Role of High Mobility Group Box-1 in the Pro-Fibrotic Response of Human Airway Smooth Muscle Cells

by

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ABSTRACT

Asthma is a chronic disorder highlighted by intermittent airway inflammation and characterized by paroxysmal dyspnea and airway hyperresponsiveness (AHR). A key feature of severe asthma is the development of airway wall remodeling, which is thought to occur through repeated rounds of inflammation and tissue repair. Remodeling includes structural changes such as increased mass of airway smooth muscle (ASM), and excessive collagen deposition. ASM cells contribute to airway remodeling via the expression and secretion of extracellular matrix (ECM) proteins. This is particularly driven by inflammatory processes, which include mediators such as transforming growth factor (TGF)-β1 and damage associated molecular pattern (DAMP) proteins, such as high mobility group box 1 (HMGB1). HMGB1 is ubiquitously expressed as a non-histone DNA-binding protein that can regulate gene expression, but can also be released in response to stress to underpin inflammation and tissue repair. In this study we tested the hypothesis that extracellular HMGB1 induces signaling pathways that control responses linked to progression of airway inflammation, remodeling and hyperresponsiveness in human ASM cells. We used primary cultured ASM cells as well as hTERT-immortalized human ASM cells. With immunoblotting we demonstrate that exogenous HMGB1 (10 ng/mL) can induce rapid and sustained phosphorylation of p42/p44 mitogen-activated protein kinase (MAPK) that is comparable to that induced by a potent mitogen, platelet derived growth factor (PDGF-BB, 10 ng/mL). We also found that TGF-β1 (2.5 ng/mL) promotes the accumulation of secreted HMGB1 in culture medium in a time line concomitant with expression of ECM proteins, collagen and fibronectin, suggesting a role for HMGB1 in pro-fibrotic effects of TGF-β1. By lentiviral delivery, we induced stable

expression of short hairpin RNA (shRNA) that silenced expression of endogenous HMGB1 or mammalian diaphanous 1 (mDia1), a cytoplasmic scaffold protein that is required for HMGB1-induced cell responses through one of its receptors, receptor for advanced glycation end products (RAGE). Immunoblot analyses revealed that silencing of mDia1 was associated with markedly decreased induction of p42/p44 MAPK phosphorylation by exogenous HMGB1. In HMGB1-silenced human ASM cells, we observed significantly reduced synthesis and secretion of collagen A1 and fibronectin in response to TGF-β1 (2.5 ng/mL, 0-48 hrs). However, exogenous HMGB1 was not sufficient to rescue ECM synthesis in response to TGF-β1 in HMGB1-silenced cells - this suggests that intracellular, but not necessarily secreted HMGB1, regulates ECM expression and secretion in response to TGF-β1. Consistent with this interpretation, exogenous HMGB1 alone was not sufficient to induce ECM synthesis or secretion in primary cultured ASM cells. In conclusion, we show that though in human ASM cells extracellular HMGB1 alone can activate MAPK signaling, likely via mDia1-dependent pathways involving RAGE. it is not capable of prompting ECM protein expression. Recombinanat exogenous HMGB1 does not appear to directly affect ECM synthesis, rather intracellular (nuclear) HMGB1 likely modulates activity of genes that are affected by TGF-β1. Overall, HMGB1 has potential to regulate tissue repair processes involving ASM through intracellular and extracellular mechanisms, thus our findings support further work to elucidate the role of HMGB1 in pathogenesis of obstructive airway disease.

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ABBREVIATIONS

ACh: acetylcholine

AGE: advanced glycation end product

ASM: airway smooth muscle

AHR: airway hyperresponsiveness

ANOVA: analysis of variance

AP-1: activator protein 1

ATP: adenosine-5'-triphosphate

BALF: bronchoalveolar lavage fluid

BSA: bovine serum albumin

Ca^{2+}: calcium

cAMP: cyclic adenosine 3’5’-monophosphate

CNS: central nervous system

CPKC: classical protein kinase C isoform

CRIDs: cytokine-release inhibitory drug

CD: cluster of differentiation

CRID: cytokine-release inhibitory drug

CRM1: the chromosome region maintenance 1 protein

CXCL: CXC chemokine ligand

DAG: diacylglycerol

DAMP: damage-associated molecular pattern

DIC: disseminated intravascular coagulation

DNA: deoxyribonucleic acid

ECM: extracellular matrix

EDTA: ethylenediarninetetraacetic acid

EGF: epidermal growth factor

EGFR: epidermal growth factor receptors

eNOS: endothelial nitric oxide synthase

ERK1/2: extracellular signal regulated kinase 1/2

FEV1: expiratory volume in one second

FITC: fluorescein isothiocyanate

GAPDH: glyceraldehydes 3-phosphate dehydrogenase

GTPase: guanosine triphosphatase

HAT: histone acetyltransferase

HMGA: high-mobility group A

HMG: high-mobility group

HMGB: high-mobility group B

HMGB1: high-mobility group box 1

HMGN: high-mobility group N

HSP: heat-shock protein

hTERT: human telomerase reverse transcriptase

ICAM-1: intercellular adhesion molecule-1

Ig: immunoglobulin

IκB: inhibitory kappa B

IL- : interleukin -

IFN-γ: Interferon gamma

iNOS: inducible nitric oxide synthase

IP3: inositol triphosphate

IPF: interstitial pulmonary fibrosis

IRAK-1: interleukin-1 receptor-associated kinase-1

JNKs: c-Jun N-terminal kinases

JNK/SAPK: c-Jun N-terminal kinase/stress-activated protein kinase

kDa: kilodalton

LABAs: long-acting β2-adrenoceptor agonists

LDH: lactate dehydrogenase

LMG: low-mobility group

LPS: lipopolysaccharide

LT: leukotrine

MAPK: mitogen activated protein kinase

MCP-1: monocyte chemottractant protein 1

mDia1: mammalian diaphanous 1

MLC20: myosin light chain

MLCK: myosin light chain kinase 8

MLCP: myosin light chain phosphatase

MMLV: moloney murine leukemia virus

mRNA: messenger ribonucleic acid

Myd88: myeloid differentiation protein 88

NHBE: normal human bronchial epithelial

NF-κB: nuclear factor κ

NK: natural killer

nM: nanomolar

NLS: nuclear localization signal

NO: nitric oxide

p53: protein 53

PAGE: polyacrylamide gel electrophoresis

PAMP: pathogen-associated molecular pattern

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PDGF: platelet derived growth factor

PG: prostaglandin

PI3K: phosphatidylinositol-3-OH kinase

PKC: protein kinase C

PLC: phospholipase C

PP1c: catalytic subunit of myosin light chain phosphatase

PRR: pattern recognition receptor

RAG1/2: recombination activating gene 1/2

RAGE: receptor for advanced glycation end-products

RBM: sub epithelial lamina reticularis

RNA: ribonucleic acid

ROCK1/2: rho kinase

RT-PCR: reverse transcriptase polymerase chain reaction

ROS: Reactive oxygen species

SABAs: short-acting β2-adrenoceptor agonists

SCF: stem cell factor

SDS: sodium dodecyl sulphate

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

siRNA: small interfering ribonucleic acid

SLPI: secretory leukoprotease inhibitor

SR: sarcoplasmic reticulum

TGF-β1: transforming growth factor beta

Tc: cytotoxic T cells

Th1: T helper 1

Th2: T helper 2

Th17: T helper 17

Treg: regulatory T cells

TLR: toll-like receptor

TNF-α: tumour necrosis factor alpha

VCAM-1: vascular cell adhesion molecule-1

CHAPTER I: INTRODUCTION

1.1.ASTHMA

1.1.Asthma: definition

The term asthma originates form the Greek word "Asthmaino", which means "gasping" and was first used by Hippocrates in 460-377 BC at the Corpus Hippocraticum. The Global Initiative for Asthma (GINA) defines asthma biology as chronic inflammation of the airways, in which many cells and cellular elements play a major role, and often involves structural remodeling (thickening) of the walls of the bronchi and bronchioles (1). The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or by treatment (1). Due to the narrowing of conducting airways in asthma, pulmonary function tests of asthmatic patients show decreased forced expiratory volume in one second (FEV1), which is reversible with β-adrenergic receptor agonist (β-agonists) administration (2). At least 50% of asthmatic patients are also atopic, which shows the importance of allergy in some forms of asthma, e.g. allergic asthma, but it is a heterogeneous disease that can be triggered by a number of non-allergic stimuli as well, e.g. exercise, cold air, aspirin and occupational pollutants (3).

