

**THE EFFECT OF OXIDATIVE STRESS AND
GLUCOCORTICIDS
ON TGF- β 1 PRODUCTION IN AIRWAY EPITHELIAL CELLS**

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A Thesis Submitted for the Degree of Doctor of Philosophy

February 2010

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ABSTRACT

Transforming growth factor- β 1 (TGF- β 1) is implicated in inflammation and airway wall remodeling in inflammatory airway disease. Oxidative stress has also been implicated in the pathogenesis of inflammatory lung disorders. Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), in the airway may activate intracellular signalling cascades and transcription factors to exaggerate lung inflammation. The balance between nuclear histone acetylation/deacetylation has also been shown to be altered by oxidative stress and may further upregulate lung inflammation.

The aim of this thesis was to determine whether ROS, alone or in combination with interleukin (IL)- 1β , can modulate TGF- β 1 expression in lung epithelial cells and whether the effect was regulated by histone methylation.

Stimulation of human airway epithelial cells (A549) with H_2O_2 or H_2O_2 plus IL- 1β enhanced TGF- β 1 mRNA and total protein expression. IL- 1β and H_2O_2 together showed a greater than additive effect on TGF- β 1 mRNA transcription, but not on the total protein level. The NF- κ B-IKK2 inhibitor AS602868 suppressed IL- 1β - and H_2O_2 plus IL- 1β -induced TGF- β 1 mRNA expression. NF- κ B p65 protein was also shown to be upregulated by IL- 1β plus H_2O_2 stimulation. Chromatin immunoprecipitation (ChIP) experiments demonstrated that p65 was recruited to the κ B3 binding site on the native TGF- β 1 promoter area. The upregulation of TGF- β 1 mRNA by IL- β plus H_2O_2 could be suppressed by fluticasone propionate (FP) although to the same extent as seen with IL- 1β -stimulated TGF- β 1 mRNA expression. This suggests that H_2O_2 induced a relative steroid insensitivity.

Both Suv39H1 protein level and mRNA expression were downregulated by IL- 1β and H_2O_2 stimulation. Knockdown of Suv39H1 with siRNA transfection induced a trend towards increased TGF- β 1 mRNA expression upon stimulation with IL- 1β and H_2O_2 . Suv39H1 was shown to be recruited to glucocorticoid receptor by co-immunoprecipitation. The anti-

inflammatory effect of FP was impaired under Suv39H1 knockdown which indicate that Suv39H1 may act as a corepressor of GR function.

DNA methylation is also involved in epigenetic regulation of gene expression. IL-1 β and H₂O₂ stimulation rapidly upregulated CpG methylation at specific sites in the TGF- β 1 promoter as demonstrated by direct sequencing after bisulphite treatment and by methylation dependent immunoprecipitation study (MeDIP). Methylation at the ATG start start induced by IL-1 β was reversed over time indicating an active demethylation.

These results indicate that H₂O₂, acting via the NF- κ B pathway, increases TGF- β 1 mRNA production and that this is regulated by FP possibly through an effect on the histone methyltransferase, Suv39H1. DNA methylation is also involved in the regulation of IL-1 β plus H₂O₂ induced TGF- β 1 mRNA expression.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Professor Ian M Adcock for his unlimited full support, and for sharing his knowledge during my studies. I would also like to thank my secondary supervisor Dr. Kaz Ito for the expert technical support he gave during troubleshooting.

I would also like to thanks the supports from my respectable colleagues, Elen, Andy, Grace, Sarah and everyone in our groups. Without their generous help, I could not have finished everything by myself.

I am grateful to Professor Fan Chung, my tutor during my PHD study, who gave me excellent advice whenever I faced difficulty.

I will never forget those who helped me when I was writing my thesis, Aristeia, Christos, Tom, Charris, and my colleagues from Chang Gung Memorial hospital, Chih-yung and Po-Jui.

Furthermore, I would like to thank Professor Kuo Han-Pin, Dr. Wang Chun-Hua, Dr. Lee Kang-Yung, and Dr. Liu Chien-Ying for their help and inspiration whenever I needed suggestions. I would like to thank Chang Gung Memorial Hospital for the financial support.

Finally, I would like to express my appreciation to my wife, and my mother, who took care of my two lovely daughters whilst I was away from Taiwan. I would also like to thank my father, although he is away from us, but I usually feel he is beside me whenever I am working alone during the night.

I would like to dedicate the thesis to all of you.

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List of Abbreviations

5-Aza	5-Azacytidine
5-MC	5-Methylcytosine
8-OH-dG	8-hydroxydeoxyguanine
AP-1	Activator Protein-1
ARDS	Adult respiratory distress syndrome
BMP-1	Bone morphogenetic protein-1
CBP	CREB binding protein
ChIP	Chromatin immunoprecipitation
COPD	Chronic obstructive pulmonary disease
CREB	cAMP response element-binding protein
Dex	Dexamethasone
DMSO	Dimethyl sulfoxide
DNMT	DNA methyltransferase
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FP	Fluticasone propionate
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Corticosteroid
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GR	Glucocorticoid receptor
GRE	Glucocorticoid responsive element

GSTP1	Glutathione S-transferase pi 1
HASM	Human airway smooth muscle
HAT	Histone acetylase
HDAC	Histone deacetylase
HMT	Histone methyl-transferase
HP1	Heterochromatin protein 1
hsp	Heat shock protein
IFN	Interferon
IkB	Inhibitors of NF-kB
IKK	IkB kinase
IL	Interleukin
IL-1RI	IL-1 receptor type I
IP	Immunoprecipitation
IRAK	Interleukin-1 receptor-associated kinase
JMJD2A	Jumonji domain containing 2; lysine (K)-specific demethylase 4A
JMJD3	Jumonji domain containing 3; lysine (K)-specific demethylase 6B
JNK	c-Jun NH2-terminal kinases
LABA	Long-acting beta-2-agonists
LAP	Latency-associated peptide
LLC	Large latent complex
LSD1	Lysine-specific histone demethylase 1
LTBP	Latent TGF- β binding protein
MAPK	Mitogen activated protein kinase
MBD	methyl-CpG binding domain
MBP	methyl-CpG-binding protein

MeCP2	methyl CpG binding protein 2
MeDIP	Methylation dependent immunoprecipitation
MEKK1	Mitogen and extracellular kinase kinase 1
MKK	MAPK kinase
MMP	Matrix Metalloproteinase
MyD88	Myeloid differentiation primary response gene (88)
NAPDH	Nicotinamide adenine dinucleotide phosphate
NF- κ B	Nuclear Factor kappa B
NLS	Nuclear localizing signals
PAGE	Polyacrylamide gel electrophoresis
PCAF	p300/CBP-associated factor
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
POMC	Pro-opiomelanocortin
QPCR	Quantitative polymerase chain reaction
RB	Retinoblastoma
RNA Pol II	RNA polymerase II
ROS	Reactive oxygen species
RT-QPCR	Reverse transcriptase-quantitative polymerase chain reaction
SAM	S-adenosylmethionine
SiRNA	Small interfering RNA
SLC	Small latent complex
SLPI	Secretory leucoprotease inhibitor
SMAD	Sma- and Mad-Related Proteins
SOB	Super optimal broth

SOC	Super optimal broth with catabolite repression
Sp-1	Specificity Protein 1
SRC	Steroid receptor coactivator - 2
SUV39H1	Suppressor of variegation 3-9 homolog 1
TAB	Transforming growth factor-activated kinase 1-binding protein
TAK1	Transforming growth factor-beta-activated kinase 1
TGF- β	Transforming growth factor- β
TNF- α	Tumor necrosis factor- α
TRAF	Tumor necrosis factor- α (TNF- α) receptor associated factor
TSP-1	Thrombospondin-1

CHAPTER ONE
GENERAL INTRODUCTION

BACKGROUND TO THE PROJECT

1.1 Chronic inflammatory airway disease

Many airway diseases are characterized by chronic inflammation resulted from multiple insults. Among them, asthma and chronic obstructive pulmonary disease (COPD) are two of the world's most prevalent diseases (O'Byrne and Postma, 1999). Both asthma and COPD are identified by the presence of characteristic symptoms and functional abnormalities with airway obstruction being the main feature. The inflammatory process in COPD is very different from that in asthma with different inflammatory cells, mediators, inflammatory effects and response to therapy (Barnes, 2000b).

In COPD, the airway obstruction is believed to result from a chronic inflammatory process mainly occurring in the small airway which leads to airway scarring and narrowing (Barnes, 2003). Reduced lung recoil, which relates to the emphysematous destruction of the lung parenchyma, is also due to the presence of chronic inflammatory which further compromises pulmonary function (Ogawa et al., 2004). In acute asthma, mucus plugging in the small airways and acute airway inflammation results from the exaggerated lower airway response to an environmental exposure leading to airway obstruction (Wark and Gibson, 2006). However, in chronic asthma, persistent airway obstruction results from airway remodelling with airway thickening due to basement membrane thickening, increased vascularity, mucous gland hypertrophy and smooth muscle hyperplasia (Vignola et al., 2003).

In most asthmatics, the occurrence of airway inflammation is in response to allergenic stimulation which is characterized by an eosinophilic inflammation with the predominant involvement of mast cells and CD4⁺ T-lymphocytes (Fabbri et al., 2003). By contrast, inflammation in COPD is a response to noxious stimuli, such as cigarette smoke, and is characterized by a neutrophilic inflammation mediated by macrophages and CD8⁺ T-lymphocytes (Barnes, 2000a).

At sites of lung inflammation and injury, numerous resident and recruited cell types,

including activated macrophages, eosinophils, platelets, epithelial cells, and fibroblasts, release TGF- β 1 (Chung, 2001) which in turn regulates many pathological changes in pulmonary disorders.

1.2 TGF- β 1 and its role in inflammatory airway disease

The TGF- β superfamily consists of a diverse range of proteins that regulate many different physiological processes including embryonic development, homeostasis, wound healing chemotaxis and cell cycle control. There are three TGF- β isoforms in humans - TGF- β 1, TGF- β 2 and TGF- β 3 - the amino acid sequences of which are 70–80% homologous but encoded by distinct genes located on chromosomes 19q13.1-q13.3, 1q41 and 14q24, respectively (Prime et al., 2004). TGF- β 1 is expressed in epithelial, hematopoietic and connective tissue cells; TGF- β 2 in epithelial and neuronal cells and TGF- β 3 primarily in mesenchymal cells (Massague, 1998). TGF- β isoforms are synthesized as latent precursors complexed with latent TGF- β binding proteins (LTBP-1, -3, and -4).

TGF- β is synthesized as a pre-pro-protein (pre-pro-TGF- β) (Fig. 1.1). After proteolytic processing in rough endoplasmic reticulum, two TGF- β monomers are dimerized via a disulphide bond to form pro-TGF- β . After cleavage by furin convertase, the mature protein forms non-covalent bonds with the latency associated peptide (LAP) to form the small latency complex (SLC). The large latent TGF- β complex (LLC) is subsequently formed by covalent attachment of SLC with the large latent binding protein (LTBP). TGF- β is activated by extracellular proteolytic cleavage of its carboxyl-terminal pro-region to which LTBP is bound thereby removing LTBP (Annes et al., 2003).

TGF- β itself cannot bind to its receptors in the latent form and must be liberated from the constraints of LAP and LTBP. *In vitro*, the activation can be achieved by using low PH (Jullien et al., 1989), heat, some proteases (Annes et al., 2003) or exposure to reactive oxygen

species (Barcellos-Hoff and Dix, 1996). *In vivo*, the mechanism is less clear, but possibly includes proteolytic activation by transglutaminase (Nunes et al., 1995), conformational change of LAP through physical interaction with thrombospondin (Crawford et al., 1998) or by mechanical traction via $\alpha v\beta 6$ integrins (Munger et al., 1999).

The TGF- $\beta 1$ promoter contains both binding sites for the redox-sensitive transcription factors activator protein (AP)-1 and nuclear factor kappaB (NF- κ B), which are both involved in the transcriptional regulation of TGF- $\beta 1$. TGF- $\beta 1$ itself can induce c-Jun and c-Fos which bind to their respective binding sites to further stimulate TGF- $\beta 1$ production in a feedforward manner (Kim et al., 1990; Kim et al., 1989). Interestingly, c-Jun-NH2-terminal kinase 1 and 2 (JNK1 and JNK2), the kinases that activate AP-1 activity, can both inhibit TGF- $\beta 1$ expression in fibroblasts (Ventura et al., 2004) which suggests that AP-1 may play a dual role in the regulation of TGF- $\beta 1$. NF- κ B is also involved in interleukin (IL)-1 β -induced transcriptional regulation of TGF- $\beta 1$ in airway epithelial cells (Lee et al., 2006). Post-transcriptional or post-translational regulation has also been reported for TGF- $\beta 1$ and this often results in discrepancies between changes in mRNA and protein levels (Allison et al., 1998; Kim et al., 1992).

TGF- $\beta 1$ is the isoform commonly implicated in inflammation and is upregulated in response to lung injury. Elevated expression of TGF- $\beta 1$ occurs in a number of pulmonary disease such as COPD (de Boer et al., 1998; Takizawa et al., 2001), asthma (Redington et al., 1997a; Vignola et al., 1997) as well as pulmonary fibrosis (Khalil et al., 1996). A significant increase in TGF- $\beta 1$ expression in the mucosa and submucosal cells in asthmatic and chronic bronchitis patients was found by immunohistochemistry and *in situ* hybridization of TGF- $\beta 1$ mRNA, (Vignola et al., 1997). The numbers of TGF- $\beta 1$ +ve cells were also associated with basement membrane thickness and fibroblast numbers. In COPD patients, it was also shown that the expression of TGF- $\beta 1$ mRNA and protein in the airway epithelium also correlate with the presence of intraepithelial macrophages (de Boer et al., 1998). These findings suggest that

TGF- β 1 is actively involved in chronic inflammatory lung disease.

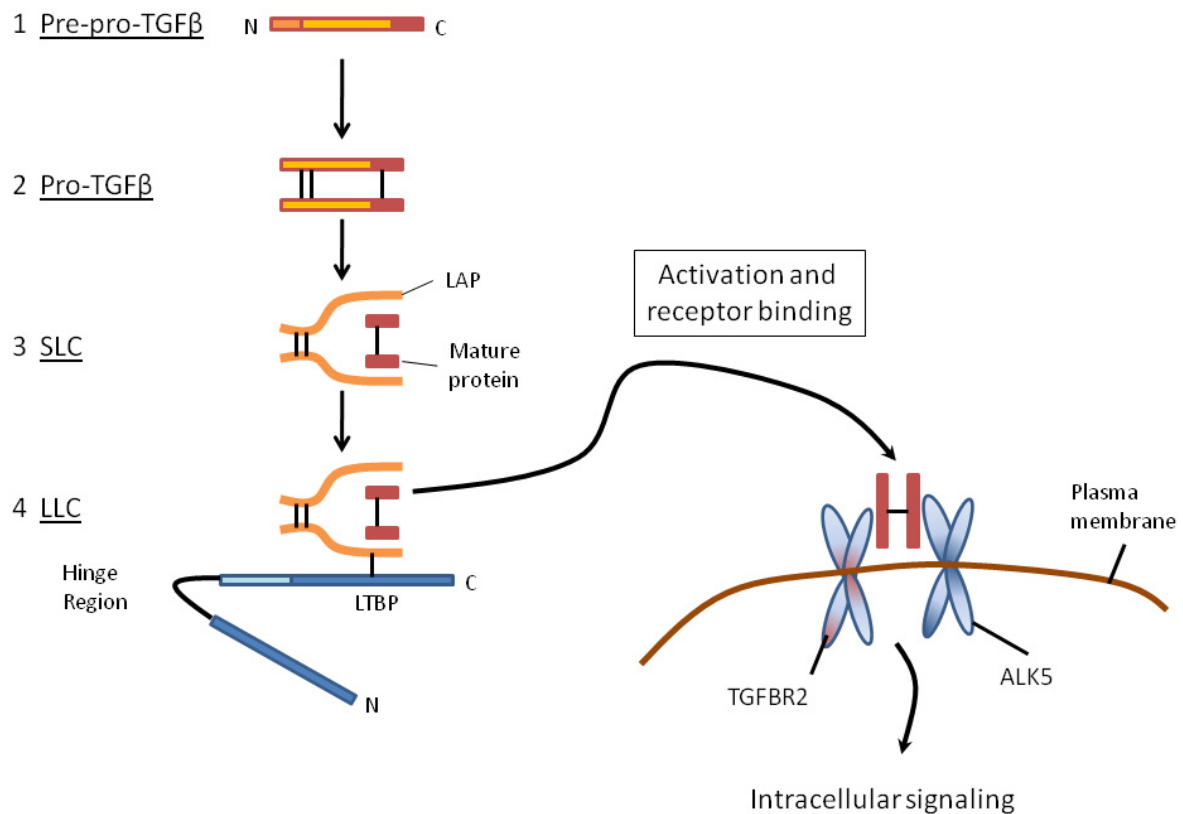


Figure 1.1 Schematic model of TGF- β regulation of synthesis and secretion. TGF- β is synthesized as a pre-pro-TGF- β form. After proteolytic processing in the rough endoplasmic reticulum, two TGF- β monomers are dimerized by the formation of disulphide bonds to produce pro-TGF- β . After cleaved by furin convertase, the latency associated peptide (LAP) and mature protein are bound together by non-covalent bonds to form the small latency complex (SLC). The large latency complex (LLC) is formed by covalent attachment of the SLC with the large latent binding protein (LTBP). Through the interaction with extracellular matrix, LLC is cleaved by proteolytic reaction allowing the release of mature TGF- β . Important molecules involved in this process including elastase, bone morphogenetic protein-1 (BMP1) and matrix metalloproteinase 2 (MMP2). TGF- β can bind to the TGFBR2 and ALK5 receptors to induce intracellular signalling.

1.3 The role of IL-1 β and NF- κ B in inflammatory lung disease

During inflammation, both resident and recruited cells can release a plethora of inflammatory mediators that result in clinical symptoms. IL-1 is one of the key cytokines involved in driving the inflammatory response in many chronic inflammatory diseases (Dinarello, 2009). Importantly, IL-1 β levels are elevated in induced sputum from stable COPD patients (Keatings et al., 1996) and also observed from alveolar macrophages from cigarette smokers and COPD patients (Lim et al., 2000). The effects of IL-1 β are mediated by the type I IL-1 receptor (IL-1RI) (Sims et al., 1988). Upon binding to IL-1RI, IL-1 stimulates the production of a series of proinflammatory proteins including enzymes, adhesion molecules, chemokines and acute-phase proteins (Dunne and O'Neill, 2003).

Currently, there are 11 members of the IL-1 family (IL-1F) of ligands (Dinarello, 2009). IL-1 β (IL-1F2) has been well studied *in vivo*, in disease, and in animal models and acts as an agonist; while IL-1Ra (IL-1F3) plays a role as a receptor antagonist for IL-1RI, which inhibits both effect of IL-1 α and IL-1 β . Both IL-1 α and IL-1 β bind to IL-1RI, which is recognized as a part as an interleukin-1 receptor/toll-like receptor superfamily and is involved in host defense and inflammation (O'Neill and Greene, 1998). There are major four protein kinase cascades which can be activated by IL-1 β namely NF- κ B, p38 mitogen activated protein kinase (MAPK), extracellular regulated kinase (ERK)1 and ERK2, and JNK pathways (Dunne and O'Neill, 2003).

The activation of NF- κ B by IL1RI is initiated by forming a complex containing myeloid differentiation primary response gene (MyD)88, IL-1R-associated kinase (IRAK)1 and IRAK-2 (Cao et al., 1996a; Wesche et al., 1997) (Fig 1.2). IRAK-1 recruits TNF receptor-associated factor (TRAF)-6 (Cao et al., 1996b) which further attracts TGF- β -activated kinase (TAK1)-binding protein (TAB-2) and forms a complex which is able to activate TAK-1 (Takaesu et al., 2000). Activation of TAK-1 triggers activation of the inhibitor of I κ B (IKK) complex and the NF κ B pathway. The IKK complex is composed of IKK α , IKK β and a

scaffold protein IKK γ (or NF- κ B essential modulator, NEMO). In most cell types, NF- κ B dimers are retained in the cytoplasm by I κ Bs, which are specific inhibitors that bind to the Rel-homology domain (RHD) and interfere with NF- κ B's nuclear localization sequence (NLS) function. IKK α and IKK β can phosphorylate I κ B α on two serines, Ser³² and Ser³⁶ (Brown et al., 1995; DiDonato et al., 1996; Traenckner et al., 1995), leading to ubiquitination of I κ B α and its subsequent proteasomal degradation. The released NF- κ B dimer (mostly the p50-p65 dimer) translocates to the nucleus, binds to specific DNA binding sites in responsive genes and activates gene expression (Ghosh and Karin, 2002). IKK complexes can also phosphorylate and regulate NF- κ B1 (p105/p50) and NF- κ B2 (p100/p52) precursor processing (Salmeron et al., 2001; Senftleben et al., 2001).

NF- κ B is ubiquitously expressed and is activated by numerous extracellular stimuli, including cytokines such as TNF- α and IL-1 β . NF- κ B activation is seen in many lung diseases including Adult respiratory distress syndrome (ARDS), systemic inflammatory response syndrome, asthma, COPD and occupational and environmental lung disease (Christman et al., 2000). Further studies also support that concept that epithelial-specific NF- κ B is important in the control of lung inflammation and injury. Thus, it is evident that IL-1 β and the subsequent synchronized activation of the NF- κ B pathway is important in lung inflammation.

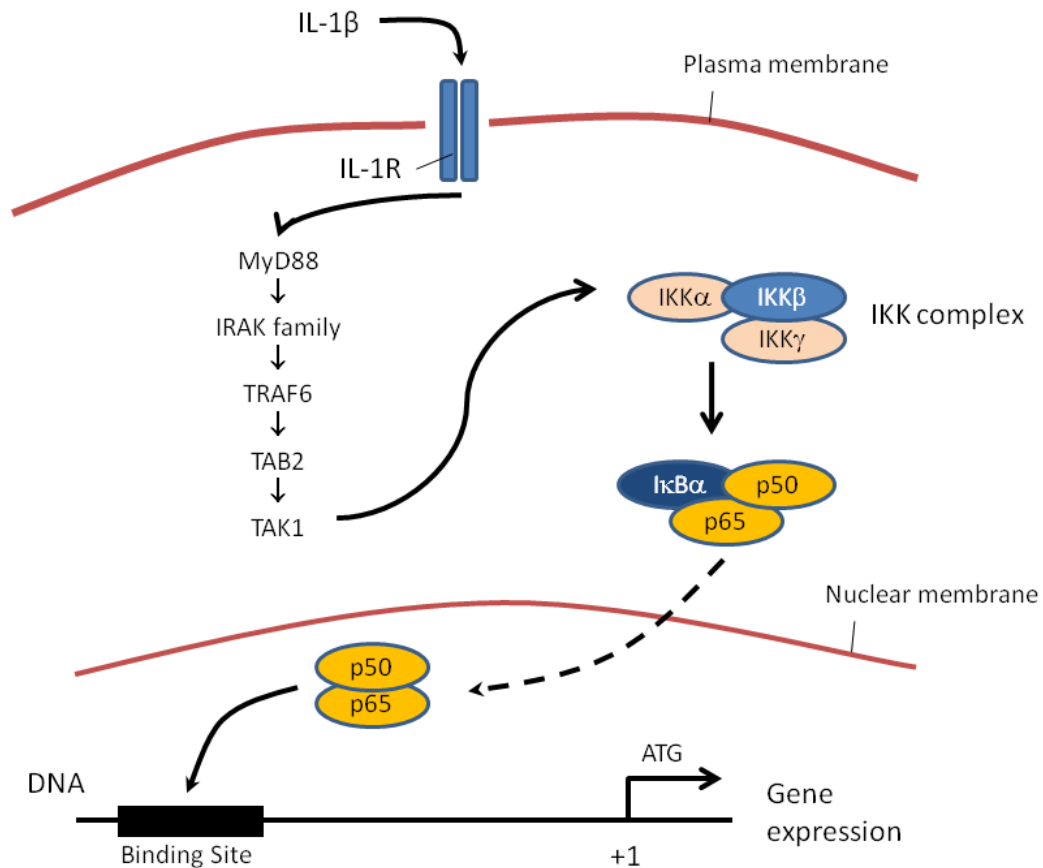


Figure 1.2 Activation of NF-κB pathway by interleukin (IL)-1β. Binding of IL-1β to the type I IL-1 receptor (IL-1RI) triggers a phosphorylation cascade involving MyD88, IRAK family members, TRAF6 and the IKK complex. Activation of IKKα and IKKβ phosphorylates IκBα leading to release and nuclear translocation of the p50/p65 complex. In the nucleus, p65 binds to DNA binding sites in the promoters of responsive genes resulting in coordinated expression of multiple genes.

1.4 Oxidative Stress

Oxidative stress refers to a state characterized by either an excess production of reactive oxygen species (ROS) and/or a reduction in antioxidant defences responsible for the metabolism and elimination of ROS (Franco et al., 2008). Biological systems are exposed to various oxidant stresses whether generated exogenously e.g. environmental insults as air pollutants or cigarette smoke, or endogenously by metabolic reactions within mitochondria (Rahman and Adcock, 2006). Biologically important reactive oxygen species (ROS) include the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^\cdot$) and hypohalous acids such as $HOCl$ (Chow et al., 2003). ROS causes oxidation of proteins, DNA and lipids, which may further cause direct cellular injury or induce a variety of cellular responses through the generation of secondary metabolic reactive species (Fig 1.3).

Glutathione (GSH) metabolism is involved in the balance between pro-oxidant and antioxidant molecules; acts as defence against constant oxidative challenge and helps to maintain the cellular redox status (Forman and Dickinson, 2003). Recently, the role of two major redox forms, reduced GSH and glutathione disulphide (GSSG) has been clarified (Rahman, 2005a). The ratio of cytoplasmic GSH/GSSG has been implicated in a range of cellular processes, such as cellular signalling and gene expression, and can be used as an indicator of the cellular redox state (Ghezzi, 2005). Glutathione peroxidase reduces H_2O_2 to H_2O by oxidising glutathione (Fig 1.4). The other enzyme, glutathione reductase, catalyzes the oxidized form of GSSG to GSH, to recycle the GSH and help to maintain the cellular redox status.

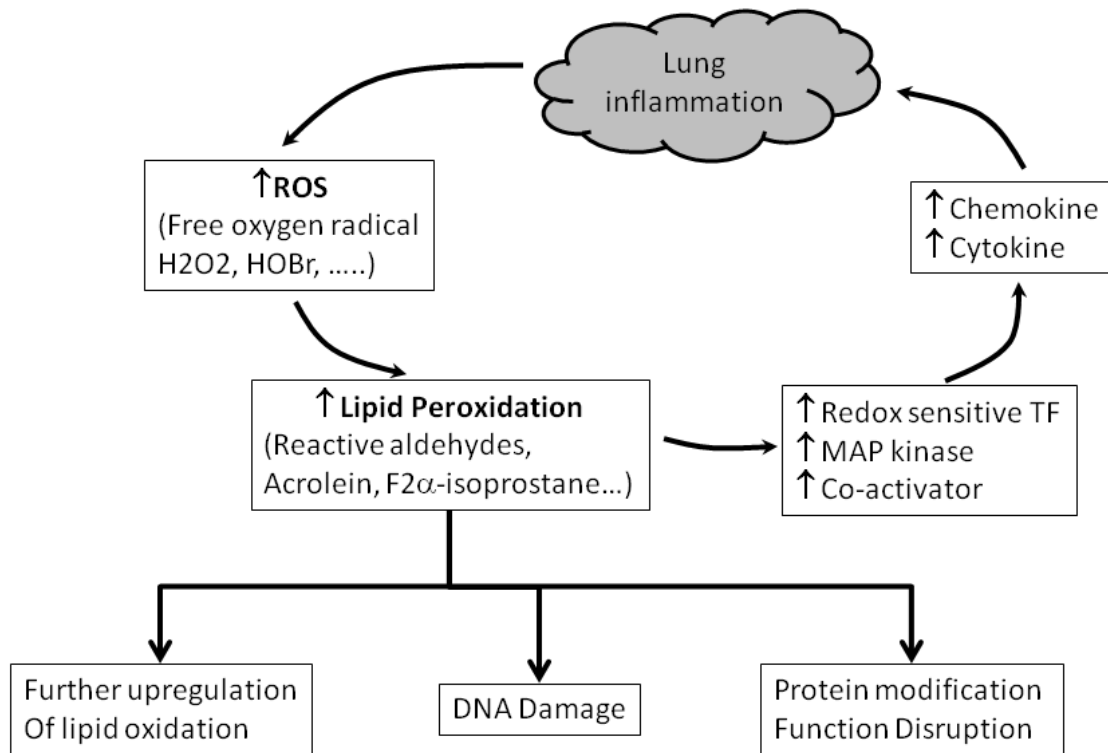


Figure 1.3 Mechanisms of reactive oxygen species (ROS)-mediated lung inflammation. The cascade of proinflammatory chemokine and cytokine release activated in lung inflammation along with increased metabolic rate may upregulate oxidative stress and generate distinct types of ROS including free oxygen radicals, H_2O_2 etc. ROS may damage cell membranes resulting in increased lipid peroxidation and its products including reactive aldehydes and acrolein which may further activate lipid oxidation. This results in a feedforward cycle leading to more lipid peroxidation products, increased DNA damage and enhanced protein modification. These lipid peroxidation products are also able to increase the expression of redox sensitive transcription factors and activate MAP kinase pathways. These changes trigger a vicious cycle, and further exaggerate lung inflammation.

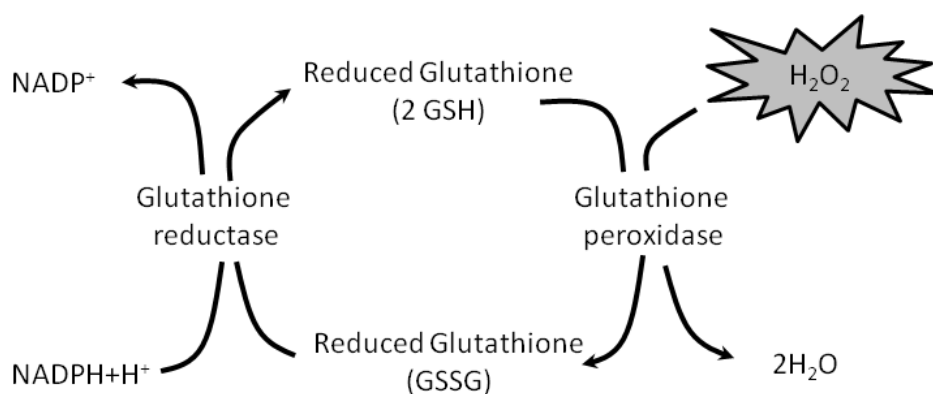


Figure 1.4 The Glutathione Oxidation Reduction (Redox) Cycle. Both nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione reductase is important for maintenance of GSH in active form.

1.5 Role of oxidative stress in inflammatory airway disease

The common source of ROS in the lung is from macrophages and neutrophils and numbers of these cells are elevated in the lung by cigarette smoke (Rahman and MacNee, 1996). Activated macrophages may release mediators which further attract neutrophils and other inflammatory cells and generate more ROS in the airways. ROS are highly reactive and, when generated close to cell membranes, can induce lipid peroxidation (Uchida et al., 1999). These lipid peroxidation products, including malondialdehyde, 4-hydroxy-2-nonenal, acrolein and F₂-isoprostanes, may abrogate cell membrane function; inactivate membrane-bound receptors and enzymes and further increase tissue permeability (Rahman, 2005b).

ROS can also lead to the activation of intracellular signalling cascades including members of the MAPK family such as the JNK and p38 MAPK kinases and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway leading to increased gene transcription (Adler et al., 1999). ROS are able to activate the redox-sensitive transcription factors such as

NF- κ B (Adcock et al., 1994; Janssen-Heininger et al., 2000) and AP-1 (Xanthoudakis and Curran, 1996), which may be responsible, at least in part, for the amplified inflammatory response in chronic airway disease (Barnes and Karin, 1997; Hart et al., 1998).

The mechanisms that lead to activation of NF- κ B by oxidative stress may be cell specific. Several mechanisms are proposed for H₂O₂-induced NF- κ B activation. H₂O₂ may enhance the activity of IKKs through phosphorylation of Ser180 in IKK1 or of Ser 181 in IKK2 in HeLa cells (Jaspers et al., 2001; Kamata, 2002). Oxidative stress may also cause ubiquitination and phosphorylation of the I κ B α complex and thereby cause subsequent I κ B α degradation (Ginn-Pease, 1996). Additionally, proteasome enzymatic activity may also be affected by oxidative stress leading to activation of NF- κ B (Okada et al., 1999). A recent study also indicates that H₂O₂ may synergise with IL-1 β in a process which involves IL-R1/Nox2 action which activates TRAF6 and leads to IKK and NF- κ B activation (Li et al., 2006c) (Fig 1.5). These findings indicate that in some cells oxidative stress induces the activation and translocation of NF- κ B to the nucleus and facilitates the binding of NF- κ B to DNA.

Recent evidence has indicated that oxidative stress is also involved in gene transcription through effects on chromatin modifications and in particular on the relative activities of histone acetylation/deacetylation (Ito et al., 2004; Moodie et al., 2004; Tomita et al., 2003). This results in enhanced transcription of specific inflammatory genes. Oxidative stress reduces histone deacetylase 2 (HDAC2) protein expression probably through proteasomal targeting (Kramer et al., 2003) and results in elevated proinflammatory gene expression and reduced glucocorticoid action (Barnes et al., 2005). The role of other histone modifications, such as histone methylation, is not clear in this condition.

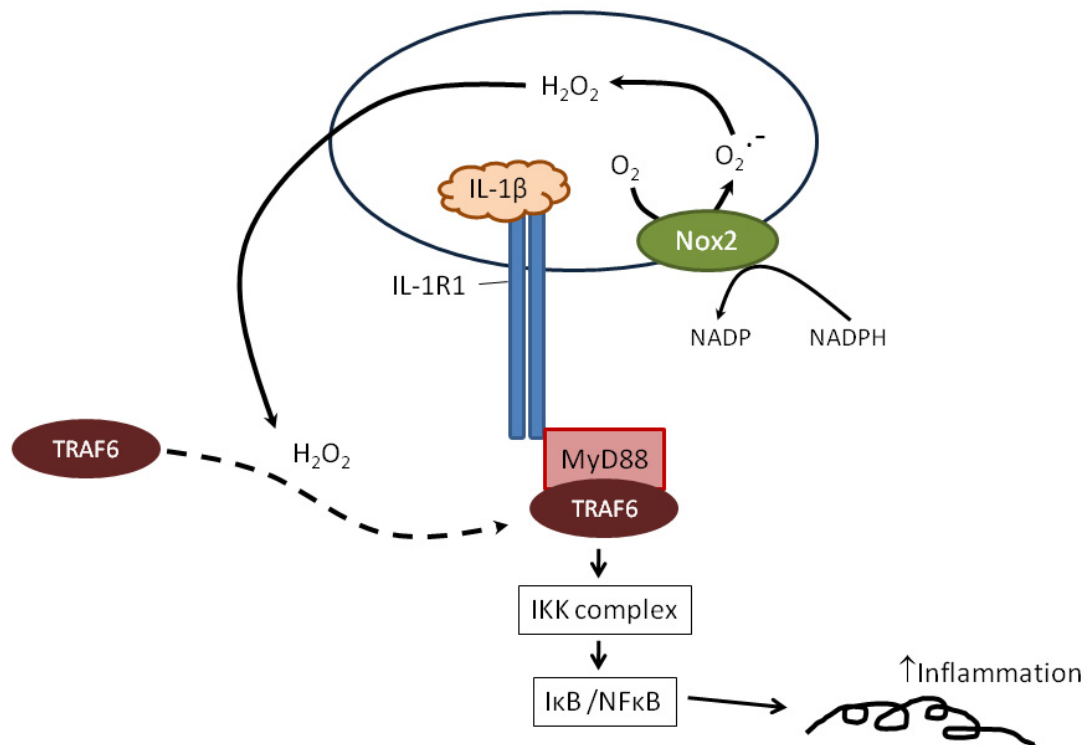


Fig 1.5 Synchronized involvement of H_2O_2 and $\text{IL-1}\beta$ in $\text{NF-}\kappa\text{B}$ activation. Upon binding of $\text{IL-1}\beta$ to the type I IL-1R (IL-1R1), it triggers a MyD88-dependent endocytosis of IL-1R1 , and endosomal ROS formation, which further triggers TRAF6 action leading to activation of the $\text{NF-}\kappa\text{B}$ pathway. This event is regulated through Nox2-dependent H_2O_2 production.

1.6 Effect of corticosteroids on regulation of lung inflammation

Corticosteroids are the most effective anti-inflammatory treatment for asthma and many other inflammatory airway diseases (Barnes and Adcock, 2003). Corticosteroids exert their effects by binding to a glucocorticoid receptor (GR) that is localized to the cytoplasm of target cells (Ito et al., 2006a). A single gene encodes human GR, but several variants e.g. GR α , GR β , GR-A and GR-B etc, generated by alternative transcript splicing and/or alternative translation initiation have been recognized and implicated in modulating the function of the major isoform GR α (Rhen and Cidlowski, 2005). GR may be modified by phosphorylation and other modifications which thereby alter the functional response to corticosteroids (Barnes, 2006). After corticosteroids have bound to GR, the activated GR-corticosteroid complex is transported to the nucleus and binds to glucocorticoid response elements (GREs) within the promoter region of responsive genes leading to increased anti-inflammatory gene transcription. This DNA-binding process has been postulated to not only induce an anti-inflammatory effect via transactivation but may also mediate some of the side effects of corticosteroids when activated GR binds to negative GREs or to GREs that cross the ATG start site (cis-repression effect) (Fig 1.6).

Corticosteroids also suppress inflammatory gene expression through trans-repression effects on pro-inflammatory transcription factors such as NF- κ B and AP-1 (Barnes and Karin, 1997). Recent studies have also indicated the GR may elicit its anti-inflammatory effects through changes in histone acetylation. Activated GRs may bind to NF- κ B which is associated with transcriptional co-activator proteins such as cAMP response element binding protein (CREB) binding protein (CBP) or other coactivators to inhibit their histone acetyltransferase (HAT) activity (Ito et al., 2000). Alternatively, GR may recruit repressor proteins such as histone deacetylase (HDAC)2 to the activated NF- κ B transcriptional complex, resulting in deacetylation of histones and thereby repressing inflammatory genes (Ito et al., 2000).

Oxidative stress due to cigarette smoking is known to affect the response to glucocorticoids especially in COPD and some asthmatic patients (Chalmers et al., 2002; Culpitt et al., 1999; Culpitt et al., 2003). ROS may affect glucocorticoid receptor activation either directly or indirectly. In primary human fibroblasts exposure to H₂O₂ can attenuate GR nuclear transport in response to dexamethasone in a time- and concentration-dependent manner (Okamoto et al., 1999). In addition, oxidative stress may also reduce the expression and activity of HDAC2 which may account for the reduced expression and activity observed in BAL macrophages from COPD patients. This effect occurs without significantly modulating HAT activity (Ito, 2004; Ito et al., 2005). The mechanism by which oxidative stress reduces HDAC2 activity may involve increased tyrosine nitration of HDAC2, which is detected in healthy smokers and COPD patients, and is inversely correlated with HDAC2 activity (Ito, 2004).

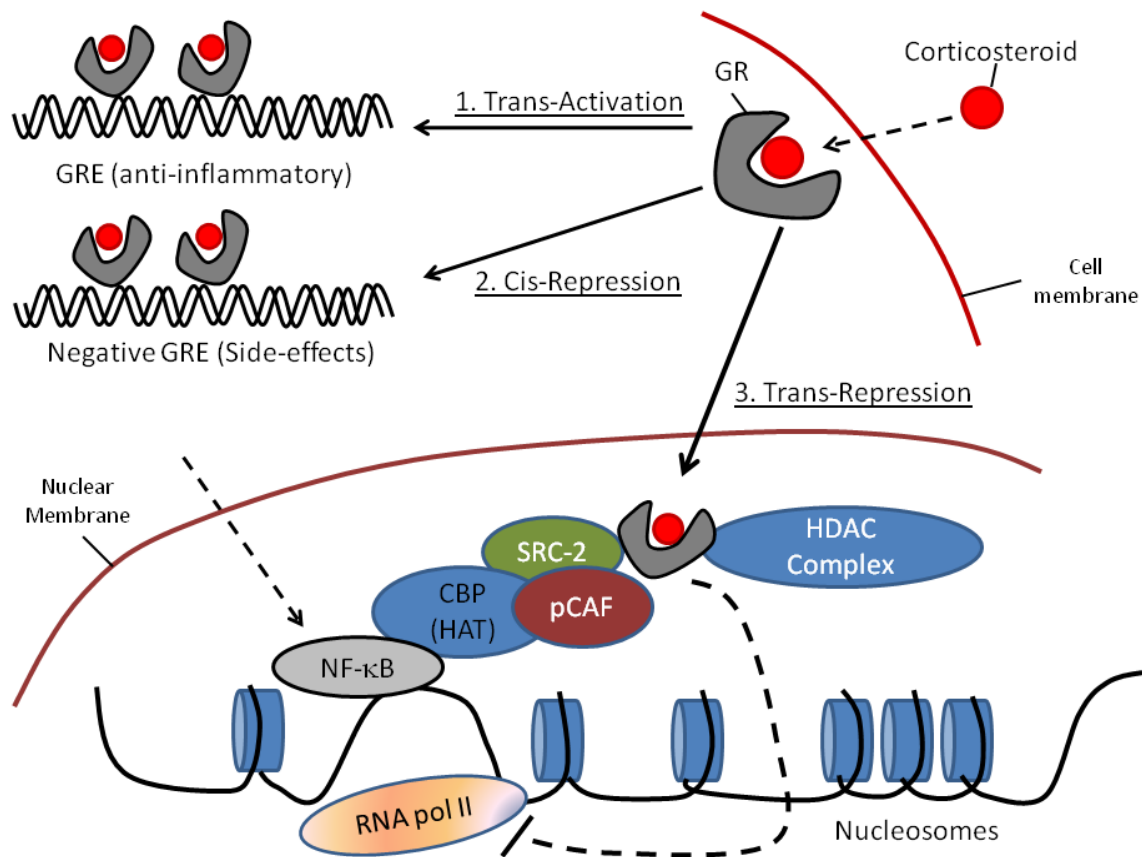


Fig 1.6 Corticosteroid actions on gene expression. Corticosteroids enter the cell and bind to and activate cytoplasmic glucocorticoid receptors (GR) which translocate into the nucleus. GR homodimers may (i) bind to glucocorticoid response elements (GREs) in the promoter region and induce upregulation of anti-inflammatory genes; or (ii) bind to negative GREs and suppress gene expression. GR may also associate with coactivator molecules, such as cAMP-response element binding protein binding protein (CBP), p300/CBP-associated factor (pCAF) or steroid receptor coactivator (SRC)-2, which have histone acetyltransferase (HAT) activity. Activated GR can inhibit HAT activity and recruit histone deacetylase (HDAC)2 which reverses histone acetylation, leading to suppression of inflammatory genes.

