40.25	7.138	5.639		
	r 2	39.54	6.348	6.229		
	r 3	37.92	5.931	6.394		
Avg	=	39.24	6.472	6.087	1 mM LNAME	6.087
%CV	=	3.049	9.473	6.516		
Std Dev	=	1.196	0.613	0.397		
6	r 1	16.65	2.828	5.887		
	r 2	0.143	0.307	0.466		
	r 3	2.793	0.981	2.847		
Avg	=	6.527	1.372	3.067	10 mM LNAME	4.367
%CV	=	135.8	95.11	88.59		

		8.862	1.305	2.717		
Std Dev	=	8.862	1.305	2.717		
7	r 1	23.59	4.549	5.187		
	r 2	35.41	5.409	6.547		
	r 3	20.54	3.42	6.006		
Avg	=	26.51	4.459	5.913	0.1mM SNAP	5.913
%CV	=	29.62	22.37	11.58		
Std Dev	=	7.853	0.997	0.685		
8	r 1	4.825	3.784	1.275		
	r 2	4.665	3.917	1.191		
	r 3	1.124	1.225	0.918		
Avg	=	3.538	2.975	1.128	1 mM SNAP	1.128
%CV	=	59.13	51.01	16.54		
Std Dev	=	2.092	1.518	0.187		
9	r 1	48.15	8.069	5.968		
	r 2	31.11	5.163	6.026		
	r 3	2.447	1.697	1.442		
Avg	=	27.24	4.976	4.478	1mM EGTA	
%CV	=	84.8	64.1	58.72		5.997
Std Dev	=	23.1	3.19	2.63		
10	r 1	53.29	8.645	6.164		
	r 2	25.96	3.768	6.888		
	r 3	5.735	2.131	2.691		
Avg	=	28.33	4.848	5.248	1uM EGTA	6.526
%CV	=	84.25	69.89	42.76		
Std Dev	=	23.87	3.388	2.244		

Experiment 4

Samples		Firefly #1	Renilla #2	Ratio		
1	r 1	0.104	0.159	0.651		
	r 2	0.075	0.156	0.482		
	r 3	0.215	0.203	1.06		
Avg	=	0.131	0.173	0.731	Negative C	0.731
%CV	=	56.32	15.17	40.68		
Std Dev	=	0.074	0.026	0.297		
2	r 1	12.06	0.204	59.12		
	r 2	11.84	0.155	76.46		
	r 3	27.02	2.313	11.68		
Avg	=	16.97	0.891	49.09	Positive	49.09

%CV	=	51.24	138.3	68.32		
Std Dev	=	8.697	1.232	33.53		
3	r 1	2.835	1.955	1.45		
	r 2	3.65	2.461	1.483		
	r 3	2.535	2.011	1.261		
Avg	=	3.007	2.142	1.398	Rat No TRT	1.398
%CV	=	19.19	12.95	8.594		
Std Dev	=	0.577	0.277	0.12		
4	r 1	0.392	0.425	0.923		
	r 2	0.613	0.517	1.185		
	r 3	0.163	0.387	0.421		
Avg	=	0.389	0.443	0.843	1 uM A23187	0.843
%CV	=	57.79	15.15	46.02		
Std Dev	=	0.225	0.067	0.388		
5	r 1	8.356	4.781	1.748		
	r 2	5.815	3.584	1.623		
	r 3	6.268	3.895	1.609		
Avg	=	6.813	4.086	1.66	1 mM LNAME	1.66
%CV	=	19.89	15.2	4.607		
Std Dev	=	1.355	0.621	0.076		
6	r 1	3.475	1.971	1.763		
	r 2	0.103	0.09	1.155		
	r 3	1.233	1.645	0.749		
Avg	=	1.604	1.235	1.223	10 mM LNAME	1.223
%CV	=	107	81.4	41.74		
Std Dev	=	1.716	1.005	0.51		
7	r 1	3.525	2.575	1.369		
	r 2	4.65	3.971	1.171		
	r 3	4.318	3.66	1.18		
Avg	=	4.164	3.402	1.24	0.1 mM SNAP	1.24
%CV	=	13.88	21.54	9.018		
Std Dev	=	0.578	0.733	0.112		
8	r 1	1.709	4.942	0.346		
	r 2	1.896	2.811	0.675		
	r 3	1.698	4.074	0.417		
Avg	=	1.768	3.943	0.479	1mM SNAP	0.479
%CV	=	6.304	27.18	36.11		
Std Dev	=	0.111	1.072	0.173		
9	r 1	0.735	1.625	0.452		

	r 2	0.468	0.62	0.756		
	r 3	0.812	0.251	3.234		
Avg	=	0.672	0.832	1.481	1 mM EGTA	0.604
%CV	=	26.82	85.49	103		
Std Dev	=	0.18	0.711	1.526		