1.2.Asthma pathogenesis

Asthma is a multi factorial disease with many mechanisms. It is characterized by: 1) airway hyperresponsiveness (4) – an exaggerated bronchoconstriction to various stimuli that do not provoke such an effect in healthy individuals; and, 2) variable airway obstruction caused by contraction of the airway smooth muscle (ASM) layer, swelling of the airway wall and mucus hyper-secretion due to inflammation (5). The pathogenesis of asthma is complex, consisting of both host factors (e.g. atopy) and environmental factors (e.g. pollutants and viruses). Due to the main observable symptom of the disease, which is reversible bronchospasm, it was thought to be disease of the airways and ASM but it is clear that T lymphocytes, as orchestrators of the inflammatory response, play a pivotal role in the pathogenesis, frequency and severity of symptoms (6). The balance between Th1 and Th2 phenotype cells is altered towards the Th2 profile is unknown, but emerging evidence links this to altered function of dendritic cells as well (7). Th2 lymphocytes in the airways express cytokines including IL-4, IL-5, IL-9 and IL-13, which can affect the function of epithelium and ASM cells (8). This complicated condition of the immune system, including both innate and adaptive immunity, causes the airway injury, which contributes to airway dysfunction and remodeling (9).

1.3.Airway Inflammation

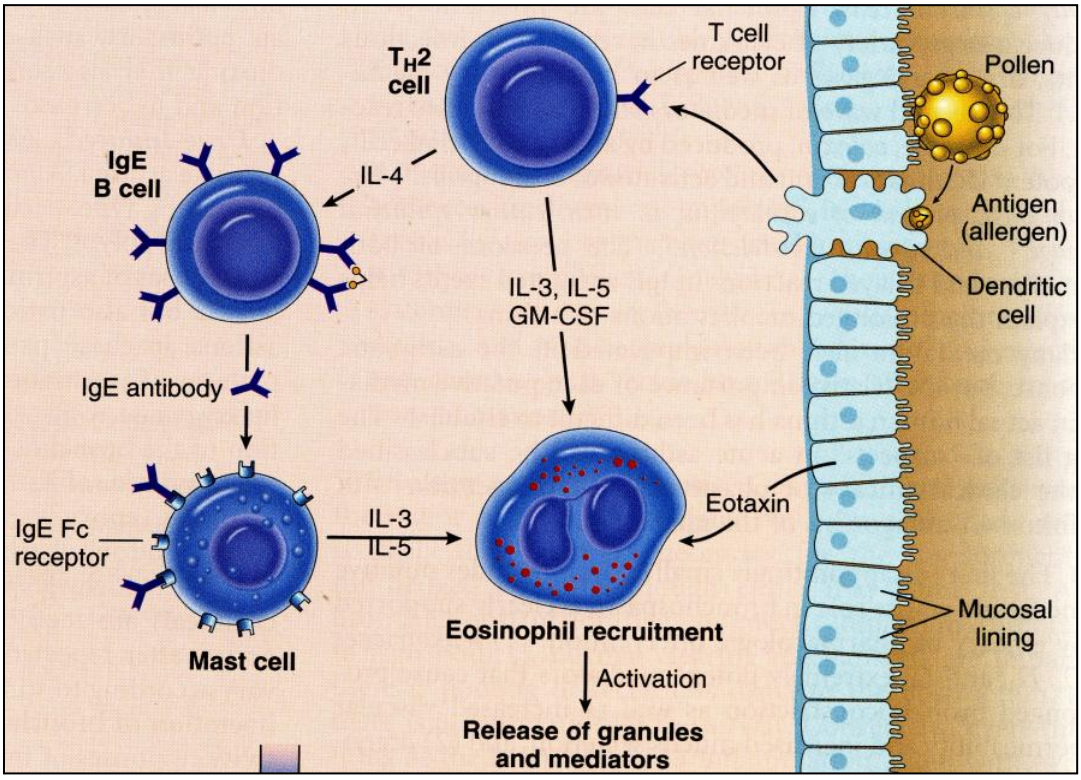
Inflammation is the body's response to any kind of injury (e.g. infection or tissue injury). It consists of three sequential domains, including: enabling

delivery of effector molecules to fight with injury and/or foreign particles (e.g. pathogens, allergens); encapsulating the inflammation area to prevent spread of infection; and, tissue repair reestablish normal structure, matrix, and cellular composition. The classical manifestations of inflammation are rubor (redness), tumor (swelling), calor (heat), and functio laesa (impaired function).

Airway inflammation is a multi-cellular process, which involves mainly Th2 lymphocytes, eosinophils, activated mast cells, neutrophils, macrophages and basophils, which release inflammatory mediators. In atopic asthma, the airway responds to airborne inhaled allergens by a Th2 response with the release of a typical array of cytokines (Th2 paradigm) (10). However, recent evidence indicates that ASM cells, in addition to their contractile role, can also actively modulate airway inflammation by secreting different cytokines (e.g. IL-8 (11), IL-5 (12) and GMCSF (13)). It also expresses receptors and adhesion molecules (e.g. ICAM1, VCAM1 and CD44 (14)), which exacerbate airway inflammation by providing docking sites for infiltrating leukocytes that release mediators which augment contractility of the myocytes (15).

In atopic asthma, the initial trigger to this cascade of inflammatory responses is sensitization to inhaled allergen. Via IgE bound to high affinity cell surface receptors, the allergen is taken up and processed by antigen presenting dendritic cells residing under the airway epithelium. Following engagement with antigen, dendritic cells migrate to local lymphoid tissue. They present processed antigen here, loaded onto MHC class II HLA molecules, to naïve T cells residing within the lymphoid follicle. The sensitized T cells produce cytokines, in

particular the interleukins IL-4, IL-5, IL-6, IL-9 and IL-13, which are elevated in the bronchoalveolar lavage fluid (BALF) and in the serum of asthma patients indicating a predominantly Th2 mediated inflammatory response (16-18). Cytokine production leads to a recruitment of secondary effectors cells such as macrophages, basophils and eosinophils into the inflammatory area while IL-4 promotes the immunoglobulin isotype switching of B cells towards IgE synthesis as shown in Fig. 1.1.



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Activation of T cells by antigen leads to their maturation into one of several different functional subsets, described below, dependent on their cluster differentiation (CD) marker status, and the inflammatory milieu. CD4+ cells have the capacity to differentiate into T helper 1 (Th1), T helper 2 (Th2), regulatory T cells (Treg) or Th17 cells. CD8+ cells develop into cytotoxic T cells (Tc). The level of IL-12 secretion by dendritic cells affects the balance of Th1 versus Th2 responses, the IL-12 shifting the balance towards the Th1 response.

Asthma is characterized by a predominantly Th2 phenotype. The early reaction to allergen inhalation is mast-cell dependent with Mast cells infiltrating the mucosa and the deeper airways (19). In chronic asthma mast cells and smooth muscle (ASM) cells are both increased in small and large airways. Mast cells are activated after binding of IgE to the high affinity IgE receptor (FCεRI) leading to the release of TNF-α, IL-4 and IL-5. Mast cells act on airway ASM cells by the release of the bronchioconstrictive mediators leukotriene (LT) D4, prostaglandin (PG) D2 and histamine, which act as potent ASM cell contractile agents (20, 21). Vice versa, ASM cells can produce stem cell factor, other chemokines, cytokines, and growth factors such as SCF, CXCL8, and CXCL10 that may act in the recruitment, differentiation, and retention of mast cells (22, 23). Also important in this context is mast cell tryptase, a protease that acts on the protease activated-receptor type 2 (PAR2) present among others ASMC and its activation induces bronchoconstriction by stimulating muscle contraction (24).