1.7 Chromatin template and histone code

Chromatin is the physiological template of the genetic information that exists in almost all eukaryotic cells. It may present as a decondensed polymer (euchromatin) in interphase or in a highly compacted state (heterochromatin) that localizes to pericentric regions (Lachner and Jenuwein, 2002). Chromatin is made up of nucleosomes (Kornberg, 1974), which are structures consisting of 147 base pairs of DNA wrapped around an octamer of two molecules each of the core histone proteins (histones 2A, 2B, 3 and 4) (Luger et al., 1997). Strings of nucleosomes line up to generate an 11nm fibre which is further compacted into a 30nm fibre whose structure is stabilised, at least partially, through the incorporation of the linker molecule, histone H1 (Cheung et al., 2000; Jenuwein and Allis, 2001; Kouzarides, 2007; Li et al., 2007).

Core histones are small, highly basic proteins whose amino-terminal tails extend through and beyond the DNA coil. These tails are subject to a variety of post-translational modifications including acetylation, phosphorylation, methylation, ubiquitination, ADP-ribosylation, sumoylation, deimination and proline isomerisation (Kouzarides, 2007). The term “histone code” has been used to describe the role of these modifications in controlling gene expression through changes in chromatin structure (Jenuwein and Allis, 2001).

The function of histone of modifications may be divided into two parts. Firstly, global changes in histone methylation for example have been linked to the establishment of global chromatin environments, such as the formation of heterochromatin, where DNA is accessible to transcription, and to that of euchromatin, where DNA is inaccessible for transcription. Secondly, histone modifications are associated with the orchestration of DNA-based biological tasks (Kouzarides, 2007).

Chromatin structure plays an important regulatory role and multiple signalling pathways converge on histones (Cheung et al., 2000). Covalent modifications of histone tails directed by DNA sequence-directed transcription factors allows the sequential recruitment of

transcriptional co-activator proteins, chromatin remodelling engines, basal transcription factors and RNA polymerase II and the initiation of gene expression. This, therefore, not only provides a mechanism to enable histone proteins to translate the information encoded by the underlying DNA sequence but also importantly extends the amount of information that regulates the genome to beyond that present in the genetic code itself (Jenuwein and Allis, 2001).

It was initially proposed that post-translational modifications on histone tails altered the charge on the histone tail thereby modulating the electrostatic interaction between the negatively charged DNA and the positively charged histones and thus opening up the chromatin structure (Collingwood et al., 1999; Wolffe et al., 2000). It is becoming clearer, however, that these post-translational marks also act as recruitment signals (e.g. bromo- and chromo-domains) for other transcriptional co-activators or remodelling enzymes that enable co-ordinated transcription to occur (Li et al., 2007).

This mechanism may also underlie, in part, the recent studies that indicate the existence of crosstalk between the various histone modifications. In addition to the synergistic effect of enzyme activity by second chromatin modification (Clements et al., 2003), other possible mechanisms include the disruption of protein binding by adjacent modifications (Fischle et al., 2005) and that the catalytic activity of the enzyme is compromised by modifications of its substrate recognition site (Nelson et al., 2006).

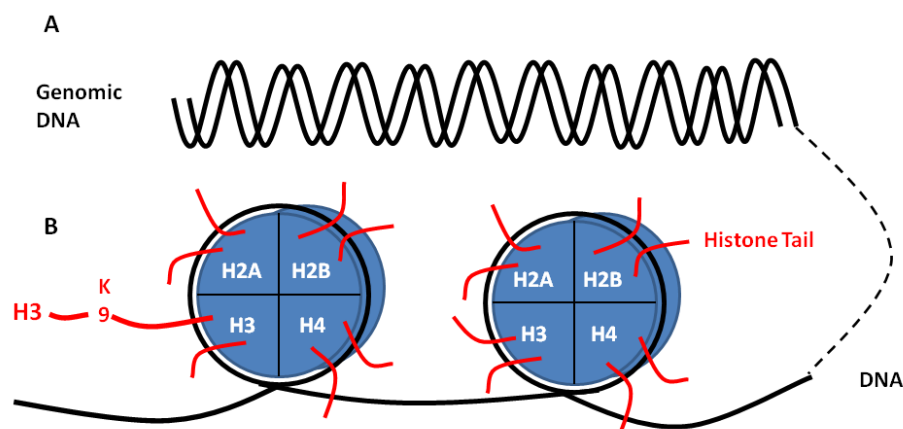


Figure 1.7 Schematic of the structure of histones in nucleosomes. The core proteins of nucleosomes are designated H2A (histone 2A), H2B (histone 2B), H3 (histone 3) and H4 (histone 4). Each histone is present in two copies, so that 147bp of DNA (black) wrap itself almost twice around an octamer of histones — the core nucleosome.

1.8 Histone acetylation and inflammatory gene expression

Transcriptional co-activators such as CBP, steroid receptor coactivators (SRC)-1 and p300/CBP associated factor (PCAF) have intrinsic histone acetyltransferase (HAT) activity and increased inflammatory/immune gene transcription is associated with an increase in histone acetylation at transcriptional start sites (Adcock et al., 2006; Wang et al., 2009). Changes in the acetylation status of specific lysine (K) residues in the N-terminal tails of histones 3 and 4 form a molecular tag for the recruitment of ATP-dependent chromatin remodelling enzymes such as Brg1 (Bilodeau et al., 2006) which allows local chromatin unwinding and the recruitment of other transcription factors, the basal transcriptional complex and RNA polymerase II (Adcock et al., 2006). This general molecular mechanism is common to all genes, including those involved in differentiation, proliferation, DNA repair and activation of cells (Adcock et al., 2006).

In general, acetylation of histones H3 and H4 along with histone H3 K4 methylation, results the formation of tags that allow subsequent recruitment of other transcriptional complexes, loosening of the local nucleosomal structure and gene transcription to occur (Wang et al., 2008). These changes occur predominantly at the transcriptional start site (Wang et al., 2009): are closely associated with transcription factor DNA binding and gene expression (Heintzman et al., 2009) and appear invariant across many cell types in contrast to the greater variability seen in histone modifications in enhancer regions (Heintzman et al.,

2009). Less frequently transcribed genes have repressive marks, remain more tightly packaged and therefore less accessible to the transcriptional mechanisms of the cell (Lee et al., 2009; Wang et al., 2008).

In contrast to the activation effect of acetylation, the removal of the acetyl group from the nucleosome, often in conjunction with a change in DNA methylation status, is associated with gene repression (Adcock et al., 2006). HDACs interact with corepressor molecules, which aid HDACs in gene repression and may provide specificity by selecting which genes are regulated by individual HDACs. The balance between HAT and HDAC activity is a key component of gene expression including inflammatory gene expression. Evidence for a key role of HDACs in inflammatory gene expression is provided by the fact that HDAC inhibitors such as trichostatin A (TSA) enhance NF- κ B-driven inflammatory gene transcription *in vitro* in different cell types with many distinct stimuli (Adcock et al., 2006). The development of selective HAT inhibitors such as anacardic acid provides further evidence for a key role of HAT activity in inflammatory gene expression in primary human cells within the airways (Wort et al., 2009).

Changes in histone acetylation are involved in the induction of numerous pro-inflammatory genes in human lung epithelial and smooth muscle cells (Adcock et al., 2006). In human airway smooth muscle (HASM) cells TNF α -stimulated eotaxin release is associated with NF- κ B binding and histone H4 acetylation of the *eotaxin* gene promoter region (Nie et al., 2005). In contrast IFN γ is able to markedly inhibit TNF α -induced expression of the NF- κ B-sensitive genes *IL-6*, *IL-8*, and *eotaxin* (Keslacy et al., 2007). IFN γ decreased TNF α -induced p65-associated HAT activity and increased total nuclear HDAC activity. Importantly, the HDAC inhibitor TSA prevented the inhibitory effect of IFN γ on TNF α -induced gene expression.

Allergic diseases are characterised by a T_H2 dominant immune response and evidence suggests that HDACs maintain pre-established T_H1-like and T_H2-like immunity in human T-

cells (Ruey-Chyi et al., 2008). Phytohemagglutinin (PHA) activation selectively stimulates antigen-driven CD45RO(+) memory T cells, eliciting recall cytokine responses (Ruey-Chyi et al., 2008). The HDAC inhibitor TSA provoked total cell hyperacetylation and led to increased T_H2-associated IL-13 and IL-5 cytokine expression and reduced T_H1-associated IFN γ and IP10-associated recall responses. In addition, IL-2 and IL-10 production was also reduced. TSA treatment shifted the T_H1:T_H2 ratio 3-8-fold, skewing the recall responses more towards a T_H2-like phenotype, independent of the stimulus used and highlighting the role of HDACs in the development and maintenance of T_H1:T_H2 immunity (Ruey-Chyi et al., 2008). Using overexpression and CAT-reporter genes, Lavender and colleagues (Jee et al., 2005) have shown that GR reduced GATA-3-mediated IL-5 and -13 promoter activity in human CD4+ T-cells. The authors postulated that local recruitment of GR may alter the ability of GATA-3 either to bind to its target site, to cause transcriptional up-regulation, or to maintain an environment that is permissive for transcription. Overall, inflammatory stimuli including environmental agents, viral infection, and allergen exposure can lead to enhanced inflammatory gene expression and T_H2 skewing as a result of changes in histone acetylation status. Enhanced histone acetylation being associated with inflammation and reduced acetylation with decreased inflammation.

This simple model is more complex as it is now clear using ChIP-seq (chromatin immunoprecipitation linked to high throughput sequencing) that HDACs are also associated with actively transcribed genes (Wang et al., 2009). The relative level of HATs and HDACs at specific promoters allows active gene expression or can pause the gene for transcription.

1.9 HDAC and HAT expression in chronic lung disease

Altered HDAC/HAT activities have been reported in chronic lung diseases (Adcock et al., 2006). Nuclear HDAC activity was reduced in PBMCs from patients with steroid

unresponsive asthma compared with steroid responsive asthmatic patients and, importantly, the reduced HDAC activity correlated directly with steroid insensitivity. In addition, Hayglass and colleagues have recently reported that reduced HDAC/HAT activity correlated with increasing bronchial hyperresponsiveness in asthmatic children (Ruey-Chyi et al., 2009). However, reduced HDAC2 expression has not been seen in all steroid responsive asthmatic patients perhaps reflecting the heterogeneity of severe asthma phenotype (Celine et al., 2006). HDAC2 expression and activity is also decreased in asthmatics, smokers, smoking asthmatics (Adcock et al., 2006), patients with COPD (Adcock et al., 2006; Barnes et al., 2005; Ito et al., 2006b) and in cystic fibrosis (Bartling and Drumm, 2009), all of which are known to be insensitive to the anti-inflammatory effects of glucocorticoids (Adcock et al., 2006). In addition, there is a negative correlation between the repressive effect of dexamethasone on cytokine production and total HDAC activity in alveolar macrophages from smokers and non-smokers (Adcock et al., 2006). Overexpression of HDAC2, but not HDAC1, in primary macrophages from COPD patients restored dexamethasone efficacy towards suppressing LPS-induced GM-CSF release to levels seen in cells from healthy control subjects (Ito et al., 2006b).

1.10 Histone lysine (K) methylation and the histone methyltransferase (HMT) Suv39H1

Lysine methyltransferases have more site specificity when compared to histone acetyltransferases. Three methylation sites on histones are implicated in activation of transcription: H3K4, H3K36 and H3K79; while another three lysine methylation sites are connected to transcriptional repression, which includes H3K9, H3K27 and H4K20.

Histone H3 K9 can be mono-, di- or tri- methylated (Shilatifard, 2006). Different HMTases have recently been found responsible for each modification state. G9a is thought to be responsible for all mono and di methylation of H3 K9 in silent regions of heterochromatin

(Tachibana et al., 2002). In contrast, Suv39H1 is thought to be responsible for the trimethylation of H3K9 found in pericentric heterochromatin (Nakayama et al., 2001). Repression of gene expression by H3K9 methylation involves the recruitment of methylating enzymes and heterochromatin protein 1 (HP1) to the promoter of repressed genes. But recently, H3K9 trimethylation and HP1 γ recruitment have been found to be enriched in the coding region of active genes (Vakoc et al., 2005). This indicates that H3K9 methylation may have a different function on gene expression when enriched in a gene promoter area or coding region.

Suv39H1, the K9 selective histone methylase in eukaryotic cells and the methyl-lysine binding protein HP1 may interact to repress transcription at heterochromatic sites (Rea et al., 2000). Lysine 9 of histone H3 is methylated by Suv39H1, creating a binding site for the chromo domain of HP1 (Bannister et al., 2001; Lachner et al., 2001). The HMT activity of Suv39H1 resides in the SET domain. Suv39H1 and HP1 are both involved in the repressive functions of the retinoblastoma (Rb) protein (Nielsen et al., 2001). For example, Suv39H1 cooperates with Rb to repress the cyclin E promoter (Nielsen et al., 2001). Thus, the Suv39H1-HP1 complex is not only involved in heterochromatic silencing but also has a role in repression of euchromatic genes by Rb and perhaps other repressor proteins (Martin and Zhang, 2005).

It was thought originally that histone methylation marks were permanent, until the discovery of the first histone demethylase lysine specific demethylase 1 (LSD1) (Shi et al., 2004). LSD1 acts to demethylate H3K4 and repress transcription. Interestingly, when LSD1 is associated with the androgen receptor, it is also able to demethylate H3K9 and activate transcription (Metzger et al., 2005). Currently, there are two known types of demethylase domain with specific catalytic reactions: the LSD1 domain and the JMJC domain (Shi and Whetstine, 2007). H3K9 can also be demethylated by other enzymes such as JMJD2C (Cloos et al., 2006) and JMJD2D (Shin and Janknecht, 2007). The interplay between HMTs and

demethylases probably plays a role in fine tuning the histone methylation (Shi and Whetstine, 2007).

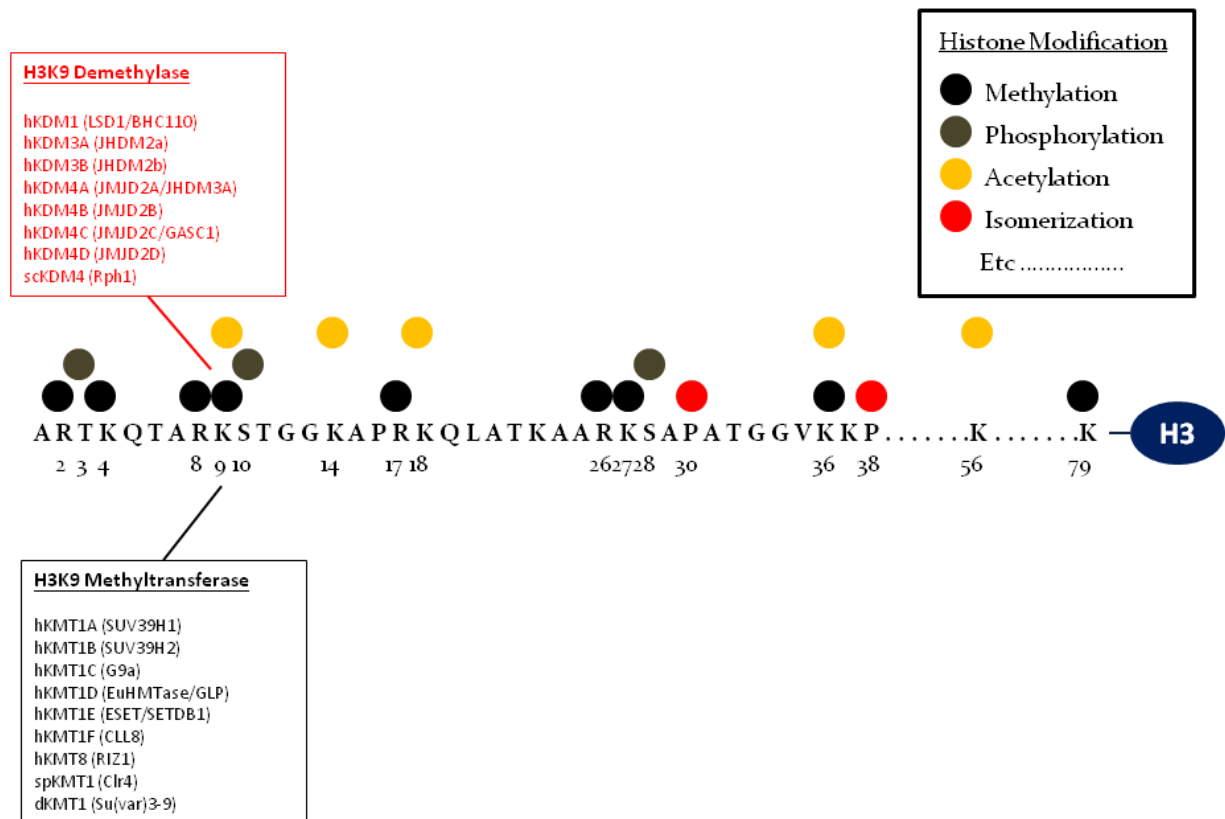


Figure 1.8 Schematic diagram of histone modification maps on Histone 3. Different histone modifications can be detected on histone tails. Different histone demethylases and methyltransferases that target the lysine (K) 9 site only are shown.

1.11 DNA methylation

Epigenetic marks confer stability of gene expression during mammalian development. Major epigenetic regulatory mechanisms include DNA methylation, covalent post-translational modifications of histone proteins and RNA-mediated gene silencing (Vaissiere et al., 2008). Cytosine (C) methylation is the only covalent DNA modification described in mammals (Weber et al., 2007). This occurs at cytosine residues clustered in sequences containing a high degree of CG dinucleotide motifs known as CpG Islands. According to commonly used criteria, a CpG Island is minimally defined as a minimum observed-expected CpG ratio of 0.65 and with a GC content greater than 55% over a distance of 500 base pair (bp) (Takai and Jones, 2002).

The chief enzyme responsible for propagating DNA methylation patterns in adult vertebrate cells is DNA methyltransferase 1 (DNMT1) (Yen et al., 1992) which is considered to responsible for the maintenance of DNA methylation. There are two other DNA methyltransferases, Dnmt3a and 3b which are primarily responsible for *de novo* DNA methylation (Okano et al., 1999) but in mouse embryonic stem cells Dnmt3a and Dnmt3b are also involved in maintenance methylation (Chen et al., 2003). Disruption of the genes Dnmt1, Dnmt3a, Dnmt3b results in embryonic lethality and is associated with global hypomethylation (Li et al., 1992). These enzymes, DNMT1, Dnmt3a, Dnmt3b, catalyze the transfer of the methyl donor group from S-adenosylmethionine (SAM) to the 5-carbon (C⁵) position of cytosine bases in CpG dinucleotides. Other Dnmts exist including Dnmt2, which have little or no DNA methylation activity *in vitro*; and Dnmt3L, which has similarities with Dnmt3 proteins, but lacks some catalytic motifs and is not expected to have DNA methylation activity (Goll and Bestor, 2005).

In mammals, methylation is restricted to CpG dinucleotides which are commonly seen in CpG islands and are highly represented in promoter areas (Weber et al., 2007). CpG sites in CpG island promoter regions are usually protected from DNA methylation, whereas CpG

sites in gene-coding or non-coding regions are usually methylated (Clark et al., 2006). Cytosine methylation can interfere with transcription factor binding, yet repression seems to occur largely indirectly via recruitment of methyl-CpG binding domain (MBD) proteins that induce chromatin changes (Klose and Bird, 2006). Approximately 70% of human genes are linked to promoter CpG islands, whereas the remaining promoters tend to be depleted in CpGs (Saxonov et al., 2006). DNA methylation does not alter the function of the gene product but provides information as to where and when the gene should be expressed (Clark et al., 2006).

DNA methylation patterns in human cancer cells are usually distorted. Cancer cells may exhibit hypermethylation within the promoter regions of many CpG island-associated tumour suppressor genes such as Rb1 (Sakai et al., 1991), glutathione S-transferase (GSTP1) (Lin and Nelson, 2003) and E-cadherin (CDH1) (Yoshiura et al., 1995). These aberrant DNA methylation marks, together with histone methylation marks, may act together with other regulatory mechanisms such as miRNA to regulate gene expression (McCabe et al., 2009).

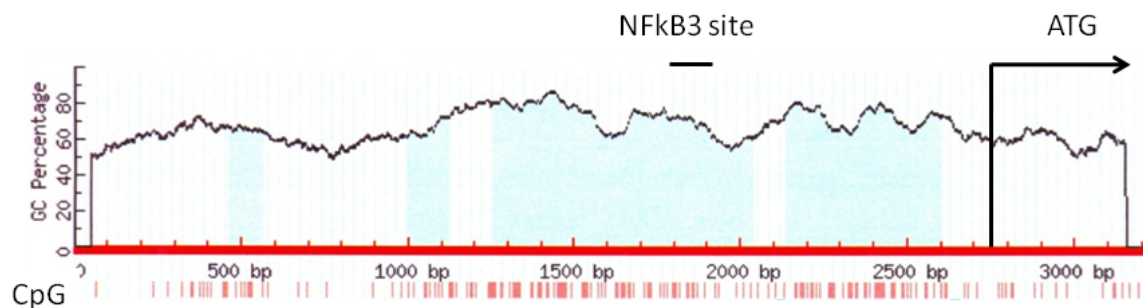


Figure 1.9 Schematic diagram of CpG island distribution on the TGF- β 1 promoter.

CpG islands are defined according to the criteria: minimum observed-expected CpG ratio of 0.65 and GC content greater than 55% over a distance of 500 base pair and are marked as a blue coloured region. Individual CpG sites are marked as red line below the X axis. Translation start site is marked as ATG. The NF- κ B binding site of interest (κ B3) is shown located in a CpG rich site.

1.12 Oxidative stress and DNA methylation

Generation of hydroxyl radicals can cause a wide range of DNA lesions including base modifications, deletions, strand breakage, chromosomal rearrangements, and further interfere with the ability of DNA to function as a substrate for the DNMTs and results in global hypomethylation (Wachsman, 1997). Additionally, the presence of 8-hydroxydeoxyguanine (8-OH-dG) in CpG dinucleotide sequences has been shown to inhibit methylation of adjacent cytosine residues (Turk et al., 1995) and further interfere with the ability of restriction nucleases to cleave DNA (Turk and Weitzman, 1995). Furthermore, O⁶-methylguanine can inhibit binding of DNA methyltransferases (Hepburn et al., 1991); spontaneously mispair with thymine (Xiao and Samson, 1993) and thereby lead to DNA hypomethylation by means of inhibiting methylation of adjacent cytosine molecules.

Incorporation of 8-OH-dG and 5-hydroxymethylcytosine into the MBP recognition sequence results in inhibition of MBP binding affinity and subsequent changes in gene expression (Valinluck et al., 2004). Additionally, repressed expression of key antioxidant enzymes may be affected by promoter hypermethylation-induced gene silencing. Decreased expression of MnSOD was detected in pancreatic carcinoma which was underlined by DNA hypermethylation-induced silencing (Hurt et al., 2007). GSTP1 gene expression was shown to be inactivated via promoter hypermethylation in hepatocellular carcinoma (Zhong et al., 2002) and prostate cancers (Millar et al., 1999).

These changes further indicate the ROS-induced oxidative stress is involved in the epigenetic regulation of genes expression and further in controlling of DNA methylation (Franco et al., 2008).

1.13 The interplay between DNA methylation and histone methylation

DNA methylation, histone deacetylation and H3K9 methylation are all associated with a repressed chromatin state. But the precise sequence of events is still unclear.

In mammals, DNA methyltransferases interact with Suv39h H3K9 methyltransferases and loss of H3K9 methylation in Suv39h-knockout embryonic stem cells decreases Dnmt3b-dependent CpG methylation at major centromeric satellites (Lehnertz et al., 2003). Additionally, H3K9 methylation and silencing of the p16^{ink4a} tumour suppressor gene can occur before CpG methylation (Bachman et al., 2003). These observations point out that DNA methylation may be a secondary event in gene silencing process. Furthermore, DNA methylation might exert a positive feedback on lysine methylation. Methyl-CpG-binding domain proteins, such as MeCP2 and MBD1, might favour H3K9 methylation in the vicinity of the methylated genes that they regulate (Fuks et al., 2003b; Sarraf and Stancheva, 2004). Some H3K9 HMTs, such as SETDB1 have built-in methyl-CpG binding domains suggesting that these histone modifying enzymes might be directly recruited to sites of CpG methylation (Li et al., 2006a).

All these observations suggest one possibility that, H3K9 methylation by an H3K9 enzyme might create a foundation for an adaptor, HP1, which would recruit DNA methyltransferases, to catalyze CpG methylation in the region of the methylated lysine. The methyl-CpG binding domain would then bind to the methylated DNA, and further attract HDAC complexes, in addition to H3K9 methyltransferase which is required to prepare H3K9 for methylation (Fuks, 2005) (Fig 1.10).

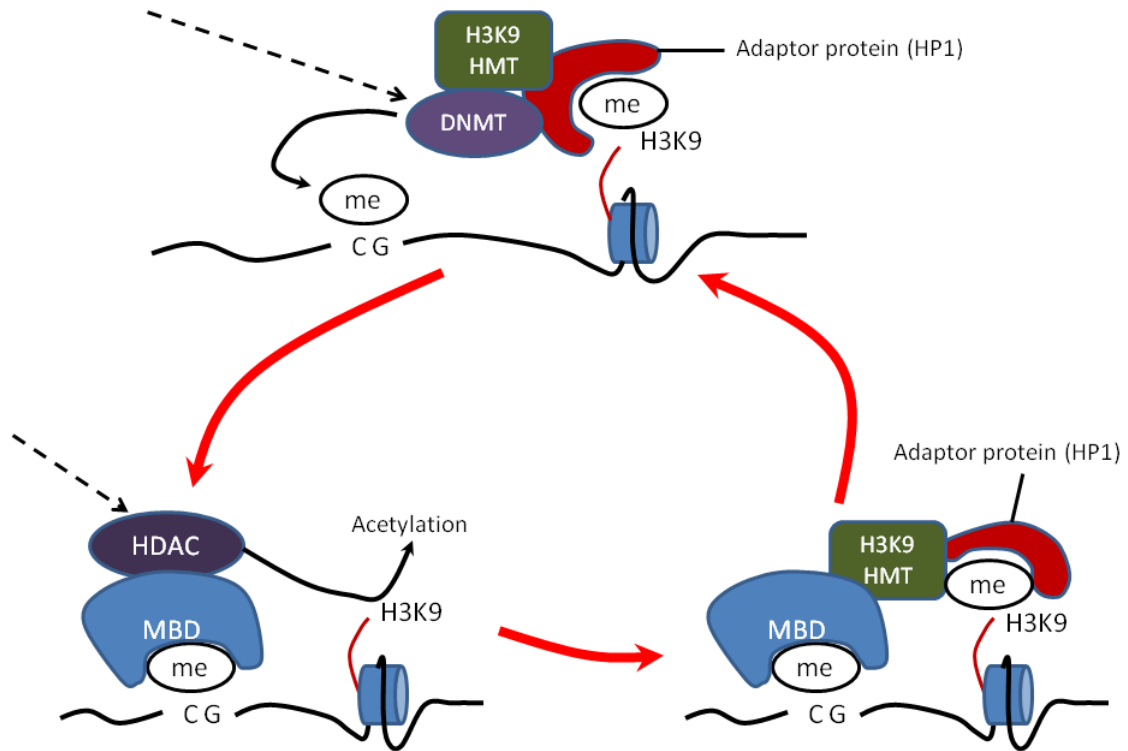


Figure 1.10 Diagram depicting the self-inducing epigenetic cycle. DNA methyltransferase (DNMT) may be attracted to the adaptor molecule (HP1) and add a methyl group to DNA where chromatin is already methylated at lysine 9 of histone H3 (H3K9). Methyl-CpG binding domain (MBD) proteins can bind to DNA due to the generation of methylated DNA by DNMT. Histone deacetylase (HDAC) can be attracted to the complex in addition to H3K9 methyltransferases, and induce H3K9 deacetylation. Histone methylation then occurs which may allow further recruitment of the HP1 adaptor protein. This self-reinforcing cycle helps to keep genes in a repressed chromatin state.

1.14 Hypothesis and aims of the thesis

We hypothesise that oxidative stress, in combination with a pro-inflammatory stimulus, may upregulate TGF- β 1 in the airways through the NF- κ B pathway. This enhanced expression of TGF- β 1 is regulated, at least in part, by the action of H₂O₂ on the expression and activity of the histone methyltransferase, Suv39H1; and the DNA methyltransferase, DNMT1. Furthermore, the enhanced expression of TGF- β 1 induced by combined oxidative stress and inflammatory stimulation results in a relatively refractory response to glucocorticoid suppression.

The specific aims were:

1. to determine the effect of H₂O₂ alone, and in combination with IL-1 β , on TGF- β 1 production by airway epithelial cells,
2. to examine the role of redox sensitive transcription factors such as NF- κ B and AP-1 on TGF- β 1 expression,
3. to elucidate the degree of TGF- β 1 mRNA suppression by fluticasone propionate (FP) under conditions of oxidative stress,
4. to examine the effect of H₂O₂ on recruitment of NF- κ B p65, Suv39H1 and DNMT1 to the native TGF- β 1 promoter area,
5. to evaluate the role of Suv39H1 in FP action, and
6. to determine the effect of H₂O₂ and IL-1 β on TGF- β 1 promoter DNA methylation status.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Cell culture

2.1.1 A549 cells

The human alveolar type II-like epithelial cell line A549 were grown in complete medium (DMEM; supplemented with 10% FCS, and 2mM L-glutamine) (Gibco, Paisley, UK). Cells were grown as monolayers in tissue culture flasks at 37°C, 5% CO₂ until 70-75% confluent, and then serum starved for 24hrs before treatment to enable the cells to synchronise cell cycle (K-Y Lee et al., JI 2006).

2.1.2 BEAS-2B cells

BEAS-2B cells were derived from cells isolated from normal human bronchial epithelium obtained by autopsy of non-cancerous individuals and subsequently infected with an adenovirus 12-SV40 virus hybrid and cloned. They retain the ability to undergo squamous cell differentiation in response to serum. BEAS-2B cells were grown in keratinocyte conditioned medium (Gibco, Paisley, UK) together with supplements (Ito K et al., BBRC 2004). Cells were grown as monolayer in tissue culture flasks at 37°C, 5% CO₂ until 70-75% confluent, and then serum starved for 24hrs in medium without EGF and bovine pituitary extracts before treatment. Both A549 and BEAS-2B cells were purchased from the European Collection of Cell Cultures (ECACC) through Sigma-Aldrich (Poole, Dorset, UK).

2.2 Dichlorofluorescein Assay

Cells were split and counted by trypan blue exclusion. Viable cells (10⁴/well) were plated into 96-well collagen-coated plates 1 day before the experiment. On the day of the experiments, after removing the medium, the cells in the plates were washed with KRH buffer (118mM NaCl, 4.8mM KCl, 1.2mM MgSO₄, 2.5mM CaCl₂, 10nM HEPES, PH 7.4), and then incubated with 100 µM DCFH-DA (2',7'-dichlorofluorescein diacetate) in serum-free medium (DMEM; supplemented with 10% FCS, and 2mM L-glutamine)(Gibco, Paisley, UK)) in 5%

CO₂/95% air at 37°C for 30 min. After DCFH-DA was removed, the cells were washed and incubated with KRH buffer and stimulants for variable times as described. Cell fluorescence from each well was measured using a Fluorescence plate reader (Synergy HT, Biotek, Winooski, VT, USA) and recorded. The excitation filter was set at 485nm and the emission filter was set at 530nm.

Unless otherwise stated all other chemicals and solutions used were purchased from Sigma-Aldrich.

2.3 Detection of alveolar epithelial cell apoptosis/late necrosis, and cell proliferation

2.3.1 Flow cytometry

Cells were collected by trypsin, and neutralized with complete medium (10% FCS, 2% L-glutamine), washed twice with cold PBS and resuspended in 1X Binding Buffer (1 part of 10X Binding buffer (0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM CaCl₂) diluted with 9 parts of distilled water) at a concentration of 1×10^6 cells/ml. 100µl of the solution (1×10^5 cells) was then transferred to a 5 ml culture tube and 5µl of Annexin V-FITC (BD Pharmingen, San Diego, CA) and 5µl propidium iodide (PI, BD Pharmingen, San Diego, CA) added. The cells were vortexed gently and incubated for 15 min at RT (25°C) in the dark. 400µl of 1X Binding Buffer were added to each tube before analysis by flow cytometry within 1 hr. Annexin-V positive cells were considered to be apoptotic if they were PI stain negative (normal permeability), or as either late apoptotic or necrotic if they were PI stain positive (increased permeability) (Geiser et al., 2004) (Fig 2.1).

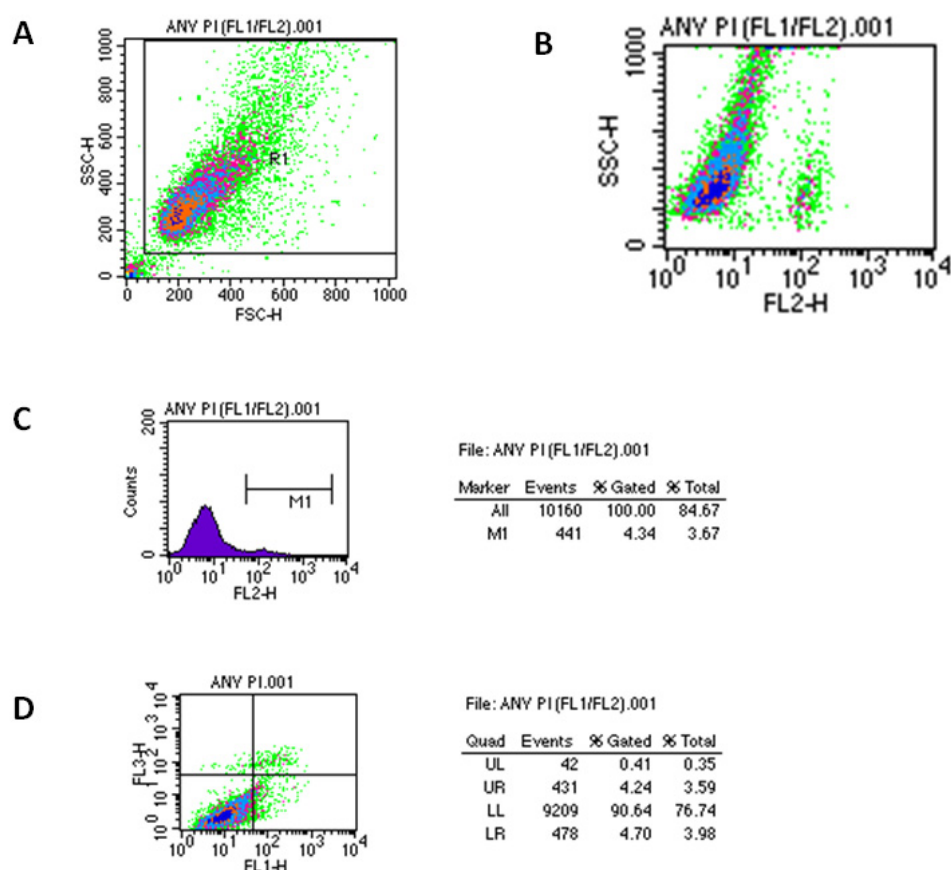


Figure 2.1 Analysis of cells labelled with annexin-V antibody and/or propidium iodide (PI) by Flow cytometry. (A) Cells were checked with forward scatter detector (FSC) and side scatter detector (SSC) and analyzed by density graph to check cell size and granularity. Fragmented cells were excluded from the study. (B) The amplitude of the detector was adjusted to separate cells positive for PI stain (FL2-H reading > 15) from those negative for PI according to the FL2 detector reading. (C) Distribution of cells over FL2 readings are analyzed by histogram. The results are shown as M1 (positive for PI) to all cells ratio. (D) After optimization for FL1 and FL2 detector setting, four quadrant density graph views were used to separate cells that were PI/Annexin V positive (right upper quadrant); Annexin-V positive, PI negative (right lower quadrant) and PI positive, annexin-V negative (left upper quadrant). Distribution of cells is shown in right table.

2.3.2 MTT assay

After cell stimulation, the supernatant was removed and A549 cells were incubated with 1 mg/ml methylthiazolyldiphenyl-tetrazolium bromide (MTT) solution at 37°C for 20-30 min. MTT solution was then removed and DMSO was added to dissolve the resulting formazan product and absorbance was measured at 550 nm.

2.4 cDNA preparation and real-time PCR

Total RNA was isolated from cultured cells using RNeasy Mini Kit by Qiagen (Crawley, East Sussex, UK). The concentration and purity of RNA was evaluated by spectrophotometry at 260 and 280 nm. The extracted RNA was reverse transcribed in RT reaction mixture (AMV-RT, Promega, Southampton, UK) with 1xRT buffer, dNTP mix, random hexamer primers and ribonuclease inhibitor (all Promega). Single stranded cDNA was synthesized by 1h incubation at 42°C and the reaction terminated by incubation at 90°C for 4min.

QPCR was performed in a Rotor-Gene 3000TM Four-Channel Multiplexing System (Corbett Research, Cambridge, UK) using a QuantiTect SYBR Green PCR Kit (Qiagen) to quantify TGF- β 1 and GAPDH or GNB2L1 control mRNAs. Cycle parameters were 95°C for 15 min to activate HotStarTaq DNA[®] Polymerase, followed by annealing and extension for 40 cycles at 94°C for 15s, 60°C for 25s and 72°C for 25s. DNA was quantified by the standard curve method of relative quantification according to instructions provided by Corbett Research. The amount of TGF- β 1 mRNA was normalized to GAPDH or GNB2L1 and all RT-QPCR data is presented as a ratio to control samples.

Table 2.1. PCR primers for real-time RT-PCR

TGF- β 1 forward	5'-CCC AGC ATC TGC AAA GCT C-3'
TGF- β 1 reverse	5'-GTC AAT GTA CAG CTG CCG CA-3'
Suv39H1 forward	5'-AAG ATC CGC GAA CAG GAA TA-3'
Suv39H1 Reverse	5'-GGA ACT GCT TGA GGA TAC GC-3'
GAPDH forward	5'-TTC CAG GAG CGA GAT CCC T-3'
GAPDH reverse	5'-CAC CCA TGA CGA ACA TGG G-3'
GNB2L1 forward	5'-CTCCGCCTCTCGAGATAAGACC-3'
GNB2L1 reverse	5'-GCAAACCTGGCCATCTGAGGA-3'
G9a forward	5'-GAC CCT GAC GAA AGG GGA C-3'
G9a Reverse	5'-AGA CTT GCT GRC GGA GTC CA-3'
Suv39H2 forward	5'-TCT TCC CCG AAT AGC ATT GT-3'
Suv39H2 Reverse	5'-TCT GAC CCT CTT TTT GGC TG-3'
DNMT1 forward	5'-GTC TGA AAG AGC CAA ATC GG-3'
DNMT1 reverse	5'-AGC GGT CTA GCA ACT CGT TC-3'

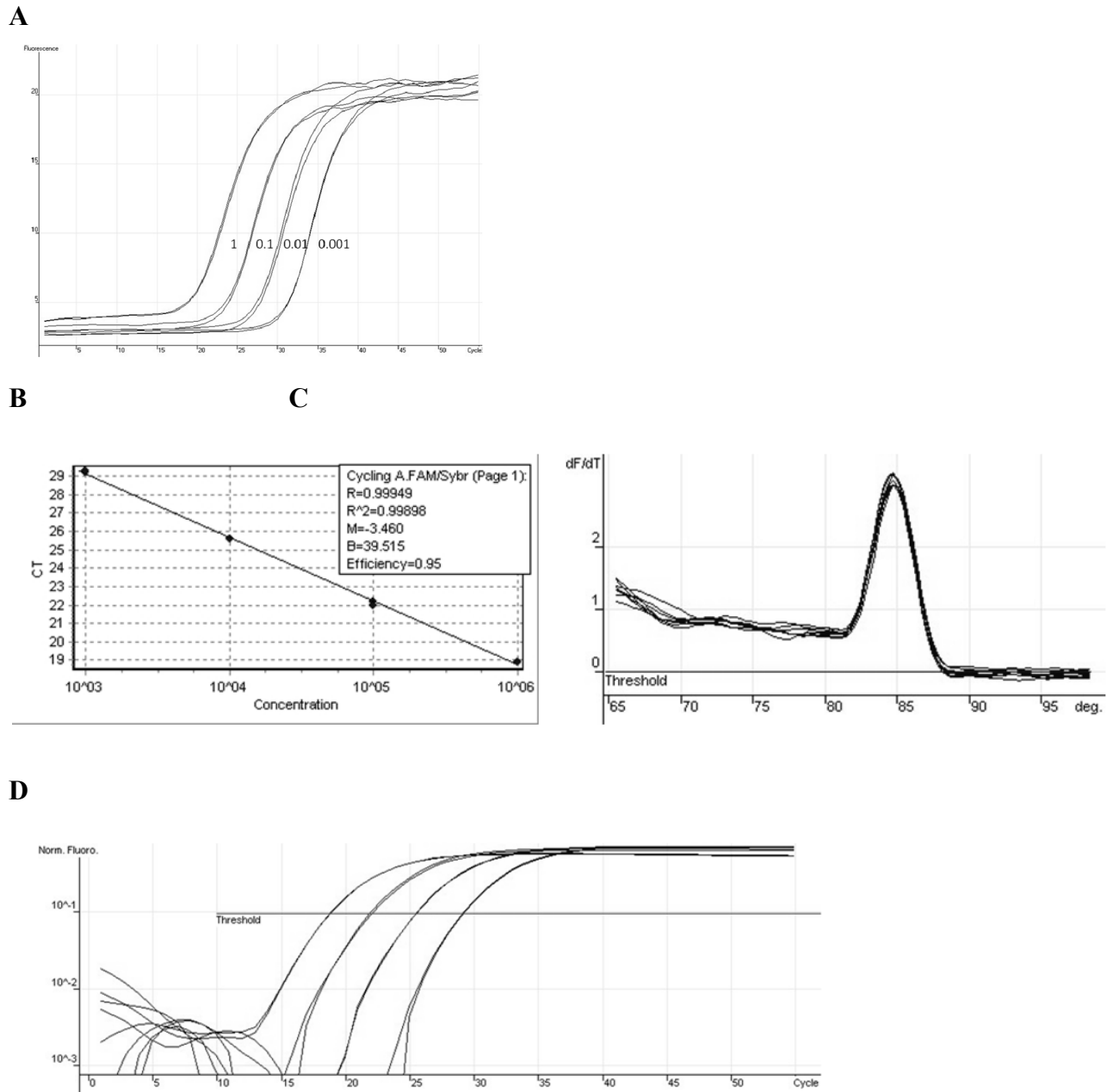


Figure 2.2 Specificity and Sensitive Quantification results by reverse transcription-quantitative PCR (RT-QPCR). (A) Raw channel graphs for RT-QPCR demonstrate serial dilution of unknown concentration DNA (1x, 0.1x, 0.01x, 0.001x) results. (B) Standard curve of the RT-QPCR result. (C) Melting curve analysis of the amplified products shows good specificity of the final amplified products by RT-QPCR. (D) Normalized fluorescence results for serial dilution of DNA of unknown concentration after normalization. The threshold cycle is marked by a red line.