10	r 1	0.294	0.258	1.138		
	r 2	1.141	0.656	1.74		
	r 3	1.043	0.44	2.373		
Avg	=	0.826	0.451	1.75	1 uM EGTA	1.439
%CV	=	56.13	44.16	35.29		
Std Dev	=	0.464	0.199	0.618		

ANALYSIS OF RESULTS

	EXP1	EXP2	EXP3	EXP4
Positive	0.89	30.61	18.81	49.09
Negative	0.261	0.271	0.192	0.731
Rat No Trt	1.527	1.433	4.579	1.398
1µM 23187	1.238	0.569	1.06	0.843
1mML-NAME	1.253	1.594	6.087	1.66
10mML-NAME	1.319	2.246	3.067	1.223
0.1Mmsnap	2.707	1.576	5.913	1.24
1Mmsnap	0.276	0.588	0.479	0.479
1mMEGTA	1.252	1.704	1.481	0.604
1µMEGTA	1.183	2.438	1.75	1.75

Stastical analysis (Normalized results)

	EXP1	EXP2	EXP3	EXP4	Average	STDV	STDER
Rat No Trt	1	1	1	1	1	0	0
1µM 23187	0.8104	0.3976	0.2311	0.6030	0.51063	0.25119	0.12559
1mML-NAME	0.8206	1.1125	1.3293	1.1874	1.11241	0.21436	0.10718
10mML-NAME	0.8638	1.5674	0.9530	0.8742	1.06491	0.33733	0.16866
0.1Mmsnap	2.9222	0.8176	1.2913	0.8868	0.99872	0.25574	0.14766
1Mmsnap	0.1804	0.3899	0.2464	0.3423	0.28977	0.09397	0.04698
1mMEGTA	0.8190	1.1891	1.3097	0.4324	0.93768	0.39628	0.19814
1µMEGTA	0.7742	1.7012	1.4253	1.0292	1.23273	0.41147	0.20579

APPENDIX 5: INVERSE PCR PROTOCOL

1. Design deletion primers to be about 21bp and Tm of 55-60°C.Try to get all Tm similar so you can mix and match all combinations. These primers will run in opposite directions to amplify the entire plasmid.

2. Set up PCR as follows:

Pfu Turbo	12.5µl
10X buffer	2.5µl
Forward primer(10uM)	0.5µl
Reverse primer(10uM)	0.5µl
Plasmid template 25-30ng	Xµl
Water	Xµl
	25µl

Do three extra PCR reactions which will serve as controls.

3. PCR program:

95°C	5 minutes
95°C	1 minute
Your annealing	30 seconds

72 °C	5 minutes
72 °C	10 minutes

Do this for 21 cycles

4. Use 2-3 µl to check PCR on 0.7% gel

5. Kinase reaction (phosphorylation reaction)

NEB T4 polynucleotide kinase	2 µl
10X buffer	5 µl
PCR product	23 µl
Water	20 µl
	50 µl

Keep one PCR reaction that you do not kinase.

6. Incubate 37°C for 1 hour

7. Cleanup kinase reaction with Qiagen PCR purification Kit resuspending in 10 µl

8. Ligation reaction:

NEB T4 DNA Ligase	2 µl
10X buffer	2 µl
Kinased PCR product	10 µl
Water	6 µl
	20 µl

Ligate one reaction that was not kinased as a control.

9. Incubate entire 20 µl ligation into XL1 Blue cells

10. Transform entire 20 µl ligation into XL1 Blue cells.

    a. Controls:

    i) PCR only

    ii) Kinased product that was not ligated

    iii) Ligated product that was not kinased

11. Plate 100 µl off top then centrifuge, aspirate to 100 µl and plate the rest.

APPENDIX 6: TRANSFECTION PROTOCOL

1. Isolate plasmid by Qiagen Maxiprep. Quantify and concentrate to 1 ug/ul.It

    may be necessary to do 2 Maxiprep per sample to obtain this high

    concentration.

2. Make media with or without serum but without antibiotics. Antibiotics will

    interfere with Lipofectamine.

3. Count cells using the hemacytometer

        a. Dilute 1:1 volumes of cells in 2X trypan blue

        b. Mix well, place cover slip on hemacytometer

        c.Load 10µl 1:1 mixture into notch on each side of hemacytometer, cell

    mixture will diffuse across hemacytometer by capillary action.

        d. Focus to see grid. Count cells in outer 4 quadrants. On both sides.

    Average all 8 numbers and multiply it by 2.This is the cell

concentration$\times 10^4$cells/ml.(If you get an average of 7,you have 14×10^4

cells/ml)

4. Dillute cells with appropriate volume of media to achieve $4-8\times10^5$cells/500µl which is the recommended concentration for transfection.