Eosinophils are a prominent cell population involved in allergic asthma (25). It is mostly Th2 cell-released IL-5 that contributes to the maturation of the

eosinophils from CD34+ precursors (26). Eosinophils are a major source of cytotoxic basic proteins, peroxidase, eicosanoides, leukotrienes and superoxide that can damage the airway epithelium. They also release TGF-β1 as well as other cytokines that can directly activate epithelial and mesenchymal cells that underpin tissue repair processes leading to airway remodeling (27). The exact role of neutrophils, monocytes and basophils in the context of asthma is poorly understood. Neutrophils are found in increased numbers in airways and sputum of patients with severe asthma and smoking induced asthma (28) (Figure 1.2) and are thought to be elemental to the steroid-insensitivity of this clinical asthma phenotype. Recent evidence from profiling of the cytokine mRNA levels in asthma subjects and controls suggest that in severe asthma there is a significant increased in the expression of the Th1 cytokine IFN-γ which also questions the primacy of the Th2 response in more severe forms of the disease (29).

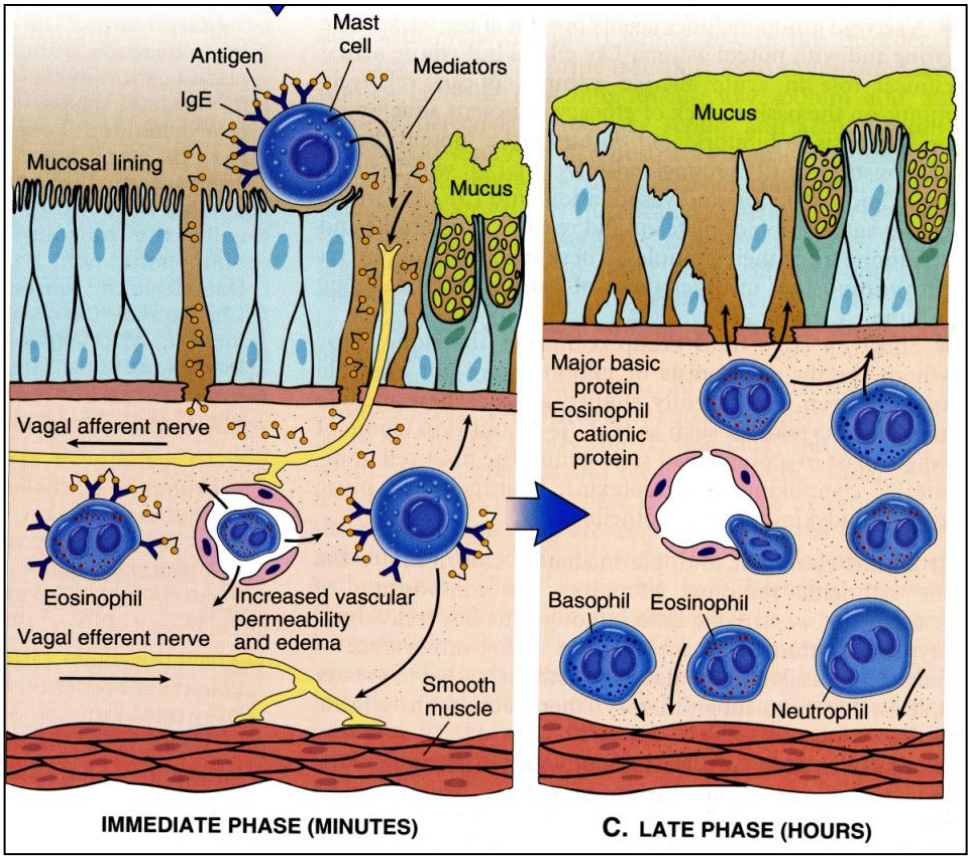


Figure 1.2: *Early and late phase reactions after allergen (pollen) inhalation and interaction with airway cells. Reproduced with permission from "Cotran, Ramzi S.; Kumar, Vinay; Fausto, Nelson; Nelso Fausto; Robbins, Stanley L.; Abbas, Abul K. 2005. Robbins and Cotran pathologic basis of disease (7th Ed.). St. Louis, MO: Elsevier Saunders"*

1.4.Airway hyperresponsiveness

AHR is an exaggerated contractile response of the airways, a characteristic functional lung abnormality of asthma that results in airway narrowing in response to a plethora of different stimuli that would be innocuous in non-asthmatic people (10, 30). As a consequence, asthma patients suffer from airflow limitation. Airway hyperresponsiveness is also linked to the degree and features of both airway inflammation and airway remodeling. AHR can be explained by

excessive contraction of the ASM cells, hence can be fully or partially reversible though the uses of bronchodilators that relax the airway smooth muscle. Acute AHR is also underpinned by local airway wall edema, mucous release and more persistent AHR is often tied to existence of thickening of the airway wall (airway remodeling). Activated inflammatory cells in asthmatic airways release pro-contractile cytokines, such as histamine, thromine and leukotrienes, in the airway. Besides elevated levels of inflammatory cytokines, such as IL-1β and TNF-α, increased expression of receptors for bradykinin and $G_{\alpha q}$ and $G_{\alpha i}$ proteins, which mediate ASM contraction, has also been described (31). Additionally, bronchoconstriction can be triggered by sensory nerves (32). The importance of the neuron-immune interactions in asthma is underlined by the fact that current anti-asthmatic medications are also directed against bronchoconstriction, such as β2 agonists and anti-cholinergics, which, in addition to the direct ASM-relaxing impact, also suppress several neural pathways that can induce contraction.

1.5. Airway remodeling

Airway remodeling refers to the irreversible changes that occur to the formed elements of the airway with chronic airway inflammation and disease progression. It involves changes in the composition, quantity, content and organization of the cellular and molecular constituents of the airway wall (33). The end result of airway remodeling is an overall thickening and stiffening of the airway wall that produces persistent, irreversible airflow obstruction, and accelerated decline in lung function over time (34, 35). Thus, as the disease

progresses and the airway remodels, bronchoconstriction and airflow limitation becomes increasingly difficult to reverse with conventional pharmacological therapies and treatment may become refractory. As there are no current therapies to prevent or reverse this remodeling process, it is importantto understand the cellular mechanisms better, so that new therapies can be developed.

Though the structural changes that occur to the airways of asthmatics, have been well documented and recognized, the cellular and molecular mechanisms that drive these changes are still not fully established. Nonetheless, remodeling is thought to be the consequence of an aberration of the dynamic process of wound repair that includes matrix production and degradation, and structural cell growth, thus leading to reconstruction of the tissue. In asthma, airway remodeling includes thickening of the walls of conducting airways and it is thought to be tied to impact of chronic local inflammation (36). More specifically, the structural changes include airway smooth muscle mass thickening due to myocyte hyperplasia and hypertrophy, mucus gland and goblet cell hyperplasia, angiogenesis, sub-epithelial fibrosis, altered deposition and composition of extracellular matrix (ECM) and proteoglycans (10, 34, 37). Thickening of the sub epithelial lamina reticularis (RBM) is a prominent and early morphological hallmark of asthma. It is due to the increased deposition of collagen I and III, and fibronectin in the sub-epithelial basement membrane and adjacent matrices (38). Exctracellular matrix that accumulates with airway remodeling is likely produced by submusosal myofibroblasts leading to so-called sub epithelial fibrosis. Accumulation of tissue matrix around ASM cells is also

evident, a feature that is linked with the capacity of the myocytes to synthesize the proteins. Airway wall thickening is greater in atopic than non-atopic forms of asthma (39). RBM thickening has been positively correlated with AHR severity, the frequency of asthma attacks, and the numbers of fibroblasts and "myofibroblasts" adjacent to the RBM (38, 40, 41).

The epithelium is dysfunctional in asthma and this is associated with up-regulation of epidermal growth factor receptors (EGFRs), impaired proliferation and reduced expression of proliferative markers (42, 43). These features leave the epithelium unable to fully repair itself, thus in a chronically injured state with repair mechanisms deregulated. The epithelium of asthmatic patients is more fragile due to the disruption of the tight junctions (10, 44) and as a consequence, the airway epithelium attains a chronic “wound scenario” that unbalances normal epithelial-mesenchymal interactions and underlies the release of pro-inflammatory cytokines and growth factors, such as epidermal growth factor (EGF) (45).