2.5 Western blotting

2.5.1 Preparation of protein samples

Proteins were extracted from A549 cells using two different protocols: whole cell protein extraction, or nuclear/cytoplasmic protein extraction depending on the application used.

For whole-cell extraction, 2×10^6 cells from a 6cm petri dish were washed twice with ice-cold phosphate buffered saline (PBS), and scraped with cell lifter. After centrifugation at 500xg for 3 mins, PBS was aspirated and the cell pellet lysed by repeated pipetting with 100 μ l ice-cold radioimmunoprecipitation (RIPA) buffer [containing PBS, pH 7.4, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (v/v) SDS, and complete protease inhibitors cocktail (Roche Applied Science, Lewes, East Sussex, UK)] on ice for 30mins. After vigorous vortexing for 10secs, lysates were collected and cell debris was removed by centrifugation for 10 min at 12,000 rpm at 4°C. The supernatants were transferred to a fresh tube and stored at -80°C until use. The efficiency of cell lysis was verified by light microscopy.

For nuclear/cytoplasmic protein extraction, subcellular fractionation was performed by using a Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA), which provides cytoplasmic and nuclear subcellular fractionation of proteins. All procedures were performed following the Manufacturer's recommended procedures. Briefly, 2×10^6 cells from a 6cm petri dish were rinsed twice with ice-cold PBS and collected by scrapping with 1ml ice-cold PBS containing phosphatase inhibitors. Cells were pelleted by centrifugation at 1,500 rpm for 5min and resuspended in 500 μ l Hypotonic Buffer, and then incubated for 15min on ice. After adding 25 μ l detergent and vortexing for 10secs, the suspension was centrifuged at 14,000 x g for 30secs, and the supernatant (cytoplasmic fraction) collected. The remaining nuclear pellets were resuspended in 50 μ l Complete Lysis Buffer (containing 1mM DTT, Lysis buffer AM1, and protease inhibitors cocktail), vortexed and incubated for 30mins at 4°C on a rocking

platform. After a further vortex, the suspension was centrifuged at 14,000 x g for 10min and the supernatant (nuclear fraction) was collected, and stored at -80°C for subsequent analysis.

2.5.2 *Bradford protein assay*

Protein concentrations were determined using Bradford's reagent (BioRad Laboratories, Hemel Hempstead, Hertfordshire, UK) before downstream application. A set of standard samples of known concentrations (0, 1, 2, 4, 6, 8, 10µg/µl) was made up by dilution of a stock 10µg/10µl BSA solution. This was used to construct a standard curve. The volume of the unknown protein samples were adjusted to ensure the measured concentration were within the range of the standard curve.

To quantify the concentration of the unknown protein, 1µl of unknown of protein samples and serial diluted standard concentrations including a blank diluent was mixed with 200µl diluted Bradford reagent (1:4 ratio with distilled water) and then added to each well of a 96-well plate. All samples were tested in duplicate and each sample was measured within 5 mins on a plate reader with absorbance at 600nm. A standard curve of absorbance against concentration was plotted, and used to establish the concentration of the unknown samples.

2.5.3 *Polyacrylamide gel electrophoresis (PAGE)*

To separate proteins of different molecular weight, pre-cast Nupage® 10%, or 4-16% Bis-Tris Gels (Invitrogen Ltd, Paisley, UK) combined with Nupage® MES or Nupage® MOPS SDS running buffer were used. These gels give an optimal separation/resolution range of 6-200 kDa. Gels were rinsed with deionised water and assembled into the gel tank. Both inner and outer chambers were filled with 1x MES SDS running buffer [50mM MES, 50mM Tris base, 0.1% SDS, 1mM EDTA, pH 7.3] or MOPS Running Buffer [50mM MOPS, 50mM Tris base, 0.1% SDS, 1mM EDTA, pH 7.7]. Protein samples for electrophoresis were mixed with 1/4 volumes of 4x Nupage LDS sample buffer [106mM Tris HCl, 141mM Tris base, 2%

LDS, 10% Glycerol, 0.51mM EDTA, 0.22mM SERVA® Blue G250, 0.175mM Phenol Red, pH 8.5], and 1/10 volume of 0.5M DTT as reducing agent was added and heated at 70°C for 10mins to denature the proteins. Samples were vortexed and centrifuged before loaded onto the gels. Electrophoresis was performed at 200 V for 50mins.

2.5.4 *Western transfer of protein to nitrocellulose membranes*

After electrophoresis was complete, the gel was taken out from the tank, and removed from the gel cassette. The gel was kept wet at all times and trimmed with a clean scalpel to the appropriate size. A piece of Nitrocellulose-ECL membrane (GE Healthcare, Little Chalfont, UK) was cut to the dimensions of the gel and equilibrated in 1x Nupage® Transfer Buffer [25mM Bicine, 25mM Bis-Tris (free base), 1mM EDTA, pH 7.2] before the transfer process. The transfer blot module was assembled as follows: cathode core, blotting pad, filter paper, gel, transfer membrane, filter paper, blotting pad, with the gel on the cathode side and the membrane on the anode side. All procedures were performed in the transfer tank filled with transfer buffer, and roller was used to ensure the removal of any air bubbles. The tank was filled with chilled 1 x Nupage® Transfer Buffer. The transfer was performed at 30 V for 1 hr.

2.5.5 *Immunodetection of specific proteins*

After the transfer process, the transfer module was disassembled and the membrane was removed and equilibrated in TBS-Tween 20 washing buffer (Tris-HCl pH 7.0, 0.5% Tween 20) briefly. Membranes were blocked in 5% nonfat milk TBS-Tween 20 for 1hr at room temperature with shaking. The blocking solution was removed and the membrane incubated with 10ml of diluted primary antibody in blocking solution overnight at 4°C with shaking. Antibodies used for Western blotting and their individual dilution ratios are shown in Table 2.2. After the incubation, membranes were rinsed and washed three times for 10mins each with TBS-Tween 20 washing buffer. After washing, membranes were incubated with

horseradish peroxidase (HRP)-conjugated secondary antibodies (raised against the host of the primary antibody) which were diluted in blocking solution for 1hr at room temperature with shaking. Membranes were washed three times with TBS-Tween 20 each time for 10mins and then rinsed twice with TBS. Finally, antigen-antibody interactions were detected using a chemiluminescence detection kit (ECL; Amersham Biosciences, Little Chalfont, UK), and the image was exposed with film with different exposure time based on signal density. The results were analyzed with image analysis software (UVP, Cambridge, UK) and were shown as integrated optical density (IOD).

2.5.6 Protein loading control

After detection with the relevant primary antibody, the blots were re-probed with an anti- β -actin antibody for cytoplasmic and whole cell lysates and with an anti-lamin A/C antibody for nuclear extracts to control for protein loading. If the protein size overlapped with a previously detected signal, membrane stripping was performed. Briefly, the membrane was washed twice for 10mins in TBS-Tween 20 with shaking, placed in 20ml Western blot stripping buffer (Perbio Science Ltd, Tattenhall, Cheshire, UK) and incubated for 10mins at room temperature. Finally, the membranes were washed as before and re-probed for the appropriate protein control.

Table 2.2 Antibodies used for Western blotting

Primary antibody (dilution)	Company (catalogue number)	Secondary antibody (dilution)	Molecular weight (kDa)
TGF- β 1	Santa Cruz (sc-146)	Goat anti-rabbit (1:2000)	12.5 (dimer at 25)
p65 (1:700)	Santa Cruz	Goat anti-mouse	65

	(sc-8008)	(1:2000)	
Suv39H1 (1:500)	Millipore (05-615)	Goat anti-mouse (1:2000)	50
GR (1:700)	Santa Cruz (sc-8992) H-300	Goat anti-rabbit (1:2000)	94
GR (1:700)	Santa Cruz (sc-1003) E-20	Goat anti-rabbit (1:2000)	94
Dimethyl-Histone H3K9 (1:2000)	Millipore (07-441)	Goat anti-rabbit (1:2000)	17
Trimethyl-histone H3K9 (1:2000)	Millipore (07-442)	Goat anti-rabbit (1:2000)	17
Lamin A/C (1:700)	Santa Cruz (sc-7292)	Goat anti-mouse (1:2000)	64
β -actin (1:10000)	Santa Cruz (sc-47778 HRP)		43
DNMT1 (1:500)	Santa Cruz (SC-20701)	Goat anti-mouse (1:2000)	170-195

2.6 TGF- β 1 ELISA

The concentrations of TGF- β 1 in supernatants were measured using human TGF- β 1 DuoSet ELISA (enzyme-linked immunosorbent assay) Development kits (R&D Systems Europe, Abingdon, UK). 96-well microplates were pre-coated with Capture Antibody (mouse anti-TGF- β 1) by incubating 100 μ l per well of the diluted Capture Antibody (20 μ g/mL in PBS) overnight at room temperature followed by blocking with 200 μ l of Block Buffer (5% Tween 20 in PBS) for 1h at room temperature. For the assay of total TGF- β 1, the supernatants prepared from cultured cells were first activated by incubating with 1N HCl for 10mins at

room temperature and neutralized (pH7.2-7.6) by 1.2N NaOH/0.5M *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonic acid. Activated samples were then transferred to the Capture antibody-precoated plates and incubated for 2hr at room temperature. After thorough washing, Detection Antibody (biotinylated chicken anti-human TGF- β 1, 300ng/ml) was added to the test wells. After further 2hr incubation at room temperature, free antibodies were removed by washing, and plates incubated with streptavidin-HRP for 20mins at room temperature. After washing, HRP Substrate Solution was added, incubated at room temperature for 20mins before the reaction was stopped with 2N H₂SO₄ and the color intensity measured with a microtitre plate reader (Synergy HT) set to 450nm corrected at 540nm. Concentrations were calculated by using a four parameter logistic (4-PL) curve-fit, created by 2-fold serial dilutions of Standard (2000pg/ml of recombinant human TGF- β 1). Samples and standards were run in duplicate and averages were used for calculation. Active TGF- β 1 in the supernatant was also determined using the same procedure without the activation step with acidification.

2.7 Suv39H1 siRNA Transfection

A549 cells were trypsinized and counted one day before transfection. The cells were diluted in complete medium (DMEM; supplemented with 10% FCS, and 2mM L-G), plated out at a density of 2.5×10^5 cells/ml in 6 well plate and incubated at 37°C, 5% CO₂. Small interfering RNA (siRNA) constructs were obtained as the siGENOME SMARTpool Suv39H1 SiRNA duplexes (M-009604-01, Dharmacon, Lafayette, CO, USA). The Suv39H1 siRNA was diluted with siRNA buffer (Dharmacon) and stored at -20 C. On the day experiment, siRNA was diluted with Opti-MEM medium (Invitrogen) with generous vortexing and then mixed with INTERFERin Kit (Polyplus transfection, Illkirch, France) with vigorous shaking before incubation for 10mins at room temperature according to the Manufacturer's instructions. During this time, each well in a 6 well plate was washed with PBS, and then changed to fresh

complete medium. The siRNA complex with Opti-MEM medium was mixed with complete medium to the final concentration 25nM added to the cells which were subsequently incubated at 37°C, 5% CO₂ for 24hrs and then in serum free media for another 24hrs before treatment.

2.8 Co-Immunoprecipitations

Whole cell lysates were prepared by incubation with immunoprecipitation (IP) buffer (50mM Tris-HCl, pH 8.0; 150mM NaCl; 0.5% NP-40; 0.5% deoxycholate; complete protease inhibitor cocktail [Roche Applied Science]) on ice for 15min before centrifugation for 10mins at 4°C. Nuclear extraction was performed with the Nuclear Extract Kit (Active Motif). The measurement of protein concentrations was performed as previously described. 500µg of whole cell lysates or 500µg of nuclear extracts were immunoprecipitated with 5µg of primary antibodies (Table 2) for 4hrs (for GR-IP) or overnight at 4°C (all other IPs) followed by a further incubation with 15ul of 50% protein A/G-Sepharose beads for 2hrs at 4°C on a rotator. The beads were then collected by brief centrifugation at 6000 rpm and washed four times with IP buffer. After a final wash, the buffer was aspirated completely and the beads resuspended in Laemmli buffer prior to being boiled at 100°C for 5mins. The immunoprecipitated proteins were fractionated by 10% or 16% SDS-PAGE) and detected by Western blotting as previously described with antibodies specific for the proteins under investigation.

Table 2.3 Antibodies used in co-immunoprecipitation

Primary antibody (dilution)	Company (catalogue number)	Secondary antibody (dilution)	Molecular weight (kDa)

p65 (1:700)	Santa Cruz (sc-8008)	Goat anti-mouse (1:2000)	65
Suv39H1 (1:500)	Santa Cruz (SC-23961)	Goat anti-mouse (1:2000)	45
GR (1:700)	Santa Cruz (sc-8992) H-300	Goat anti-rabbit	94
DNMT1	Santa Cruz (SC-20701)	Goat anti-mouse (1:2000)	170-195

2.9 *In-vitro NF- κ B/TGF- β 1 Promoter binding assay*

In-vitro NF- κ B/TGF- β 1 Promoter Binding Assays were an ELISA based assay based on the same principle in TransAM Kit. This assay measures the binding affinity of NF- κ B proteins to specific sequences in the TGF- β 1 promoter (Fig. 2.3). DNA oligonucleotides containing a putative NF- κ B or AP-1 site in the TGF- β 1 promoter and mutated oligonucleotides were prepared by mixing equal amounts of 100 μ M sense and anti-sense oligonucleotides in annealing buffer (10mM Tris-HCl pH7.5, 50mM NaCl, 0.1% Tween 20, 2.7mM KCl) in a preheated block (95°C) before leaving at room temperature for 60min. Streptavidin coated microplates (Thermo Labsystems, Helsinki, Finland) were immobilized with 0.25 μ M biotinylated DNA oligonucleotides containing a TGF- β 1 promoter NF- κ B binding site in annealing buffer (10mM Tris-HCl pH7.5, 50mM NaCl, 1mM EDTA) overnight at 4°C. The binding reaction was performed by incubating nuclear extracts (20 μ g/20 μ l) from A549 cells with 30 μ l of binding buffer (4mM HEPES pH7.5, 120mM KCl, 8% glycerol, 1% BSA, 2mM DTT, 10 μ g/ml Herring sperm DNA) for 1h at room temperature in each well of the DNA oligonucleotide-immobilized microtitre plate. In competitive binding experiments, non-biotinylated wild-type (0.2 or 2 μ M) or mutated oligonucleotides (2 μ M) were added to the 30 μ l binding buffer. After washing with washing buffer [10mM PB (Phosphate Buffer; 1.16g

Na₂HPO₄, 0.2g KCl, 0.2g KH₂PO₄/L) pH7.5, 50mM NaCl, 0.1% Tween 20, 2.7mM KCl], DNA oligonucleotide-bound protein was detected with anti-p65 (1:1000, Active Motif and an HRP conjugated goat anti-rabbit secondary antibody (1:500, Dako, Cambridge, UK) diluted in buffer (10mM PB, pH7.5, 50mM NaCl, 0.1% Tween 20). The colorimetric reaction was performed with 100µl of substrate reagent (R&D Systems Europe), stopped with 50µl stop solution (2N H₂SO₄) and was measured at 450 nm with a reference wavelength of 550nm. The oligonucleotides employed in these studies are listed in Table 2.4.

Table 2.4 TGF-β1 promoter oligonucleotides used in *in vitro* NF-κB/TGF-β1 Promoter Binding Assays

Name	Oligonucleotide sequence
NF-κB site	
κB1 Sense	5'-agg ctg ggt TGG AAA CTC Tgg gac aga a-3'
Antisense	5'-ttc tgt ccc aga gtt tcc aac cca gcc t-3'
κB2 Sense	5'-agg agt ctg GTC CCC ACC Cat ccc tcc t-3'
Antisense	5'-agg agg gat ggg tgg gga cca gac tcc t-3'
wκB3 Sense	5'-ggg ggc agg GGG GAC GCC Ccg tcc ggg g-3'
Antisense	5'-ccc cgg acg ggg cgt ccc ccc tgc ccc c-3'
mκB3 Sense	5'-ggg ggc agg GGG GAG CCC Ccg tcc ggg g
Antisense	5'-ccc cgg acg ggg gct ccc ccc tgc ccc c-3'

2.10 Production of Suv39H1 plasmid, and Suv39H1 overexpression

Full length Suv39H1 cDNA was cloned from a human cDNA library (Origene, Rockville, MD, USA) with specific Suv39H1 primers which include CACC for forward primers with a stop codon for reverse primers to produce blunt-end PCR products using an Invitrogen Platinum Pfx DNA polymerase system (Invitrogen). 100ng full length Suv39H1

cDNA which was to be inserted into the overexpression vector (pcDNA 3.1/V5-His-TOPO vector) was mixed with Pfx Amplification buffer (1X), dNTP mixture (0.3mM), MgSO₄ (1mM), Primer mix (0.3μM each) and 1.5 units Platinum Pfx DNA polymerase® (all Invitrogen). Autoclaved nuclease-free water was added to the mixture to a final volume of 50μl. The cycling conditions for the PCR reaction were: 94°C for 5mins, 94°C for 15secs and 55°C for 30secs with a final extension step of 68°C for 2mins. The cycling was repeated for 40 cycles and included a final step of 72°C for 10mins. The final product was analyzed by 1% agarose gel electrophoresis to check if there are nonspecific products. The pcDNA 3.1 Directional TOPO expression kit (Invitrogen) was used to make the Suv39H1 overexpression plasmid exactly according to the Manufacturer's instructions. Briefly, 5ng fresh PCR product from the previous PCR reaction was mixed with salt solution, TOPOR (pcDNA 3.1 vector), and sterile water was added to a final volume of 6μl. The reaction was mixed gently and incubated for 5mins at room temperature. 2μl of the TOPOR Cloning reaction was added to a vial of Chemically Competent E. coli (One Shot® Top10 Chemically Competent cells) and mixed gently before incubating on ice for 30mins. Cells were then heat shocked for 30secs at 42°C in a pre-warmed water bath without shaking and tubes immediately transferred to ice. 250μl of SOB (Super optimal broth) or SOC (Super optimal broth with catabolite repression; 2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) medium kept at room temperature was added and the tube shaken horizontally (200rpm) at 37°C for 1hr. Transformed cells were spread (50-200μl) onto pre-warmed selective LB plates containing ampicillin and incubated overnight at 37°C. The next morning 5 colonies were picked from each plate for further analysis.

Plasmid mini-preps were isolated from colonies using a plasmid purification kit (Qiagen). The presence of the correct product in the correct orientation was confirmed using restriction enzymes and subsequent sequence analysis by Cogenics (Takeley, Essex, UK).

Table 2.5 Primers for cloning Suv39H1 cDNA

Suv39H1 forward	5'-CACCATGGCGGAAAATTTAAAAGGC-3'
Suv39H1 reverse	5'-TCTAAGGGCTAGAAGAGGTATTTGC-3'

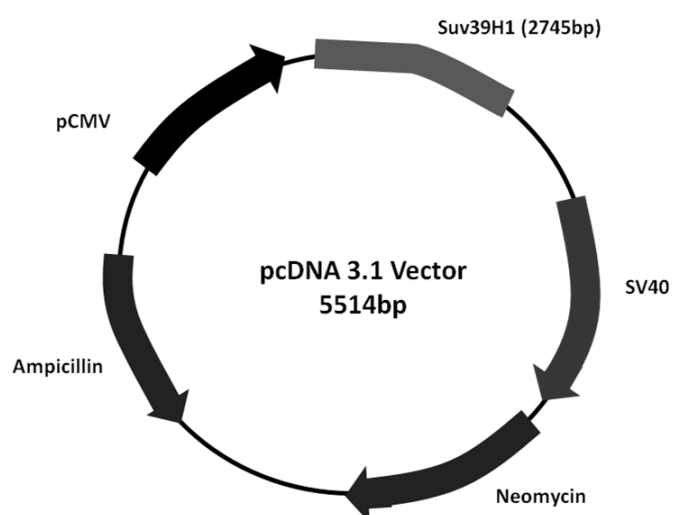


Figure 2.3 Structure of Suv39H1-pcDNA 3.1 overexpression plasmid. Schematic diagram of Suv39H1-pcDNA 3.1 overexpression plasmid. pCMV: CMV promoter; SV40: SV40 origin of replication; Neomycin: Neomycin resistance gene; Ampicillin: Ampicillin resistance gene.

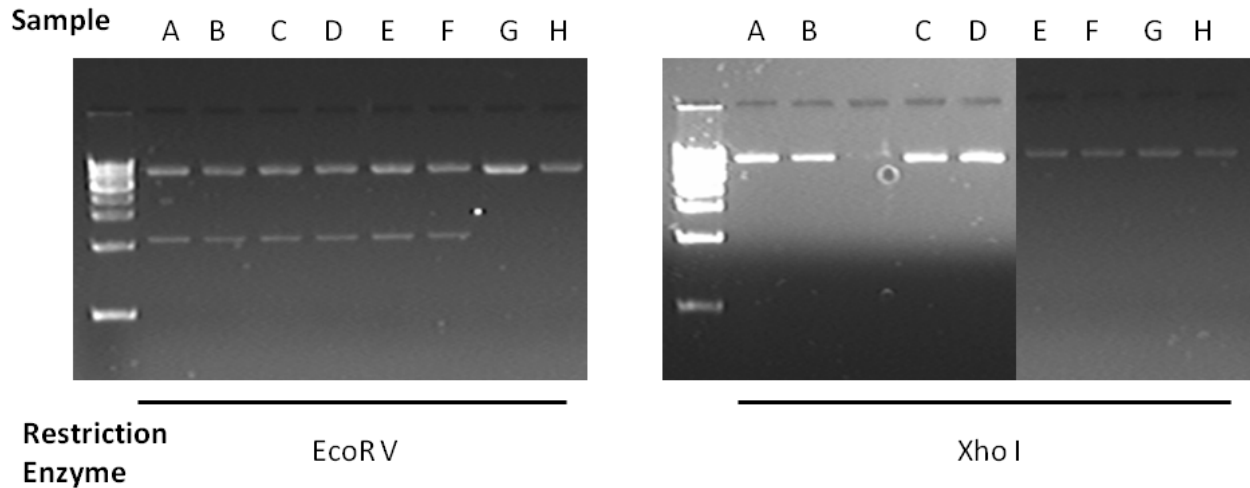


Figure 2.4 Validation of successfully transfected vector. Colonies of transformed *E. coli* (BL21 Star (DE3) One Shot Competent) grown successfully on Ampicillin-containing LB plates were picked and cultured overnight in S.O.C medium containing 50µg/ml ampicillin. Plasmid DNA was isolated, and analyzed by restriction digest (EcoR V: two cutting sites; Xho I: one cutting site) to confirm the presence and correct orientation of the insert. The digested DNA was run on a 1% agarose DNA gel and stained with ethidium bromide. Samples A, B, C, D, E & F were transformed successfully, with two fragments after cutting by EcoR V, and one fragment only by Xho I. Samples G & H were not transformed successfully, with one fragment after cutting by both EcoR V and Xho I.

2.11 Transfection of *Suv39H1* plasmid

Transfection was performed using the Roche FuGene 6® system (Roche Applied Science). Briefly, A549 cells were incubated in a 6-well plate and growing up to 50-60% confluence. Fugene 6 reagents (6µl) were pipetted into 100µl serum-free media and the mixture vortexed for 1sec before incubation for 5mins in room temperature. *Suv39H1* overexpression plasmid (2µg) was added and the mixture vortexed for 1sec before incubation at room temperature for a minimum of 15mins. The transfection reagent/DNA complex was finally added to each well of the 6-well plate and complete fresh medium added to give a total 2 ml in each well. The

cells were incubated with plasmid for 24hrs before stimulation.

2.12 *Chromatin immunoprecipitation*

A549 cells from 2 wells of 6-well plate (2×10^6 cells) were collected for each experiment. After stimulation, protein-DNA complexes were cross-linked by formaldehyde (1% final concentration) for 10mins at 37°C before the reaction was stopped by the addition of glycine (0.125M final concentration). Cells were washed twice with ice-cold PBS and scrapped with ice-cold PBS containing complete protease inhibitors cocktail (Roche Applied Science). Cells were pelleted by centrifugation at 4000 rpm for 5min and resuspended in 200µl SDS lysis buffer (50mM Tris, pH 8.1, 1% SDS, 5mM EDTA, complete protease inhibitor mixture) and subjected to sonication on ice with 5 cycles of 10secs pulses. This sonication is optimal for shearing DNA to 150-1000 bp pieces (500 bp on average, Fig. 2.4). Sonicated samples were centrifuged at 13,000 rpm for 10mins 8°C to spin down cell debris. The soluble chromatin solution (200µl) was further diluted with ChIP dilution buffer (0.01%SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl, pH8.1, 167mM NaCl, complete protease inhibitor) to a final volume of 2ml. 20µl (1%) of the ChIP dilution solution was kept as input control. The remained diluted solution was pre-cleared by incubating with 80µl salmon sperm DNA/protein A agarose-50% slurry (Millipore, Billerica, MA, USA) for 30min at 4°C on a rotator.

After brief centrifugation, the supernatant was separated into two microcentrifuge tubes (900µl each). Each sample was immunoprecipitated by incubation with 1µg antibodies specific for protein of interest (see Table 5) overnight at 4°C with rotation and followed by 1hr incubation with 60µl salmon sperm DNA/protein A agarose-50% slurry at 4°C on a rotator. Protein-bound immunoprecipitated DNA was sequentially washed once with 1ml low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH8.1, 150mM NaCl), 1ml high salt immune complex wash buffer (0.1% SDS, 1%

Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH8.1, 500mM NaCl), 1ml LiCl immune complex wash buffer [0.25M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid (sodium salt), 1mM EDTA, 10mM Tris-HCl, pH8.1] and twice with 1ml TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The immune complexes were then eluted by adding 250µl fresh elution buffer (1% SDS, 0.1 M NaHCO₃) twice followed by incubation for 4hrs at 65°C in 200mM NaCl to reverse cross-links between proteins and DNA. The solution was subsequently incubated with 70µg/ml proteinase K (Sigma-Aldrich) for 1h at 45°C to digest proteins. DNA was finally extracted with an equal amount (500µl) of phenol/chloroform/isoamyl alcohol (25:24:1; Invitrogen), precipitated with 1ml ethanol/0.3M CH₃COONa/20µg glycogen and resuspended in 100µl nuclease free water. QPCR was performed with 3µl of DNA sample and the primer sets listed in Table 6 for quantification. Data were normalized to input control samples. A no antibody control was also used to show the specificity of the IP antibodies used; QPCR with primer sets amplifying DNA sequences away from the transcription factor binding sites under study in the TGF-β1 gene was also used to demonstrate the specificity of DNA enrichment.

Table 2.6 Antibodies used in ChIP assay experiments

Antibody	Host	Company (catalogue number)
p65 (C-20)	Rabbit	Santa Cruz (sc-372)
Suv39H1	Mouse	Millipore (05-615)
DNMT1	Mouse	Santa Cruz (SC-20701)
RNA PolII	Mouse	Abcam (ab5408)
GR (H300)	Rabbit	Santa Cruz (sc-8992)

Table 2.7 Sequences for all primers used in QPCR for ChIP analysis

Name	Sequence	Product size
κ B Site		
κ B1 Forward	5'-GCT GCT GTG TGG GGA TAG AT-3'	95
Reverse	5'-GGC CCA GTC TTT TCC TCT CT-3'	
κ B2 Forward	5'-AAC CCA GAG AGG AAA AGA CT-3'	116
Reverse	5'-TGC AGG AAA GGA GAG AGA G-3'	
κ B3 Forward	5'-GCC ATC TCC CTC CCA CCT-3'	153
Reverse	5'-CCT CGG CGA CTC CTT CCT-3'	

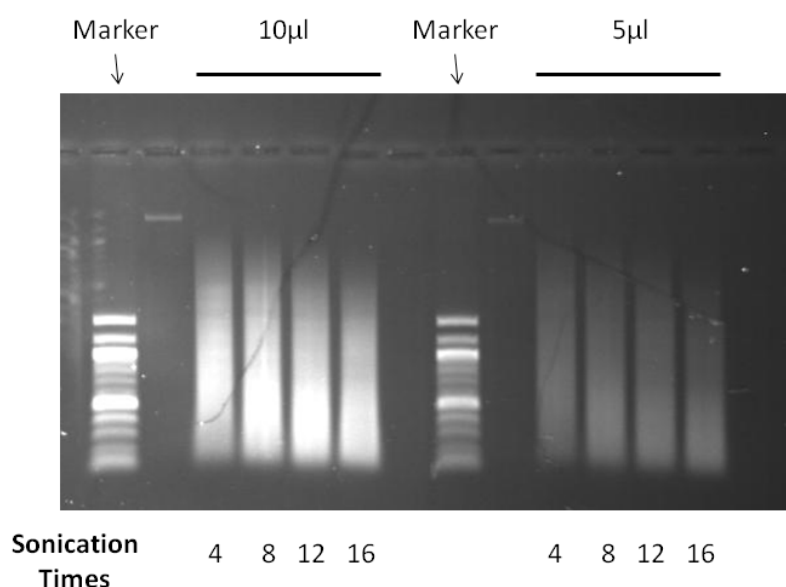
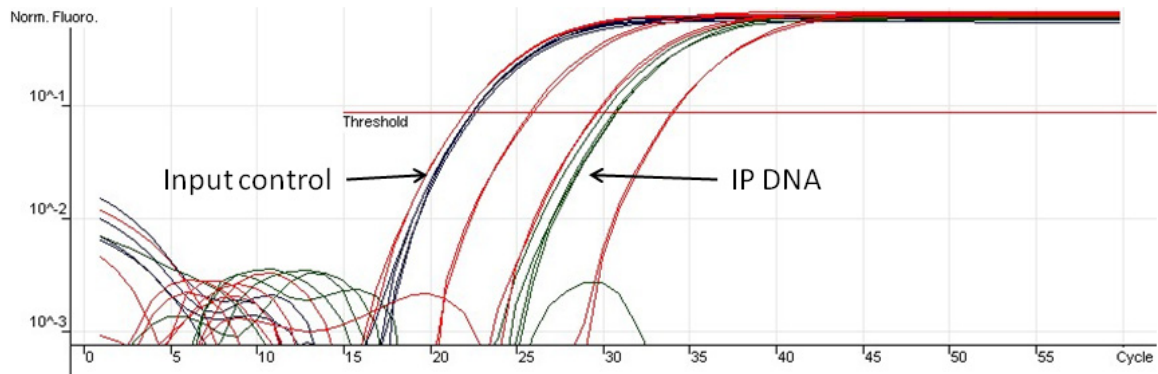
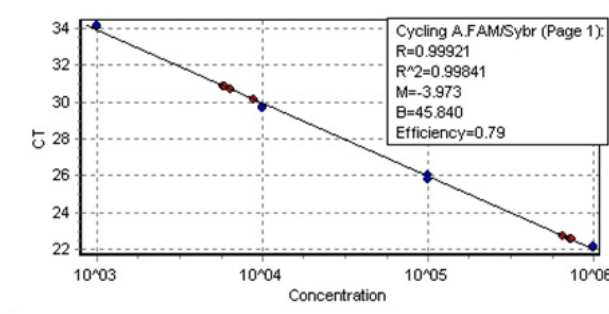


Figure 2.5 Gel analyses for optimization of sonication setting. A549 cells were fixed for 10mins with 1% formaldehyde (final concentration) and chromatin sheared with 4, 8, 12, or 16 pulses at 40% power for 10sec in 200µl SDS lysis buffer. The chromatin samples were subjected to cross-link reversal, treated with proteinase K followed by phenol/chloroform extraction and finally precipitated with alcohol. Air-dried DNA was dissolved in 50µl nuclease free water and two aliquots (5µl, 10µl) separated by 1% agarose gel electrophoresis. DNA sonicated for 8 pulses were suitable to be used in ChIP assays showing a chromatin smear between 200-1500bp.

A



B



C

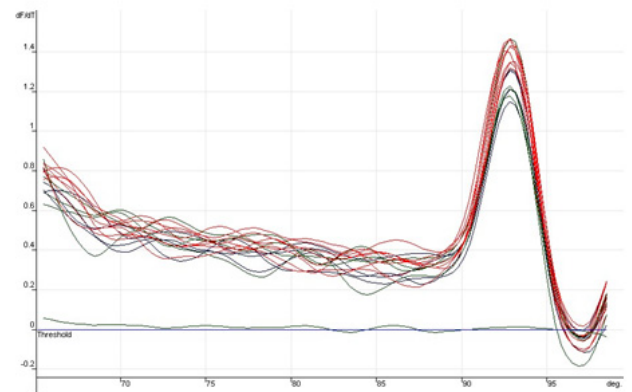


Figure 2.6 Specificity and Sensitivity quantification by QPCR in Chromatin immunoprecipitation experiments. (A) Raw channel data for QPCR demonstrates serial dilution of unknown concentration DNA (1x, 0.1x, 0.01x, 0.001x) results. The C_T values for both input control (blue curve) and IP DNA (green curve) were in the range between 1x and 0.001x the unknown concentration sample. (B) Standard curve of the QPCR result. (C) Melting curve analysis of the amplified products shows a single peak demonstrating good specificity of the final product.

2.13 GR-IP and NF- κ B-IP histone methyl transferase (HMT) activity assay

HMT activity assay was modified from a previous report (Li et al., 2002). GR was immunoprecipitated from 500 μ g of nuclear extracts by incubating in 200 μ l of immunoprecipitation buffer (IP buffer; 25mM Tris [pH 8.0], 150mM NaCl, 1mM DTT, 0.1% NP-40, and 1mM PMSF) with a specific anti-GR antibody (5 μ g, H-300, Santa Cruz Biotechnology, Santa Cruz, CA). After 1hr incubation in a cold room with rotation, 15 μ l of 50% protein A/G-Sepharose beads was added to each tube and further incubated for 2hrs. The beads were then collected by brief centrifugation and washed five times with IP buffer and once with HMT buffer (50mM Tris pH 8.0, 1mM PMSF, and 0.5mM DTT). The HMT assay was performed by incubating the washed beads with 2.5 μ g of calf thymus core histones (Roche Molecular Biochemicals) as substrate along with S-(5'-Adenosyl)-L-methionine chloride (SAM, 100 μ M) as a methyl donor in 25 μ l of the HMT buffer at 30°C for 1hr. Histones were fractionated by 16% SDS-PAGE). H3K9-directed HMT activity was detected by Western blotting analysis with an antibody against trimethyl-H3K9 (Upstate Biotechnology, Buckingham, U.K.).

2.14 GR and NF- κ B activation assay

This procedure essentially followed that of the active motif TransAM® Kit protocol. Briefly, 10 μ g nuclear extracts were collected using the Nuclear Extraction Kit from Active Motif (Active Motif) as previously described for the assay. NF- κ B activation was measured with TransAm NF- κ B family kit (Active Motif) according to the manufacturer's instructions. 30 μ l complete binding buffer (2 μ M DTT, 10ng/ μ l Salmon sperm DNA in binding buffer AM3) was added to each well of 96 well plate. 10 μ g nuclear extracts dissolved in 20 μ l complete lysis buffer (1mM DTT, 0.2 μ l protease inhibitor cocktail, in lysis Buffer AM1) into each well. Blank wells containing complete lysis buffer or binding buffer only were included. Samples

were incubated at room temperature for 1hr with mild agitation. 200µl wash buffer was used for washing wells before adding 50µl diluted NF-κB p65 antibody to each well being used, including the blank wells. The plate was then incubated for 1hr at room temperature. After washing, 50µl diluted HRP-conjugated antibody was added to each well and incubated at room temperature for 1hr. At the end of this time, each well was washed 4 times before 50µl Developing solution was added with minimal exposure of the plate to light. Stop solution (2N H₂SO₄) was added after 10mins as the blue colour developed and chemiluminescence was read using a plate reader.

2.15 *Bisulphite sequencing*

Genomic DNA was extracted using phenol/chloroform as described above (Section 2.12). DNA concentration was determined using a nanodrop machine (Invitrogen). Bisulphite conversion was performed exactly as described in the MethylSEQR bisulfite conversion kit (Applied biosystem, Foster City, CA, USA). 250ng of genomic DNA (gDNA) was combined with 5µl methylSEQR® Denaturation Buffer to a final volume of 50µl and mixed thoroughly before incubation at 37°C for 15mins. 100µl methylSEQR® Conversion Reagent was mixed with the denatured gDNA and incubated in the dark at 50°C for 16hrs. The mixture was then diluted with 250µl deionized water and purified on a purification column. 350µl 0.1M sodium hydroxide was added to column to start the conversion reaction and the converted DNA washed off with TE buffer.

The converted DNA was then processed by PCR using specific primers spanning the gene promoter region of interest. The primers used for PCR amplification and sequencing are listed in the Table within Fig 2.7. 0.4ug DNA was mixed with PCR master mix [consisting of: Green GoTag® Flexi Buffer, 2mM MgCl₂, 0.2mM each dNTP, 0.2µM for both sense and antisense primer, 1.25unit GoTaq® Hot Start Polymerase](All Promega). After an initial 2mins denaturing step at 94.5°C, 40 cycles of PCR were performed (94°C for 30secs, 72°C

for 1min). After product size confirmation using gel electrophoresis, samples were purified with a Qiagen PCR purification kit and sent for sequencing by Cogenics (Takeley, Essex, UK).

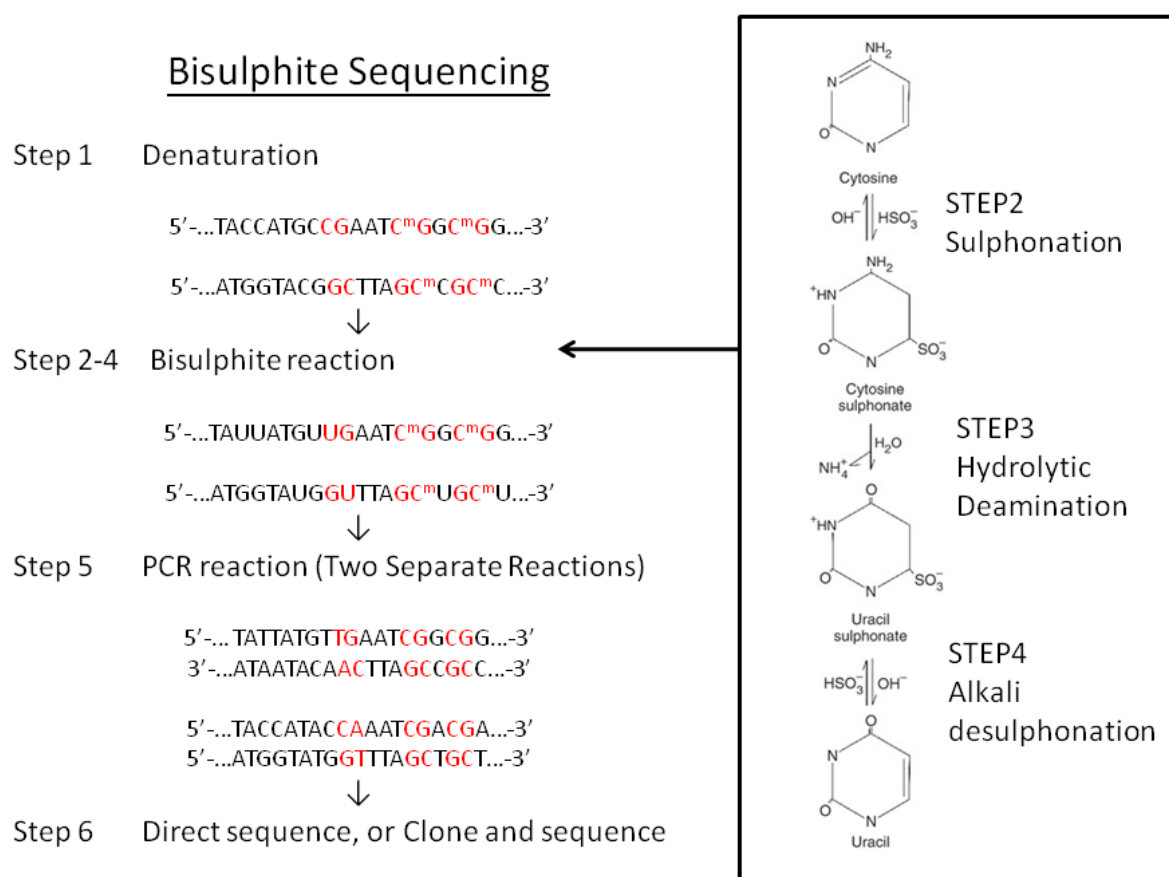


Figure 2.7 Schematic diagram of the steps involved in the bisulphite conversion and PCR amplification reaction. Genomic DNA was denatured to obtain single stranded DNA. The DNA then underwent the bisulphite conversion reaction. In this method unmethylated C residues are transformed to a T residue and methylated C residues remain as C residues. Specific DNA regions were amplified using sequence-specific primers. After PCR amplification, the methylation status of the CpG sites can be determined by direct sequencing, or by cloning and sequencing.