5. Seed cells into appropriate plate at density of $4-8\times10^5$cells/500µl, taking into account the volume you use in the plate.

    a. volume of 24 well plate in 0.5ml (most common size for transfections)

    b. volume of 12 well plate in 1 ml.

    c. volume of 6 well plate in ? ml

    d. volume of 10 cm plate in 10 ml

    e. plates used for C2C12 must be gelatin coated.

6. If cells are already at appropriate concentration, they need 4-6 hours to sit down on the plate. However if they are slightly less concentrated than desired, you may allow them to differentiate 24 hours.

7. Make master mix of plasmid and optimem (1 ug plasmid and 50 ul per well) Mix the plasmid and optimem in a 15 ml conical vial.

8. Make a second master mix of Lipofectamine and optimem (2ul Lipofectamine and 50ul optimem per well).Mix in 15ml conical vial.

9. Mix the plasmid/optimem and the Lipofectamine/optimem in 15ml conical vials and incubate 5 minutes. Combine 1:1 volumes of the 2 mixtures and incubate for 20-30 minutes. DO NOT leave any longer than 30 minutes.

This is the time the plasmid is forming lipid complexes with the Lipofectamine so that the plasmid can diffuse across the cell membrane.

10. During incubation, aspirate old media from each well. If media used to seed the wells contained antibiotics or excess serum, wash with PBS.Replace media with 500μl appropriate media without antibiotics.

11. Once the incubation period is over, aliquot 100ul of the 1:1 plasmid/Lipofectamine mixtures to each well.

12. Swirl plate to mix well

13. Place in 37°C incubator

    a. For myofibroblasts, cells must be recovered after 5-6 hours with by adding 500ul 20% media (to make 1 ml of 10%media/well)

    b. If treating cells, treat12 to 24 hours post-transfection then assay 48 hours post-treatment.

14. Harvest cells 48 hours post-transfection

15. Aspirate media and wash 2 X with PBS

16. Dillute Passive Lysis Buffer 1:5 and 100ul PLB in each well.

17. Either put plate in -80°C freezer or place on a shaker for 1 hour and assay.

18. Assay cells using the Promega Dual Luciferase Assay.

APPENDIX 7: PASSAGING OR SPLITTING CELLS PROTOCOL

1. Clean hood. You should always clean hoods before and after use.

2. Place all dry supplies under UV light: pipettes, pipette tips, Styrofoam tube holders etc.

3. Prewarm media, PBS and thaw 10X trypsin in 37°C water bath.

4. Label plates with name of cell line, your initials, and date and passage number.

5. Aspirate media with Pasteur pipette. Always be sure to aspirate 10% bleach after using a Pasteur pipette.

6. Wash with Phosphate Buffered Saline (PBS) by pippetting enough PBS onto the plate to cover the bottom. Rock back and forth gently.

7. Aspirate PBS and wash again

8. Dillute one 10X trypsin aliquot(-20) with 9 ml PBS. Relabel tubes as 1X trypsin and put your initials and date on it. 1X trypsin should be refrigerated for up to 1 week. Trypsin cleaves the glycogen bonds the cells have made to adhere to the plastic.

9. Pipette 1 ml 1X trypsin dropwise and for myofibroblasts allow trypsin to sit on plate 1 minute while rocking plate to ensure trypsin covers plate

10. Place plates, which now have a thin layer of trypsin on them, into the $37^{o}C$ incubator for 2-3 minutes. The high temperature allows trypsin to work more efficiently. Be careful not to place plates in incubator if they have media on them.

11. This is a good time to clean out the hood. Remove all supplies you will not need anymore.

12. After incubation, look at the plate under the inverted scope to check for"balling". If all the cells have formed balls, they are ready. You will always want to be careful not to over trypsinize cells or they will die.

13. If the cells are balled, whack plate on counter very hard to further release the cells from the plastic.

14. Place plate back in hood and immediately pipette 10ml 10% NGM on plate. The serum in the media stops the trypsin activity.

15. Tip plate and wash the cells down plate using the 10ml media. Use the pipette tip to scrape cells while washing.

16. Pipette cells into a 50 ml conical vial. Add appropriate amount of media such as 40ml media for 1:5 dilution. If you are doing 1:10 dillution, pipette 5 ml cells in each of 250 ml conical vials and add 45 ml media in each. Myofibroblasts should not be split more than 1:6

17. Once cells have been diluted with new media, pipette up/down gently to mix.

18. Pipette 10ml diluted cells on new, labeled plates.

19. Place plates in incubator.

APPENDIX 8: CULTURING MYOFIBROBLASTS FROM LIQUID NITROGEN

1. Prepare hood; warm media

2. Take cells directly from N2 and quick thaw in $37^{\circ}C$ water bath(2-3 min)

3. Transfer cells for a 15 ml conical vial in sterile hood.

4. Add 9 ml normal growth media (DMEM+10%FBS) on top of thawed cells-dropwise at first until about 3-4 ml, and then continue as normal.