Under the effects of epithelial-derived growth factors, mesenchymal cells produce collagen, reticular, elastic fibers, as well as proteoglycans and glycoproteins of the extracellular matrix (ECM), all of which contribute to the thickened airway wall of asthmatic subjects and all lead to an enhanced proteoglycan deposition in the sub epithelial tissue layer wall (46). It is therefore likely that ECM production and deposition is under the control of the epithelial-mesenchymal unit, leading to the development of structural alterations localized in the inner tissue layer of the airway wall (the tissue between the luminal surface

and the smooth muscle layer). The volume increment of the inner airway wall may have dramatic functional consequences in terms of luminal changes in response to a given stimulus by smooth muscle contraction.

Fibroblasts and myofibroblasts can contribute to tissue remodeling by releasing ECM components such as elastin, fibronectin and laminin (47). An increased number of sub-mucosal myofibroblasts are found in the airways of asthmatic patients. Also, mast-cell derived serine protease, which is a potent stimulant of fibroblast and ASM cells proliferation, can stimulate synthesis of type I collagen by human fibroblasts and ASM cells (48).

Increased mass of ASM is an important hallmark of airway remodeling and it is a consequence of both hyperplasia and hypertrophy of ASM cells (49). This may occur in response to different mitogens, inflammatory mediators and changes in local matrix properties (50). As originally described by Lambert et al. (51), thickened airway in asthmatic patients is due to the increased ASM volume and ECM deposition, which result in further narrowing of the airway and lumen obstruction. Besides, ASM cells of asthmatic patients show enhanced force generation and contraction compared to non-asthmatic (52).

Airway wall remodeling in asthma also includes increased vascularity; evidence suggests that the number and size of bronchial vessels is increased in patients with asthma compared with normal controls (47). A model of airway remodeling in asthma can be found in figure 1.3.

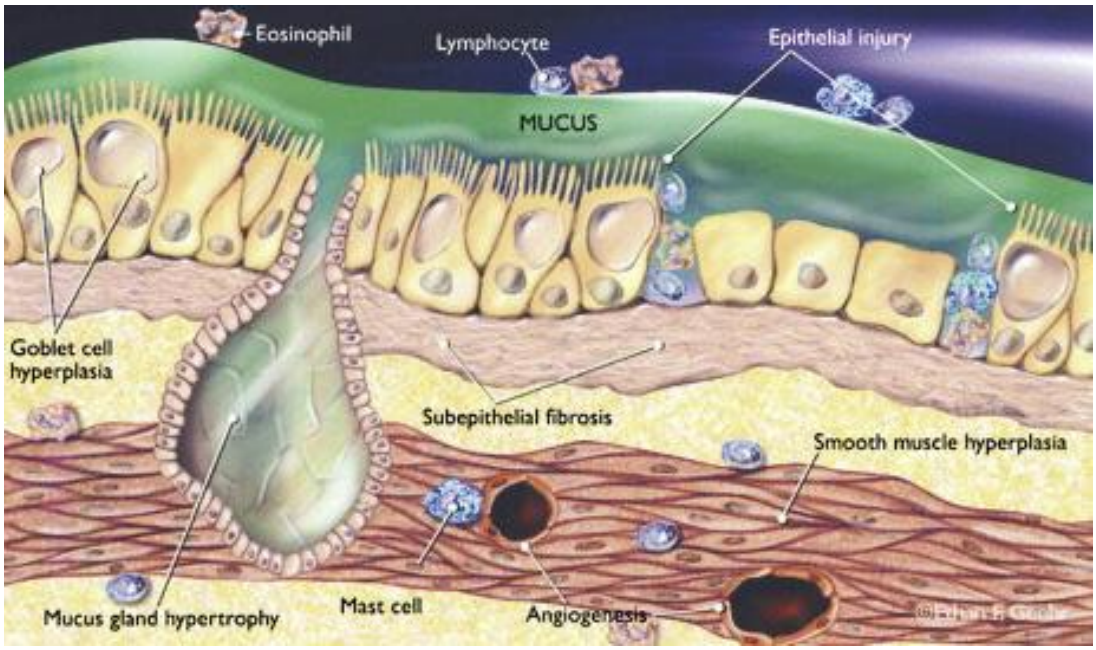


Figure 1.3: *Model of the airway remodeling with inflammatory cells, epithelial damage, goblet cell hyperplasia, sub epithelial fibrosis, mucus hyper secretion and smooth muscle cell hypertrophy and hyperplasia. Reproduced with permission from "Panettieri RA Jr, Covar R, Grant E, et al. Natural history of asthma: persistence versus progression-does the beginning predict the end? J Allergy Clin Immunol. 2008 Mar;121(3):607"*

1.6.Asthma and Airway Smooth Muscle (ASM)

1.6.1. Role of ASM in asthma pathogenesis

It was proposed as early as in 1868 that ASM has a key role in the pathogenesis of asthma (47). The idea that inflammation is the cause of asthma led to concepts that ASM cells are passive players in asthma, only at the receiving end of stimuli resulting in enhanced contraction of the airways. Over the past two decades the paradigm has shifted towards a paradigm in which ASM cells are active players in the inflammatory process and remodeling observed in asthma. This view on the pathogenesis of asthma arises from studies showing

that ASM cells are not only involved in contraction, but possess dynamic phenotypic capacity that supports them having an active role in the remodeling process and inflammation of the airways. One of the most striking aspects of the pathology of airway remodeling in asthma is the increased number and size of airway smooth muscle (ASM) cells (53). (Figure1.4). Increased ASM mass in asthma is accompanied by increased abundance of contractile protein markers, which underpins increased contractility of ASM tissue in asthma and in asthma models (49, 54-56).