2.16 *Methylation dependent immunoprecipitation (MeDIP) assay*

The assay was modified from a previous report (Zhang et al., 2006). A549 cells (2×10^6) from 2 wells of 6-well plate were used in each experiment. DNA sonication conditions were the same as used in the ChIP protocol above (section 2.x) i.e. 8x 10secs at 40% amplitude. Genomic DNA was extracted using phenol/chloroform and after air drying was dissolved in 100ul of DEPC water. 10ul gDNA was stored as input control. The remaining 90ul was incubated with 110ul FB buffer [consisting 10mM Tris-HCl, PH 7.5, 50mM NaCl, 1mM EDTA) with 1.8μg 5meC-specific antibody (Calbiochem, , Nottingham, UK) overnight at 4°C on a rotator. Antibody-DNA complexes were isolated by addition of 35ul protein A/G beads (Santa Cruz Biotechnology) for 6hrs at 4°C. Beads were washed five times in 100ul FB buffer and DNA eluted by vortexing in 100ul TE buffer containing 1.5%, 0.5% and 0.1% SDS sequentially. Elutes were pooled and DNA purified by phenol/chloroform extraction. The amount of methylated DNA in each sample was analyzed by QPCR with specific primers and compared with input control DNA for each sample.

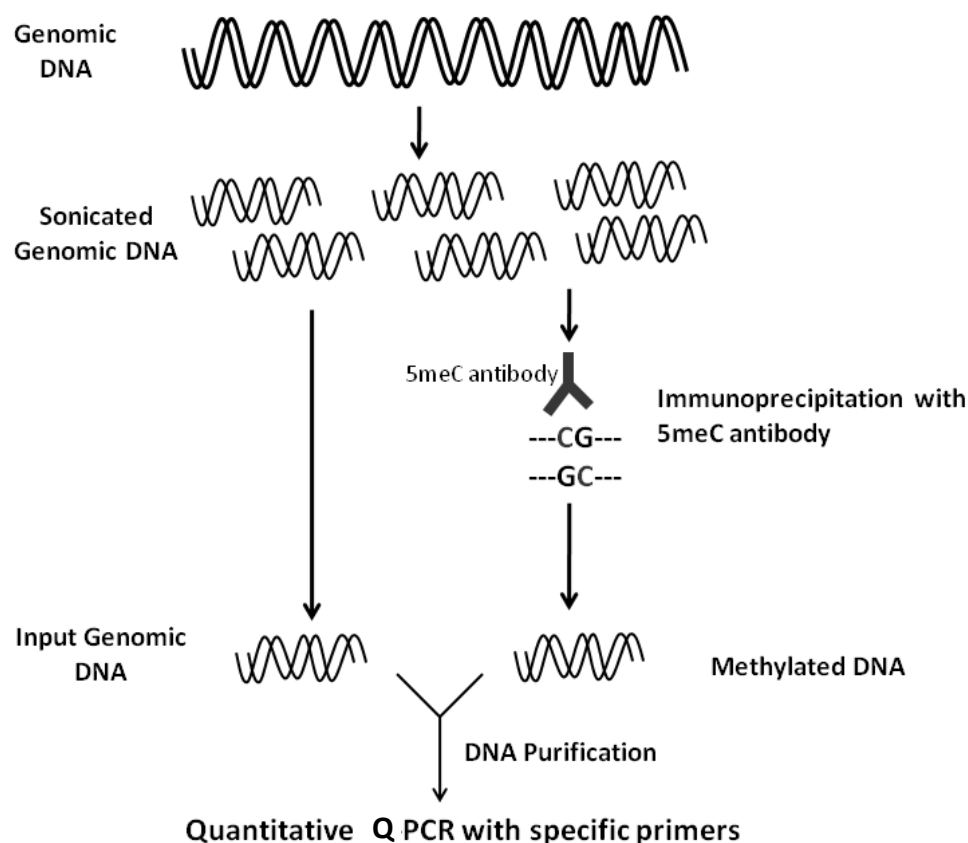


Figure 2.8 Schematic diagram of the steps involved in the Methylation dependent immunoprecipitation (MeDIP) assay. Cells were lysed in SDS lysis buffer and genomic DNA (gDNA) fragmented by sonication. 1/20 of sonicated gDNA was used as input control. All remaining sonicated DNA was immunoprecipitated with 5meC antibody overnight and purified by phenol/chloroform extraction. Methylated DNA was quantified by QPCR with primers specific for regions of interest in selected promoters as a ratio to input control.

2.17 Statistics

Data points are values in the text and figure legends represent the mean S.E.M of at least 3 independent determinations taken from different cell preparations. Groups of data were compared using the Mann-Whitney test. Statistical significance was set at $P < 0.05$.

CHAPTER THREE
THE EFFECT OF OXIDATIVE TRESS ON TGF- β 1 TRANSCRIPTION
IN A549 CELLS

3.1 Rationale

Chronic inflamed tissues are characterized by a complex milieu of pro-inflammatory cytokines (e.g. IL-1 β and TNF α), and ROS (e.g. H₂O₂) (Enesa et al., 2008). In smokers (Nowak et al., 1996), asthmatic patients (Antczak et al., 1997), and COPD patients (Nowak et al., 1998) also tend to have higher levels of exhaled H₂O₂ than non-smokers, and levels are even higher during exacerbations of COPD (Dekhuijzen et al., 1996). Other markers of oxidative stress including lipid peroxidation products and changes in redox capacity are also abnormal in the lungs and biopsies of COPD patients and subjects with severe asthma (Rahman review). Oxidative stress, like H₂O₂, may activate intracellular cascades through redox sensitive transcription factors such as NF- κ B and AP-1 (Gloire et al., 2006) to exaggerate lung inflammation in the airways. Oxidative stress has been implicated in the pathogenesis of inflammatory lung disorders (Rahman and Adcock, 2006). IL-1 β is a proinflammatory cytokine which is secreted from monocytes/macrophages, neutrophils, and airway epithelial cells (Chung, 2001). Increased IL-1 β is reported in induced sputum from stable COPD patients (Keatings et al., 1996) and greater IL-1 β release is observed from alveolar macrophages from cigarette smokers and COPD patients (Lim et al., 2000). Upon binding to IL-1 receptors on the cell surface, IL-1 β stimulates the expression of a large number of proinflammatory proteins, including enzymes, adhesion molecules, chemokines, and acute phase proteins (Dunne and O'Neill, 2003). Transcription factor NF- κ B is one of the key signalling molecules activated by IL-1 β , and leads to increased transcription of target genes (Barnes and Karin, 1997).

Elevated expression of TGF- β 1 occurs in a number of pulmonary diseases such as COPD (de Boer et al., 1998), asthma (Minshall et al., 1997; Redington et al., 1997b), as well as pulmonary fibrosis (Khalil et al., 1996). Analysis of the 5' end of the human TGF- β 1 gene revealed that the promoter region is very G+C rich, containing binding sites for AP-1 (Kim et al., 1990; Kim et al., 1989), and NF- κ B. Increased recruitment of NF- κ B p65 to the TGF- β 1

promoter is found following IL-1 β stimulation of A549 cells (Lee et al., 2006). However, information concerning the transcriptional regulation of TGF- β 1 under conditions of oxidative stress remains unclear.

Since TGF- β 1 is upregulated in some chronic inflammatory lung disease, it is interesting to investigate whether oxidative stress, together with IL-1 β , are involved in this transcriptional regulation, and whether the redox-sensitive transcription factor NF- κ B is involved in IL-1 β and H₂O₂ induced TGF- β 1 transcription.

3.1.1 Hypothesis and aims of this Chapter

We hypothesise that oxidative stress, in combination with IL-1 β , may upregulate TGF- β 1 in the airways. And the upregulated TGF- β 1 can be suppressed by glucocorticoid.

The specific aims were:

1. To determine the effect of H₂O₂, and in combination with IL-1 β , on TGF- β 1 production by A549 cells, and BEAS-2B cells.
2. To evaluate the role of redox sensitive transcription factors, such as NF- κ B, on TGF- β 1 expression.
3. To examine the effect of FP treatment on H₂O₂ stimulation alone, and in combination with IL-1 β induced upregulation of TGF- β 1 production.

3.2 Experiment Protocols

3.2.1 *Cell Survival and Apoptosis study under oxidative stress*

85-95% confluent A549 cells were cultured in serum-free DMEM medium supplemented with L-glutamine for 48hrs before stimulation. Cells were stimulated with IL-1 β (10ng/ml), H₂O₂ (40, 200, 400 and 2000 μ M) and fluticasone propionate (10⁻⁸ and 10⁻¹⁰M) for 6h. Cells were collected following treatment with trypsin and neutralized with complete medium (10% FCS, 2% L-glutamine). Cells were subsequently washed twice with cold PBS and then

resuspend in 1X Binding Buffer at a concentration of 1×10^6 cells/ml. 100 μ l of the solution (1×10^5 cells) were transferred to a 5ml culture tube. 5 μ l of Annexin V-FITC and 5 μ l PI were added to the solution. The cells were vortexed gently and incubated for 15mins at RT (25°C) in the dark. 400 μ l of 1X Binding Buffer were added to each tube before analysis by flow cytometry within 1h. Annexin-V positive cells were considered to be apoptotic if they stained negative for PI (normal permeability) or as either late apoptotic or necrotic if they stained positive for PI (increased permeability) (Geiser et al., 2004).

3.2.2 *Measurement of intracellular oxidative stress by Dichlorofluorescein Assay*

A549 cells were split and counted by trypan blue exclusion. Viable cells (10^4 /well) were plated into 96-well collagen-coated plates 1 day before the experiments. On the day of the experiments, after removing the medium, the cells were washed with KRH buffer (118mM NaCl, 4.8mM KCl, 1.2mM MgSO₄, 2.5mM CaCl₂, 10nM HEPES, PH 7.4), and then incubated with 100 μ M DCFH-DA (2',7'-dichlorofluorescein diacetate) in loading medium in 5% CO₂/95% air at 37°C for 30mins. After DCFH-DA was removed, the cells were washed and incubated with KRH buffer and IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated for various times. The cell fluorescence from each well was measured by Fluorescence plate reader (Synergy HT) and recorded. The excitation filter was set at 485nm and the emission filter was set at 530nm.

3.2.3 *TGF- β 1 release from stimulated cells*

85-95% confluent A549 cells were cultured in serum-free DMEM medium supplemented with L-glutamine for 36hrs before stimulation. Cells were stimulated with IL-1 β (10ng/ml), with or without H₂O₂ 200 μ M, or left untreated for variable times (0, 8, 16 and 24hrs). Total TGF- β 1 in the supernatant was determined by ELISA.

3.2.4 *Effect of AS602868, SP600125 and actinomycin D on active TGF- β 1 release*

A549 cells were pre-treated with AS602868 (10 μ M, an IKK β inhibitor), SP600125 (10 μ M, a JNK inhibitor) or Actinomycin D (ACD, 10 μ g/ml, an inhibitor of transcription) for 1hr before incubation with medium alone or IL-1 β , with or without H₂O₂, for 6hrs. Supernatants were collected and assayed for TGF- β 1 by RT-QPCR.

3.2.5 *TGF- β 1 mRNA expression*

Cells were incubated with medium or IL-1 β (0.01, 0.1, 1, 5 and 10ng/ml), with or without H₂O₂ (40, 100, 200, 1000 and 2000 μ M), for 4, 8, 16 and 24hrs. RNA was isolated and levels of TGF- β 1 mRNA were quantified by RT-QPCR as a ratio of control housekeeping mRNA (β -actin or GNB2L1).

3.2.6 *TGF- β 1 protein production*

Cells were incubated with medium or IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), for 8 or 24hrs. Protein extracts were obtained and TGF- β 1 detected by Western blotting.

3.2.7 *Effect of oxidative stress on BEAS-2B cells*

90% confluent BEAS-2B cells were serum-starved for 24hrs before stimulating with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated and samples collected after 6hrs. Total RNA was isolated and levels of TGF- β 1 mRNA were quantified by RT-QPCR with primers specific for TGF- β 1. Relative TGF- β 1 mRNA expression levels were normalized to GNB2L1 mRNA expression.

3.2.8 *NF- κ B DNA binding activity*

Cells were left untreated or were stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), for 1hr. Nuclear protein was extracted and NF- κ B DNA binding activity was

determined by TransAm NF- κ B p65 assay kit (Active motif, Belgium).

3.2.9 NF- κ B p65 occupancy at the native TGF- β 1 promoter

A549 cells were untreated, or stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), for variable times (0, 30, 60 and 120mins). NF- κ B p65 recruitment to the TGF- β 1 promoter was determined by ChIP assay with a specific p65 antibody. Enrichment of DNA was quantified by QPCR with primer pairs amplifying DNA sequences around the putative κ B sites.

3.2.10 In vitro p65 TGF- β 1 promoter binding affinity

A549 cells were treated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated for 1h. NF- κ B p65 binding affinity to specific TGF- β 1 sites was determined by an in vitro p65 DNA binding assay with a specific antibody against p65. Competitive experiments for the κ B-3 site were carried out by incubating samples with wild-type and mutant competitor DNA oligonucleotides (w κ B3 and m κ B3).

3.2.11 Effect of Fluticasone propionate (FP) on oxidative stress induced TGF- β 1 up-regulation

A549 cells were serum-starved for 48hrs and then pre-treated with variable concentrations of FP (10⁻¹² - 10⁻⁶M) for 30mins. The cells were stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated for 4hrs. Total RNA was isolated and levels of TGF- β 1 mRNA were quantified by RT-QPCR and expressed relative to control mRNA.

3.2.12 Effect of Mifepristone (RU486) on FP-suppression of oxidative stress-induced TGF- β 1 mRNA expression

A549 cells were serum starved for 48hrs, pre-treated with Mifepristone (10⁻¹⁰ - 10⁻⁶M) for

30mins, followed by addition of FP (10^{-8} M) for 30mins. The cells were then stimulated with IL-1 β (10ng/ml) plus H₂O₂ (200 μ M) for 6hrs. Total RNA was isolated, and levels of TGF- β 1 mRNA were quantified by RT-QPCR. Relative expression level was normalized with GNB2L1 mRNA expression level as the internal control.

3.2.13 Effect of IL-1 β and H₂O₂ on IL-8/CXCL8 and GMCSF mRNA expression.

A549 cells were serum starved for 48h before pre-treatment with FP (10^{-8} M) for 30mins. The cells were then stimulated with IL-1 β (10ng/ml) plus H₂O₂ (200 μ M) for 6hrs. Total RNA was isolated, and levels of IL-8/CXCL8 and GM-CSF mRNA were quantified by RT-QPCR with specific primer pairs. Relative expression levels were normalized to GNB2L1 mRNA expression as an internal control.

3.3 RESULTS

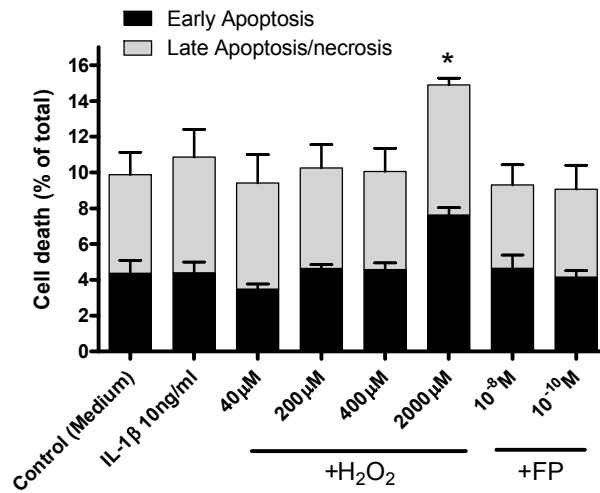
3.3.1 Effect of hydrogen peroxide (H₂O₂), IL-1 β , and fluticasone propionate (FP) on cell survival and cell proliferation

H₂O₂ can affect cell apoptosis and death (Geiser et al., 2004; Miyoshi et al., 2006) so we investigated the effect of H₂O₂ on cell apoptosis by flow cytometry using Annexin-V and PI-staining. H₂O₂-induced cell death was only observed at the highest concentration of H₂O₂ examined (2000 μ M) (Fig 3.1A). Both early apoptosis (PI-negative cells) and late apoptosis or necrosis (PI-positive cells) contributed to cell death at this concentration of H₂O₂. At lower concentrations of H₂O₂ no significant effect on cell death or apoptosis was seen in medium control, IL-1 β - or FP-treated groups.

In order to determine the effect of H₂O₂, IL-1 β , and FP on cell proliferation, we perform MTT assays after 16hrs of incubation with the different treatments (Fig 3.1B). Although there

was a trend towards a reduction in cell proliferation with H_2O_2 alone this did not reach statistical significance and was not seen in the presence of either IL-1 β or FP.

A.



B.

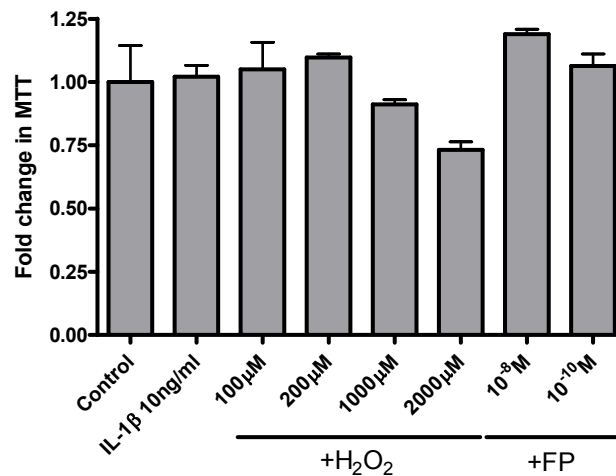


Figure 3.1 Effect of hydrogen peroxide (H_2O_2), IL-1 β , and fluticasone propionate (FP) on cell survival and cell proliferation. (A) Effect of treatment on cell survival and apoptosis. A549 cells were treated with H_2O_2 , IL-1 β , and FP. Early apoptosis [annexin V positive, propidium iodide (PI) negative] and late apoptosis/necrosis (annexin V positive, PI positive) was determined after 16h by annexin V/PI staining and flow cytometry. H_2O_2 increased apoptosis at the highest concentration of H_2O_2 investigated (2000 μ M). Results are shown as mean $\pm$ SEM. * $P < 0.05$ compared with medium control (n=4). (B) Effect of treatments on cell proliferation as determined by MTT assay. A549 cells were treated with H_2O_2 , IL-1 β , and FP for 16hrs. Results are shown as mean $\pm$ SEM (n=3).

3.3.2 Effect of IL-1 β and H₂O₂ on intracellular oxidative stress

To examine whether H₂O₂ is able to induce intercellular oxidative stress in our model, A549 cells were stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated for variable times (1, 2, 4, 6 and 24hrs) (Fig 3.2). Upon stimulation with IL-1 β , there was no difference in intracellular oxidative stress when compared to the basal fluorescence reading. H₂O₂ stimulation effectively increased intracellular oxidative status when compared to the basal level. In addition, IL-1 β plus H₂O₂, in combination, further enhanced intracellular oxidative stress. This demonstrates that IL-1 β stimulation may enhance the ability of exogenous H₂O₂ to trigger intracellular oxidative stress. The data also suggests that a component of the IL-1 β signalling pathway is redox sensitive.

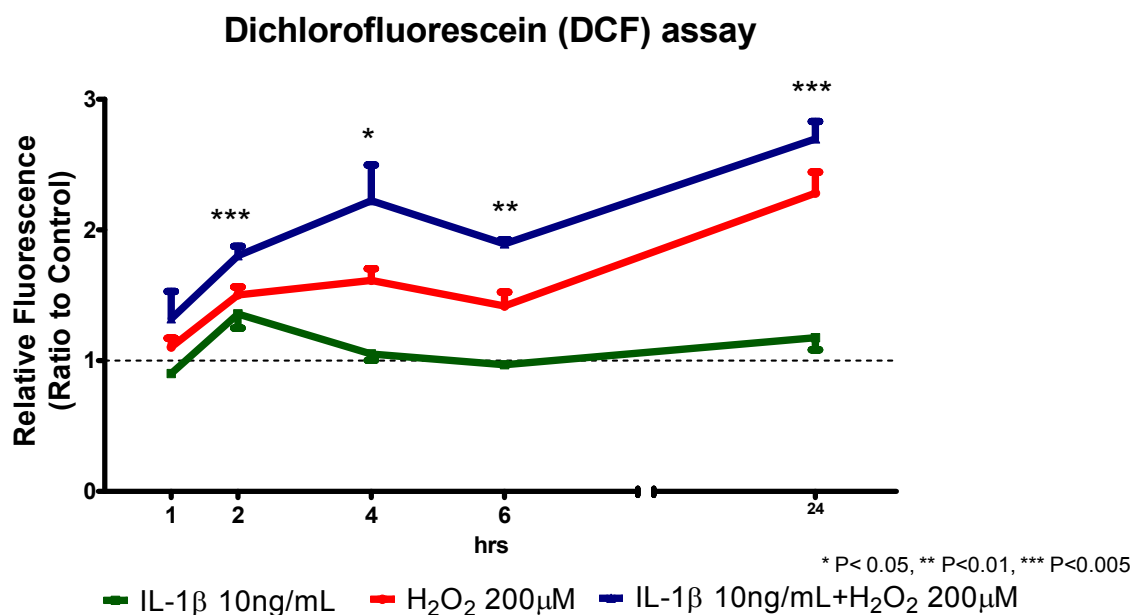


Figure 3.2 Effect of IL-1 β and hydrogen peroxide (H₂O₂) on intracellular oxidative stress. Cells were washed with KRH buffer and then incubated with 100 μ M DCFH-DA in the loading medium in 5% CO₂/95% air at 37°C for 30 mins. DCFH-DA was removed, and cells were washed and incubated with KRH buffer and finally stimulated with IL-1 β , with or without H₂O₂, or left untreated for variable times (1, 2, 4, 6 and 24hrs). Results are shown as mean $\pm$ SEM. * p<0.05, ** p<0.01, *** p<0.005 when compared to basal status at the same time point. N = 3 independent experiment.

3.3.3 Effects of IL-1 β on TGF- β 1 mRNA

The effects of IL-1 β on gene expression of TGF- β 1 were analyzed by quantitative real-time (RT-QPCR) with TGF- β 1 specific primers. A549 cells were serum starved for 24hrs before being stimulated with various concentrations of IL-1 β (0.01 - 10ng/ml) for 6hrs. The results demonstrated a concentration-dependent induction of TGF- β 1 mRNA by IL-1 β . Levels of TGF- β 1 mRNA increased with a maximal 1.6-fold induction ($P<0.05$) achieved at 10ng/ml IL-1 β (Fig. 3.3A).

The kinetics of mRNA expression demonstrated a time-dependent induction of TGF- β 1 mRNA by IL-1 β (Fig. 3.3B). The level of TGF- β 1 in mRNA revealed a 1.5-fold increase ($P<0.05$) when compared with media control at 6hrs, and reached a maximal response at 16h (1.7 fold, $P<0.005$) which was maintained for at least 24hrs (1.5 fold, $P<0.01$).

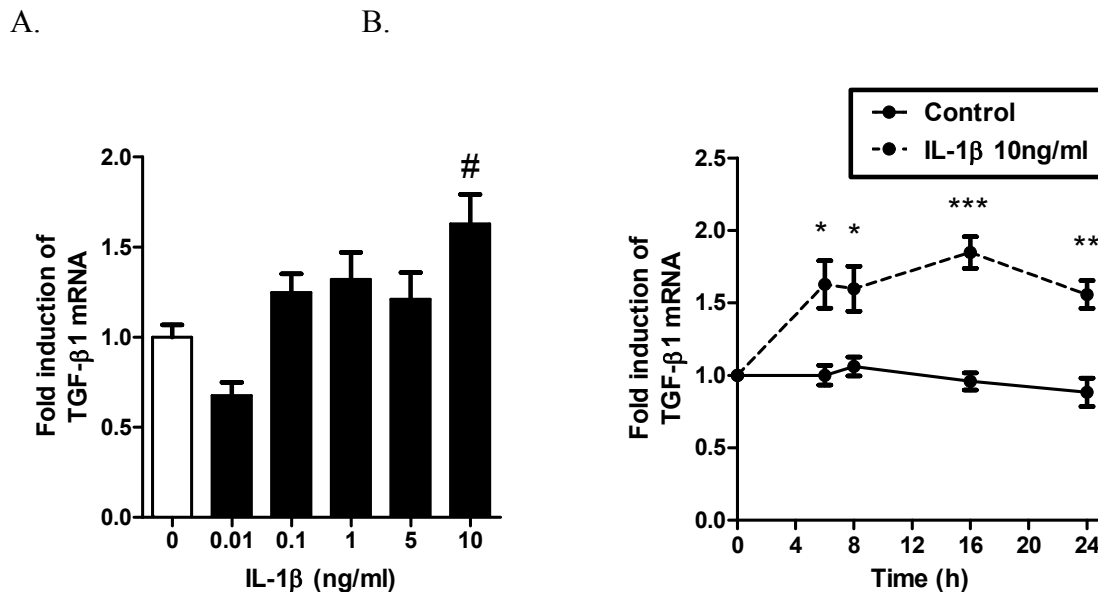


Figure 3.3 Effect of IL-1 β on TGF- β 1 mRNA induction. Cells were incubated with medium or IL-1 β (0.01 - 10ng/ml) for 6hrs (A) or IL-1 β (10ng/ml) for 6, 8, 16 and 24hrs (B). Levels of TGF- β 1 mRNA were quantified by RT-QPCR and were normalized to GAPDH mRNA levels. # $P<0.05$ when compared with no treatment. * $P<0.05$; ** $P<0.01$; *** $P<0.005$ when compared with non-treatment (n=at least three independent experiments).

3.3.4 Effects of H₂O₂ on TGF-β1 message RNA expression

H₂O₂ alone had a small but significant 1.3 fold induction of TGF-β1 mRNA expression at 200μM only (P<0.05) (Fig. 3.4A) but this was not concentration-dependent (Fig. 3.4A).

This induction of TGF-β1 mRNA was time-dependent (Fig. 3.4B). The level of TGF-β1 mRNA induced by 200μM H₂O₂ revealed a slight increase from 6hrs, and reached a maximal 1.6 fold (P<0.05) increase compared with media control at 16hrs. However, the induction ratio returned to baseline by 24hrs (Fig. 3.4B).

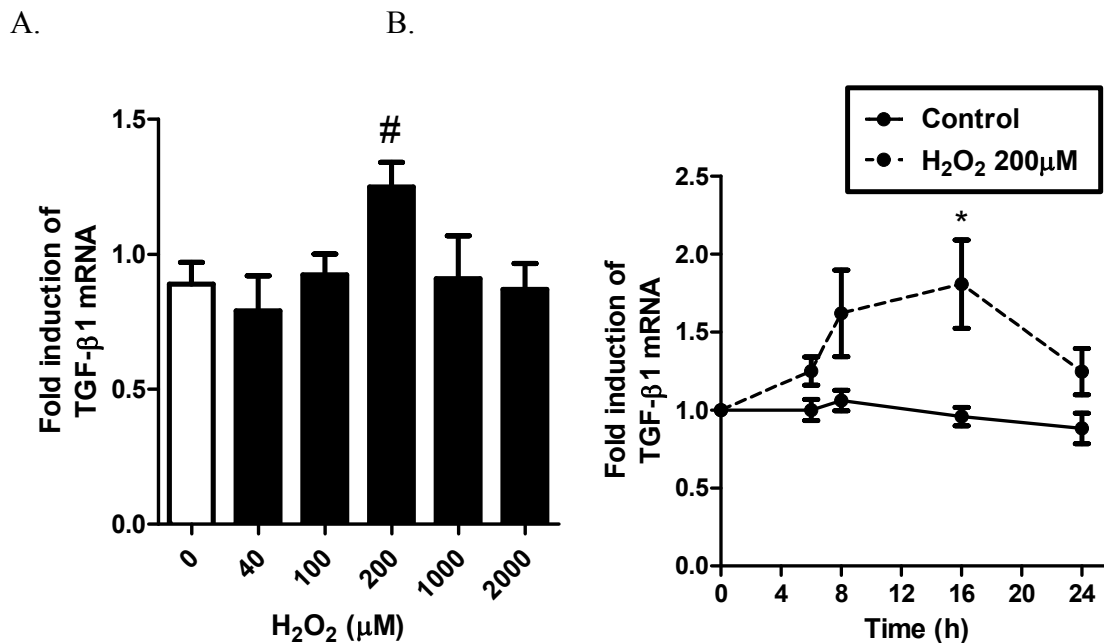


Figure 3.4 Effect of hydrogen peroxide (H₂O₂) on TGF-β1 mRNA induction. Cells were incubated with medium or H₂O₂ (40 - 2000μM) for 6h (A) or H₂O₂ (200μM) alone for 6, 8, 16 and 24hrs (B). Levels of TGF-β1 mRNA were quantified by RT-QPCR and normalized to GAPDH mRNA levels. [#]P<0.05 when compared versus non-treatment. *P<0.05 when compared with non-treatment (n=at least three independent experiments).

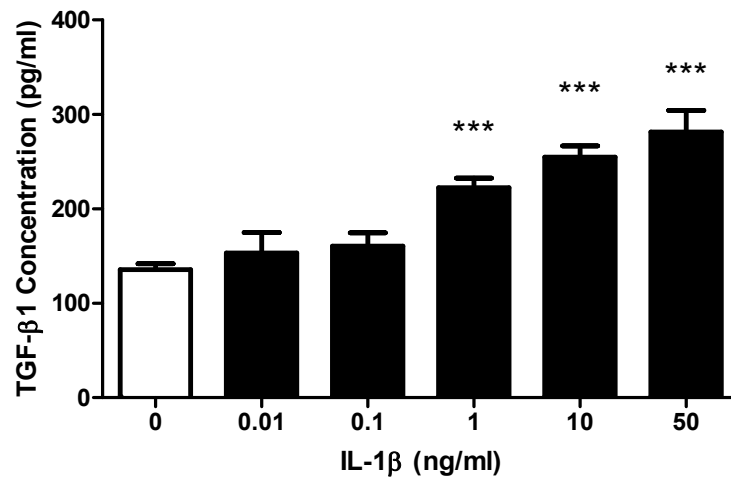
3.3.5 Effect of IL-1 β and hydrogen peroxide (H₂O₂) on TGF- β 1 release

TGF- β 1 protein levels in the conditioned media of cultured A549 cells were measured by ELISA following IL-1 β (Fig. 3.5A) or H₂O₂ treatment (Fig. 3.5B). Inasmuch as most cells secrete TGF- β 1 in latent form which can be activated upon extreme acidification, heat or protease digestion (Annes et al., 2003), total TGF- β 1 was determined by acidification of conditioned media.

The results demonstrated a significant concentration-dependent elevation of total TGF- β 1 levels in response to IL-1 β (Fig 3.5A). This stimulation reached a maximal 1.7-fold induction (281.2 ± 23.1 pg/ml vs 135.7 ± 6.3 pg/ml, $P < 0.005$) at 50ng/ml IL-1 β (Fig. 3.5A), which is similar to the effect on TGF- β 1 mRNA levels (Fig. 3.3A).

In the H₂O₂ alone treated group, total TGF- β 1 levels increased up to 1.2 fold (167.1 ± 10.1 pg/ml vs 135.7 ± 6.3 pg/ml, $P < 0.05$) after treatment with 200 μ M H₂O₂ (Fig 3.5B). Interestingly, in contrast to the increases seen in TGF- β 1 mRNA levels, treatment with 2000 μ M H₂O₂ produced a decrease in the levels of total TGF- β 1 by 1.5 fold (91.9 ± 10.94 pg/ml vs 135.7 ± 6.3 pg/ml, $P < 0.05$).

A.



B.

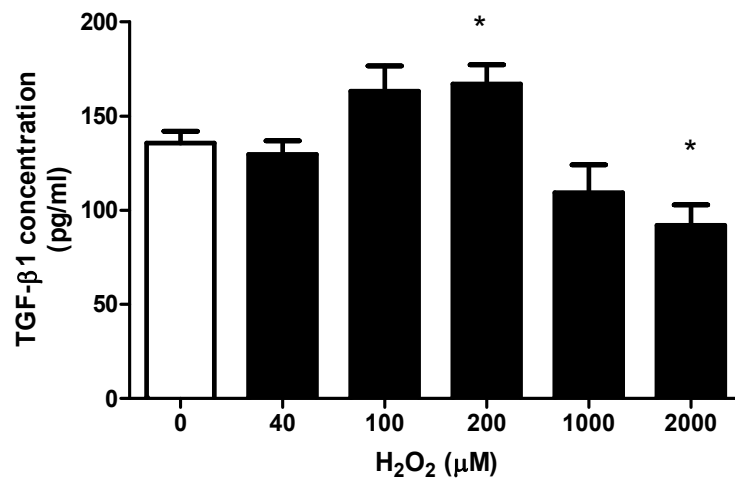


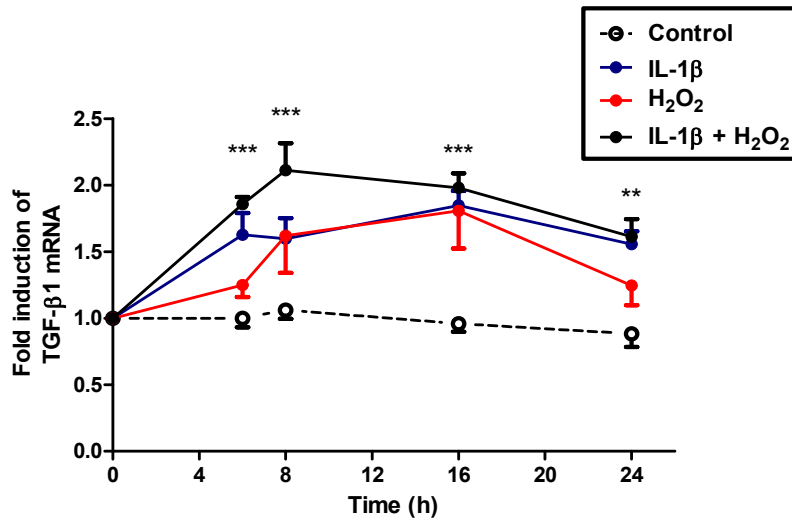
Figure 3.5 Effect of IL-1β (A) and hydrogen peroxide (H₂O₂)(B) on total TGF-β1 release. Cells were incubated with medium alone, IL-1β (0.01 - 50ng/ml) or H₂O₂ (40 - 2000μM) for 8h. Supernatants were collected and assayed for TGF-β1 by ELISA. *P<0.05; *** P<0.005 versus non-treatment. Results are expressed as means ± the SEM (n=at least three independent experiments.)

3.3.6 Hydrogen peroxide (H₂O₂) augments the effect of IL-1 β on TGF- β 1 release

The kinetics of TGF- β 1 mRNA expression demonstrates a time-dependent induction by IL-1 β plus H₂O₂ (Fig. 3.6A). Upon IL-1 β (10ng/ml) plus H₂O₂ (200 μ M) stimulation, levels of TGF- β 1 mRNA increased 1.7 fold (P<0.005) after 6h and reached a maximum 2.0 fold increase (p<0.005) after 8hrs when compared with media control. Although the levels of TGF- β 1 mRNA were still elevated at 16 and 24hrs there was no additional effect above that of IL-1 β over this time (1.6 fold, P<0.05). Furthermore, the enhanced effect of IL-1 β plus H₂O₂ treatment evident at 6 and 8hrs was lost by 16-24hrs.

Upon IL-1 β (10ng/ml) plus H₂O₂ (200 μ M) stimulation, measurement of TGF- β 1 by ELISA (Fig. 3.6B) demonstrated elevated levels of total TGF- β 1 protein when compared with medium alone at 8hrs (164.6 $\pm$ 7.3 pg/ml vs 135.7 $\pm$ 6.3 pg/ml, P<0.05) and 16hrs (316.1 $\pm$ 11.4 pg/ml vs 264.8 $\pm$ 40.9 pg/ml, P<0.05). However, in contrast to the effect seen on TGF- β 1 mRNA levels there was no additional effect of IL-1 β plus H₂O₂ treatment when compared with IL-1 β or H₂O₂ induction alone at any time point examined.

A.



B.

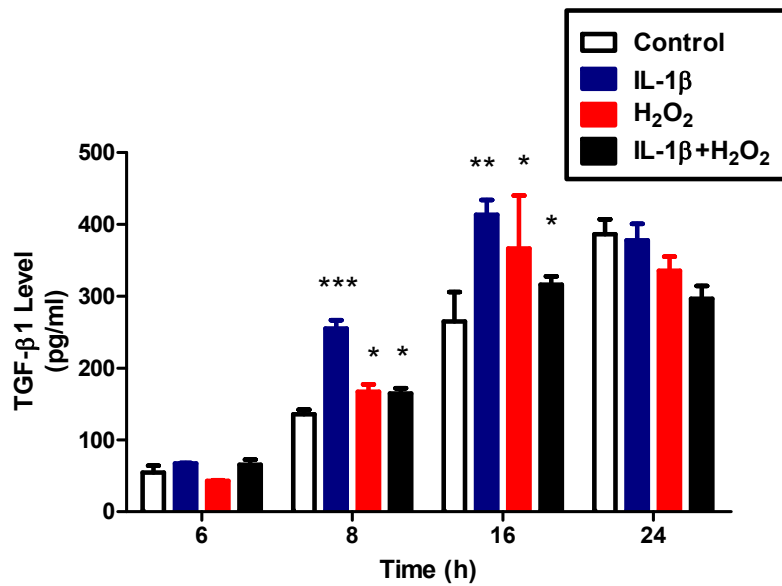


Figure 3.6 Effect of IL-1β plus hydrogen peroxide (H₂O₂) on TGF-β1 mRNA and total protein. Cells were incubated with medium alone, IL-1β (10ng/ml), H₂O₂ (200μM), or IL-1β plus H₂O₂ for 6, 8, 16 or 24hrs. (A) TGF-β1 mRNA levels were quantified by RT-QPCR and were normalized to GAPDH mRNA levels. (B) Supernatants were collected and assayed for total TGF-β1 by ELISA. *P<0.05; **P<0.01; ***P<0.005 versus medium alone. Results are expressed as means ± SEM (n=at least three independent experiments).

3.3.7 Effect of H₂O₂, IL-1 β , and H₂O₂ plus IL-1 β on TGF- β 1 production

In an initial attempt to determine whether IL-1 β -induction of TGF- β 1 release involves *de novo* TGF- β 1 production rather than release from pre-formed stores, Western blot analysis of whole cell lysates was performed. Using a specific TGF- β 1 antibody a clear immunoreactive band at 25kd was detected after 8h of incubation with medium alone (Fig 3.7). This was elevated in the presence of 10ng/ml IL-1 β (1.5 fold, P<0.01) and H₂O₂ plus IL-1 β (1.4 fold, P<0.05) but not by 200 μ M H₂O₂ alone (Fig 3.7B). IL-1 β alone also increased intracellular TGF- β 1 at 24 hrs (1.3 fold, P<0.01)(Fig 3.7C).