5. Gently pipette up and down to mix cells

6. Pipette 10ml of cells +media into 100mm dish

7. Place cells at $37^{\circ}C$ IN CO2 incubator.

APPENDIX 9: SEQUENCING PROTOCOL

1. Quantify DNA by gel and gel.(Usually need 10-20ng for sequencing up to 2000bp however see page 2-6 in Big Dye Terminator v3.1 Cycle Sequencing Protocol manual for exact concentrations)

2. Be sure DNA is clean. If needed, clean up Qiagen kit o gel purification.

3. Set up sequencing reaction as follows:

Sterile water	Xµl
5X sequencing buffer(ABI)	2µl
Sequencing juice	2µl
Forward or reverse primer(3.2pmol)	0.5µl
DNA template(10ng)	Xµl
	10µl

4. Load reaction on thermacycler using the following program:"ABI seq"

Initial Denature	96	1 minutes
Denature	96	30 seconds(10s)
Anneal	50	15 seconds(5s)
Extension	60	4 minutes

Run this for 25 cycles.

5. Perform sequencing cleanup as follows:

    a. Add 40µl 75% isopropanol

    b. Incubate at room temperature 20 minutes

    c. Centrifuge 20 minutes at maximum speed

    d. Carefully remove supernatant, avoid sides of the tube as pellet is invisible.

    e.Add 250µl 75% isopropanol

f. Centrifuge 10 minutes at maximum speed.

g. Carefully remove supernatant, avoid sides of the tube as pellet us invisible.

h. Dry in speed vac 15 minutes with no heart.

i. Re-dissolve in 20µl formamide buffer.

6. Load on samples on ABI prism sequencer as follows:

a. Password is 3130USER

b. Click on "foundation Data Collection"

c. Click on "Plate Manager" in the menu

d. Click on "new".

e. Enter a name for your plate that contains the date of the run

f. Under application, choose "sequencing analysis"

g. You have to enter something for both owner and operator fields (it doesn't matter what you put here.

h. Press the "Tray" button on the machine to remove the plate.Plates are used until all wells have been used. The septa have a dot on it at A1.Be sure to keep the dot at A1 or the samples will be contaminated with the other samples it has been in contact with. If the plate is full, get a new plate and clean septa.

i. Determine which wells have not been used and load the entire 20µl sample in the appropriate wells. Replace the septa and be sure the holes line up.

j. Place the white plate until it clicks

k. Place the tray back in the machine.One side has a notch in it, so it will only fit in the machine one way. Be sure it clicks into place.

l. Press the "Tray "button again to load the plate.

m. Once you have determined which wells to use, go back to the plate setup and name the appropriate wells with a unique name for each sample.

n. Beside the name, in the "instrument protocol"field, choose Tia_sequencing.

o. In the "results group"field, choose N_Rice_Results_Group.This is the folder in which your final results will be stored.

p. In the analysis protocol field, there should only be one option, 3130POP7_BDTv3..

q. Save the plate.

r. Press "OK" on the plate set up screen.

s. On the menu, click "Run scheduler". Click "find all". This lists all the plates saved on the computer. The picture of a plate to the right should be yellow meaning the plate is loaded. If it gray, the plate is not loaded.

t. Find your plate (whatever you named it in 7.e.). Click on it. The picture to the left should turn from yellow to green.

u. In the upper left corner in the toolbar, click the neon green triangle (play) button. You should then receive a message that "you are about to start a run". This indicates that you have successfully started sequencing your plate.

The green light on the sequencer should be flashing and should remain flashing until all samples are finished. You may now leave the machine running. It takes approximately 2 hours/4 samples.

8. Retrieval of sequences off the ABI prism software

a. Once the run is complete, click on "shortcut to data"

b. Find results in the Rice folder or go to ga3130.This folder contains all results ever run. Go to the date you ran your sequences and find yours.

c. Change names of files to reflect names of your clones

d. Highlight all files and drag them over the "sequence analysis" icon on the desktop. Application will open.

e. Change the "dye set primer "to POP7_BDTv3

f. Click green "play "button

g. When finished, go to file and "save all samples"

h. Yellow=no red, Blue=poor quality=good quality

i. Click on view and sequence

j. At the bottom, click on the electropherogram tab to view the sequence. Blue columns indicate reliable results, Red is not so good.

k. Open notepad and copy sequence from the sequence view into a separate file for each sequence.

l. Save to a USB disk

m. View on vector NTI.