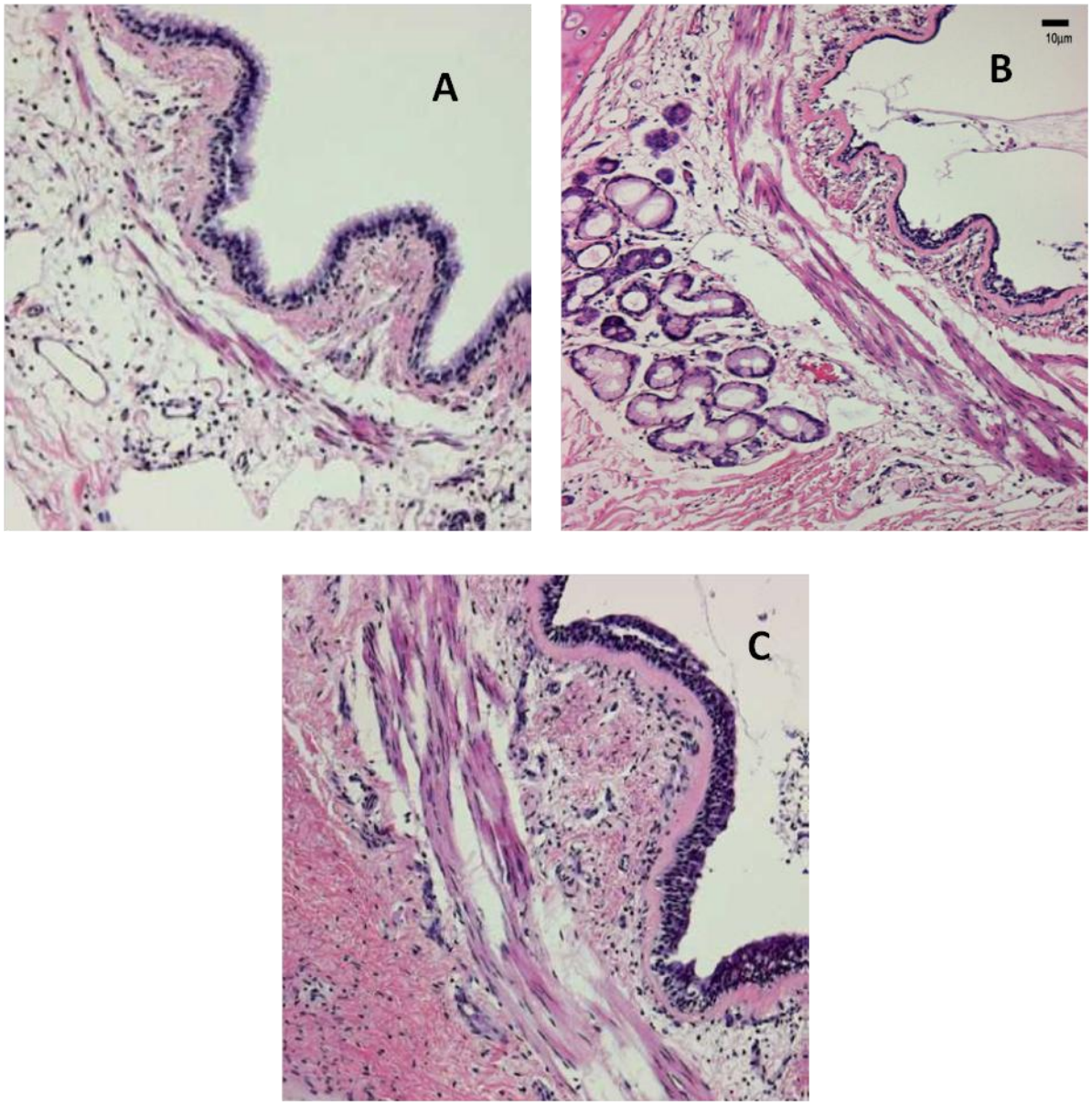


Figure 1.4*: Histology of a representative airway of A non-asthma control (A), a patient with mild-to moderate asthma (B), and a patient with asthma who died of status asthmaticus (C). "The asthmatic airways present a thickening of the basement membrane and the increased mass of smooth muscle cells. Reproduced with permission from "*Borger P, Tamm M, Black JL, Roth M. *Asthma: is it due to an abnormal airway smooth muscle cell? Am J Respir Crit Care Med. 2006 Aug 15;174(4):367-72"*

The multifunctional capacity of ASM cells enables them to contribute directly both to reversible, intermittent bronchial spasm, but also to the structural changes in the airway wall. The later have been implicated in the development of fixed loss of lung function in patients with obstructive airways diseases (57). Other factors, such as swelling of airway wall compartments and mucus plugging also worsen airway the narrowing (58).

ASM cells have the capacity to synthesize and release pro-inflammatory cytokines in response to a variety of stimuli, thus exhibiting a synthetic/proliferative phenotype that contributes to their role in asthma pathogenesis (15, 59). Asthmatic ASM cells also produce an altered composition and abundance of ECM proteins compared to cells from healthy individuals, leading to fibrotic changes associated with asthma that affect airway and lung function (15).

1.6.2. ASM phenotype

Freshly isolated ASM cells are contractile, but upon culture in serum-rich conditions, ASM cells modulate from a “contractile” phenotype to a “synthetic-proliferative” phenotype, which lacks responsiveness to contractile agonists and has reduced expression of contractile proteins, such as smooth muscle myosin heavy chain (smMHC), smMLCK, and smooth muscle α-actin (αSMA)(60). The remarkable plasticity of these cells allows them to migrate, proliferate, and secrete extracellular matrix (ECM), growth factors, cytokines, and chemokines (61, 62). “Synthetic-proliferative” cells can mature into “contractile” cells during lung

development and this process can be mimicked in vitro by prolonged serum deprivation of ASM cells leading to a "hyper contractile" phenotype with increased expression of αSMA, smMHC, SM22, desmin, calponin and M3 muscarinic receptor (62, 63). This modulation and maturation is known as phenotype switching (Figure 1.5). The contractile function of ASM cells was long seen as the most important function of these cells. However, studies with "synthetic-proliferative" cells have shown that ASM cells are a source of a wide variety of inflammatory mediators, including cytokines (e.g. IL-6), lipid mediators (e.g. prostaglandin E2), ECM (e.g. collagen, fibronectin) and chemokines (e.g. IL-8) (64-66).

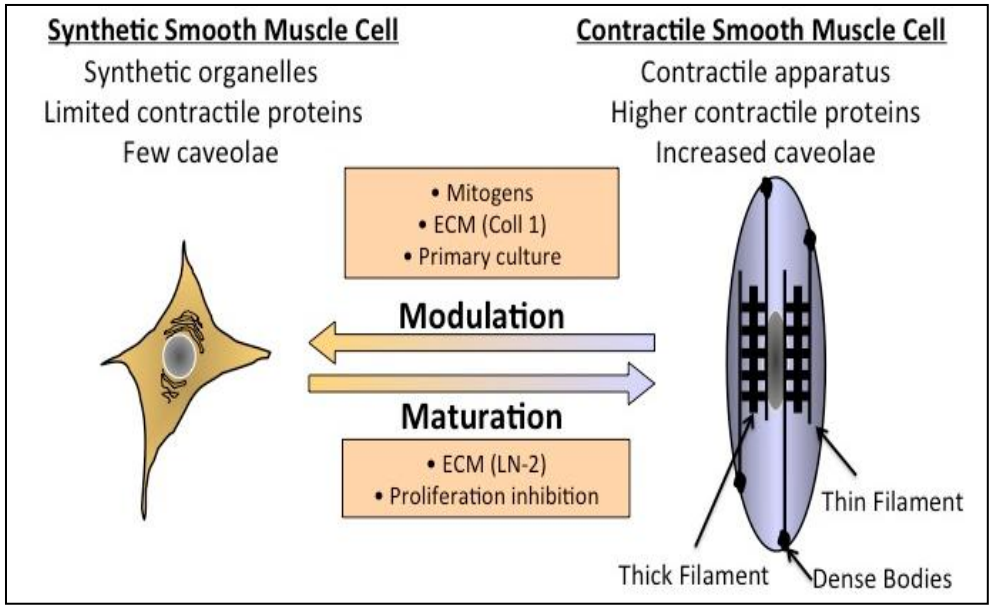


Figure 1.5. Schematic representation showing the phenotype plasticity of smooth muscle*.Phenotypic plasticity results from reversible modulation and maturation of smooth muscle cells (SMC) between a synthetic and contractile state as defined by cell response to defined stimuli and by the presence of molecular and ultrastructural markers. Reproduced with permission from "Phenotype and functional plasticity of airway smooth muscle: role of caveolae*

and caveolins"; Volume 5, pp. 80--88, 2008 Proceedings of the American Thoracic Society, HighWire Press, USA.

1.6.3. Increased ASM mass

Increased airway smooth muscle mass in the lungs from cats that were infected by a nematode worm was first described by William Stirling in 1878 (67). The degree of ASM mass increase seems to be related to the severity of asthma (55, 68). These data suggest that increased ASM mass is not the consequence of asthma. The novel view suggests that increased ASM mass can present before symptoms of asthma develop and therefore it may be (one of) the underlying pathobiological mechanisms for asthma. The increase in ASM mass in different sized airways appears to result, to different degrees, from an increase in cell size (hypertrophy) and/or cell number (hyperplasia), respectively. The cause of ASM thickening is not fully understood but may occur in response to the presence of mitogens, decreased apoptosis of ASM cells, and/or recruitment of stem cells or transformation of mesenchymal cells (37, 69).

1.6.4. Altered extracellular matrix

In addition to increased ASM mass, alterations in the extracellular matrix (ECM) contribute to the thickening of the airway wall. In asthmatics, the ECM is altered compared to that of healthy subjects with increased deposition of collagen I, III,V, fibronectin, tenascin, hyaluronan, versican and laminin (70, 71). The composition of the ECM is tightly regulated by a balance between de novo synthesis and degradation and this composition of the ECM, on which ASM cells

grow, influences their proliferation rate (72). These data suggest that the ECM profile determines the proliferation, synthetic capacity and phenotype of ASM cells. Also the ECM produced by ASM cells is important in remodeling of the airways.