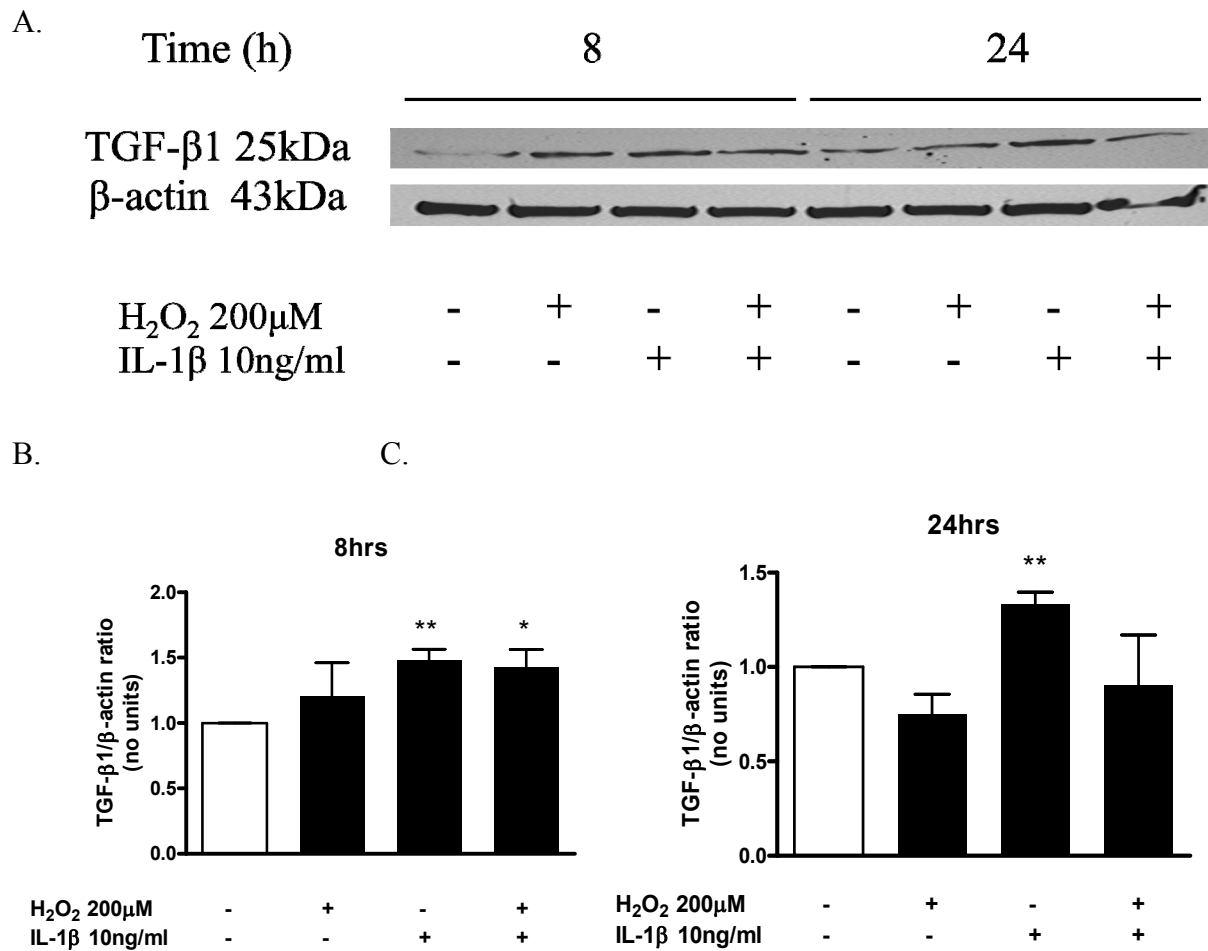


Figure 3.7 Effect of hydrogen peroxide (H₂O₂), IL-1 β and H₂O₂ plus IL-1 β on TGF- β 1 production. Cells were incubated with medium, H₂O₂ (200 μ M), IL-1 β (10ng/ml), or H₂O₂ plus IL-1 β for 8 and 24h. TGF- β 1 in whole cell lysates was examined by Western blotting (A). Bar graphs show quantitative analysis of TGF- β 1 expression at 8h (B) and 24h (C). Results are expressed as mean $\pm$ SEM of the TGF- β 1/ β -actin loading control ratio versus medium control at 8h and 24h. (n=3).

3.3.8 Determination of the signalling pathways involved in H₂O₂, IL-1 β , and H₂O₂ plus IL-1 β induction of TGF- β 1 mRNA

To test whether NF- κ B, JNK or MAPK pathways are involved in the induction of TGF- β 1 mRNA the selective IKK2 inhibitor AS602868 (10 μ M), the selective JNK inhibitor SP600125 (10 μ M) and the relatively selective inhibitor of p38 α / β SB203580 (10 μ M) were added 1hr before IL-1 β stimulation and incubated for a further 6hrs (Fig.3.8A). Only AS602868 suppressed IL-1 β -induced TGF- β 1 mRNA induced by IL-1 β and by H₂O₂ plus IL-1 β . SP600125 and SB203580 had no effect at this time point. Pretreatment with the transcriptional inhibitor actinomycin D (10 μ g/ml) prevented induction of TGF- β 1 mRNA by both IL-1 β alone and by IL-1 β plus H₂O₂ confirming a role for transcription in the increase in TGF- β 1 mRNA expression. However, actinomycin D had no significant effect on H₂O₂-induced TGF- β 1 mRNA expression. This suggests that other regulatory mechanisms such as mRNA stability must be involved in this induction of TGF- β 1 mRNA. We further validated the total TGF- β 1 protein in supernatants by ELISA (Fig. 3.8B). Pretreatment with the IKK2 inhibitor reduced both basal and stimulated levels of secreted TGF- β 1 protein. These data emphasized the key role of the IKK2/NF- κ B pathway in mediating IL-1 β - and IL-1 β plus H₂O₂-induced TGF- β 1 gene expression but not TGF- β 1 gene expression induced by H₂O₂ alone.

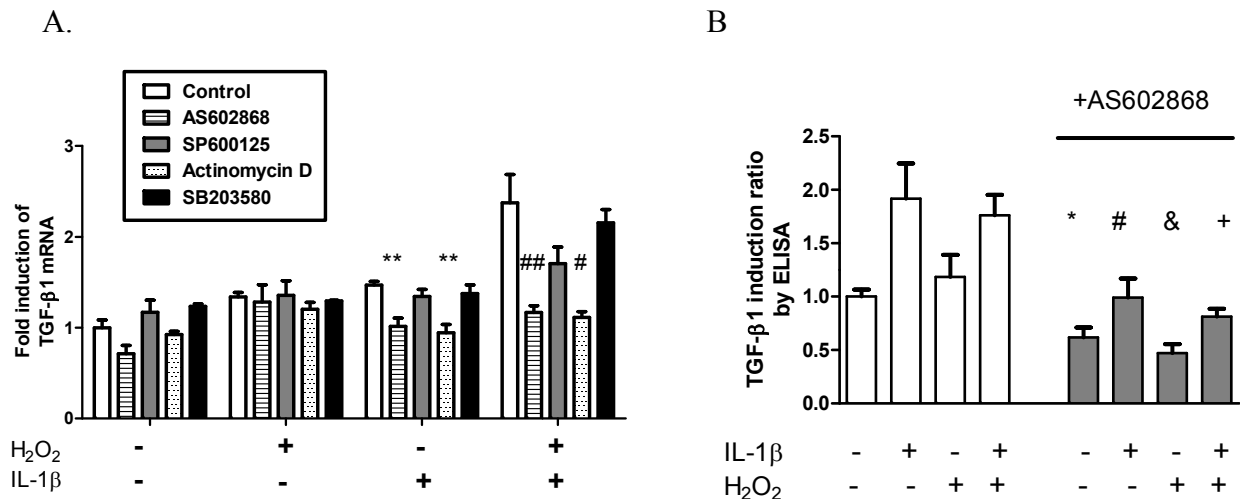


Figure 3.8 Effect of AS602868, SP600125, SB203580 and Actinomycin D on IL-1 β , hydrogen peroxide (H₂O₂) and IL-1 β plus H₂O₂ induced TGF- β 1 gene expression. (A) Cells were pretreated with AS602868 (10 μ M), SP600125 (10 μ M), SB203580 (10 μ M) or actinomycin D (10 μ g/ml) with or without treatment with IL-1 β (10ng/ml), H₂O₂ (200 μ M), or IL-1 β plus H₂O₂ for 6h. Levels of TGF- β 1 mRNA were quantified by RT-QPCR and normalized to GAPDH mRNA levels. **P<0.01 versus IL-1 β alone; #P<0.05; ###P<0.01 versus IL-1 β plus H₂O₂. (B) Cells were pretreated with AS602868 before stimulation with IL-1 β , with or without H₂O₂, for 6hrs. Supernatants were collected and TGF- β 1 levels measured by ELISA. All levels are shown as an induction ratio compared to control. Results are expressed as mean $\pm$ SEM of fold induction versus medium control. * P<0.05 versus control; # P<0.05 versus IL-1 β stimulation; &P<0.05 versus H₂O₂ stimulation; + P<0.01 versus IL-1 β plus H₂O₂ stimulation.

3.3.9 NF- κ B protein and activity is upregulated by IL-1 β and H₂O₂

To confirm whether the amount of NF- κ B p65 protein was increased following IL-1 β and H₂O₂ stimulation, A549 cells were stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), for 1hr. Total cell proteins were extracted and the amount of p65 protein detected by Western blotting with a specific p65 antibody. The result demonstrates a small but significant increase in NF- κ B p65 protein expression following stimulation of the cells with IL-1 β , H₂O₂ and IL-1 β plus H₂O₂ although the effect of the combination treatment was not significantly greater than that seen with either single treatment alone (Fig 3.9A & B).

We further checked to see whether the activation status of NF- κ B p65 was also increased by IL-1 β and H₂O₂ by examination of p65 DNA binding using a TransAM NF- κ B p65 kit. Cells were stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), for 1hr. Nuclear protein was collected and activation status measured. NF- κ B p65 DNA binding activity was significantly enhanced by both IL-1 β and by H₂O₂ albeit the effect of IL-1 β was much greater than that of H₂O₂ (Fig 3.9C). IL-1 β plus H₂O₂ increased NF- κ B p65 DNA binding to a similar extent as seen with IL-1 β alone. The data demonstrates that although H₂O₂ can enhance p65 protein to the same extent as IL-1 β the functional response (DNA binding) to IL-1 β stimulation is much greater. This clearly demonstrates that protein levels *per se* are not the only drivers for NF- κ B p65 activation.

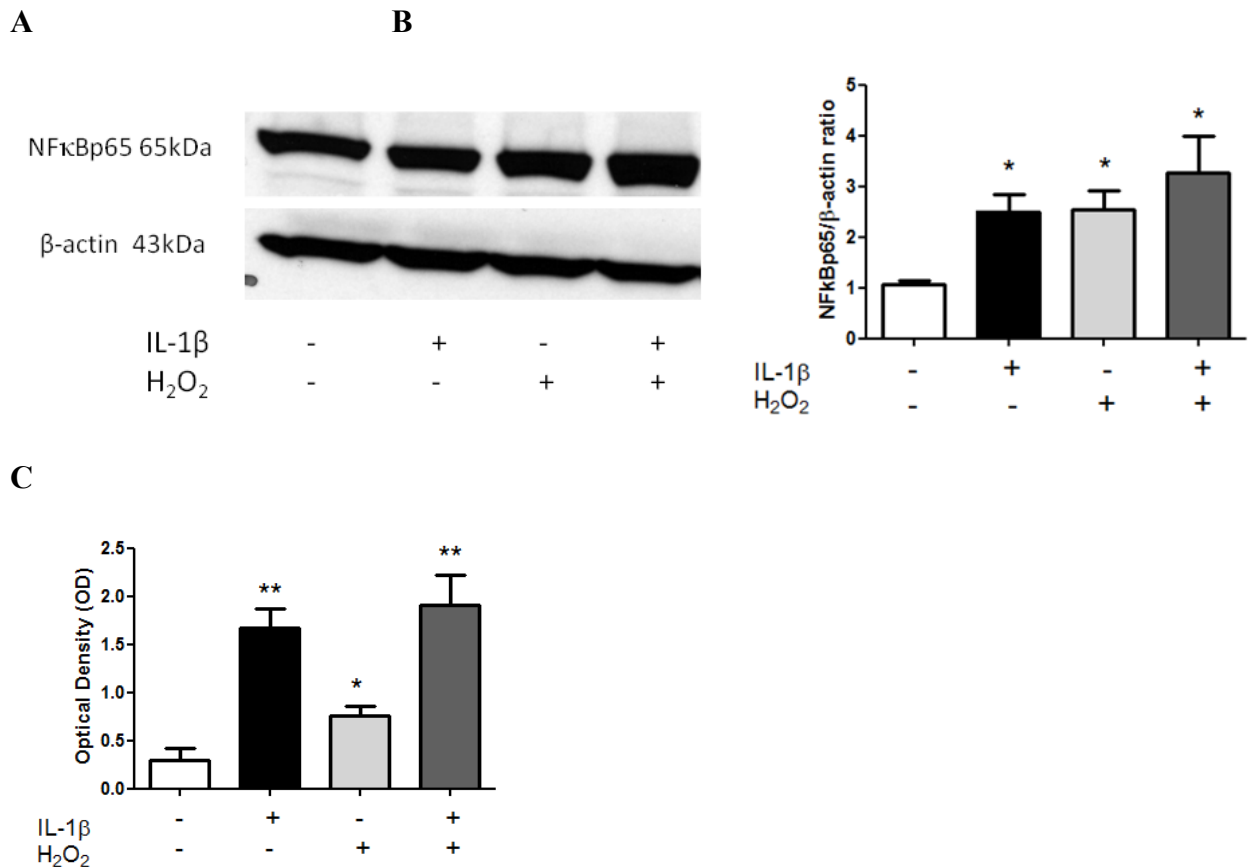


Figure 3.9 Effect of IL-1 β and hydrogen peroxide (H₂O₂) on NF- κ B protein expression and DNA binding. (A) Cells were pretreated with IL-1 β , with or without H₂O₂, or left untreated for 1hr. Whole cell proteins were extracted, the amount of NF- κ B p65 was by Western blotting and compared to that of β -actin as a loading control. (B) Results of densitometric analysis are presented graphically in (B). Results are shown as mean $\pm$ SEM. * p <0.05 when compared to basal status. N = three independent experiments. (C) Cells were pretreated with IL-1 β , with or without H₂O₂, or left untreated for 1hr. Nuclear protein was extracted and activation of NF- κ B p65 was detected with TransAM NF- κ B p65 DNA binding kit. Results are shown as mean $\pm$ SEM. * p <0.05, ** p <0.01, *** p <0.005 when compared to basal status. N = three independent experiments.

3.3.10 Binding of p65 to the TGF- β 1 κ B3 site *in vitro*

An ELISA based DNA binding assay was used to investigate whether the binding of p65 to the κ B3 site was DNA sequence-specific. Nuclear extracts were added to a well of a microtitre plate previously coated with immobilized κ B1, κ B2 or κ B3 oligonucleotides (Fig. 3.10A). Protein binding to specific oligonucleotides was then detected with a p65-specific antibody as with a standard TransAm assay. Recruitment of p65 to the κ B3 oligonucleotides, but not to the κ B1, or κ B2 oligonucleotides, increased significantly with stimulation by IL-1 β plus H₂O₂, the (Fig. 3.10B). These results indicate that p65 binding to the TGF- β 1 κ B3 site may be due to different intrinsic affinities of the different sites for p65.

To determine whether p65 binding was κ B3 specific we then performed a competitive study with wild type (w κ B3) and mutant type κ B3 (m κ B3) oligonucleotides. The binding activity of p65 to the κ B3 oligonucleotide was decreased after addition of excess w κ B3 in a concentration-dependent manner (Fig. 3.10C) ($P < 0.01$ for 2 μ M w κ B3) but not with the mutant m κ B3 oligonucleotide. To further confirm the specific response of IL-1 β and H₂O₂ on p65 recruitment to the κ B3 binding site, cells were stimulated with IL-1 β , with or without H₂O₂ (200 μ M). The results demonstrates that the DNA binding activity of p65 was upregulated in IL-1 β ($P < 0.01$) and IL-1 β plus H₂O₂ ($P < 0.01$) stimulated cells (Fig. 3.10D) but not in cells stimulated with H₂O₂ alone.

A.

κB1	agg ctg ggt TGG AAA CTC Tgg gac aga a
κB2	agg agt ctg GTC CCC ACC Cat ccc tcc t
wκB3	ggg ggc agg GGGGAC <u>G</u> CCC cgt ccg ggg
mκB3	ggg ggc agg GGGGAG <u>C</u> CCC cgt ccg ggg

(Only the sense oligonucleotide sequence is shown)

B.

C.

D.

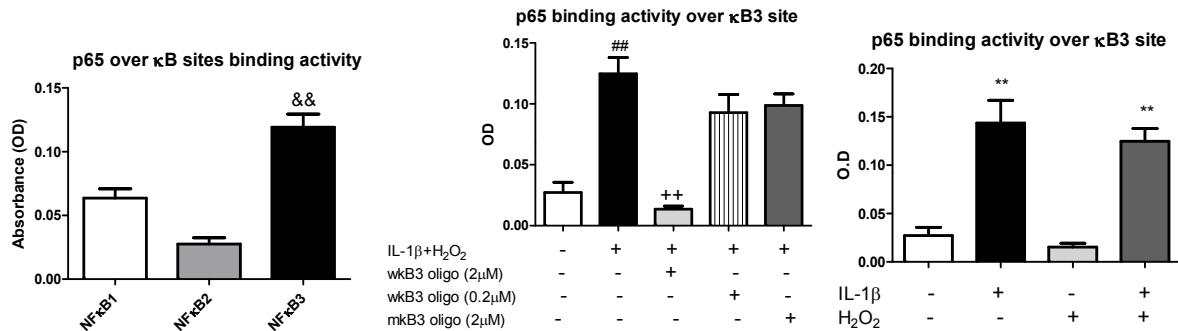


Figure 3.10 Binding of p65 to consensus NF-κB binding sites in the TGF-β1 promoter

in vitro. (A) Sequence of the DNA oligonucleotides including the κB1, κB2, κB3 or mutated (m) oligonucleotides used in the NF-κB promoter binding assays. Mutations from wildtype (w) κB3 sites are underlined. (B) Cells were treated with IL-1β (10ng/ml) plus H₂O₂ (200μM) for 1hr. Nuclear extracts were incubated with different oligonucleotides in 96-well plates previously coated with κB1, κB2, or κB3 nucleotides. Binding activities were detected with a specific p65 antibody. (C) Cells were incubated with wκB3, or mκB3 for competitor assays. (D) Cells were treated with IL-1β, with or without H₂O₂, or left untreated. Results are mean ± the SEM of ratio to basal levels. &&P<0.01 versus κB1, κB2. ##P<0.01 versus control. ++P<0.01 versus IL-1β plus H₂O₂. **P<0.01 when compared with control. n = three independent experiments.

3.3.11 Selective recruitment of p65 to κ B response elements in the native TGF- β 1 promoter

In order to determine the role of NF- κ B pathways in the induction of TGF- β 1 transcriptional activation, ChIP assays with a p65 specific antibody were performed to measure p65 association with the native human TGF- β 1 promoter. TGF- β 1 promoter was analyzed by Alibaba ([Http://www.gene-regulation.com/pub/programs/alibaba2](http://www.gene-regulation.com/pub/programs/alibaba2)), and the results showed 3 putative NF- κ B binding sites (κ B1, -2105 to -2096; κ B2, -2014 to -2005; κ B3, -789 to -780, from the translation start site) (Fig.3.11A). Primers pairs with QPCR products were designed to amplify the DNA corresponding to the putative κ B sites, in order to measure the DNA fragments immunoprecipitated by specific p65 antibody by QPCR. A set of primers amplifying a distal site 3' to the translation start site, 25665-25797 from the translation start site were used as a negative control.

The time course of p65 ChIP analysis showed that IL-1 β and IL-1 β plus H₂O₂ stimulation resulted in p65 recruitment to the κ B3 site from 60min and up to 120min (Fig.3.11B) but not to the κ B1 or κ B2 site (Fig.3.11C). There was no significant effect of H₂O₂ on p65 association with any of the TGF- β 1 promoter κ B sites. ChIP experiments also confirmed that the results were selective as none of the stimuli used at any time point examined had any effect on p65 association with the 3' distal end of the TGF- β 1 promoter. These results indicate that IL-1 β and IL-1 β plus H₂O₂ induce association of p65 to the κ B3 binding site but that H₂O₂ stimulation alone does not.

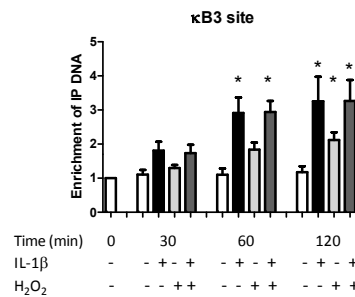
These results further demonstrate that p65 binding to κ B3 sites on the TGF- β 1 promoter is sequence specific following either IL-1 β or IL-1 β plus H₂O₂ stimulation. In addition, the presence of H₂O₂ does not enhance p65 TGF- β 1 promoter binding. This cannot, therefore, explain the increased NF- κ B-dependent TGF- β 1 mRNA transcription that occurs with IL-1 β /H₂O₂ stimulation.

A



DNA Sequence for κB1 site: GAAACTCT. κB2 Site: GTTCCCCACCC; κB3 Site: GGGGACGCCC

B



C

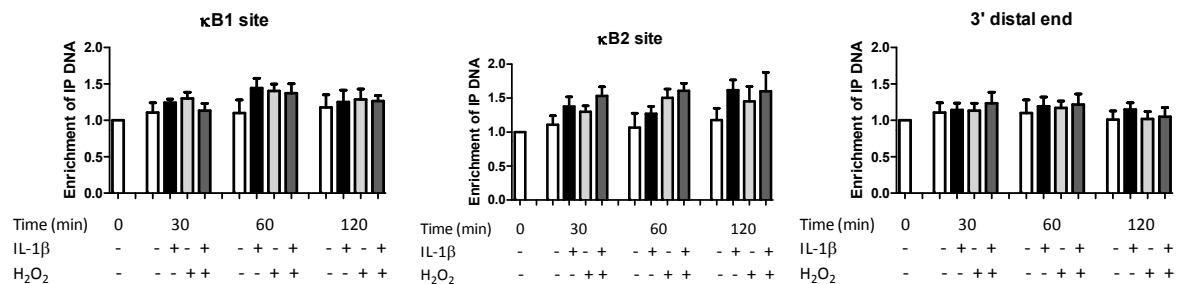


Figure 3.11 Effect of IL-1β and hydrogen peroxide (H₂O₂) on p65 recruitment to the native TGF-β1 promoter. (A) Schematic diagram of the TGF-β1 promoter - numbers indicate positions in relation to the translation start site (ATG). NF-κB binding sites (κB1, κB2 and κB3) and a distal 3' site are indicated. (B) Time course of p65 recruitment to the κB3 site following stimulation with IL-1β (10ng/ml), with or without H₂O₂ (200μM), for 0, 30, 60 and 120mins. The p65-associated DNA was quantified by QPCR with primer pairs specific for the κB3 site. (C) Time course of p65 recruitment to the κB1 site, κB2 and the 3' distal sites. Associated DNA was quantified by QPCR. Values were normalized to input DNA. Results are mean ± the SEM of ratio to basal levels. * denotes P<0.05 versus basal level (same time point). n = three independent experiments.

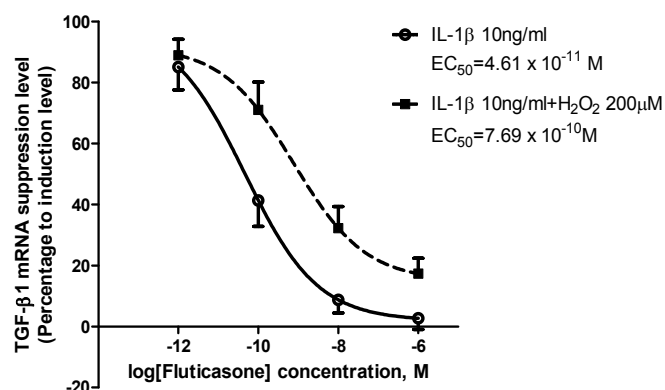
3.3.12 Effect of fluticasone propionate (FP) on oxidative stress induced TGF- β 1 mRNA expression in airway epithelial cells

Glucocorticoids are used extensively for their potent anti-inflammatory effects in a number of chronic inflammatory lung diseases including asthma (Barnes and Adcock, 2003), COPD (Singh et al., 2002) and some other mild forms of interstitial lung disease (Pujols et al., 2004). We therefore, examined the ability of a potent synthetic glucocorticoid, fluticasone propionate (FP) to suppress IL-1 β -stimulated TGF- β 1 mRNA expression in the presence of an oxidative stress (200 μ M H₂O₂).

The effect of FP on TGF- β 1 gene expression was analyzed by RT-QPCR. The result demonstrated that FP reduced IL-1 β -induced TGF- β 1 mRNA levels in a concentration-dependent manner (Fig. 3.12A). The EC₅₀ for FP inhibition of IL-1 β -induced TGF- β 1 was 4.61 x 10⁻¹¹M. In contrast, the EC₅₀ for FP inhibition of IL-1 β plus H₂O₂-induced TGF- β 1 mRNA expression was shifted to the right 17-fold (4.61 x 10⁻¹¹M versus 7.69 x 10⁻¹⁰M). The results confirmed that the potency of FP towards TGF- β 1 is reduced in these cells in the presence of H₂O₂. Furthermore, FP is also unable to reduce the level of IL-1 β /H₂O₂-stimulated TGF- β 1 mRNA down to basal levels (Fig 3.12A). This further suggests that the addition of H₂O₂ may impair the ability of FP to suppress TGF- β 1 mRNA expression.

We then confirmed that the effect of FP on TGF- β 1 mRNA expression was mediated by GR. A549 cells were pretreated with the GR antagonist RU486 (Garside et al., 2004) (10⁻¹⁰ - 10⁻⁶M) and FP (10⁻⁸M) in the presence of IL-1 β (10ng/ml) plus H₂O₂ (200 μ M) for 6hrs (Fig. 3.12B). RU486 antagonized the suppressive effects of FP on IL-1 β plus H₂O₂ induced TGF- β 1 mRNA expression in a concentration-dependent manner with complete abrogation of FP effects seen with 1 μ M RU486. These results demonstrate that the inhibitory effects of FP on IL-1 β /H₂O₂-stimulated TGF- β 1 mRNA expression were mediated through GR.

A.



B.

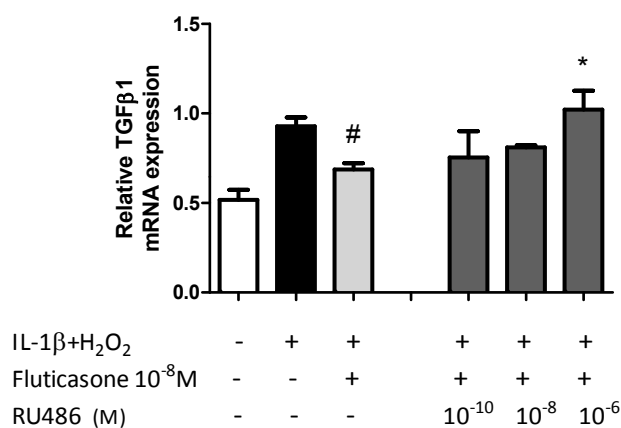


Figure 3.12 Effect of hydrogen peroxide (H₂O₂) on fluticasone propionate (FP)-induced suppression of TGF-β1 mRNA expression. (A) Cells were pretreated with variable concentrations of FP (10⁻¹² - 10⁻⁶M) for 30min before stimulation with either IL-1β (10ng/ml) alone or IL-1β plus H₂O₂ (200μM) for 6hrs. (B) Cells were pretreated with variable concentrations of RU486 (10⁻¹⁰ - 10⁻⁶M) for 30min before exposure to FP (10⁻⁸M) for 30mins and subsequent stimulation with IL-1β (10ng/ml) plus H₂O₂ (200μM) for 6h. Total RNA was collected, quantified by RT-QPCR with primers specific for TGF-β1 and relative expression levels corrected for GNB2L1 mRNA as control. Results are shown as means ± the SEM. [#]p<0.05 versus IL-1β plus H₂O₂. *P<0.05 versus IL-1β plus H₂O₂ plus FP. N = at least three independent experiments.

3.3.13 Effect of IL-1 β and hydrogen peroxide (H₂O₂) on TGF- β 1 mRNA expression in BEAS-2B cells

To examine whether the induction of TGF- β 1 by IL-1 β plus H₂O₂ is found in other airway/lung epithelial cells, BEAS-2B cells were pretreated with or without variable concentrations of FP (10^{-10} - 10^{-6} M) for 30 mins before stimulation with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), for 6 hrs (Fig 3.13). The results demonstrated a small but significant increase in TGF- β 1 mRNA expression by IL-1 β (1.28 ± 0.09 versus control: 0.96 ± 0.08), H₂O₂ (1.25 ± 0.07 versus control) and IL-1 β plus H₂O₂ (1.40 ± 0.11 versus control). Unlike the effects seen in A549 cells, the increase in TGF- β 1 mRNA with IL-1 β plus H₂O₂ was suppressed back to basal levels by FP (10^{-8} M and 10^{-6} M). These results indicate that although IL-1 β and H₂O₂ enhancement of TGF- β 1 mRNA expression is similar in both cell types and the functional response to FP is distinct. This may reflect a difference in anti-oxidant capacity between the two cell lines. How primary human epithelial cells respond is an obvious area for future research.

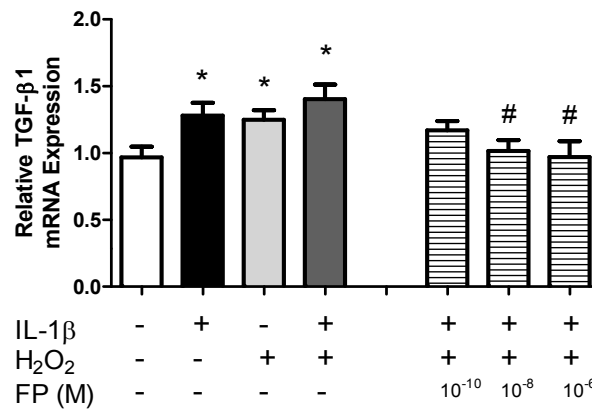


Figure 3.13 Effect of IL-1 β , hydrogen peroxide (H₂O₂) and fluticasone propionate (FP) on TGF- β 1 mRNA expression. Cells were pretreated with FP (10⁻¹⁰ - 10⁻⁶) for 30mins followed by stimulation with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), for 6h. Total RNA was collected and the level of TGF- β 1 mRNA was quantified with specific primers and expression levels corrected for control GNB2L1 mRNA. Results are shown as mean $\pm$ the SEM. *p<0.05 when compared with control; #p<0.05 when compared with IL-1 β plus H₂O₂ stimulation. N = four independent experiments.

3.3.14 Effect of IL-1 β , H₂O₂ and FP on GM-CSF and IL-8/CXCL8 mRNA in A549 cells

We further checked whether the combination of IL-1 β and H₂O₂ also enhanced the expression of two NF- κ B associated cytokines GM-CSF and IL-8/CXCL8. Cells were pretreated with FP (10⁻⁸M) for 30mins before stimulation with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), for 6hrs. GNB2L1 The results demonstrate distinct effects of IL-1 β /H₂O₂ treatment on GMCSF mRNA and on IL-8/CXCL8 mRNA expression. IL-1 β and H₂O₂ stimulation induced a greater than additive enhancement of GMCSF mRNA expression whilst this trend was not seen for IL-8/CXCL8 mRNA expression. In the case of IL-8/CXCL8 mRNA, both IL-1 β and H₂O₂ significantly enhanced mRNA expression but the effect of IL-1 β was much greater than

that of H₂O₂. In addition, the combination of IL-1 β and H₂O₂ did not further enhance IL-8/CXCL8 mRNA expression. In both cases, FP effectively reduced mRNA levels but, as with TGF- β 1 mRNA, these did not return to basal levels. A full concentration response curve was not performed and this may provide important additional information.

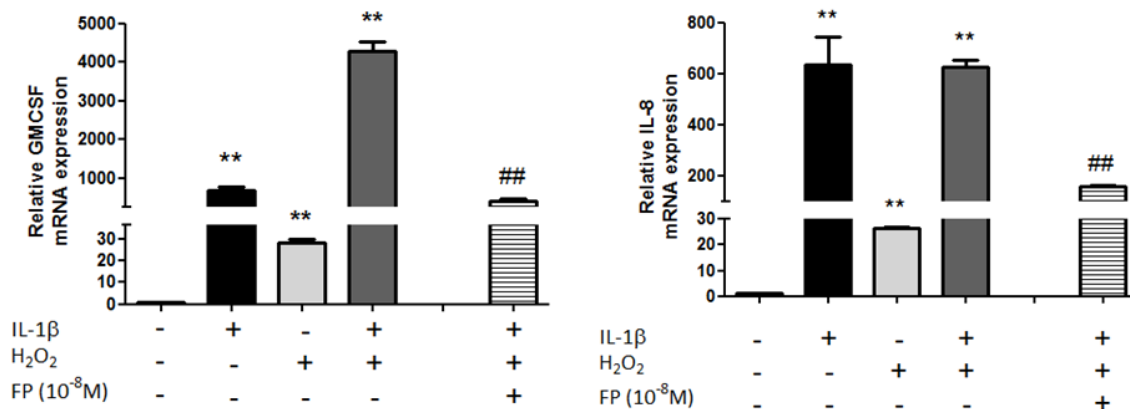


Figure 3.14 Effect of IL-1 β , hydrogen peroxide (H₂O₂) and fluticasone propionate (FP) on GM-CSF and IL-8/CXCL8 mRNA expression. Cells were pretreated with FP (10⁻⁸M) for 30mins before stimulation with IL-1 β (10ng/ml) and/or H₂O₂ (200 μ M) for 6hrs. Total RNA was extracted and relative expression of GM-CSF and IL-8/CXCL8 mRNA expression quantified by RT-QPCR and normalized to GNB2L1 mRNA expression. Results are shown as mean $\pm$ SEM. **p<0.01 compared to baseline; ##p<0.01 compared to IL-1 β plus H₂O₂ stimulation. N = at least three independent experiments.

3.4 Discussion

The results presented here demonstrate that IL-1 β , H₂O₂, and IL-1 β plus H₂O₂ can induce TGF- β 1 mRNA expression and protein from A549 cells. H₂O₂ stimulation alone is not a strong stimulus for TGF- β 1 mRNA expression but it can augment the effect of IL-1 β on TGF- β 1 mRNA expression. TGF- β 1 induction was mediated through the NF- κ B pathway. Global NF- κ B activation was activated by both IL-1 β and by H₂O₂ although this effect was gene specific as H₂O₂ was unable to cause recruitment of NF- κ B p65 to the activating κ B3 promoter site *in vitro* or at the native TGF β promoter. Further evidence for a gene-specific effect of H₂O₂ on NF- κ B activity and mRNA expression was provided by the differential induction of TGF- β 1, GM-CSF and CXCL8/IL-8 mRNA by the same levels of NF- κ B activation.

We further demonstrate that the potent synthetic corticosteroid fluticasone propionate (FP) can downregulate the enhanced TGF- β 1 mRNA expression induced by IL-1 β and H₂O₂ albeit with a lower efficacy and to a lesser extent than that seen with TGF- β 1 mRNA stimulated with IL-1 β alone.

It is believed that the combination of inflammation and oxidative stress may be important in the pathogenesis of many diseases (Koh et al., 2009; Rahman and Adcock, 2006; Rodriguez-Iturbe et al., 2004). We therefore exposed cells to an acute inflammatory stress using IL-1 β to mimic the inflammatory conditions encountered in asthma and COPD and other airways diseases in the presence or absence of H₂O₂ which was used as a model of oxidative stress. We were unable to demonstrate a direct effect of IL-1 β on intracellular oxidative stress but importantly the combination of IL-1 β with H₂O₂ gave a greater intracellular oxidant stress than H₂O₂ alone. This suggests that further investigation of the ability of IL-1 β to enhance intracellular oxidative stress should be performed using more sensitive assays in combination with selective inhibitors or siRNA knockdown of specific oxidant/anti-oxidant molecules. In disease, cells are exposed chronically to both low levels of

inflammation and oxidative stress and perhaps a more chronic model of these events would show a greater enhancement of TGF- β 1 responsiveness as well as effects on exaggerated cell apoptosis and cell death (Hildeman et al., 1999).

TGF- β 1 expression shows a concentration-dependent upregulation by IL-1 β with peak effects at $\sim 50\mu\text{g/ml}$ for both protein and mRNA expression. H_2O_2 stimulation alone enhances TGF- β 1 mRNA and protein secretion with a maximal effect at $\sim 200\mu\text{M}$. Simultaneous treatment of A549 cells with IL-1 β (10ng/ml) and H_2O_2 ($200\mu\text{M}$) induces a greater than additive increase in TGF- β 1 mRNA expression but not on secreted or intracellular TGF- β 1 protein. The uncoordinated expression of TGF- β 1 mRNA and protein level possibly indicates the post-transcriptional or post-translational regulation may affect the TGF- β 1 protein production (Allison et al., 1998; Kim et al., 1992).

To determine whether this effect was specific for the A549 cell line, we also checked the response of TGF- β 1 to IL-1 β plus H_2O_2 in BEAS-2B cells. The results demonstrated a similar response to IL-1 β and H_2O_2 , which possibly indicates that it is a universal finding on epithelial cells that TGF- β 1 mRNA expression may be upregulated upon inflammation and oxidative stress. It is important to perform similar studies in primary human airway epithelial cells to confirm this.

Using the IKK2 inhibitor, AS602868, we were able to confirm that stimulated and basal TGF- β 1 was under the control of the NF- κ B pathway. Measurement of NF- κ B activation in response to IL-1 β , H_2O_2 , and IL-1 β plus H_2O_2 demonstrated enhancement of p65 expression by Western blot analysis at 1h by all three treatments. Furthermore, p65 DNA binding showed a similar increase in DNA binding at a consensus κ B site and the specific κ B3 TGF β 1 promoter site in response to IL-1 β and IL-1 β plus H_2O_2 stimulation at 1h. The response to H_2O_2 stimulation was less pronounced as compared to the ratio difference when measured by Western blotting. In addition, specific recruitment of p65 to the κ B3 site was confirmed by ChIP assay which also showed that H_2O_2 alone or in combination with IL-1 β did not affect

p65 TGF β 1 promoter binding. The reasons for the discrepancies in NF- κ B activity measures probably reflect gene specificity and the precise aspect of NF- κ B biology that each technique measures. To study whether IL-1 β and H₂O₂ will regulate other NF- κ B regulated cytokines, total RNA was quantified for IL-8 and GM-CSF mRNA expression study. Upregulation of IL-8 and GM-CSF mRNA were also identified. But the result showed a differential pattern, in which IL-1 β and H₂O₂ stimulation induced a greater than additive effect on GM-CSF mRNA expression, but this enhanced effect was not seen with CXCL8/IL-8 mRNA expression. These findings support that oxidative stress, simulated by IL-1 β and H₂O₂, may induce a differential response on gene expression. Further studies using a combination of gene arrays and/or ChIP-seq analysis may further delineate this.

We further examined whether the synthetic corticosteroid, FP, was able to downregulate the expression of TGF- β 1 mRNA induced by IL-1 β and H₂O₂. FP downregulates TGF- β 1 mRNA expression at concentrations higher than 10⁻¹⁰M. Compared to stimulation with IL-1 β alone, IL-1 β plus H₂O₂ stimulation results in a relative corticosteroid insensitivity, with a reduced EC₅₀ (4.61 x 10⁻¹¹M versus 7.69 x 10⁻¹⁰M) and a reduced maximal suppression.

The data clearly suggests that the enhanced NF- κ B-dependent TGF β 1 mRNA transcription rate is not due to clear changes in p65 promoter association but may reflect changes in transcriptional co-activator or chromatin remodeling factor recruitment perhaps as a consequence of H₂O₂-induced changes in p65 post-translational modifications (Perkins, 2007). The histone code hypothesis (Jenuwein & Allis Science 2001; Li, Carey & Workman Cell 2007) postulates that changes in histone modifications are key to transcription. Previous work has indicated a role for changes in histone acetylation in TGF β 1 mRNA induction by IL-1 β (Lee et al., 2006) but there is no published data concerning the possible influence of histone methylation of TGF β 1 expression or whether this can affect FP modulation of TGF β 1 mRNA.

CHAPTER FOUR

**THE ROLE OF HISTONE METHYLATION IN OXIDATIVE STRESS-
INDUCED TGF- β 1 EXPRESSION**

4.1 Rationale

Most inflammatory genes are regulated by proinflammatory transcription factors such as NF- κ B and AP-1 (Barnes and Adcock, 1998). DNA is compacted around eight histone molecules (2 each of histones 2A, 2B, 3 and 4) within chromosomes and post-translational modification of these core histones is able to switch on or off the gene expression in a process known as epigenetic regulation (Adcock et al., 2006). There are at least eight known modifications found on histones (Kouzarides, 2007). Among them, histone acetylation and deacetylation are the most studied mechanism involved in regulating inflammatory lung diseases (Barnes et al., 2005). In bronchial biopsies from asthma patients there is a marked increase in HAT activity which was reversed in patients on inhaled corticosteroids (Ito et al., 2002) whilst in COPD patients there is a marked reduction in HDAC activity which correlated well with disease severity (Ito et al., 2005).

Compared with histone acetylation, the enzymes responsible for histone lysine methylation, lysine methyltransferases (HMTs) have enormous substrate site specificity compared to the non-selective HATs (Kouzarides, 2007). Methylation of different lysine residues on histones results in distinct functions: H3K4, H3K36 and H3K79 trimethylation is associated with activation of gene transcription whilst H3K9, H3K27 and H4K20 trimethylation is associated with transcriptional repression.

Suv39H1, a member of the suppressor of variegation 3-9 homolog family, can specifically trimethylate K9 on histone H3 using monomethylated H3 K9 as a substrate. Current research indicates that HMTs, such as Suv39H1, may team up with DNA methyltransferases to silence genes (Fuks, 2005). Thus, SUV39H1 may direct DNA methylation to pericentric heterochromatin (Lehnertz et al., 2003), and may also be associated with DNA methyltransferases through the adaptor protein HP1 (Fuks et al., 2003a). In the same way that histone acetylation is reversed by HDACs the balance of histone methylation on specific K residues is controlled by HMTs and histone demethylases such as

JMJD2A, JMJD2B and JMJD3 (Shi and Whetstine, 2007). Hence, the relative activity of HMTs and HDMTs may be able to fine tune the methylation status of chromatin and subsequent gene expression profiles.

Preliminary data from our laboratory (Kang-Yung Lee, PhD Thesis 2006) indicated that changes in TGF β 1 expression were associated with modifications in H3K9 methylation and that K9-specific methylases and demethylases such as LSD1 and G9a were not involved. Currently the effect, if any, of oxidative stress on the regulation of histone methylation is not clear.

4.1.1 Hypothesis and aims of this Chapter

We hypothesise that oxidative stress, in combination with IL-1 β , may modify the H3K9 methylation, and further affect the TGF- β 1 production through the recruitment of H3K9 trimethylase Suv39H1 to TGF- β 1 promoter.

The specific aims were:

1. To determine the effect of H₂O₂, and in combination with IL-1 β , on global H3K9 trimethylation status on A549 cells.
2. To evaluate the effect of H₂O₂, and in combination with IL-1 β on Suv39H1 production.
3. To examine the effect of Suv39H1 on GR function under oxidative stress.

4.2 Experimental protocols

4.2.1 *Effect of oxidative stress on H3K9 trimethylation*

90% confluent A549 cells were cultured in serum-free DMEM medium supplemented with L-glutamine for 48h before stimulation. Cells were stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M) for 1hr. Nuclear proteins were extracted and the level of H3K9 trimethylation was detected using a specific anti-trimethylated H3K9 antibody (Millipore).

4.2.2 *Effect of oxidative stress on Suv39H1 mRNA expression*

A549 cells were serum-starved for 48hrs, and stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated for 4hrs. At the end of stimulation, total RNA was isolated and Suv39H1 mRNA was determined by RT-QPCR.

4.2.3 *Kinetics of the Suv39H1 promoter occupancy under oxidative stress*

A549 cells were serum-starved for 48h before stimulation with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated. At the end of the stimulation, proteins and DNA were cross-linked with 1% formaldehyde and Suv39H1-TGF- β 1 promoter association was determined by Suv39H1 ChIP assay using a specific anti-Suv39H1 antibody (Millipore). Enrichment of IP DNA was quantified by RT-QPCR with primer sets amplifying NF- κ B binding sites, and a distal 3' site in the TGF- β 1 gene.

4.2.4 *The association between Suv39H1 and GR*

A549 cells were serum-starved for 48h before stimulation with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated. Whole cell proteins were extracted and were subsequently immunoprecipitated with an anti-GR-specific antibody (H-300, Santa Cruz Biotechnology). The associated Suv39H1 was determined by Western blotting with a specific antibody against Suv39H1.

4.2.5 *GR-associated histone H3K9 HMT activity*

A549 cells were stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated for 2hrs in the presence or absence of FP (10⁻⁸M) pretreatment for 30mins. GR was immunoprecipitated from the nuclear extracts with a specific anti-GR antibody. GR-associated H3K9 HMT activity was investigated by *in vitro* HMT assay with core histones as

the substrate, and SAM as the methyl group donor. H3K9 HMT activity was determined by Western blotting with a specific antibody against trimethyl H3K9.

4.2.6 Effect of Suv39H1 knockdown on oxidative stress induced TGF- β 1 mRNA expression

50% confluent A549 cells were transfected with 25nM Suv39H1 siRNA (Dharmacon, USA) with transfection reagent (FuGene 6, Roche) for 24hrs in DMEM medium with 10% FCS and L-glutamine supplementation. The culture medium was then changed to serum-free DMEM for 48h. After this time A549 cells were about 85-90% confluent. These cells were pretreated with variable concentrations of FP (10^{-12} to 10^{-6} M) 30min before stimulation with IL-1 β (10ng/ml) in the presence or absence of H₂O₂ (200 μ M) or left untreated. At the end of stimulation period total RNA was isolated and TGF- β 1 mRNA was determined by RT-QPCR.

4.2.7 Effect of Suv39H1 overexpression on oxidative stress induced TGF- β 1 mRNA expression

50% confluent A549 cells were transfected with 1ng Suv39H1 overexpression plasmid with transfection reagent (FuGene 6) for 24hrs in DMEM medium with 10% FCS, L-glutamine, and then the medium were changed to serum-free DMEM for starvation for 48hrs. About 85-90% confluent A549 cells were yielded, and were stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated. At the end of stimulation, total RNA was isolated and TGF- β 1 mRNA was determined by RT-QPCR.

4.2.8 Effect of Suv39H1 knockdown on oxidative stress induced GRE activation

50% confluent A549 cells were transfected with 25nM Suv39H1 siRNA (Dharmacon) with Interferin (Polyplus) reagent for 24hrs in DMEM medium with 10% FCS and L-glutamine supplementation. The medium was then changed to serum-free DMEM for 48hrs. After this

time A549 cells were about 85-90% confluent and were stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated. At the end of stimulation, nuclear proteins were extracted. 10ng nuclear extract was used for GRE activation study (Active motif, Belgium).

4.2.9 *NF- κ B p65-associated histone H3K9 HMT activity*

A549 cells were stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated for 2hrs. Experiments were repeated in the presence or absence of FP (10⁻⁸M) pretreatment for 30mins. NF- κ B p65 was immunoprecipitated from the nuclear extracts with a specific anti-p65 antibody (Santa Cruz Biotechnolgy). NF- κ B p65-associated H3K9 HMT activity was investigated by an *in vitro* HMT assay with core histones as the substrate and SAM as the methyl group donor. H3K9 HMT activity was determined by Western blotting with a specific antibody against trimethyl H3K9.

4.2.10 *Effect of Suv39H1 knockdown on oxidative stress induced NF- κ B p65 activation*

50% confluent A549 cells were transfected with 25nM Suv39H1 siRNA (Dharmacon) with chemical transfection reagent Interferin (Polyplus) for 24h in DMEM medium with 10% FCS and L-glutamine supplementation. The medium was changed to serum-free DMEM for 48hrs. After this time A549 cells were about 85-90% confluent and were stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated. At the end of stimulation, nuclear proteins were extracted. 10ng nuclear extract was used for NF- κ B p65 activation/DNA binding analysis (Active motif, Belgium).

4.2.11 *Effects of oxidative stress induced G9a, Suv39H2, JMJD2C, and JMJD3 mRNA expression*

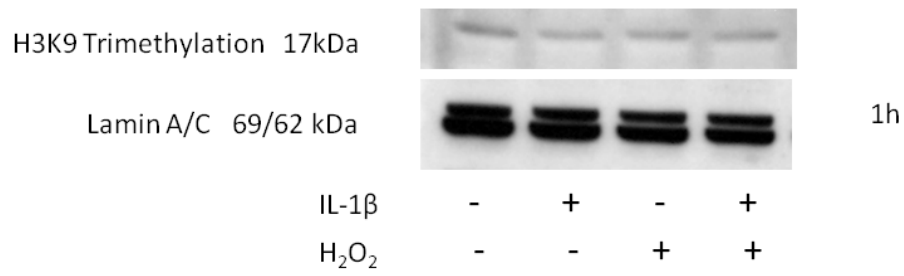
A549 cells were serum starved for 48hrs before stimulation with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated for 6h. At the end of the stimulation, total RNA was isolated, and individual mRNA expression was determined by RT-QPCR with specific primers. The relative expression level was normalized by GNB2L1 mRNA expression.

4.3 RESULTS

4.3.1 *IL-1 β and H₂O₂ reduces histone 3 lysine 9 trimethylation*

In order to the effect of oxidative stress on H3K9 trimethylation, A549 cells were serum-starved, and stimulated with IL-1 β , with or without H₂O₂ stimulation. Nuclear proteins were isolated after 1hr and 16hrs and Western blot analysis performed with antibodies specific for H3K9 trimethylation. The global H3K9 trimethylation status was reduced by IL-1 β , H₂O₂, and IL-1 β plus H₂O₂ stimulation after 1hr (Fig. 4.1.A). Interestingly, global H3K9 trimethylation was upregulated after 16hrs by H₂O₂ alone or in combination with IL-1 β . In contrast, IL-1 β alone reduced global trimethyl H3K9 (Fig. 4.1.B). These results demonstrate initial decreased trimethylation status after stimulation with IL-1 β and H₂O₂ but suggest that there may be different regeneration or cycling mechanisms for H3K9 trimethylation at later time points following either IL-1 β or H₂O₂ stimulation.

A.



B.

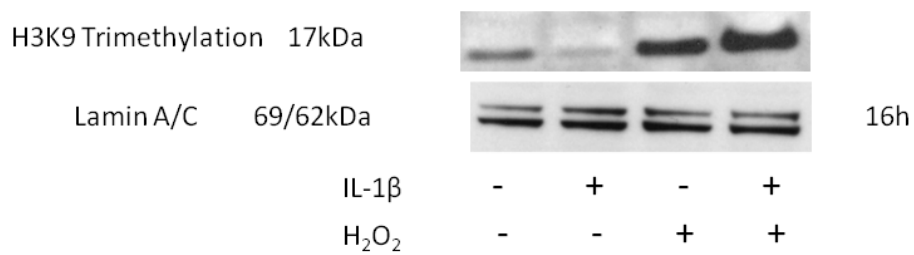
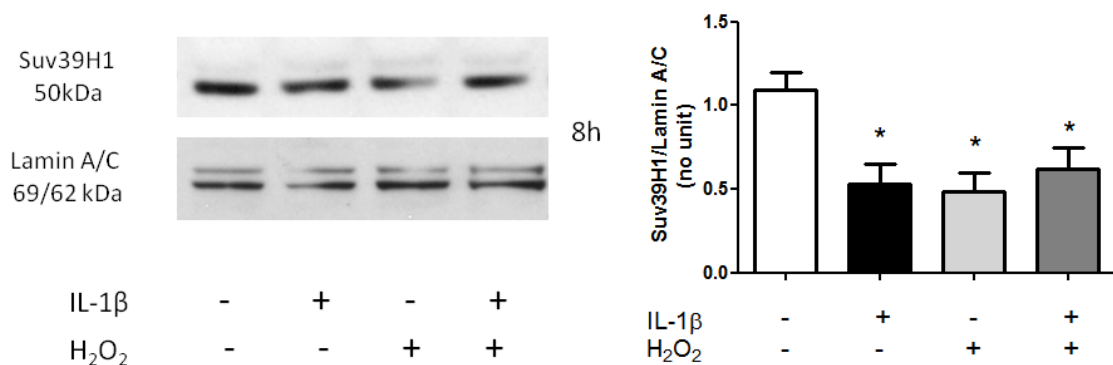


Figure 4.1 Effect of IL-1 β and H₂O₂ stimulation on H3K9 trimethylation status. Cells were incubated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated for 1hr (A) and 16hrs (B). Nuclear extracts were obtained and H3K9 trimethylation detected by Western blotting. Data are examples of two independent experiments.

4.3.2 Oxidative stress inhibits Suv39H1 mRNA expression

In order to determine whether the H3K9 trimethylase Suv39H1 was downregulated by oxidative stress, RT-QPCT quantification of Suv39H1 mRNA was performed along with Western blot analysis (Fig 4.2). The results demonstrate decreased expression of Suv39H1 mRNA and protein which paralleled the reduced H3K9 trimethylation level.

A.



B.

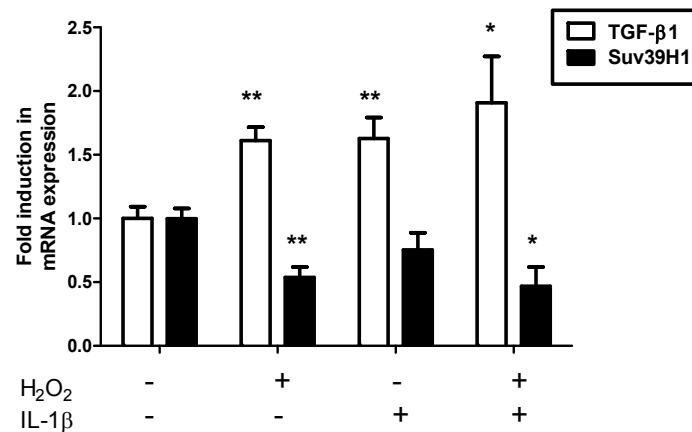


Figure 4.2 Effect of hydrogen peroxide (H₂O₂), IL-1 β and H₂O₂ plus IL-1 β stimulation on Suv39H1 protein and mRNA expression. Cells were incubated with medium alone or H₂O₂ (200 μ M), IL-1 β (10ng/ml), and H₂O₂ plus IL-1 β for 8h (A). (B) Level of TGF- β 1 and Suv39H1 mRNA were normalized with GAPDH mRNA levels. *P<0.05; **P<0.01 versus non-treatment. Results are mean $\pm$ the SEM of fold induction versus medium control (n=at least three independent experiments).

4.3.3 *Suv39H1 is recruited to the TGF- β 1 promoter under oxidative stress*

To examine if Suv39H1 is recruited to the TGF- β 1 promoter, ChIP assays with a specific antibody against Suv39H1 were carried out. Cells were stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated for variable time (0, 30mins, 60mins, 120mins) (Fig 4.3).

The results demonstrated that Suv39H1 was recruited to the TGF β 1 promoter κ B3 binding site following IL-1 β and H₂O₂ stimulation for 60min and this effect was lost by 2hrs. In contrast, Suv39H1 association with the control TGF β 1 promoter 3' distal site was unaltered by any stimulation.

4.3.4 *Suv39H1 is associated with GR under IL-1 β and H₂O₂ stimulation*

In theory a change in H3K9 methylation status could be associated with gene repression. Since FP is able to suppress the upregulation of TGF- β 1 under oxidative stress, we then tested if Suv39H1 was associated with GR under oxidative stress conditions and thereby acted as a GR corepressor. Cells were pretreated with FP in the presence of IL-1 β , with or without H₂O₂ for 2h and 4h. Whole cell extracts were immunoprecipitated with an anti-GR antibody and the presence of Suv39H1 detected with an anti Suv39H1 antibody. Figure 4.4.A demonstrates an increase in GR-associated Suv39H1 with H₂O₂ and IL-1 β plus H₂O₂. The trend is similar at both 2h and 4h (Fig 4.4A).

We then confirmed these results using reverse co-immunoprecipitations using an anti-Suv39H1 antibody for immunoprecipitation and an anti-GR antibody for Western blotting. The results demonstrate an increased association of GR with Suv39H1 under IL-1 β , and H₂O₂ stimulation, and a further increased association were seen in FP pretreatment, or FP pretreatment plus IL-1 β +H₂O₂ (Fig 4.4B). These results indicate that GR is physically associated with Suv39H1 under oxidative stress.

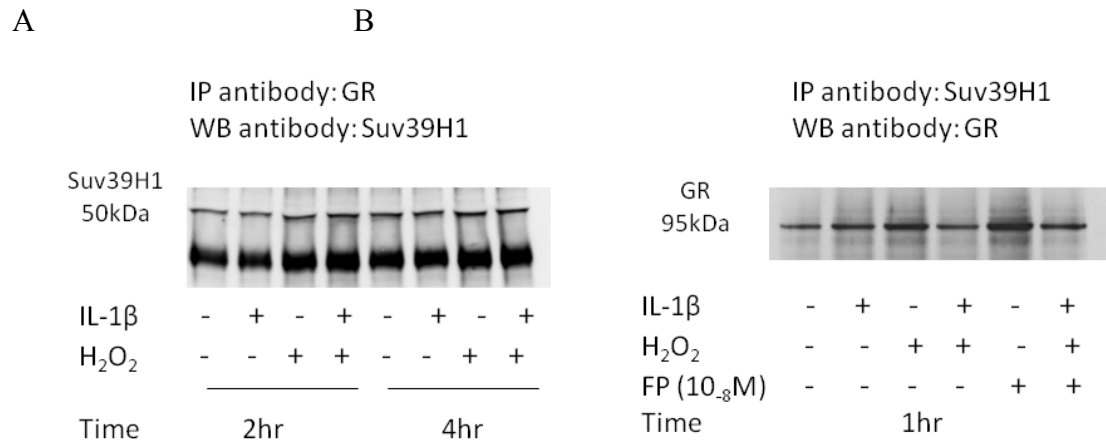


Figure 4.4 Effect of IL-1 β , hydrogen peroxide (H₂O₂) and fluticasone propionate (FP) on Suv39H1 recruitment to GR. (A) Cells were stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated for 2h or 4h. GR was immunoprecipitated with a specific antibody against GR from whole cell extracts, and the associated Suv39H1 protein level is detected with an antibody against Suv39H1. (B) Cells were pretreated with or without FP (10⁻⁸M), then stimulated with IL-1 β , with or without H₂O₂, or left untreated for 1hr. Cells lysates were immunoprecipitated with antibody against Suv39H1, and the associated GR level was detected with antibody against GR. Data are examples from at least two independent experiments with similar results.

4.3.5 GR is associated with HMT activity

To investigate whether GR-associated Suv39H1 has enzymic activity, we performed an *in vitro* HMT assay. Cells were pretreated with FP in the presence and absence of IL-1 β and/or H₂O₂ for 2h. Nuclear extracts were immunoprecipitated with anti-GR antibody and an HMT reaction performed by incubating the immunoprecipitated proteins in HMT buffer with core histones as the substrate and SAM as the methyl donor. HMT activity, was determined by Western blotting with an anti-trimethyl H3K9-specific antibody. A significant increase in H3K9 trimethylation was detected in cells following IL-1 β , H₂O₂, IL-1 β plus H₂O₂, and FP stimulation (Fig 4.5). Together with previous co-immunoprecipitation results, it is possible that Suv39H1 may be responsible for this GR-associated HMT activity.

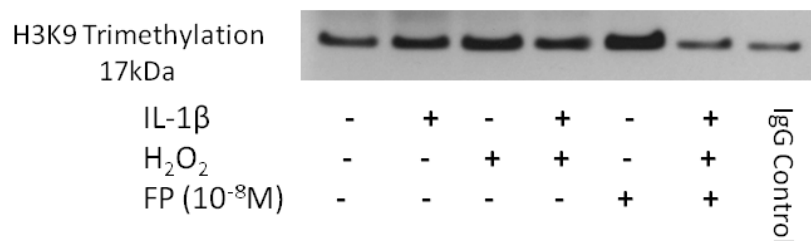


Figure 4.5 GR is associated with HMT activity. (A) Cells were pretreated with or without fluticasone propionate (FP, 10⁻⁸M) for 30min and then stimulated with IL-1 β (10ng/ml), with or without hydrogen peroxide (H₂O₂, 200 μ M) for 1h. GR was immunoprecipitated from nuclear extracts with a specific antibody against GR. The *in vitro* HMT assay was performed with core histones as substrate and SAM as the methyl donor. H3K9 trimethylation HMT activity was determined by specific antibody against H3K9 trimethylation. Data are examples from two independent experiments with similar results.

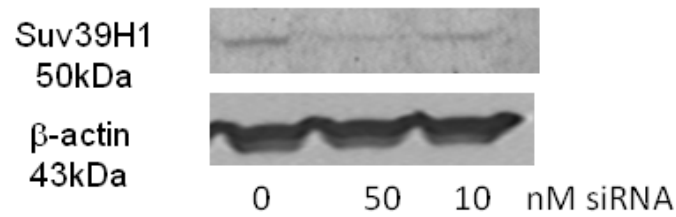
4.3.6 *Knockdown effect of Suv39H1 siRNA on Suv39H1 mRNA and protein*

To test whether Suv39H1 siRNA produced an effective knockdown after transfection, A549 cells were pre-treated with Suv39H1 siRNA for 48hrs. Western blotting analysis of whole cell lysate was then performed. Using a specific Suv39H1 antibody, a band at 53kd was detected in the medium control group, but reduced immunoreactivity was seen in the Suv39H1 siRNA treated group in a concentration-dependent manner (Fig. 4.6A).

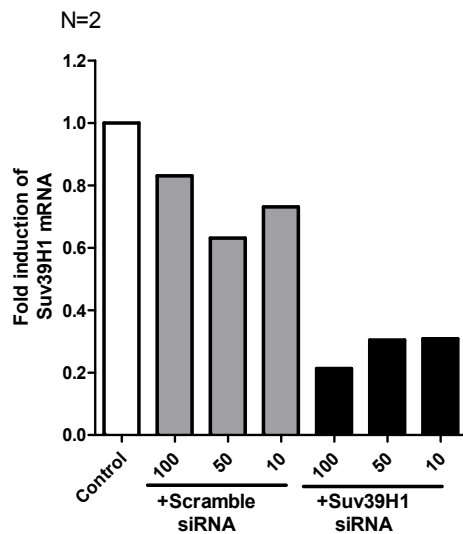
Suv39H1 siRNA transfection also resulted in reduced Suv39H1 mRNA levels with a 75%-80% knockdown seen after 24hrs (Fig. 4.6B). In contrast, scrambled siRNA (10, 50, 100nM) had no effect on Suv39H1 mRNA expression (Fig. 4.6B).

To investigate whether the various stimuli to be used had any effect on the ability of Suv39H1 siRNA to knockdown Suv39H1 expression, cells were treated with FP (10^{-8} M), H₂O₂ (200μM) and/or IL-1β (10ng/ml) in the presence of 50nM suv39H1 siRNA. Suv39H1 gene expression was analyzed by quantitative RT-QPCR and the result showed a similar degree of knockdown (70%-80%) after Suv39H1 siRNA treatment in the absence of cell stimulation (Fig. 4.6C).

A.



B.



C.

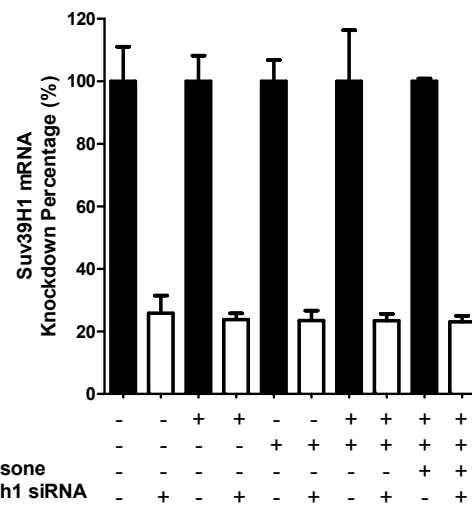


Figure 4.6 Knockdown effect of Suv39H1 siRNA on Suv39H1 mRNA and protein level.

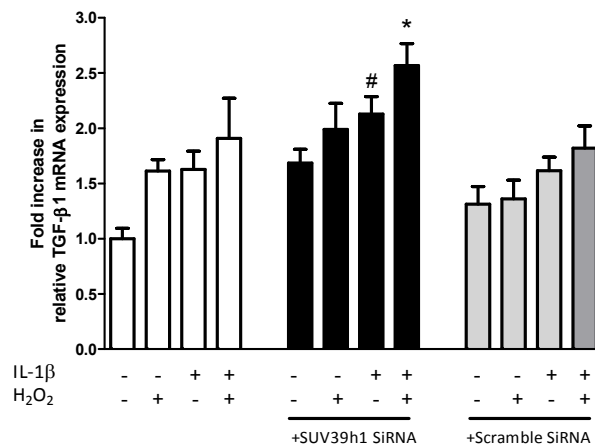
(A) Cells were incubated with medium or Suv39H1 siRNA (50, or 10nM) for 48hrs. Whole cell extracts were obtained and examined for Suv39H1 by Western blotting. (B) Cells (n=2) were incubated with medium, scrambled siRNA (100, 50, or 10nM) or Suv39H1 siRNA (100, 50, or 10nM). Levels of Suv39H1 mRNA were quantified by RT-QPCR and were normalized to GNB2L1 mRNA levels. (C) Cells were pretreated with Suv39H1 siRNA (50nM) for 24hrs, then serum-starved for a further 24hrs before being treated with medium alone, hydrogen peroxide (H₂O₂, 200μM), IL-1β (10ng/ml) or H₂O₂ plus IL-1β. Suv39H1 mRNA level was quantified with RT-QPCR and normalized with respective to GAPDH mRNA levels. Results are expressed as mean ± SEM of percentage versus no siRNA treated group (n=at least three independent experiments).

4.3.7 Knockdown of Suv39H1 further upregulates IL-1 β and H₂O₂-induced TGF- β 1 mRNA expression

To investigate whether Suv39H1 is involved in regulation of TGF- β 1 mRNA expression cells were pre-treated with Suv39H1 siRNA (Fig 4.7). Knockdown of Suv39H1 resulted in enhanced basal TGF β 1 mRNA expression and a further enhancement of TGF- β 1 mRNA levels were seen in the Suv39H1 siRNA treated group under stimulation with IL-1 β , and IL-1 β plus H₂O₂ (Fig 4.7.A). The scrambled siRNA pre-treated group showed no effect on TGF- β 1 mRNA levels when compared with the control group (Fig 4.7A).

Previous experiments demonstrate that oxidative stress-induced upregulation of TGF- β 1 mRNA was downregulated by FP. We therefore checked whether the suppressive effect of FP was modified by Suv39H1 knockdown (Fig 4.7B). A549 cells were pretreated with medium alone, scrambled siRNA (50nM) or Suv39H1 siRNA (50nM) for 48hrs and then serum starved for a further 24hrs. Cells were subsequently incubated with FP (10⁻⁸M) for 30mins, then stimulated with medium, H₂O₂ (200 μ M), IL-1 β (10ng/ml) or H₂O₂ plus IL-1 β for 6h (Fig 4.7B). The results demonstrate that in the presence of FP (10⁻⁸M), TGF- β 1 mRNA expression was further upregulated after Suv39H1 knockdown indicating that Suv39H1 is somehow involved in GR regulation of TGF- β 1 mRNA transcription.

A



B.

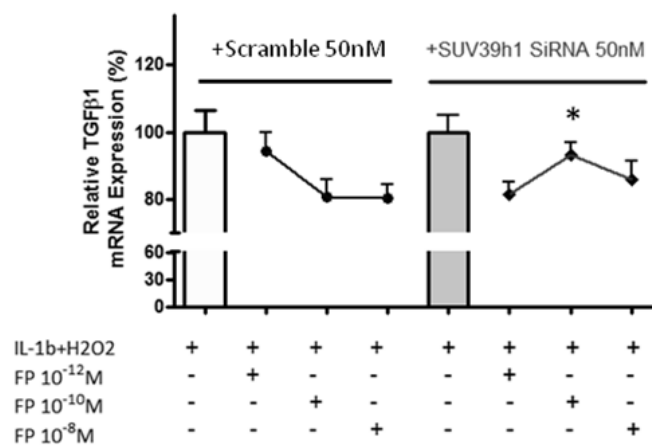


Figure 4.7 Effect of Suv39H1 siRNA knockdown on TGF-β1 message RNA. A549 cells were incubated with medium alone, scrambled siRNA or Suv39H1 siRNA for 48hrs and subsequently serum starved for a further 24hrs. The cells were then incubated with FP for 30mins followed by medium, hydrogen peroxide (H₂O₂, 200μM), IL-1β (10ng/ml), or H₂O₂ plus IL-1β for 6hrs. Levels of TGF-β1 mRNA were quantified by RT-QPCR and were normalized with respective to GNB2L1 mRNA levels. Results are mean ± the SEM of fold induction versus medium control (n= at least 4 independent experiments). (A) # P<0.05 versus IL-1β (pretreated with Suv39H1 scrambled siRNA); *P<0.05 versus IL-1β plus H₂O₂ (pretreated with Suv39H1 scrambled siRNA). (B) *p<0.05 versus scrambled siRNA plus IL-1β+H₂O₂.

4.3.8 *Overexpression of Suv39H1 has no effect on TGF- β 1 mRNA expression*

In order to confirm the siRNA results we then performed an overexpression study with an Suv39H1 plasmid to determine whether oxidative stress-induced TGF- β 1 mRNA expression could be downregulated by Suv39H1 overexpression.

Two different amounts of Suv39H1 overexpression plasmid (1 μ g, 2 μ g) were tested to determine optimum transfection efficiency. After transfection with Suv39H1 overexpression plasmid, Suv39H1 protein was increased as determined by Western blotting with a specific Suv39H1 antibody (Fig 4.8A). The effect on Suv39H1 mRNA upregulation was much more pronounced with a 10-20 thousand-fold induction when compared to the vector alone group (Fig 4.8B).

To investigate whether Suv39H1 overexpression could regulate the effect of IL-1 β and H₂O₂ on TGF- β 1 mRNA expression, cells were pretreated with FP (10⁻⁸M) for 30mins and then stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated for 6h. TGF- β 1 mRNA expression was enhanced following IL-1 β +H₂O₂ stimulation in the vector alone treated cells but this did not reach statistical significance (Fig 4.8C). Furthermore, there was no difference between cells treated with the Suv39H1 overexpression plasmid or with vector alone in the ability of FP to regulate TGF- β 1 mRNA expression.

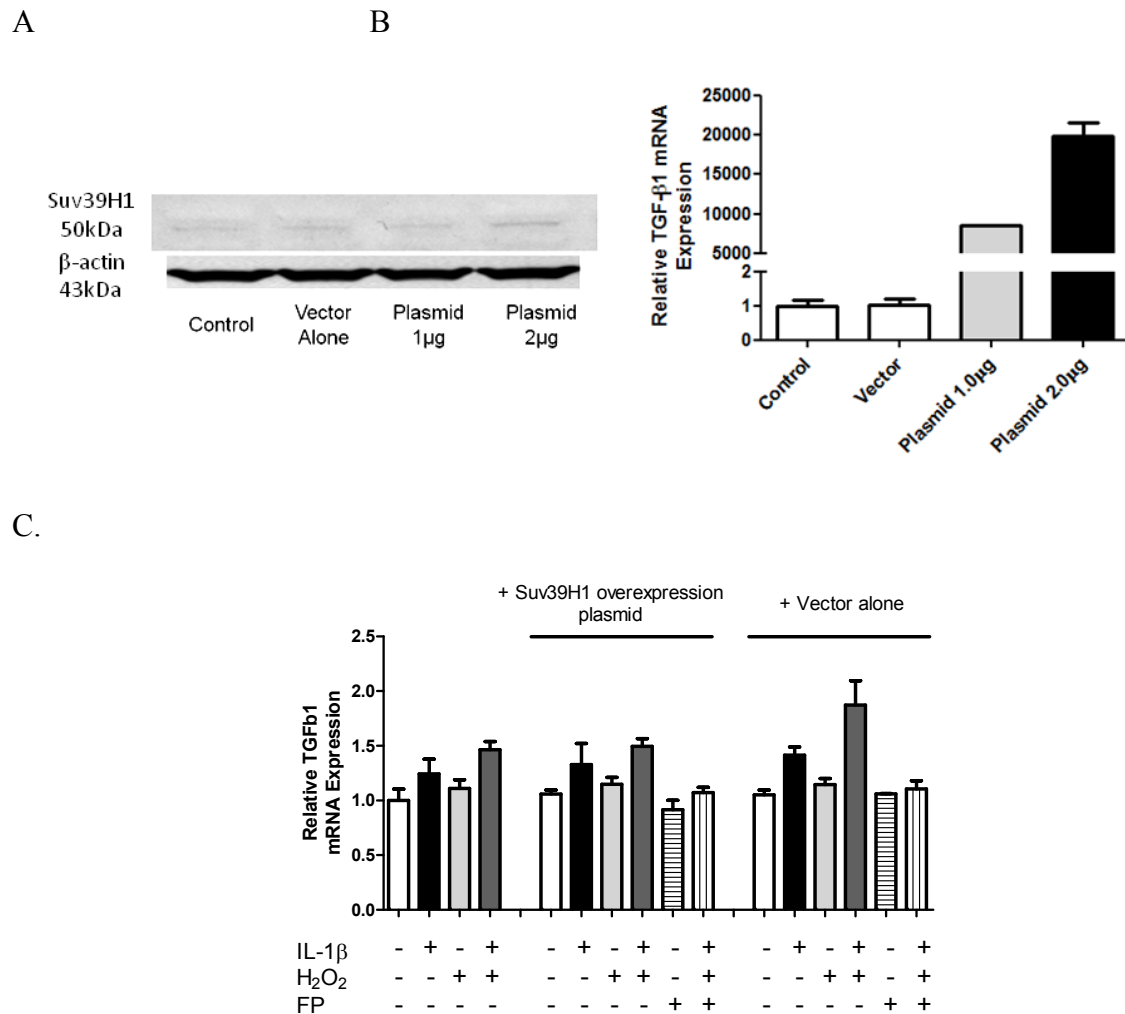


Figure 4.8 Effect of Suv39H1 overexpression on IL-1 β , hydrogen peroxide (H₂O₂) and Fluticasone propionate (FP) induced TGF- β 1 mRNA expression. Cells were pretreated with medium only, vector alone, or Suv39H1 overexpression plasmid (1 μ g, 2 μ g) for 24h. Whole cell protein extracts (A) and total RNA (B, C) were collected. Suv39H1 protein was detected by Western blotting with a Suv39H1-specific antibody (A). Relative expression of TGF- β 1 was quantified by RT-QPCR with primers specific for TGF- β 1 (B). Cells were preincubated with medium alone, vector, or plasmid for 24hrs, then serum-starved for 24hrs. The cells were pretreated with FP for 30mins and then stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), for 6hrs. TGF- β 1 mRNA expression was quantified by RT-QPCR with specific primers. Results are mean $\pm$ the SEM. N= at least two independent experiment for (A) and (B). N=at least three independent experiments in (C).

4.3.9 Knockdown of Suv39H1 by siRNA on IL-1 β and H₂O₂ induced GMCSF mRNA expression

IL-1 β and H₂O₂ were shown to have a greater than additive effect on the induction of GMCSF mRNA in the previous experiment. We then investigate whether Suv39H1 also regulates GMCSF mRNA expression and its suppression by FP.

A549 cells were pretreated with medium alone, scrambled siRNA (50nM) or Suv39H1 siRNA (50nM) for 48hrs and then serum starved for 24hrs. Cells were subsequently incubated with FP (10⁻⁸M) for 30mins, then stimulated with medium, H₂O₂ (200 μ M), IL-1 β (10ng/ml) or H₂O₂ plus IL-1 β for 6hrs. The scrambled siRNA pre-treated group showed no effect on GMCSF mRNA levels when compared with the control group (Fig 4.9A). In contrast, increased GMCSF mRNA levels were seen in the Suv39H1 siRNA treated group following stimulation with IL-1 β plus H₂O₂ when compared to scrambled siRNA treated group (Fig 4.9A).

We further compared the ability of FP to downregulate GMCSF mRNA expression in the absence of Suv39H1 (Fig 4.9B). The ability of FP to IL-1 β plus H₂O₂-induced GM-CSF mRNA expression was impaired to a small but significant extent in the Suv39H1 siRNA treated group when compared to the scrambled siRNA group. The results also indicate that Suv39H1 may involve in the FP regulation of GMCSF mRNA expression under IL-1 β plus H₂O₂. A full concentration response curve should be performed to confirm this.

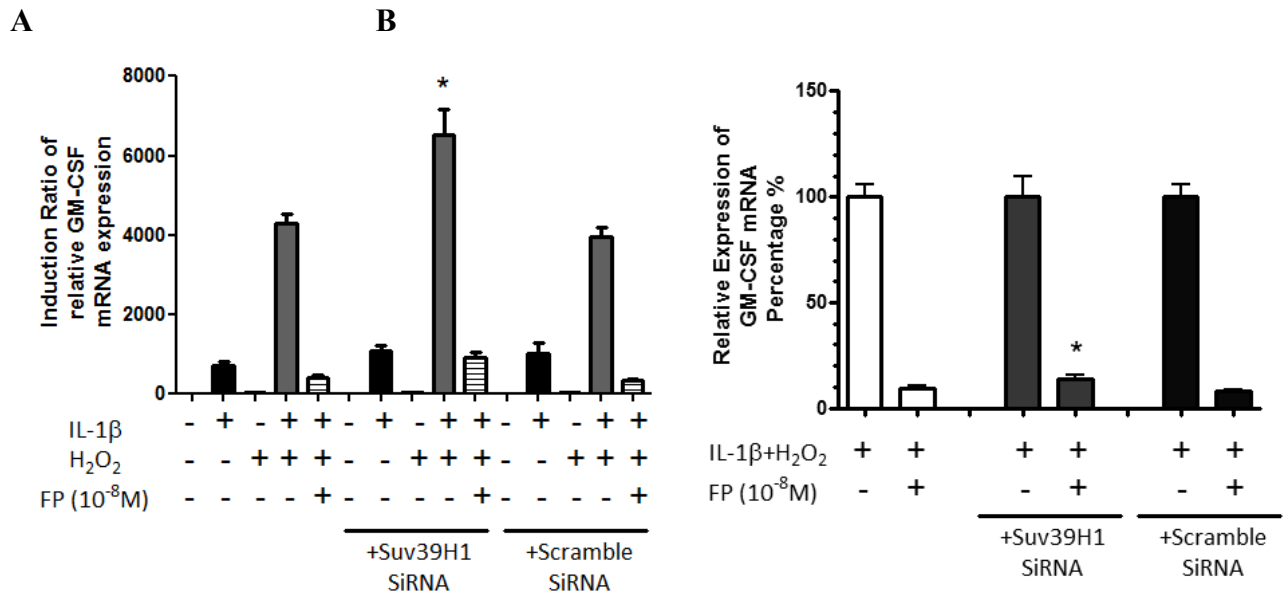


Figure 4.9 Effect of Suv39H1 knockdown by siRNA on IL-1 β , hydrogen peroxide (H₂O₂) and fluticasone propionate (FP)-induced GM-CSF mRNA expression. Cells were incubated with Suv39H1 siRNA, scrambled siRNA, or medium alone for 48hrs. Then cells were pretreated with FP for 30min followed by stimulation with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated for 6h. Total RNA was quantified by RT-QPCR with primers specific for GM-CSF. Relative mRNA expression was normalized by GNB2L1 mRNA expression as an internal control. Results are shown as mean $\pm$ the SEM. (A) * p <0.05 when compared to scrambled siRNA plus IL-1 β +H₂O₂. (B) * p <0.05 when compared with scrambled siRNA plus FP+IL-1 β +H₂O₂. N = at least three independent experiments.

4.3.10 *Suv39H1 knockdown downregulates GR-GRE activation under oxidative stress*

To investigate whether Suv39H1 directly affects the GR-GRE activation, cells were preincubated with Suv39H1 siRNA, or scramble siRNA, then pretreated with FP followed by IL-1 β , with or without H₂O₂, or left untreated. Nuclear protein was extracted for GR-GRE activation assay. GR-GRE activation was not affected by IL-1 β , or H₂O₂. However, FP-stimulation of GR-GRE binding was decreased after knockdown of Suv39H1 by siRNA (OD 0.162 $\pm$ 0.011 vs . 0.24 $\pm$ 0.023, P <0.05)(Fig 4.10). These data suggest that Suv39H1 is actively involved in FP-induced GR-GRE DNA binding.

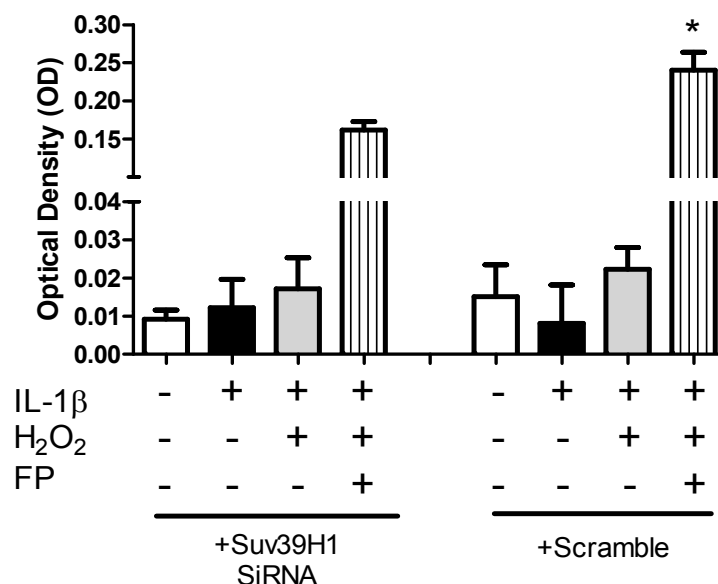


Figure 4.10 Suv39H1 knockdown decreases GR-GRE binding. Cells were incubated with Suv39H1 siRNA, or scramble siRNA for 24hrs. Cells were then treated with fluticasone propionate (FP, 10⁻⁸M) for 30mins followed by stimulation by IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated for 1hr. Nuclear proteins were extracted for GR-GRE activation assay. Results are mean $\pm$ the SEM. N = three independent experiments. * P <0.05 versus IL-1 β +H₂O₂ plus FP with Suv39H1 siRNA knockdown.

4.3.11 *Suv39H1 associates with NF-κB p65 following IL-1β and H₂O₂ stimulation*

To study whether Suv39H1 has a direct association with NF-κB p65, cells were pretreated with FP (10⁻⁸M) for 30mins, then stimulated with IL-1β (10ng/ml), with or without H₂O₂ (200μM) for 1hr. Whole cell proteins were collected and Suv39H1 was immunoprecipitated with a specific anti-Suv39H1 antibody. The associated p65 was determined with an anti-p65 specific antibody. A strong NF-κB p65-specific band was detected in the Suv39H1 immunoprecipitate (Fig 4.11). The results demonstrate that the recruitment of p65 to Suv39H1 was increased under IL-1β, H₂O₂ and IL-1β plus H₂O₂. Interestingly, the association of NF-κB p65 to Suv39H1 was decreased in the presence of FP (Fig 4.11).

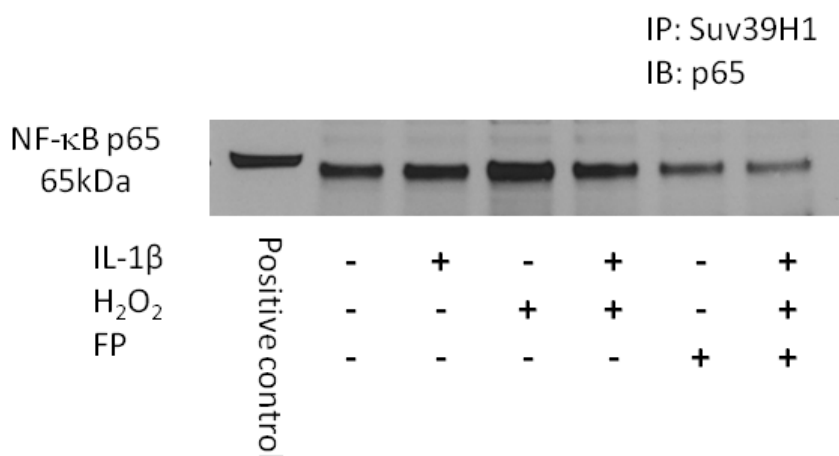


Figure 4.11 Effect of IL-1β and hydrogen peroxide (H₂O₂) on Suv39H1 association with NF-κB p65. Cells were stimulated with IL-1β (10ng/ml), with or without H₂O₂ (200μM), for 1hr. Suv39H1 was immunoprecipitated with a specific antibody against Suv39H1 from whole cell extracts. In some experiments cells were pretreated with FP (10⁻⁸M) for 30mins before stimulation with IL-1β plus H₂O₂. The associated p65 protein was detected by Western blotting with an antibody specific for NF-κB p65. Data are examples from two independent experiments with similar results.