1.7. Asthma treatment

There is no curative therapy for asthma. Today's standard therapy consists mainly of inhaled glucocorticosteroids such as budenoside, ciclenoside and fluticasone that control airway inflammation (73) their regular use has beneficial effects in terms of asthma symptoms: improvement of lung function (74) and decreased airway hyperresponsivness (75, 76). The anti-inflammatory action of glucocorticoids is through a transactivation of anti-inflammatory mediators and suppression of pro-inflammatory mediator release. The binding of activated glucocorticoid receptor homodimers to glucocorticoid regulatory elements (GRE) in the promoter region of steroid-sensitive genes leads to transcription of genes encoding anti-inflammatory mediators (annexin-1, secretory leukoprotease inhibitor (SLPI), IL-10, and the inhibitor of nuclear factor kB (IkBα). In addition, the glucocorticoid receptor–corticosteroid complex interacts with large co-activator molecules, such as the regulators of intrinsic histone acetyltransferase (HAT) activity, which are activated by pro-inflammatory transcription factors, such as NF-kB or AP-1, thus switching off the expression of inflammatory genes (77, 78).

Inhaled β2-adrenoceptor agonists are divided into two classes: short-acting and long-acting β2-adrenoceptor agonists (SABAs and LABAs, respectively). SABAs, such as salbutamol and turbutaline bind to the β2-adrenoceptor, activate adenylate cyclase and increase intracellular cyclic adenosine 3'5'-monophosphate (cAMP) that activates protein kinase A. This leads to muscle relaxation and relieves bronchoconstriction through the phosphorylation of myosin light-chain kinase and opening of Ca^{2+} channels to reduce intracellular Ca^{2+} concentration. LABAs, such as formoterol and salmeterol, induce bronchial dilatation for at least 12 hours (79). The combination of LABAs and corticosteroids represent the main therapy in asthma today.

2. HMGB1

2.1. High-mobility group protein family

Most DNA-related activities, such as transcription, replication, recombination, and repair need changes in the structure and organization of the DNA. Some of these structural changes are facilitated by a family of abundant non-histone nuclear proteins which belong to the high-mobility-group (HMG) family. The term "high-mobility group" (HMG) was coined as these proteins have a higher mobility in polyacrylamide gels than trichloroacetic acid-precipitated proteins, which in turn are called low-mobility group (LMG) – proteins (80).

HMG proteins have been subdivided into three distinct structural families: HMGA (the HMG-AT-hook family), HMGN (the HMG-nucleosome binding

family), and HMGB (the HMG-box family). Members of each family are abundantly and ubiquitously expressed in most eukaryotic cells, and possess different structures and unique DNA or chromatin-binding motifs, but they all affect chromatin architecture. Association of HMG proteins is not confined to specific DNA sites, being rather dynamic, as the proteins “scan” for potential chromatin binding sites and move from one chromatin site to another in a “ hit and run” fashion (81, 82). All HMG proteins have intracellular, mainly nuclear localization. However, under certain conditions, some HMG proteins may have an extra nuclear localization (81, 83, 84). HMG proteins associated with DNA have been implicated in regulation of numerous processes such as cell cycle transit and gene transcription through condensing or unfolding of chromatin (85).

2.2. High-mobility group box 1

High mobility group box 1 (HMGB1), formerly named HMG1, but also referred as amphoterin was identified as an intra nuclear protein over three decades ago (80). HMGB1 is a 25 kDa protein that migrates as a 30 kDa band in SDS-PAGE, perhaps because of the high number of positively charged amino acids contained in the protein (86). HMGB1 is abundantly expressed in the nucleus by nearly all cell types, but cellular levels differ between cell types (87). Although HMGB1 is primarily a nuclear protein, its location can be variable, for example lymphoid cells contain significant HMGB1 stores in both the cytoplasm and nucleus (88), and cells in liver and brain tissues contain HMGB1 predominantly in the cytoplasm (89). Further, it is present on the cell surface of

neurons and platelets (90, 91). HMGB protein can be found even in ancient small multinuclear animals and evolved over 1,000 million years ago, which shows importance of these proteins during evolution (92). HMGB1 is constitutively expressed in quiescent cells and commonly localized to the nucleus due to the presence of a neutral lysine-rich NLSs. (93). Knockout mice ($HMGB1^{-/-}$) die soon after birth due to hypoglycemia which shows that HMGB1 is essential for mammal life. Phenotypic features include ruffled, small size and disorganised fur, absence of fat and long hind paws. Cells lines deficient in HMGB1 have an abnormal gene expression of different genes, such as the glucocorticoid receptors, but display a normal growth. Besides its originally described nuclear functions, more recently additional roles for HMGB1 have been revealed, for instance extracellular HMGB1 (released by non-classical mechanisms in various conditions) induces migration, recruits stem cells, possesses antibacterial functions and HMGB1 induces cytokine production (94). Recent evidence also suggests that secreted HMGB1 may contribute to modulated senescence of cells, a process that drives development of a hypersecretory phenotype in somatic cells such as fibroblasts, and that may serve as an anti-oncogenic mechanism (95).

2.3. HMGB1 structure

Structurally, HMGB1 consists of 214 amino acids and the sequence is highly conserved among species (with 99% amino acid sequence identity among mammals) (96). HMGB1 is a member of the high mobility group box (HMGB) family of proteins that includes three proteins, HMGB1, HMGB2, and HMGB3.

These members share a common structure. HMGB1 comprises a single polypeptide chain of 214 amino acids (the gene encodes for 215 amino acids residues, but the initial methionine would be removed). Two DNA-binding domains exist in HMGB1, denoted as the A and B boxes respectively, and though these do not associate with specific sequences per se. These are each made up of approximately 80 amino acid residues arranged in three alpha helices which serve to localize the protein to the minor groove. (Fig 2.1) and are strongly positively charged. The A and B boxes (DNA-binding) are followed by a highly acidic 30 amino acid tail containing only aspartic and glutamic acids which can interact with and fold over the HMG boxes and may thereby interfere with their intermolecular activity (97).

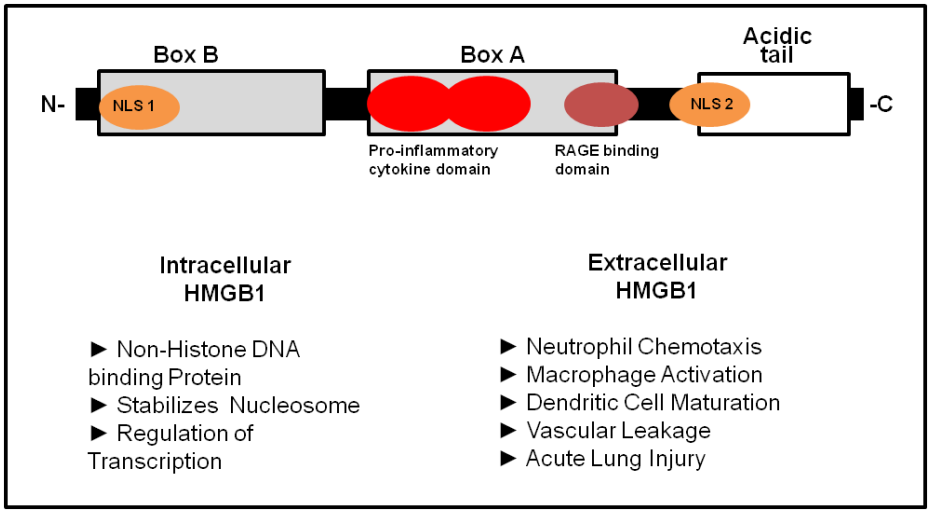


Figure 2.1. *Schematic representation showing the structure and function of HMGB1, denoting its two DNA binding domains (the A and B boxes) and the acidic C-terminal tail. HMGB1 residues 150–183 interact with RAGE to mediate chemotaxis, proliferation and differentiation. Intracellular shuttling of HMGB1 between the cytoplasm and nucleus is regulated by the acetylation of lysine residues in the NLS domains. Intracellular HMGB1 can bind to DNA as an architectural element, once released from cells during activation and death processes, displays*

immunological activities. Reproduced with permission from "Ulloa L, Messmer D. High-mobility group box 1 (HMGB1) protein: friend and foe. Cytokine Growth Factor Rev. 2006 Jun;17(3):189-20."

2.4. Post-translational modifications of HMGB1

HMGB1 can exist in several different forms or conformations generated through post-translational modifications. HMGB1 actively secreted from monocytes can be acetylated in all positions, but HMGB1 derived from necrotic cells is acetylated on lysines at positions 2 and 11 (93). Hypermethylation, acetylation and phosphorylation of HMGB1 weaken its binding capacity to DNA and prevent its reentry to the nucleus (98). The accumulation of HMGB1 in the cytoplasm results in formation of secretory lysosomes for subsequent HMGB1 secretion (93, 99).