4.3.12 *NF-κB p65 is associated with HMT activity*

To study whether NF-κB p65 is associated with Suv39H1, a preliminary *in vitro* HMT activity assay was performed with nuclear extracts from IL-1β and H₂O₂ stimulated samples (Fig 4.12). Nuclear extracts were immunoprecipitated with an anti-p65 antibody and an HMT reaction performed by incubating the immunoprecipitated proteins in HMT buffer with core histones as substrate and SAM as the methyl donor. HMT activity was determined by Western blotting with an anti-trimethyl H3K9 specific antibody. Increased H3K9 trimethylation was detected in IL-1β, H₂O₂, and FP pretreated cells. The sample from IL-1β plus H₂O₂ treated cells seems to be lost in this experiment and must be repeated later. This preliminary data suggests that NF-κB p65 can associate with Suv39H1 and that Suv39H1 may contribute, at least in part, for the NF-κB p65 associated HMT activity.

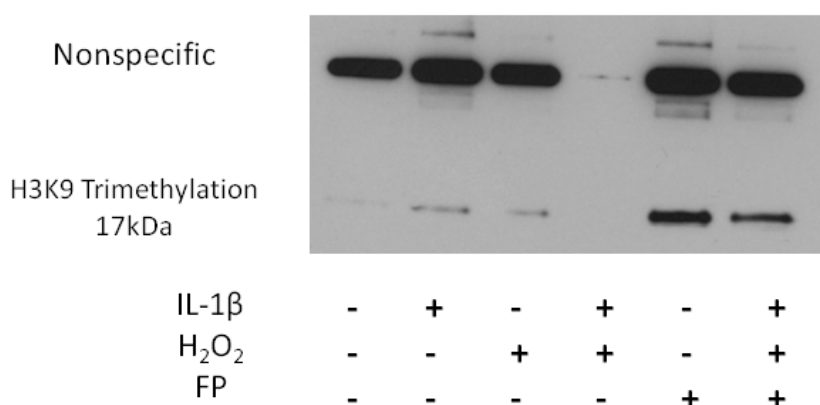


Figure 4.12 Effect of IL-1β, hydrogen peroxide (H₂O₂) and Fluticasone propionate (FP) on NF-κB p65 associated histone methyltransferase (HMT) activity. Cells were pretreated with FP for 30mins then stimulated with IL-1β (10ng/ml), with or without H₂O₂ (200μM), or left untreated. Whole protein was extracted and p65 was immunoprecipitated with a specific anti-p65 antibody. The *in vitro* HMT assay was performed with core histones as substrate and SAM as the methyl donor. H3K9 trimethylation was determined using a specific antibody against trimethylated H3K9. Data is from 1 experiment.

4.3.13 G9a mRNA is downregulated by oxidative stress

We also checked whether other H3K9 HMTs were affected by oxidative stress. A549 cells were serum-starved, then pretreated with variable concentrations of FP (10^{-6} , 10^{-8} , 10^{-10} , 10^{-12} M) for 30min followed by stimulation with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M) for 6h. Total RNA was quantified by RT-QPCR with primers specific for TGF- β 1. The results demonstrate that the expression of G9a mRNA is also downregulated by IL-1 β plus H₂O₂ (Fig 4.13) and that FP has no effect on the repression of G9a mRNA levels (Fig 4.13). We have also tried to detect the expression of Suv39H2 in A549 cells, but the levels were too low to be detected by RT-QPCR which may be due to a cell specific expression pattern. These results indicate that G9a and Suv39H1 have similar responses following exposure to oxidative stress.

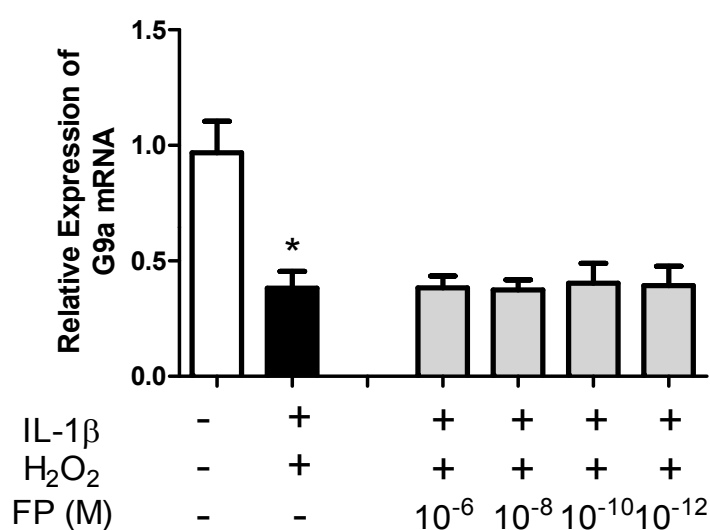


Figure 4.13 Effect of IL-1 β plus hydrogen peroxide (H₂O₂) on G9a mRNA expression.

Cells were pretreated with fluticasone propionate (FP) for 30min followed by stimulation with IL-1 β (10ng/ml) plus H₂O₂ (200 μ M) or left untreated for 6h. Total RNA was quantified by RT-QPCR with primers specific for G9a. Results are shown as mean $\pm$ the SEM. N = 3 independent experiments. * P<0.05 compared to medium only.

4.3.14 Effect of IL-1 β and H₂O₂ on the histone demethylases JMJD2C and JMJD3

To examine whether IL-1 β and H₂O₂ may affect histone demethylase expression, A549 cells were stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated for 6h. Total RNA was extracted and mRNA expression for JMJD2C, a H3K9 demethylase, and JMJD3, a H3K27 demethylase, measured (Fig 4.14) quantified by RT-QPCR. The relative expression of JMJD2C and JMJD3 mRNA expression was normalized to GNB2L1 mRNA.

The expression of JMJD3 mRNA was increased by IL-1 β and H₂O₂, both alone and in combination, and subsequently the induced JMJD3 mRNA expression was downregulated by FP treatment (Fig 4.15A). Modulating the expression of Suv39H1 using Suv39H1 siRNA knockdown (Fig 4.15B) or Suv39H1 overexpression (Fig 4.15C) had no effect on JMJD3 mRNA expression in response to IL-1 β , H₂O₂ or FP in comparison with scrambled siRNA.

In contrast, the expression of JMJD2C exhibited a different response compared to JMJD3. JMJD2C mRNA expression was decreased by IL-1 β and to a lesser extent by H₂O₂ stimulation (Fig 4.15D), a similar trend to that observed for Suv39H1.

These results demonstrate that IL-1 β and H₂O₂ induce different profiles of histone demethylases. How HDM protein expression and activity is affected under these conditions needs examination.

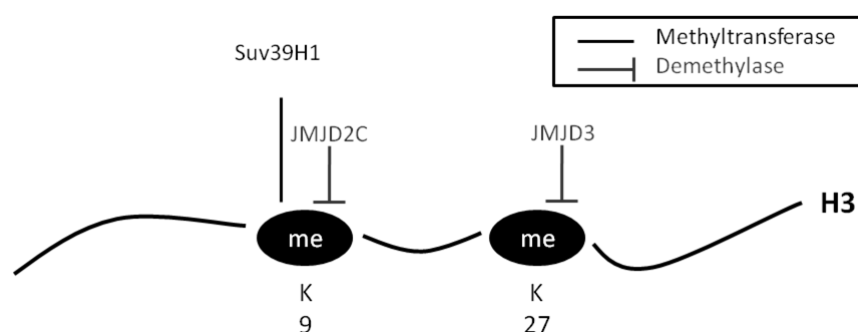


Figure 4.14 Relationship between histone methyltransferase Suv39H1 and the demethylases JMJD2C and JMJD3 on the N-terminal tail of histone 3.

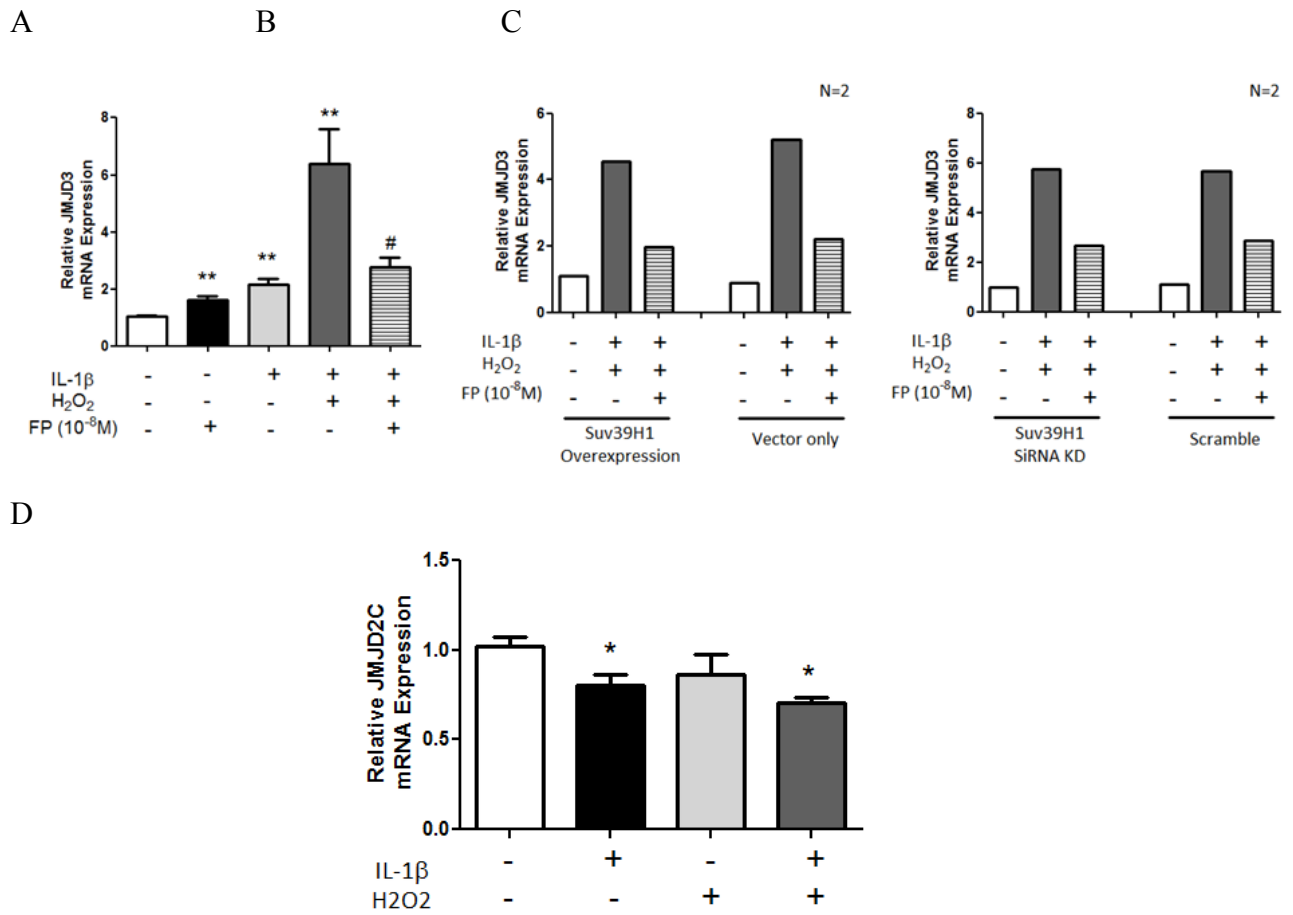


Figure 4.15 Effect of IL-1 β and hydrogen peroxide (H₂O₂) on histone demethylase JMJD2C and JMJD3 mRNA expression. Cells were pretreated with or without fluticasone propionate (FP) for 30mins followed by stimulation with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M) for 6h. Total RNA was collected and quantified by RT-QPCR with primers specific for JMJD3 (A-C) or JMJD2C (D). The relative mRNA expression level was normalized with GNB2L1 mRNA expression. (B) Cells were incubated with Suv39H1 overexpression plasmid or vector for 24h followed by stimulation with IL-1 β and H₂O₂. (C) Cells were incubated with Suv39H1 siRNA or scrambled siRNA for 48h and then stimulated with IL-1 β and H₂O₂. Results are shown as mean $\pm$ the SEM. *p<0.05, **p<0.01 when compared to the basal level. # p<0.05 when compared to the IL-1 β plus H₂O₂ group. (A) and (D) N = at least three independent experiments. (B) and (C) N = two independent experiments.

4.4 Discussion

Our current results show that both IL-1 β and H₂O₂ decrease H3K9 trimethylation status at 1hr. However, differential regulation of H3K9 trimethylation is suspected as differential effects on H3K9 trimethylation were seen at 16hrs with IL-1 β and H₂O₂. Suv39H1 mRNA expression was downregulated under IL-1 β and H₂O₂ stimulation, which possibly accounts for the decreased amounts of H3K9 trimethylation. By ChIP analysis, Suv39H1 was seen to be recruited to the κ B3 binding site in the TGF- β 1 promoter at 1hr, and that recruitment was decreased after 2hrs. Knockdown of Suv39H1 expression with siRNA resulted in enhanced TGF- β 1 mRNA expression with IL-1 β plus H₂O₂ stimulation when compared to scramble siRNA. However, a 10,000-fold increase in Suv39H1 mRNA expression using an overexpression vector did not affect IL-1 β and H₂O₂-induced TGF- β 1 mRNA expression. It will be important in future to examine the effect of overexpression on HMT enzymic activity.

As TGF- β 1 mRNA expression is under the control of NF- κ B we checked the association of NF κ B p65 with Suv39H1. Using co-immunoprecipitation, we demonstrated that NF- κ B p65 was associated with Suv39H1 under conditions of IL-1 β , H₂O₂, and IL-1 β plus H₂O₂ stimulation. This association may account for the H3K9 HMT enzymic activity pulled down with NF κ B p65 in this model system.

Interestingly, Suv39H1 siRNA also increased TGF- β 1 mRNA expression following FP treatment which reduced TGF- β 1 expression to a greater extent under control conditions. This suggests that Suv39H1 may modulate GR function. Indeed, Suv39H1 and GR were shown to be part of the same complex following IL-1 β and H₂O₂ stimulation in co-immunoprecipitation studies. This may account for the increased GR-associated H3K9 HMT activity seen with IL-1 β , H₂O₂, IL-1 β plus H₂O₂ and FP treatment. Importantly, NF- κ B p65-associated H3K9 HMT enzymic activity was reduced by the presence of FP. Furthermore, GR transactivation as determined by GR-GRE DNA binding assay was also attenuated in Suv39H1 siRNA-treated cells. Further temporal analysis of p65, GR and Suv39H1

recruitment to the native TGF- β 1 promoter κ B3 site would be important to perform in future studies along with a comparison of GR/Suv39H1 recruitment to a native GRE using re-ChIP experiments.

This effect of Suv39H1 knockdown was not restricted to TGF- β 1 as Suv39H1 siRNA was also able to upregulate GMCSF mRNA expression following IL-1 β and H₂O₂ stimulation. In addition, the suppressive effect of FP on GMCSF mRNA expression was also impaired with Suv39H1 mRNA knockdown. The combination of ChIP-seq and gene arrays may also give a clearer idea as to the generality of the effects described here. Since the changes in H3K9 methylation shown here occur prior to changes in AcH3 And AcH4 (Lee et al., 2006), it would be interesting to determine whether these methylation and acetylation marks were linked by examining whether Suv39H1 siRNA affected changes in histone acetylation status.

We further examined whether other H3K9 methyltransferases, G9a and Suv39H2 (Rice et al., 2003), and their associated histone demethylases, JMJD2C and JMJD3 (Shi and Whetstine, 2007; Shin and Janknecht, 2007), were modulated by IL-1 β and/or H₂O₂ in this model system. We were not able to detect Suv39H2 mRNA expression in A549 cells but RT-QPCR quantification indicated that both G9a and JMJD2C show a similar downregulation of their respective mRNAs following treatment of cells with IL-1 β and H₂O₂. However, in contrast, JMJD3 mRNA expression was upregulated in response to IL-1 β and H₂O₂ treatment. In addition, IL-1 β and H₂O₂ stimulated JMJD3 mRNA expression was downregulated by FP.

Our results demonstrate the H3K9 methylation may be able to regulate specific gene expression in A549 cells. H3K9 trimethylation is thought to be associated with gene repression (Kouzarides, 2007). But under oxidative stress conditions, the level of H3K9 trimethylation was decreased, which may account for increased gene expression. Although we have focussed on Suv39H1 in this thesis, G9a and Suv39H1 also showed a similar pattern

of downregulation in response to IL-1 β and H₂O₂ treatment. It would be interesting to determine if other HMTs are also regulated in the same manner and also the mechanism for this apparently co-ordinated response. Are the differences in HMT and HDM mRNA expression linked to changes in protein expression and, more importantly, enzymic activity? In addition, what is the mechanism for this action i.e. whether mRNA stability/transcription rate or posttranslational modifications induced by oxidative stress or inflammation alone can affect the expression and activity of these HMTs?

The results also demonstrated differential regulation of H3K9 trimethylation at 16h with inflammatory and oxidative stress stimuli. The effect of these stimuli on the temporal recruitment of HMTs to the κ B3 site in the native TGF- β 1 promoter may confirm this. The HMT activity assay used in this thesis is able to detect HMT activity associated with an immunoprecipitated protein complex. It is possible that the HMT activity may not be directly associated with the immunoprecipitated protein of interest but is pulled down as a passenger protein as part of a larger complex. Mass spectroscopic analysis of the immunoprecipitated complexes in combination with HMT assays following increasing stringency of washes may reveal whether there is a direct protein-HMT interaction. BRET analysis may also provide evidence for a direct association (Pfleger and Eidne, 2006).

It is unclear exactly how an inflammatory stimulus or an oxidative stress can target Suv39H1 association with p65 and/or GR. Data from our group has examined this in part for the recruitment of HDAC2 to p65 and GR as demonstrated that specific post-translational modifications were important (Ito et al., 2006b). Similar studies may reveal the same process occurring for Suv39H1.

Interestingly, we also demonstrated that GR- and p65-immunoprecipitated HMT-containing complexes had a differential response to FP. NF- κ B p65-immunoprecipitated HMT activity was downregulated with FP treatment which was not the case with GR-associated HMT activity. This may be a result of GR and NF- κ B p65 having a different affinity for

Suv39H1 and may also underlie the different effects on gene expression and promoter-specific H3K9 methylation seen with various treatments.

To our knowledge there is only one published study that has reported an effect of GR on H3K9 methylation (Islam and Mendelson, 2008). Surfactant protein A (SP-A) mRNA is induced by IL-1 β and by dibutyl cAMP and this enhanced response is completely repressed by dexamethasone in primary type II alveolar cells. SP-A expression in these cells is regulated by NF- κ B complexed with another transcription factor Thyroid Transcription Factor (TTF)-1. Using ChIP analysis the authors reported that dexamethasone increased H3K9 dimethylation at the TTF-1 binding element (TBE) in the SP-A promoter. This was associated with recruitment of heterochromatin protein 1 (HP-1).

The H3K27 demethylase JMJD3 mRNA showed a different response to IL-1 β and H₂O₂ stimulation to that seen for JMJD2C and, in addition, the increased JMJD3 mRNA expression can be suppressed by FP. H3K27 is another site which was thought to be related to gene repression (Kouzarides, 2007). Upregulation of JMJD3 can demethylate previous methylation marks around this site (Hong et al., 2007; Swigut and Wysocka, 2007), and further antagonize the effect of H3K9 methyltransferase. But this is not known whether demethylation of the repressive H3K27 mark is associated with gene activation (De Santa et al., 2007; Lee et al., 2007). It will be interesting to check if JMJD3 has cross-activity with other target proteins which may affect gene activation/repression. The effect of IL-1 β and/or H₂O₂ on H3K27 methylation status at the κ B3 site on the native TGF- β 1 promoter may also be informative as would the recruitment of specific H3K27 HMTs.

After transfection with Suv39H1 overexpression plasmid Suv39H1 mRNA expression was significantly upregulated. However, in contrast to Suv39H1 knockdown, we were unable to show a difference between TGF- β 1 mRNA expression nor an effect on regulation of GR function. This may simply reflect the non-linear relationship between mRNA and protein expression we saw for Suv39H1 expression (Fig 4.8A and B) or a non-significant effect on

enzymic activity (not measured) or H3K9 methylation status (not measured). Alternatively, it is possible that the natural level of Suv39H1 expression produces the maximal effect on TGF- β 1 mRNA expression and GR function possible in these cells and Suv39H1 overexpression may not, therefore, be able to modulate these effects.

There is evidence in the literature that changes in histone methylation and the recruitment of HMTs are linked to changes in DNA methylation (Fuks et al., 2003a; Lehnertz et al., 2003). We, therefore, examined whether IL-1 β and/or H₂O₂ could modulate DNA methylation status.

CHAPTER FIVE

**THE EFFECT OF OXIDATIVE STRESS ON DNA METHYLATION AND
UPREGULATION OF TGF- β 1 mRNA EXPRESSION**

5.1 Rationale

DNA methylation is one of the important covalent modifications involved in epigenetic regulation of gene repression (Fuks, 2005). DNA methylation occurs at the carbon-5 position of cytosine (mC^5) and is catalysed by the DNA methyltransferases DNMT1, DNMT3a and DNMT3b using the methyl donor S-adenosyl-L-methionine (Yen et al., 1992). Approximately 50% of protein coding genes have GC-rich areas of DNA in their promoters (CpG islands) consisting of regions of >500 base pairs with a GC content greater than 55% and methylation of these islands usually silences gene expression. In cancer, aberrant CpG island methylation in tumour suppressor gene is associated with cancer progression (McCabe et al., 2009). More recently, it has become evident that DNA methylation can occur outside of promoter CpG sites at sites known as DNA shores (Irizarry et al., 2009).

DNA methylation was for many years considered a static or invariant mark that could not be removed (Gehring et al., 2009; Ramchandani et al., 1999). However, a recent study described cyclical methylation of the ps2 and ER α promoters (Kangaspeska et al., 2008). These data indicates that the CpG island methylation may be not be permanently fixed and that DNA methylation and demethylation is a dynamic process that potentially regulated by specific enzymes. The search for specific enzymes that regulate demethylation of DNA is ongoing (Gehring et al., 2009). Active DNA demethylation underlies key facets of reproduction in flowering plants and mammals and serves a general genome housekeeping function in plants. A family of 5-methylcytosine DNA glycosylases catalyzes plant demethylation via DNA base-excision-repair process. However, mammals lack counterparts to the plant demethylases. Recent results have suggested that DNA repair is also a plausible mechanism for global DNA demethylation in mammals (Gehring et al., 2009). Alternative methods of causing active DNA demethylation have also been proposed utilising active transcriptional processes and histone acetylation (D'Alessio et al., 2007) and the methyl-CpG binding domain protein MBD3 (Brown et al., 2008).

Histone methylation is thought to be related to DNA methylation, and a reinforcing cycle was described to aid in gene repression (Fuks, 2005). It is unclear whether histone methylation provides a mark to enable the recruitment of a DNA methyltransferase or whether DNA methylation provides a scaffold for HMT recruitment (Fuks et al., 2003b; Kouzarides, 2002). The precise order of events may be both gene- and context-specific (Bird, 2002; Fischle, 2008)

It has been reported that DNA methylation was affected by oxidative stress mainly through the actions of lipid secondary metabolic products. This results in the formation of an impaired DNMT1 binding site (Turk et al., 1995), or through the impaired function of MBP (Valinluck et al., 2004).

Among the various DNA methyltransferase, we focus on the DNMT1, the maintenance DNA methyltransferase, since it is important in re-establishing DNA methylation after the methylation mark in one of the parent strand of DNA. Additionally, DNMT1 is directly associated with Suv39H1 (Fuks et al., 2003a). Thus, the DNA methyltransferase, DNMT1, might be a good candidate to study the effect of oxidative stress on DNA methylation.

5.1.1 Hypothesis and aims of this Chapter

We hypothesise that oxidative stress, in combination with IL-1 β , may change the methylation status of CpG island on the TGF- β 1 promoter.

The specific aims were:

1. To determine the effect of H₂O₂, and in combination with IL-1 β , on CpG island methylation on A549 cells.
2. To evaluate the effect of H₂O₂, and in combination with IL-1 β , on DNMT1 production, and the recruitment of DNMT1 to the NF- κ B binding site on TGF- β 1 promoter.

3. To examine the effect of H₂O₂ alone, and in combination of IL-1 β on CpG island methylation on TGF- β 1 promoter by Bisulphite sequence

5.2 Experiment Protocols

5.2.1 *Effect of 5-aza on oxidative stress induced TGF β 1 mRNA expression*

90% confluent A549 cells were cultured in serum-free DMEM medium supplemented with L-glutamine for 48hrs before stimulation. Cells were pretreated with the DNA methylation inhibitor 5-Aza (1ng/ul) for 30 mins before stimulation before stimulation with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M) or left untreated for 4h. RNA was isolated, and levels of TGF- β 1 mRNA were quantified by RT-QPCR.

5.2.2 *Bisulphite sequencing*

90% confluent A549 cells were cultured in serum-free DMEM medium supplemented with L-glutamine for 48hrs before stimulation. Cells were then stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated for variable times (0, 1hr). Cells were collected, and DNA was extracted according to protocol described in Chapter 2. 500ng genomic DNA was obtained and taken forward for bisulphite treatment. The bisulphite-treated DNA was sent for sequencing.

5.2.3 *Effect of oxidative stress on DNMT1 mRNA expression*

Cells were incubated with medium or IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), for 6hrs. RNA was isolated and levels of DNMT1 mRNA were quantified by RT-QPCR.

5.2.4 *Effect of oxidative stress on DNMT1 protein expression*

Cells were incubated with medium alone or IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), for 1hr. Protein extracts were obtained and examined for DNMT1 by Western blotting.

5.2.5 *The association between DNMT1, Suv39H1 and NF- κ B p65*

Cells were incubated with medium alone or IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), for 1hr. Cell lysates were immunoprecipitated by a specific antibody against DNMT1. The associated Suv39H1 and NF- κ B p65 were determined by Western blotting using specific antibodies.

5.2.6 *Enrichment of mC⁵ at the NF- κ B binding site on TGF- β 1 promoter*

Cells were incubated with medium alone or IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), for variable times (1 and 2hrs). DNA was extracted and incubated with 1 μ g antibody specific for 5mC antibody (Calbiochem, UK) overnight. The immunoprecipitated DNA was purified with proteinase K, and phenol/chloroform/alcohol method. The enrichment of 5mC at the NF- κ B binding site was quantified by comparing amplified DNA by RT-QPCR with primers specific for κ B3 binding site.

5.2.7 *Effect of IL-1 β and H₂O₂ on TGF- β 1 promoter occupancy by DNMT1*

A549 cells were serum-starved for 48hrs before incubating with medium alone or IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), for variable times (0, 30 mins and 1hr). At the end of stimulation, proteins and DNA were cross-linked with 1% formaldehyde and DNMT1-TGF- β 1 promoter association was determined by DNMT1 ChIP assay with specific antibody against DNMT1 (H300, Santa Cruz Biotechnology). Enrichment of IP DNA was quantified by RT-QPCR with primer sets amplifying the κ B3 site.

5.2.8 Effect of FP on IL-1 β and H₂O₂ induced DNMT1 gene expression

90% confluent A549 cells were cultured in serum-free DMEM medium supplemented with L-glutamine for 48hrs before stimulation. Cells were then pretreated with FP (10⁻⁸M) for 30mins before being stimulated with IL-1 β (10ng/ml), with or without H₂O₂ (200 M), or left untreated for 6hrs. At the end of stimulation, total RNA was isolated and DNMT1 mRNA was determined by RT-QPCR. The relative expression level of DNMT1 mRNA was normalized with GNB2L1 mRNA as an internal control.

5.3 Results

5.3.1 5-aza upregulates the expression of TGF- β 1 mRNA

To investigate whether DNA methylation affects the regulation of TGF- β 1 mRNA, cells were preincubated with the DNA methylation inhibitor, 5-aza (10 μ M), 30min before stimulation with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated for 6h. Upon 5-Aza pretreatment, TGF- β 1 mRNA expression was further upregulated when stimulated with IL-1 β alone (P<0.05, 1.35 $\pm$ 0.05 versus 1.70 $\pm$ 0.08), H₂O₂ alone (P<0.05, 1.15 $\pm$ 0.06 versus 1.45 $\pm$ 0.08) and or IL-1 β plus H₂O₂ (P<0.05, 1.52 $\pm$ 0.08 versus 1.94 $\pm$ 0.12)(Fig 5.1). The result demonstrates that DNA methylation involved in regulation of TGF- β 1 mRNA expression. These cells were not proliferating and so intercalation of 5-Aza into de novo DNA does not account for the effects observed.

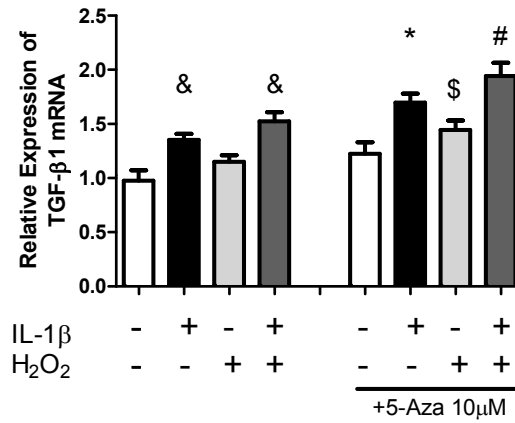


Figure 5.1 Effect of 5-aza-cytidine (5-Aza) on IL-1 β plus hydrogen peroxide (H₂O₂)-induced TGF- β 1 mRNA expression. Cells were pretreated with 5-Aza (10 μ M) for 30mins, followed by stimulation with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), or left untreated. TGF- β 1 mRNA expression was quantified by RT-QPCR with primers specific for TGF- β 1, and compared with GNB2L1 mRNA as internal control. Results are shown as mean $\pm$ the SEM. N equal to three independent experiments. &p<0.5 when compared to basal level; *p<0.5 when compared to IL-1 β ; \$p<0.5 when compared with H₂O₂; #p<0.5 when compared to IL-1 β plus H₂O₂.

5.3.2 IL-1 β and H₂O₂ induced differential changes of mC⁵ at the κ B3 binding site and the ATG start site of the TGF- β 1 promoter

To determine the effect of IL-1 β and H₂O₂ on the methylation status of the TGF- β 1 promoter CpG island, we have sequence two areas of the TGF- β 1 promoter site, one includes the κ B3 binding site, which represents a high density CpG island area; and the other includes ATG starting site, which represents a low CpG island density area (Fig 5.2A). Unfortunately, due to technical difficulties we were not able to sequence the region which includes the κ B3 binding site due to the very high C to T changes seen after bisulphite treatment (Fig 5.2B).

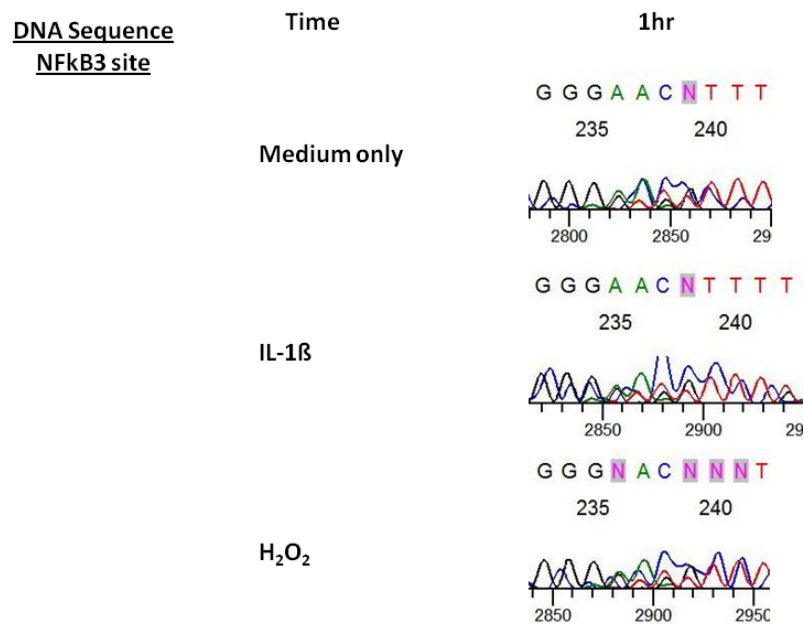
Interestingly, we were able to demonstrate that there are variable methylation changes occurring around the ATG starting site area. There were significant rapid (60 mins) T to C changes after both IL-1 β and H₂O₂ stimulation, when compared to medium alone (Fig 5.2C) demonstrating that there is a rapid methylation of residues occurring at this site. Interestingly, in the case of IL-1 β , there was a reversal of this methylation process by 2hrs indicating that an active demethylation event was also occurring at this site (Fig 5.2C). Further investigation of this effect with IL-1 β and with H₂O₂ is required as is the possible effect of FP.

We have repeated this experiment but again were unable to obtain sequencing data at the κ B3 site possibly suggesting interference from variable methylation status of the CpG islands on TGF- β promoter after acute stimulation with IL-1 β or H₂O₂. This indicates that there are possibly two or more different amplified DNA sequences present. We are currently in discussion with the Sequence provider, Cogenics, with the aim of obtaining a better signal/noise ratio for this sequence.

A



B



C

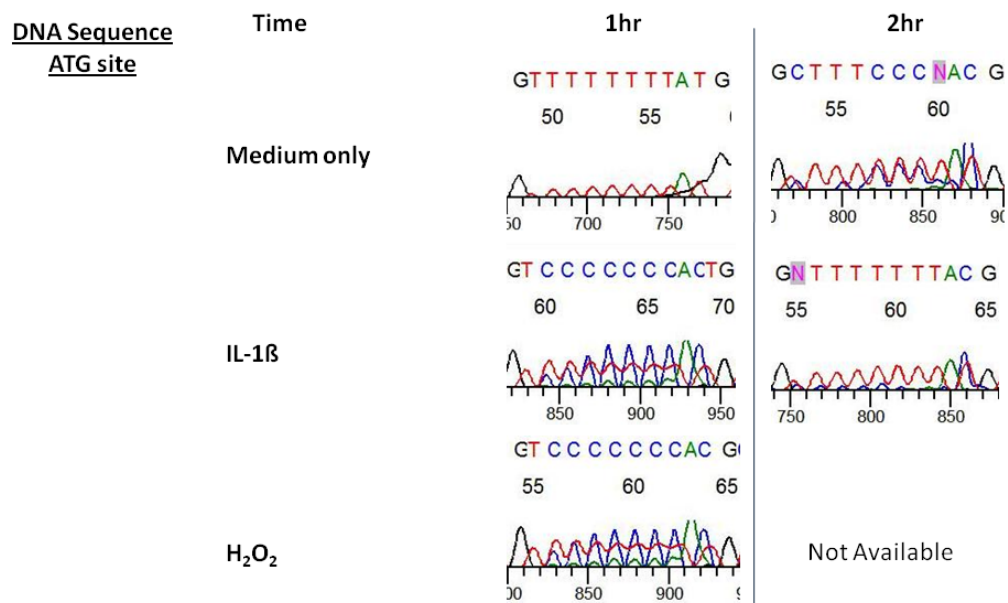


Figure 5.2 Effect of IL-1β and hydrogen peroxide (H₂O₂) on mC⁵ at the TGF-β1 promoter. (A) Schematic diagram of DNA sequence for κB3 site and ATG site. Numbers

represent distance for ATG translation start site. Focused area for sequencing was underlined. Cells were stimulated with IL- β (10ng/ml), H₂O₂ (200 μ M), or left untreated for 1hr or 2hrs. DNA was collected, and treated with Bisulphite for transformation of non-methylated C to T bases. DNA was sequenced with specific primers for areas near the κ B3 binding site, which represents a high density CpG island area (B), and for a region near the ATG translation start site (C). On the sequence graph, green colour represents A base, red colour represent T base, blue colour represents C base; while black colour represent G base. Different height of the graph means density by sequence for individual base. N = 1 in this study.

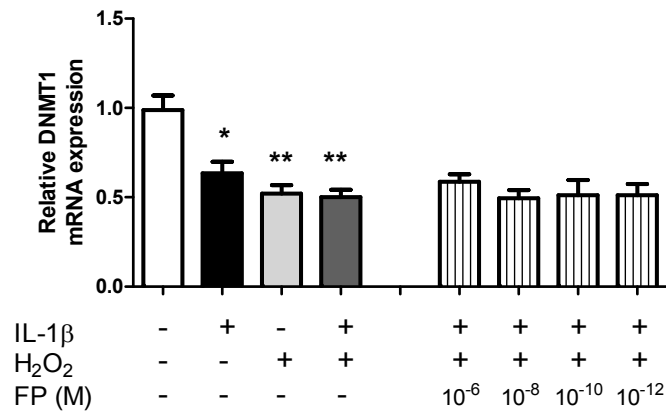
5.3.3 *IL-1 β and H₂O₂ downregulate the production of DNMT1*

To investigate whether oxidative stress and inflammation have effects on the DNMT1 mRNA expression, A549 cells were pretreated with IL-1 β with or without H₂O₂. The RT-QPCR results demonstrate that the expression of DNMT1 mRNA was downregulated by IL-1 β , with or without H₂O₂ stimulation, after 4hrs (Fig 5.3A).

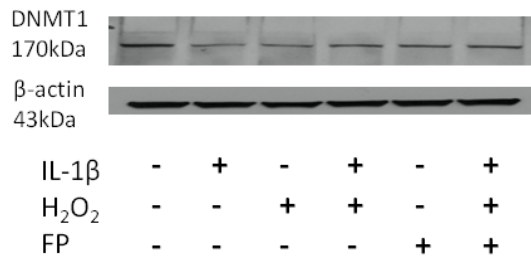
To confirm that DNMT1 protein production was also decreased, Western blot analysis of whole cell lysates was performed. Using a specific anti-DNMT1 antibody, a trend to a decrease in DNMT1 expression (band at 105kDa) was seen after 1 and 4hrs of incubation (Fig 5.3B). The loss in DNMT1 protein expression was prevented by the presence of the proteasomal inhibitor MG-132 (Fig 5.3C).

FP had no apparent effect on IL-1 β + H₂O₂-repressed DNMT1 mRNA expression (Fig 5.3A) although some attenuation in the loss was seen for DNMT1 protein expression (Fig 5.3B). However, further experiments are required to confirm this result.

A



B



C

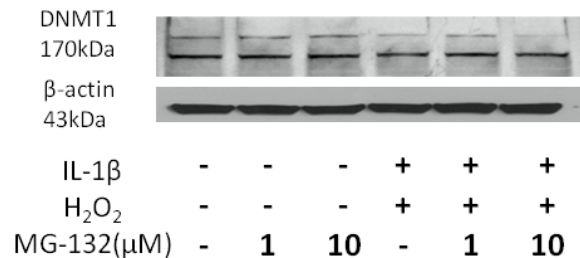


Figure 5.3 Effect of IL-1β and hydrogen peroxide (H₂O₂) on DNMT1 mRNA and protein expression. (A) Cells were pretreated with fluticasone propionate (FP, 10⁻⁸M) for 30mins, then stimulated with IL-1β (10ng/ml), with or without H₂O₂ (200μM), or left untreated for 4hrs. Total RNA was collected and DNMT1 mRNA expression was quantified by RT-QPCR with primers specific for DNMT1 and normalized to GNB2L1 mRNA. Results are shown as mean ± SEM. N = 3 independent experiments. * p<0.05, ** P<0.01 as compared to basal level. (B) Cells were pretreated FP (10⁻⁸M) for 30mins, then stimulated with IL-1β, with or without H₂O₂, or left untreated for 1hr. Whole cell protein was extracted. The amount of DNMT1 protein was detected by Western blotting with DNMT1 antibody. (C) Cells were pretreated with variable concentration of MG-132 (1, 10μM) for 1hr, then stimulated with IL-1β plus H₂O₂ or left untreated for 1hr. Whole cell protein was extracted, and amount of DNMT1 protein was detected by DNMT1 antibody. N = 1 experiment.

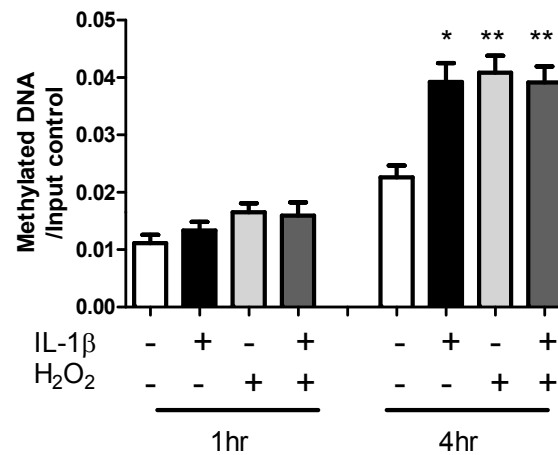
5.3.4 Exaggerated methylation of CpG islands on κ B3 binding site in the TGF- β 1 promoter area

We further performed a Methylation dependent immunoprecipitation assay (MeDIP assay), using an anti-mC⁵ antibody to pull down the methylated Cytosine residue. QPCR experiments with primers specific for the κ B3 binding site on TGF- β 1 promoter area indicated that both H₂O₂ and IL-1 β induced an increase in C⁵ methylation at the κ B3 binding site at 1h when compared to control alone (Fig 5.4A). This increase reached significance at 4hrs (Fig 5.4A).

We also examined the effect of the DNA methyltransferase inhibitor 5-Aza on DNA methylation status at the κ B3 and ATG start sites in the presence of IL-1 β plus H₂O₂ (Fig 5.3B). There was a rapid (30mins) decrease in DNA methylation at the ATG start site in response to 5-Aza in the presence and absence of IL-1 β + H₂O₂ (Fig 5.4B). This effect was reduced over time and no clear effect was seen at 4hrs. In contrast, there was no clear effect pattern of methylation changes seen at the κ B3 site in response to 5-Aza. Further experimentation are needed to confirm these results.

The apparent discrepancy between the data sets for the κ B3 site in the experiments shown in Fig 5.4A and B (the ratio between 5-Aza and control sample is lower in B than in A) may be due to the solvent used for 5-Aza. DMSO has been reported to upregulate CpG island methylation (Iwatani et al., 2006). Further experimentations will be required to resolve this.