Two highly positively charged segments of HMGB1 confer its preferential nuclear positioning as these regions serve as nuclear localization signals (NLSs). Neutralization of these NLSs results in relocation of HMGB1 from the nucleus into the cytoplasm (93). Phosphorylated HMGB1 is also translocated into the cytoplasm and prevented from reentering the nucleus. However, phosphorylated HMGB1 has not been demonstrated in extracellular fractions (100). Last, mono-methylation of HMGB1 on lysine 42 can occur in neutrophils. This methylated HMGB1 can be both relocated into the cytoplasm and secreted (99). All these modifications diminish HMGB1-chromatin interactions, but it is not clear whether they are required for lysosomal and plasma membrane passage or not. HMGB1 from metabolically stressed cells can also be translocated to the cytoplasm

without undergoing modification, which makes the role of these post translational changes less clear (101).

2.5. Intracellular HMGB1

In the nucleus, HMGB1 plays an important role in transcription regulation, modifying the structure of DNA and stabilizing nucleosomes (97, 102). HMGB1 binds the minor groove of DNA without specifying the sequence and induces bends, but has a preference for binding sharply bent structures (103). Binding of HMGB1 to DNA facilitates physical interactions between DNA and transcription factors, including p53, homeobox-containing proteins, steroid hormone receptors and recombination activating gene 1/2 (RAG1/2) proteins, which are needed for VDJ recombination in T and B lymphocytes (104, 105). There is an intriguing report about HMGB1 transactivating the human IL1-β gene (105, 106). There is also one report demonstrating that HMGB1 may directly bind to TNF promoter in osteoclasts to activate TNF transcription (106). HMGB1 interaction with undamaged DNA is a rapid and transient process, while HMGB1 binds tightly to sites of distorted DNA (107). HMGB1 constantly shuttles between the nuclear and cytoplasmic compartments (108). This may be because the two nuclear localisation signals in HMGB1 can also bind to calmodulin, which partition HMGB1 to the cytoplasm and the nucleus (93, 100, 109). Export of HMGB1 from the nucleus to the cytoplasm is independent of protein synthesis and is mediated by the chromosome region maintenance 1 protein (CRM1) (100, 110).

2.6. Extracellular HMGB1

HMGB1 plays an important role in migration, mediating cellular neurite outgrowth and tumor formation (90, 111). When added to normal and dystrophic mouse muscles, HMGB1 attracts mesoangioblasts, supporting a role for HMGB1 as a chemoattractant (112). In addition, HMGB1 promotes angiogenesis, the process leading to formation of new blood vessels during development, growth, tissue repair and tumor growth (113-115). HMGB1 has been placed in the antibacterial barrier defence system, as HMGB1 in purified form (isolated from human adenoid glands) eliminates bacteria within a few minutes in cultures (116).

2.6.1. HMGB1 release

Extracellular release of HMGB1 can be passive or active. Active release of HMGB1 from monocytes occurs via a non-classical pathway that involves secretory lysosomes (117). As previously described hypermethylation, acetylation and phosphorylation of HMGB1 weaken its capacity to bind DNA, preventing nuclear re-entry and leading to accumulation in the cytoplasm and causing formation of secretory lysosomes (93, 99)

HMGB1 release into the extracellular milieu is a prerequisite for its pro-inflammatory effects. Levels of HMGB1 significantly increase in the serum of patients with sepsis (118). In healthy animals and normal human subjects, HMGB1 is present at a very low plasma level of 5 ng/mL, but HMGB1 levels increase to levels as high as 25.2 to 83.7 ng/mL in patients with septic shock, respectively (119). HMGB1 can be released from activated

monocytes/macrophages 18–24 hours after exposure to endotoxin or pro-inflammatory cytokines such as TNF-α, IL-1β, or IFN-γ (118-121).

Despite its clinical importance, the exact mechanism of HMGB1 release has largely remained unknown. Some progress has been made recently, revealing that HMGB1 can be released from cells in either an active or passive ways (122-124).

2.6.2. Active secretion of HMGB1

HMGB1 lacks a classic leader peptide and does not pass through pathways involving the endoplasmic reticulum and the Golgi apparatus; rather large amounts of HMGB1 are released into the extracellular space via non-classical mechanisms by inflammatory cells such as monocyte/macrophages (117, 125). Indeed, active release of HMGB1 has been studied in different cell types that include monocytes, macrophages, dendritic cells, NK-cells, osteoblast, osteclasts, hepatocytes, enterocytes, endothelial cells, T-cells, neurons, smooth-muscle cells, osteoclasts, and intestinal epithelial cells (84, 93, 126-131).

Recent evidence suggests that the secretion of HMGB1 involves at least three steps (117):

(a) Exit from the nucleus into the cytoplasm;

(b) Translocation from the cytosol into cytoplasmic organelles (secretory lysosomes); and,

(c) Exocytosis.

In macrophages and monocytes activated by endotoxin or other pro-inflammatory cytokines, HMGB1 undergoes acetylation at its NLS, thus leading to translocation of nuclear HMGB1 into cytoplasmic vesicles and subsequent release into the extracellular milieu (93, 117, 126). Precise mechanisms for LPS or TNFα-stimulated release of HMGB1 are yet to be known. LPS stimulates macrophages to release HMGB1 by hyper-acetylation, partly through TNF-dependent pathways (100, 132, 133). Other studies have indicated that HMGB1 needs to be phosphorylated for secretion in response to LPS. HMGB1 phosphorylation is induced by the classical protein kinase C isoforms (CPKC) and leads to secretion by a calcium-dependent mechanism (134).

2.6.3. Passive release of HMGB1

Passive release of HMGB1 from necrotic cells is absolutely confirmed (135). HMGB1 is bound loosely to chromatin throughout the cell cycle, but "leaks" into the surrounding medium when membrane integrity is lost in necrotic or damaged (permeabilized) cells (136-138). Necrotic cells from HMGB1$^{-/-}$ mice have a reduced ability to promote inflammation, suggesting a primary pro-inflammatory mechanism in response to tissue damage (137). In contrast, apoptotic cells are not able to release HMGB1, even after undergoing secondary necrosis, and thus fail to trigger inflammation (135). In apoptotic cells, HMGB1 remains bound to chromatin; because it is not acetylated, and thus not released into extracellular milieu (135, 139). However, recent studies show that some apoptotic cells may release HMGB1 passively, likely during late apoptosis (140-

142). This suggests that the originally understood dichotomy between necrosis and apoptosis may not be entirely correct. Furthermore, Wang and co-workers found that apoptotic cells can stimulate macrophages to release HMGB1 in mice with severe sepsis, and splenectomy protects against sepsis lethality by reducing serum HMGB1 levels (143, 144).