A



B

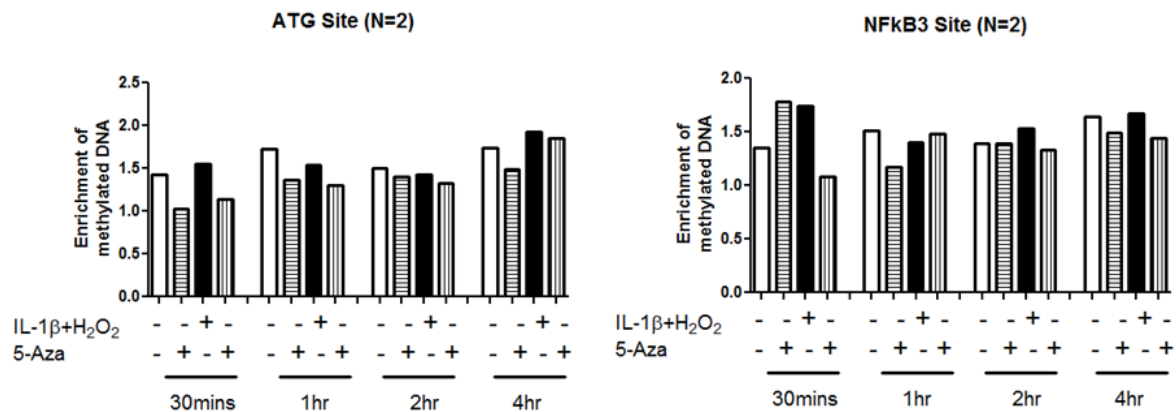


Figure 5.4 IL-1β and hydrogen peroxide (H₂O₂) upregulate DNA methylation at the TGF-β1 promoter. (A) Cells were stimulated with IL-1β (10ng/ml), with or without H₂O₂ (200μM), for 1 and 4hrs. Genomic DNA was extracted and sonicated to <2000bp. Methylated C⁵ residues were immunoprecipitated with a specific anti-mC⁵ antibody overnight and the immunoprecipitated DNA purified. DNA enrichment was quantified by QPCR with primers specific for the κB3 site and normalized by input DNA. Results are shown as mean ± SEM. N = three independent experiment. *p<0.05, **p< 0.01 compared to unstimulated cells. (B) Cells were pretreated with 5-aza-cytidine (5-Aza, 10μM) for 1hr and then stimulated with IL-1β plus H₂O₂ for variable time (30mins, 1hr, 2hrs, 4hrs). Genomic DNA was collected and sonicated, then immunoprecipitated with antibody specific for mC⁵. The immunoprecipitated

DNA was purified, and quantified by QPCR with primers specific for the κ B3 and ATG sites. Enriched DNA was normalized to input DNA. Results are shown as mean $\pm$ SEM. N = two independent experiments.

5.3.5 *Suv39H1 is associated with DNMT1*

To study whether Suv39H1 has increased recruitment to DNMT1 under oxidative stress, cells were pretreated with FP (10^{-8} M), then stimulated with IL-1 β , with or without H₂O₂, or left untreated for 1 hr. Whole cell proteins were extracted and immunoprecipitated with a specific anti-DNMT1 antibody before detection of Suv39H1 by Western blot analysis. The results demonstrate that Suv39H1 was recruited to DNMT1 (Fig 5.5), but that this association was independent of cell stimulation. The reverse co-IP was also performed. The results also indicated that the association of both proteins occurred independent from cell stimulation – compare Ab control control lanes with basal non-stimulated cells in Fig 5A and 5B.

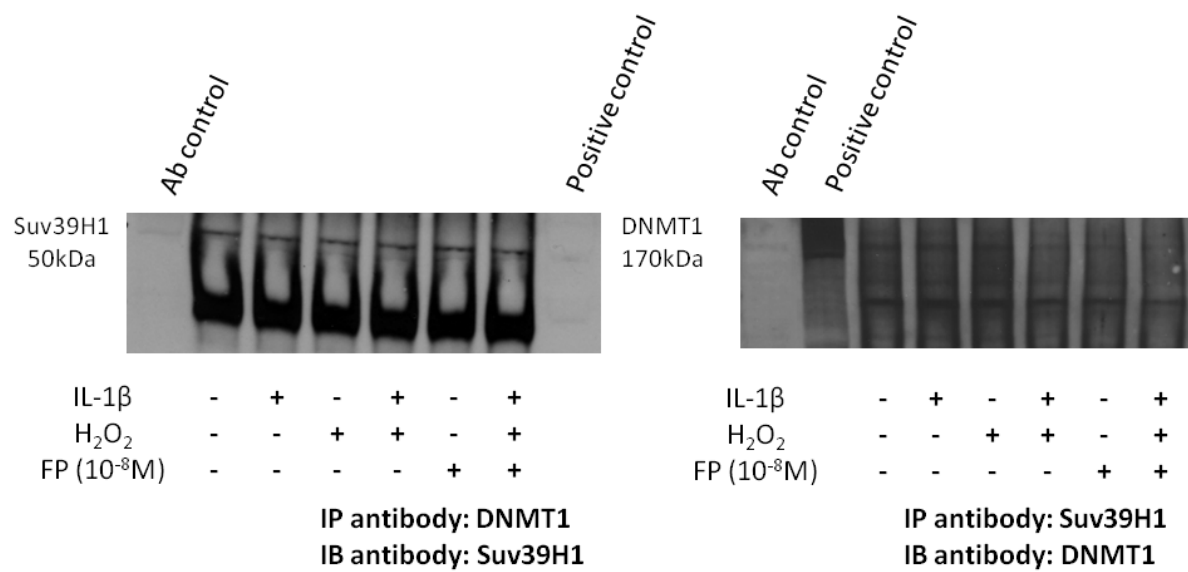


Figure 5.5 Suv39H1 is associated with DNMT1. Cells were pretreated with or without fluticasone propionate (FP, 10⁻⁸M) for 30mins and then stimulated with IL-1β (10ng/ml) and/or hydrogen peroxide (H₂O₂, 200μM) for 1hr. Total protein was extracted, and the associated protein was checked with antibody specific for interested protein. Blots are representative of N = 2 independent experiments.

5.3.6 *NF-κB p65 is associated with DNMT1*

To study whether NF-κB p65 was recruited to DNMT1 under IL-1β and H₂O₂ stimulation a co-immunoprecipitation experiment using whole cell extracts was used and demonstrated an increased association of p65 with DNMT1 in H₂O₂ and IL-1β plus H₂O₂ stimulated cells at 60 mins (Fig 5.6, lanes 4 and 5). The control experiment using immunoprecipitation with pre-immune serum showed no association between p65 and DNMT1 (Fig 5.6 last lane). These results indicate that DNMT1 is actively associated with NF-κBp65 under IL-1β and H₂O₂ stimulation.

Pre-treatment of cells with FP (10⁻⁸M, 30 mins) attenuated complex formation between p65 and DNMT1 induced by combined H₂O₂ and IL-1β (Fig 5.6, lane 7). In contrast, FP alone had no effect on basal complex association. Further experiments are required to confirm these data with different DNMT1 antibody.

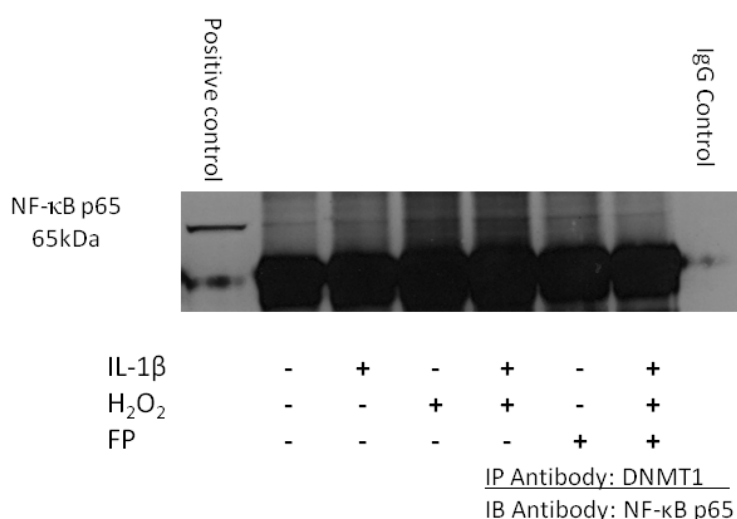
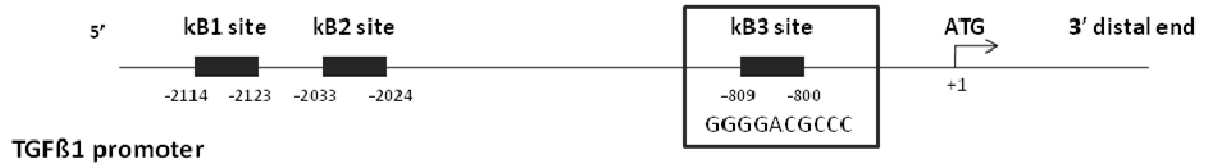


Figure 5.6 NF-κB p65 is complexed with DNMT1. Cells were pretreated with fluticasone propionate (FP, 10⁻⁸M) for 30min and then stimulated with IL-1β (10ng/ml), with or without H₂O₂ (200μM), or left untreated for 1h. DNMT1 protein was immunoprecipitated with a specific anti-DNMT1 antibody and the associated NF-κB p65 detected by Western blotting. Results are representative of N = 2 independent experiments showing a similar trend.

5.3.7 *DNMT1 is recruited to the TGF- β 1 promoter under oxidative stress conditions*

Analysis of the TGF- β 1 promoter had previously revealed consensus NF- κ B binding sites of which κ B3 was associated with p65 DNA binding to the native promoter (Fig 5.7A). We therefore examined whether DNMT1 is recruited to NF- κ B binding sites in the TGF- β 1 promoter area using ChIP assays. DNMT1 was recruited to κ B3 site in a time-dependent manner with a significant effect seen at 30mins with IL-1 β only (Fig 5.7B). Association of DNMT1 with the κ B3 site was not affected by H₂O₂ or IL-1 β + H₂O₂ at any time point studied (Fig 5.7B). These results demonstrate that DNMT1 is recruited to the TGF- β 1 promoter possibly by tethering to NF- κ B. Further experiments looking at recruitment to the ATG start site may reveal distinct patterns.

A



B

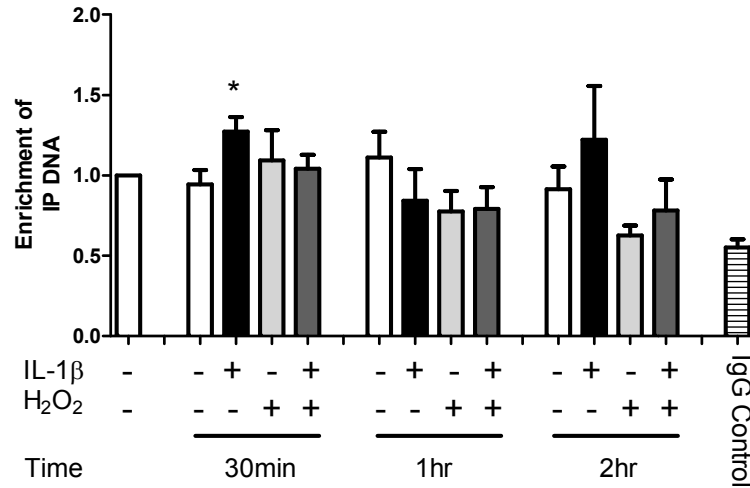


Figure 5.7 Effect of IL-1 β and hydrogen peroxide (H₂O₂) on DNMT1 recruitment to TGF- β 1 promoter. (A) Schematic diagram of the TGF- β 1 promoter. The numbers indicate positions in relation to the translation start site (ATG). Consensus NF- κ B binding sites including the κ B3 site are indicated. (B) Cells were pretreated with IL-1 β (10ng/ml), with or without H₂O₂ (200 μ M), for various times (0, 30mins, 1hr & 2hrs). DNMT1 recruitment was determined by ChIP assay and the associated DNA quantified by quantitative RT-QPCR with primer pairs specific for the putative κ B3 site. The values were normalized to input DNA. Results are mean $\pm$ the SEM of ratio to basal levels. * denotes P<0.05 versus basal level (same time point). n = three independent experiments.

5.4 Discussion

The present study examined the effect of oxidative stress and IL-1 β on the methylation status at a CpG island and at the ATG start site in the native TGF- β 1 promoter. The DNMT inhibitor 5-Aza further enhanced IL-1 β and H₂O₂ upregulation of TGF- β 1 mRNA which suggests that DNA methylation plays a role in the regulation of TGF- β 1 mRNA expression. 5-Aza is classically thought to act in a delayed manner by intercalating into newly synthesised DNA (Christman, 2002; Palii et al., 2008) but this is unlikely to be the case in these experiments as the cells are not proliferating and the time-course of the effect is too rapid. It is possible that 5-Aza blocks the actions of DNMTs by sitting in the active site of the enzyme.

Direct sequencing of genomic DNA after bisulphite treatment enabled us to examine DNA methylation at the ATG transcriptional start site but not at the CpG Island associated with the κ B3 site. The later was due to technical reasons due to the very high methylation status around the κ B3 binding site and a subsequent failure to obtain primers that could produce an effective sequence read. Direct sequencing in future may provide a better way of analysing this region of the TGF- β 1 promoter. We observed a rapid (60 mins) induction of C⁵ methylation at the ATG start site with both IL-1 β and H₂O₂ and a restoration of the basal demethylated status at 120 mins with IL-1 β .

We also performed MeDIP analysis to confirm the DNA sequencing data. The results demonstrated that CpG island methylation at the κ B3 site was enhanced at 4h, but not at 1h, after stimulation with IL-1 β and H₂O₂. We have also tried to use 5-Aza to inhibit DNMT function, but the ratio between 5-Aza and control sample was lower than with our previous results. The solvent, DMSO, which was used to dissolve 5-Aza has been reported to increase CpG island methylation (Iwatani et al., 2006). This may explain the disparity between the results as a trend for upregulated CpG island methylation was seen in our initial experiment.

It will be important to measure the effects of 5-Aza on the mC⁵ status throughout the TGF- β 1 promoter.

This data raises several issues relating to the site of cytosine methylation and the rapid demethylation of the ATG start site that occurs between 0 and 60 mins. DNA methylation is considered a stable epigenetic mark that is heritable but there is now clear evidence that DNA methylation marks can be removed although this process is not well understood (Gehring et al., 2009). Methylation can be lost passively through a failure to maintain methylation status following DNA replication or via a more active process whereby mC⁵ is enzymatically removed (Gehring et al., 2009). This process is clearly occurring in the context of our experiments looking at the TGF- β 1 promoter. In plants, this process is driven by DNA glycosylases which are normally associated with DNA repair but there are no mammalian homologues for these enzymes (Gehring et al., 2009). Alternative methods of causing active DNA demethylation have also been proposed utilising active transcriptional processes and histone acetylation (D'Alessio et al., 2007) and the methyl-CpG binding domain protein MBD3 (Brown et al., 2008). However, it is becoming evident that active DNA demethylation in vertebrates can be mediated through other DNA excision repair enzymatic machineries that promote coupling of deamination, base and nucleotide-excision repair in the process of DNA demethylation (Ma et al., 2009). What the mechanism is for targeting the rapid DNA demethylation seen at the ATG but not the κ B3 site would be interesting to investigate further. Whether these changes occur at other gene start sites or elsewhere in the genome and if the acute response is altered with chronic exposure to ROS is also important to investigate.

Analysis of 8-OH-dG presence in the TGF- β 1 promoter may reveal a potential mechanism that could affect DNMT1 DNA binding and subsequent recruitment of MeCP2 (Turk et al., 1995; Valinluck et al., 2004). These are both valid areas of future research particularly as 8-OH-dG provides a link to DNA excision repair processes (Jackson and Bartek, 2009) that have been implicated in DNA demethylation events (Gehring et al., 2009) and see below).

For many years it has been assumed that changes in DNA methylation occur in CpG islands but this concept has recently been challenged, at least in cancer. Recent evidence from Fienberg and colleagues (Irizarry et al., 2009) has reported that most methylation changes in colon cancer do not occur at CpG Islands, or even in promoters, but can occur in sequences up to 2 kb distant from CpG Islands at regions termed 'CpG island shores'. This group strongly correlated CpG island shore methylation with gene expression. Subsequently, several other studies have also reported that non-CpG DNA methylation is associated with changes in gene expression and cell function. *Dlx5* is an important transcription factor that regulates *runx2* whose expression is a hallmark of osteogenesis. 5-Azacytidine enhanced *dlx5* expression in mesenchymal stem cells and this was associated with reduced hypermethylation in the *dlx5* promoter "CpG island shore". In contrast, the *dlx5* promoter CpG Island remained in a hypomethylated state (Zhou et al., 2009). In addition, the oestrogen receptor (ER)-associated CpG shore ESR1 promoter C, rather than the CpG islands, showed a greater variability in methylation status in breast cancer cells and an inverse associations with ER α and progesterone receptor expression (Gaudet et al., 2009).

It will be interesting to perform an extended and more complete time course to examine DNA methylation and demethylation at the κ B3 and ATG sites in response to IL-1 β , H₂O₂ and FP. Furthermore, when the biochemistry of DNA demethylation is better understood we will be better able to investigate this process in relation to the model system used here or ideally in primary human airway epithelial cells.

As a first step to understanding the DNA methylation occurring at the TGF- β 1 promoter we examined whether NF- κ B p65 was able to recruit DNMT1 to the TGF β 1 promoter by ChIP assay. Initial experiments indicated that NF- κ B p65 was able to associate with DNMT1 and that this complex formation was increased in the presence of oxidative stress. We then demonstrated that DNMT1 was recruited to the κ B3 binding site at 30min and that this recruitment was decreased with time. These findings suggest that other DNMTs may also be

important in inducing the longer lasting methylation seen with the MeDIP assay. Recruitment of other DNMTs to the TGF- β 1 promoter by NF- κ B along with a functional analysis of the enzymic activity of p65-associated DNMTs under IL-1 β and H₂O₂ stimulation should be undertaken. Intermediate time points between those already studied should also be performed.

Analysis of enzymic activities are particularly important as we were able to demonstrate that IL-1 β and H₂O₂ stimulation down-regulated the expression of DNMT1 protein and mRNA. The loss of protein expression was prevented by the proteasome inhibitor MG-132 implicating a possible role for the various ubiquitin-associated pathways to regulate DNMT1 expression. Further investigation of the pathways important in DNMT1 degradation and the involvement of other possible post translational modifications of DNMT1 induced by IL-1 β and/or H₂O₂ may also provide fruitful areas of research.

We showed that Suv39H1 was associated with DNMT1 and NF- κ B p65 in unstimulated cells by co-immunoprecipitation experiments. However, there were no differences in the relative protein amounts following cell stimulation. This surprising result may possibly relate to the antibody used to immunoprecipitate the protein i.e. it was not possible to immunoprecipitate one of the proteins as a binding epitope was blocked by the other antibody. The other possibility remains that, the amount of DNMT1 protein was downregulated by oxidative stress, thus it is not possible to show the difference between various stimulations. It is also possible that an MBP such as MeCP2 could act as an intermediate protein. Determination of a full time-course for these experiments and a measure of enzymic activity may resolve these later issues. The use of tagged proteins and subsequent co-immunoprecipitation or BRET experiments may also prove informative. As with all these studies translation of these effects into primary cells will ultimately determine whether the results reported here may have any clinical relevance.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE DIRECTIONS

TGF- β 1 is a pleiotropic cytokine that regulates physiological processes including cell proliferation, differentiation, apoptosis, early embryonic development, and extracellular matrix process (Gawaziuk et al., 2007; Li et al., 2006b; ten Dijke and Arthur, 2007). It is actively involved in pathological changes in airway disease, like COPD (de Boer et al., 1998), asthma (Redington et al., 1997a) and interstitial lung disease (Khalil et al., 1996). Systemic oxidative stress is enhanced in asthma, smoker and COPD patients (Rahman et al., 1996). H₂O₂, among one of the key mediator of oxidative stress, is also involved in COPD patients (Dekhuijzen et al., 1996) and is associated with asthma severity (HORVATH et al., 1998). However, there are few studies examining the transcriptional regulation of TGF- β 1 under conditions of oxidative stress. Here, we would like to summarize our novel findings concerning the roles of the transcription factor NF- κ B; histone H3K9 methyltransferase, Suv39H1; and DNA methylation over a CpG island and at the ATG start site in the TGF- β 1 promoter on upregulation of TGF- β 1 under oxidative stress.

IL-1 β and IL-1 β plus H₂O₂ induce TGF- β 1 mRNA and protein expression

Our current findings demonstrate that IL-1 β and H₂O₂, which represent a model of inflammation and oxidative stress leading to exaggerated intracellular oxidative stress, can upregulate the expression of TGF- β 1 mRNA expression, secreted TGF- β 1 and intracellular TGF- β 1 levels in A549 cells. H₂O₂ alone has minimal effects on the expression of TGF- β 1, but in combination with IL-1 β stimulation, it acts in an additive manner to induce TGF- β 1 mRNA expression. From our results, there was no additive effect on TGF- β 1 protein production in supernatants by ELISA or of intercellular protein by Western blot. This may be due to the limitation of the need for supernatant acidification in order to measure total TGF- β 1 or as a result of post-translational modification of TGF- β 1 under oxidative stress. Similar effects on TGF- β 1 under IL-1 β - and IL-1 β plus H₂O₂-stimulation can also be demonstrated on BEAS-2B cells which indicates that this upregulation could be a universal phenomenon.

This upregulation of TGF- β 1 gene expression by IL-1 β and H₂O₂ stimulation could also be seen with GMCSF and IL-8 mRNA expression. However, the response to the combination of IL-1 β and H₂O₂ is different. A greater than additive effect of the combination of IL-1 β plus H₂O₂ can only be identified on GMCSF mRNA expression but not on IL-8 mRNA expression. This needs to be confirmed in primary cells.

The involvement of NF- κ B in TGF- β 1 production under IL-1 β and IL-1 β plus H₂O₂ stimulation

Using the IKK2 inhibitor AS602868 we demonstrated that the upregulation of TGF- β 1 by IL-1 β or IL-1 β plus H₂O₂ is predominantly NF- κ B-dependent. In contrast, there was a much smaller effect seen on TGF- β 1 by both the JNK inhibitor (SB600125) and the P38 inhibitor (SB203580). However, induction of TGF- β 1 mRNA by H₂O₂ is not fully inhibited by the IKK2 inhibitor indicating that other pathways are likely to be important. Using Western blot analysis we were able to show that NF- κ B p65 production was also enhanced under IL-1 β , H₂O₂ or IL-1 β plus H₂O₂ exposure.

Using CHIP analysis, we clearly demonstrated that NF- κ B p65 was recruited to the κ B3 site in the TGF- β 1 promoter upon stimulation with IL-1 β and H₂O₂ and this was associated with the enhanced TGF- β 1 mRNA expression. There are three possible NF- κ B binding sites on TGF- β 1 promoter but only the proximal one, the κ B3 site, which is 867 bp away from the translation start site, is a target for NF- κ B binding to the native promoter. This was demonstrated by both CHIP and in vitro binding assays. The reason for the selective recruitment to the κ B3 site is currently unclear but may involve sequence-specific changes in DNA binding site:NF- κ B p50 affinity. Although also seen with in vitro assays where no histones are involved there may also be an important role for localised chromatin structure in the native promoter.

The histone methyltransferase, Suv39H1, involved in GR effect on suppression TGF- β 1 induced by IL-1 β , or IL-1 β plus H₂O₂

Our study indicates that the upregulation of TGF- β 1 under IL-1 β , or IL-1 β plus H₂O₂, can be suppressed by FP in a concentration-dependent manner. The effect of oxidative stress on enhanced TGF- β 1 production induces a rightward shift in the dexamethasone IC₅₀ which indicates a relatively steroid resistant model. Current concepts relating to the induction of relative dexamethasone resistance under oxidative stress conditions suggest that oxidative stress may affect various aspects of the GR activation pathway including nuclear translocation (Adcock et al., 2005; Xu and Chu, 2008), altered cross-talk with pathways linked to proinflammatory cytokines and intracellular signalling (Adcock et al., 2005) and/or reduction in GR-corepressor proteins such as HDAC2 (Ito et al., 2005; Ito et al., 2001).

In this thesis we were able to demonstrate that GR has an associated HMT activity and that this activity can be induced by IL-1 β or IL-1 β plus H₂O₂. In particular, we demonstrated that GR is associated with the H3K9 methyltransferase, Suv39H1. Knockdown of Suv39H1 expression using siRNA results in the upregulation of TGF- β 1 mRNA expression following IL-1 β and H₂O₂ stimulation and a reduction in the ability of FP to suppress TGF- β 1 mRNA expression. In addition, Suv39H1 knockdown significantly downregulates GR-GRE DNA binding, a measure of GR transactivation, following IL-1 β and IL-1 β plus H₂O₂ stimulation. These findings support the concept that Suv39H1 may act as a corepressor on GR function.

We also examined the effect of overexpressing Suv39H1 using an overexpression plasmid. Although we induced a significant upregulation of Suv39H1 mRNA and protein following transfection of the Suv39H1 overexpression plasmid there were no effects on GR associated suppression of TGF- β 1 function. This failure to confirm the knockdown data may be due to a number of reasons including overwhelming basal histone methylation as a consequence of cell activation or an insufficient induction of Suv39H1 expression and importantly activity.

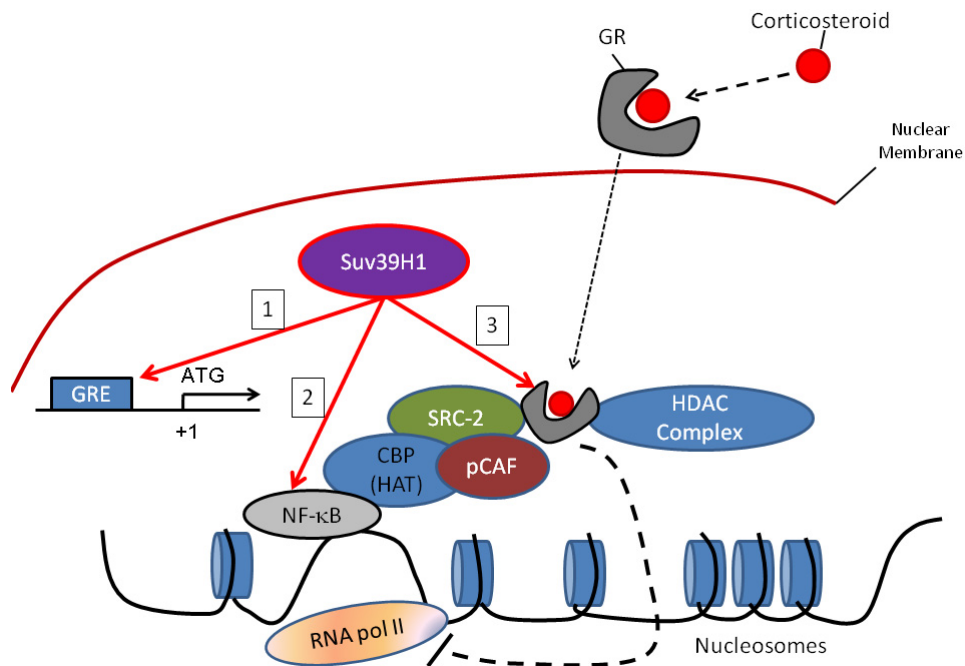


Fig 6.1 Possible model for H3K9 methyltransferase, Suv39H1 on TGF- β 1 gene

regulation. Inside the nucleus, Suv39H1 may directly associate with NF- κ B p65 and after stimulation of cells with IL-1 β or IL-1 β plus H₂O₂ Suv39H1 is recruited to the κ B3 site (2) and thereby change the local H3K9 trimethylation status. Dexamethasone stimulation results in binding to, and activation of, cytoplasmic glucocorticoid receptors (GRs) in the cytoplasm which then translocate into the nucleus. Within the nucleus Suv39H1 may directly associate with GR (3), form part of a complex with GR and NF- κ B or associates with GR at a GRE (1) to inhibit GR-mediated transactivation.

The H3K9 histone methyltransferase G9a, Suv39H2, and the H3K9 demethylase JMJD2C, and H3K27 demethylase under IL-1 β , and IL-1 β plus H₂O₂ stimulation

Competition between methyltransferases and demethylases acting at the same histone residue may control gene regulation (Kouzarides, 2007; Shi and Whetstine, 2007). We have also examined the expression of two other H3K9 histone methyltransferases, G9a and Suv39H2. Suv39H2 is undetected in our cell model, and G9a shows a similar response as Suv39H1 being downregulated upon stimulation with IL-1 β and H₂O₂.

The expression of the H3K9 demethylases JMJD2A and JMJD2C mRNA were also checked, and the results demonstrated decreased mRNA expression under IL-1 β and IL-1 β plus H₂O₂ stimulation. Surprisingly, the H3K27 histone demethylase, JMJD3, exhibits a distinct response to IL-1 β and H₂O₂. JMJD3 is reported to be bind to Polycomb group (PcG) proteins, and regulates H3K27me3 levels and transcriptional activity (De Santa et al., 2007). It can be upregulated by LPS and TNF- α through the effect of NF- κ B (De Santa et al., 2007). Our study demonstrated that upregulation of JMJD3 mRNA expression was seen after IL-1 β and H₂O₂ stimulation and that JMJD3 mRNA expression can be downregulate by FP treatment. Since H3K27 trimethylation is also associated with gene repression (Hong et al., 2007), it is possible that the upregulation of JMJD3 by IL-1 β and H₂O₂ may elicit an H3K27 response which favours gene expression. The suppressive effect of FP on JMJD3 mRNA expression raises the possibility that it may be also play a role in FP gene repressive actions.

CpG island methylation on TGF- β 1 promoter induced by IL-1 β and H₂O₂ associates with TGF- β 1 mRNA expression

CpG island methylation in promoter area of normal cells is associated with X-chromosome inactivation (Hansen et al., 1996), control of imprinted genes (Paulsen and Ferguson-Smith, 2001) or cell-specific gene expression (Esteller, 2007). Methylation can be

lost through the enzymatic removal of mC⁵ or a failure to maintain methylation status following DNA replication (Gehring et al., 2009).

To investigate CpG island methylation changes at the TGF- β 1 promoter, we initially performed bisulphite sequencing. Unfortunately, we are not able to direct sequence the κ B3 site directly due to the high CpG percentage around that area and we were not able to design specific primer pairs to span that area. Using an alternative method to analyse C⁵ methylation, the MeDIP assay, and demonstrated that the mC⁵ levels increased at the κ B3 under with IL-1 β , H₂O₂, and IL-1 β plus H₂O₂ exposure and reached a peak at 4h. Our results also demonstrate that DNMT1 directly associates with NF- κ B, but failed to show a difference under different stimulatory conditions. This may be due to problems with the antibody pairs used in the experiments and when other antibodies become commercially available these will be tested in the model system. The effect on DNMT1 activity associated with p65 under various stimulations should also be investigated as it is possible that although p65:DNMT1 association doesn't change there may be a change in enzymic activity associated with the complex following ROS stress. Overall, we postulate that DNA methyltransferases are potentially involved in methylation at the κ B3-associated CpG island in the TGF- β 1 promoter, that these events can be upregulated under oxidative stress and that this results in the modulation of gene expression.

The rapid changes in DNA demethylation are interesting. Our preliminary results demonstrated that IL-1 β and H₂O₂ stimulation can induce rapid induction of methylation change around ATG translation start site area around 1h and that the demethylated state was restored back to normal after 2h. This data suggests that there possibly is a short-term site specific demethylation event occurring at the TGF- β 1 start site between 60 and 120 minutes post IL-1 β stimulation. It will be important to investigate whether this has any functional effect on TGF- β 1 expression and whether the changes are more permanent under chronic ROS stimulation.

Epidemiological studies have indicated that exposure to traffic pollution or cigarette smoke can have a marked effect on the profile of promoter methylation with specific gene hypermethylation e.g. tumour suppressors and global hypomethylation ((Baccarelli et al., 2009; Breton et al., 2009)). Experiments to examine the effect of cigarette smoke or diesel particles/PM10s on the rapid changes seen here may indicate a mechanism underlying the epidemiological data.

Interplay between histone methylation and DNA methylation on TGF- β 1 promoter

Since histone methyltransferase Suv39H1 (Fuks et al., 2003a) or G9a (Estève et al., 2006) also directly associates with DNA methyltransferase, it would be interesting to see if histone modifications may act alone or in combinations with DNA methylation to initiate or gene transcription.

Using co-immunoprecipitations, we are able to demonstrate that DNMT1 is associated with Suv39H1 but we unable to show differential recruitment with the various stimulations. This may reflect the problems with the antibody pairs as discussed previously. In ChIP experiments, DNMT1 was recruited to the κ B3 site by IL-1 β after 30min. We were not able to show differences, however, with H₂O₂, or IL-1 β plus H₂O₂ stimulation. Further experiments with different antibody pairs may resolve whether this is a result of a decrease in total DNMT1 protein as we demonstrated with H₂O₂ or if there was a change in recruited DNMT1 activity associated with Suv39H1.

Our results show that both IL-1 β and H₂O₂ decreased H3K9 trimethylation at 1h and that this is linked temporally to the changes in DNA methylation that we have also seen. Further analysis of the time-courses of these events may provide a better indication as to the linkage between the two events at the TGF- β 1 promoter. By ChIP analysis, Suv39H1 was seen to be recruited to the κ B3 binding site in the TGF- β 1 promoter at 1h presumably whilst complexed with NF- κ B. Knockdown of Suv39H1 expression with siRNA resulted in

enhanced TGF- β 1 mRNA expression and reduced FP responsiveness. A similar effect was seen with GM-CSF and ideally a combination of ChIP-seq and gene arrays may also give a clearer idea as to the generality of the effects described here under Suv39H1 control. Indeed, Suv39H1 and GR were shown to be part of the same complex which also modulated GR-GRE DNA binding. Further temporal analysis of p65, GR and Suv39H1 recruitment to the native TGF- β 1 promoter κ B3 site would be important to perform in future studies along with a comparison of GR/Suv39H1 recruitment to a native GRE using re-ChIP experiments.

Future directions

Currently, due to time limitations, there are some experiments which have been performed only once or twice or require further optimisation. It will be important to increase the n number of certain experiments to validate the initial results and enable valid statistical analysis.

Throughout the whole study our model has focussed on an alveolar type II cell line - A549 cells. The finding of upregulation of TGF- β 1 upon stimulation with IL-1 β and H₂O₂ was also shown in another epithelial cell line, BEAS-2B cells, which may suggest that this regulation is a universal phenomenon in epithelial cells. But ideally these findings need to be repeated in primary cells and in cells from patients with disease e.g. severe asthma or COPD. Analysis of differences in H3K9 methylation status and the expression of HMT e.g. Suv39H1 and HDMs such as JMJD2 in primary cells from patients with asthma, particularly severe asthma, or COPD should be investigated.

Furthermore, we would examine in greater biochemical detail the links between histone and DNA methylation and how this is modulated by oxidative stress. A more stringent analysis of DNA methylation across the TGF- β 1 promoter over time along with determination of the mechanism of active DNA demethylation would greatly enhance this area. An extensive Western blot-co-immunoprecipitation or LC-MS/MS analysis of protein-protein interactions linked to analysis of enzyme activity could also be undertaken to examine the time-course of complex formation. In order to determine whether the various changes in DNA methylation, histone methylation and/or acetylation are co-ordinated, knockdown of DNMT1 for example may be examined for its ability to affect the ability of IL-1 β and/or H₂O₂ to modulate histone methylation and acetylation. Similar experiments could be conducted in the presence of FP to examine whether there is an essential early 'hit' for GR function. Additionally, the balance between HMTs and HDMs can also be studied to understand its role under oxidative stress (Shi, 2007).

In addition to histone modifications, ATP requiring histone remodelling complex members such as Brg-1 (Bilodeau et al., 2006) could be assessed as potential targets for GR function in these cells. Furthermore, we can also use Luciferase reporter assays to study GR function. Our results also indicate that the H3K27 demethylase, JMJD3, is possibly involved in IL-1 β - and H₂O₂-induced inflammation. JMJD3 may also be involved in GR actions in suppressing inflammation. Further investigation to study the role of JMJD3 with ChIP for study for timely recruitment to NF- κ B binding site, and its association with GR, would be interesting to delineate the function of this demethylase.

Our results also demonstrate that, GR, Suv39H1, and NF- κ B are recruited to κ B3 site at similar time point (1hr). Co-immunoprecipitation can show the spatial protein-protein relationship under stimulation, but it is not able to show the functional importance regarding the recruitment to binding site on promoter area. It will be better to do sequential ChIP study, to check the co-occupancy of transcriptional factor binding site, which can elicit the upregulation or downregulation of specified gene response.

We have demonstrated that DNMT1 is actively recruited to the κ B3 site by ChIP analysis. Recently, other related DNA methyltransferase, such as DNMT3b, and methyl-group binding proteins (MBD), in addition to DNMT1, also involved hormone-induced transcriptional derepression (Kim et al., 2009). It would be interesting to study broadly these other DNA methyltransferases under IL-1 β , and IL-1 β plus H₂O₂ stimulation.

We had problems with the specificity and selectivity of some of the antibodies used. This lack of useful commercial tools has restricted much of the work in the epigenetics field although the production of selective high affinity antibodies produced by phage display by the Structural Genomics Consortia (SGC) based in Oxford, Toronto and Stockholm may resolve this problem. Until these tools become available in an open-access manner, alternative approaches to study protein-protein interactions are needed. We can design overexpression plasmids with different tags for proteins of interest in order to show protein-

protein interactions either by immunoprecipitation or by BRET analysis. In addition, deletion mutants may reveal interaction sites in vitro between the various proteins of interest. Alternatively, protein-protein interactions between NF- κ B p65 and DNMTs and HMTs may be investigated using LC-MS/MS analysis.

It will also be important to determine the effect of oxidative stress on DNA and histone methylation status in a more global manner. Linking global analysis of DNA methylation and H3K9 methylation status as measured by ChIP-seq with transcriptomic and/or proteomic arrays will allow a better picture of the potential functional consequences of oxidative stress on inflammatory gene expression and the possible involvement of methylation changes.

Since the changes in H3K9 methylation shown here occur prior to changes in AcH3 And AcH4 (Lee et al., 2006), it would be interesting to determine whether these methylation and acetylation marks were linked by examining whether Suv39H1 siRNA affected changes in histone acetylation status. In addition, it is unclear exactly how an inflammatory stimulus or an oxidative stress can target Suv39H1 association with p65 and/or GR. Data from our group has examined this in part for the recruitment of HDAC2 to p65 and GR as demonstrated that specific post-translational modifications were important (Ito et al., 2006b). Similar studies may reveal the same process occurring for Suv39H1.

Possible therapeutic opportunities for novel drugs that alter histone or DNA modifications

Current evidence supports that changes in histone methylation, through the recruitment of methyl-binding proteins and adaptor proteins, are linked to the changes in DNA methylation (Fuks, 2005). These epigenetic changes help to keep genes in a repressed status. Recent advances have connected DNA methylation to chromatin-remodelling enzymes, and understanding this link will be central to the design of new therapeutic drugs.

A growing number of human diseases have been found to be associated with aberrant DNA methylation, and histone modifications. For example, evidence supports that mutation in methyl-CpG binding protein 2 (MeCP2) have been linked to the human mental retardation disorder Rett syndrome (Fan and Hutnick, 2005), and various changes in DNA methylation are associated with human neurodevelopmental syndromes and development (Okano et al., 1999; Robertson, 2005; Robertson and Wolffe, 2000). These links between disease and epigenetics changes confirm the changes are epigenetics through drug modification possible have its role in disease treatment.

To study the possible therapeutic opportunities for the development of novel drugs, we may need to use the in vitro model cell line system first to test the optimal dosing, and interval of treatment to obtain the desired result of the novel drugs. For example, if the novel drugs exhibit the effects on the DNA CpG island methylation, we may focus on interested promoter area which are disease relevant and do methylation specific PCR after Bisulfite treatment, or methylation dependent immunoprecipitation assay using antibodies which are 5MeC or MeCP2 specific to study the methylation changes after the specific drug. A global study of methylation changes can also be performed after immunoprecipitation of the DNA first with 5MeC antibody or MeCP2 antibody followed by DNA microarrays to analyze the promoter area of interested genes. If the drug has its effect on histone methylation, we may test the effect of global histone methylation changes with western blot study, or further using Chromatin immunoprecipitation study to confirm the specific changes on promoter area.

After the in vitro results are available, it will be interesting to test the effect of the novel drugs with the in vivo model. Using the in vivo model, we may further test the effect of the novel drugs on epigenetic changes whether based on DNA CpG island methylation or histone methylation changes depends on its presumed effects. First, we may investigate effects of the drugs on bone marrow cells, or peripheral blood monocytes with the aforementioned methods to detect the changes on histone methylation or DNA methylation, and further effects or gene

activation/inactivation using different dosing and timing of treatment. Furthermore, we may use other drugs which are already proved to have different effect on the epigenetic gene silencing, and possibly to have the synchorized effect with the novel drug, to investigate whether this combination is able to reverse or enhance the gene activation or inactivation status. We may go further to check the effects of the novel drugs based on the established disease model on animals. For example, we may use the ovalbumin-induced airway hyperresponsiveness model to check if these novel drugs have effects on the progression of asthma change.

Recently, the combined DNA methyltransferase and histone deacetylase inhibition has been shown to have the clinical response in the myeloid neoplasms (Gore et al., 2006). Huang et al. also described the novel oligoamine analogues which inhibits LSD1 could induce reexpansion of epigenetically silenced genes (Huang et al., 2009). These firm evidences suggest that, drugs which modulate the epigenetics changes may possibly play a role in controlling disease progression. Aggressively investigation of the drugs with the well established models will offer the opportunity to find the novel drugs which possess the effects on epigenetic modification for therapeutic purpose.

STATEMENT OF ORIGINALITY

I confirm that these experiments and experimental protocols were designed and carried out by myself.

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