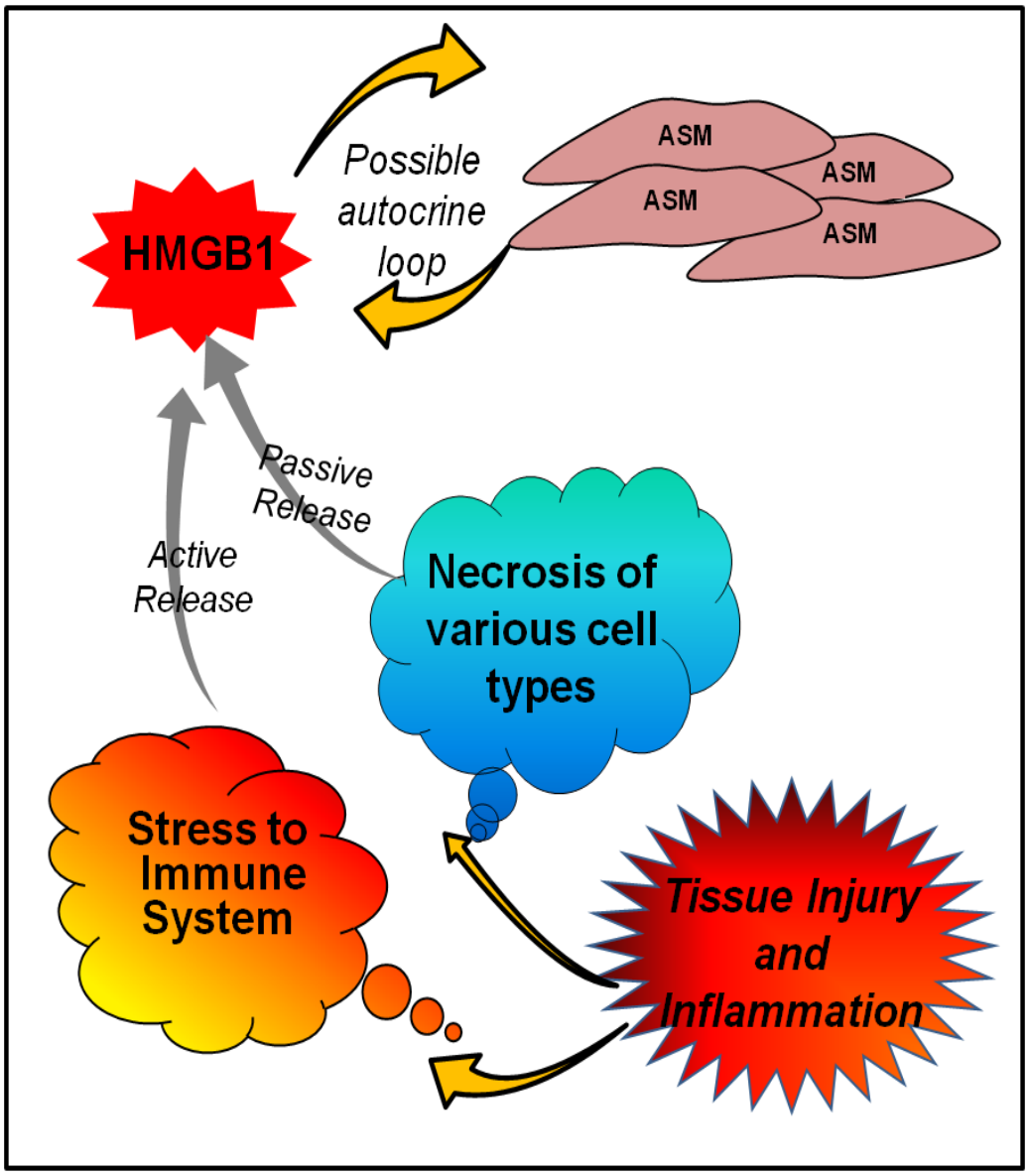


Figure 2.2. *Schematic representation showing release of HMGB1. HMGB1 "passively" releases into extracellular milieu during cellular damage following necrosis or "actively" from different*

cells in response to tissue injury and damage. The released form of HMGB1 can affect ASM cells during activation and death processes, displays immunological activities. Reproduced with permission from "Ulloa L, Messmer D. High-mobility group box 1 (HMGB1) protein: friend and foe. Cytokine Growth Factor Rev. 2006 Jun;17(3):189-20."

2.7. HMGB1 biologic effects

In the cell nucleus, HMGB1 binds to DNA and can modify chromatin structure to modulate transcriptional regulation (145). Also HMGB1 can regulate transcription, when it interacts with other transcription factors via protein-protein interactions (146). HMGB1's role is so crucial that $HMGB1^{-/-}$ mice die because of hypoglycaemia, and these mice show other phenotypic problems, such as growth retardation and blindness (147). Secreted/released HMGB1 can cause chemotaxis of myeloid cells, release of pro-inflammatory cytokines, and MMP production by monocytes and macrophages, and it modulates maturation of dendritic cells (118, 121, 148-150). HMGB1 causes the activation, and cheomtaxis of neutrophils (150, 151) and promotes differentiation of T-cells to a Th1 phenotype (152). HMGB1 stimulates endothelial cells to release pro-inflammatory cytokines and the upregulation of adhesion molecules (153, 154). HMGB1 also reportedly plays a crucial role in tissue repair process by promoting proliferation, differentiation and migration of stem cells and smooth muscle cells (112, 125, 155). HMGB1 is also involved in nervous tissue repair processes by promoting neuronal outgrowth (156), growth, chemotaxis, pro-inflammatory cytokine production and MMP activity in astrocytes (157).

In the same year that HMGB1 was first identified in the blood of patients with sepsis, Polly Matzinger proposed the idea that the immune system responds to "danger signals"(158). This model offered an explanation for how the immune response can be triggered in different scenarios (e.g., infection-induced vs. sterile inflammation) (159-161). The Matzinger model proposed built upon the "infectious no-self model" developed by Charles Janeway in the late 80's (162). The Janeway model suggested that the immune system recognizes a conserved molecular motif called a pathogen-associated molecular pattern (PAMP), which characterizes pathogens but is not normally found externally on host cells. These can include lipopolysaccharide, peptidoglycans, nonmethylated CpG, and double-stranded RNA. PAMPs are generally invariant and the cellular receptors , which recognize them, called Pattern Recognition Receptors (PRRs), are evolutionarily conserved. The first PRR was identified in 1997: the Toll-like receptors (TLRs) (163, 164). The "Danger" model is supported by the subsequent discovery of several endogenous molecules that are released during infectious and/or sterile inflammation; this includes HMGB1, heat shock proteins (HSPs), S100 proteins, and hyaluronan. These molecules have been implicated as possessing the capacity to trigger the immune system through PRRs, much like PAMPs (118, 165, 166). The signals are normally intracellular cell constituents but can be released, either passively by necrotic cells or actively by stressed cells in response to cellular injury. While the term PAMP is restricted to patterns located on pathogens, endogenous released PAMPs such as HMGB1 are more specifically termed alarmins (167). Exogenous PAMPs and alarmins are

subgroups of the larger category of danger signals termed Damage Associated Molecular Patterns (DAMPs) (168, 169). Alarmins are of particular interest, because of their role in both infectious and sterile inflammation. They are present either locally during inflammation or systemically in severe sepsis, burns, infection, and cancer (84, 118, 170, 171).

2.8. Receptors for HMGB1

The first described receptor for HMGB1 was the receptor for advanced glycation end (RAGE) products (172, 173). However, in several studies blocking antibodies for RAGE only partially suppress the effects of HMGB1, and in RAGE-deficient mice HMGB1 stimulation was not fully abrogated, strongly suggesting that RAGE is not the only functional receptor for extracellular HMGB1 (174-178). More recently, toll-like receptors (TLR) 2 and 4 have been suggested to interact with HMGB1 (175, 177, 179), as well as β2-integrin (Mac-1) (180).

RAGE belongs to the immunoglobulin (Ig) super family, being comprised of three extracellular immunoglobulin domains, a single trans-membrane segment and a short cytoplasmic tail (181). RAGE appears to interact with several ligands, such as amyloid-β, multiple members of the S100 protein family, and advanced glycation end products (182, 183). RAGE is the only receptor constitutively expressed at high levels in the lung and skin, but is markedly up-regulated in almost every other tissue examined (184). RAGE-ligand interaction evokes central changes in key biological properties of cells, including proliferation,

generation of inflammatory mediators, and migration. Although RAGE-dependent signal transduction is dependent on its short cytoplasmic domain, all ligand-stimulated RAGE signaling requires mDia1 (also known as Dia1, Drf1 for Diaphanous-related formin-1, Diaphanous1 Diaph1). It has been shown that mDia1 interacts with RAGE to activate RAGE-dependent signaling pathways (185, 186). In fact, RAGE, directly binds to ERK through mDia1 and regulates p-ERK1/2 (187, 188). It is possible that cytoplasmic HMGB1 interacts with the cytosolic domain of the receptor for advanced glycation end products, and mediates concomitant ERK1/2 phosphorylation (188-190). Collectively, these findings indicate that the interaction of the RAGE cytoplasmic domain with mDia1 is required to transduce the extracellular environmental cues evoked by binding of RAGE ligands, such as HMGB1. Binding of HMGB1 to RAGE appears to have two main consequences (191, 192):

i) Activation of intracellular signal transduction pathways through proximal effectors such as Cdc42, Rac1 and other guanosine triphosphatases (GTPase);

ii) ii) Activation of mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B (NF-κB).

In macrophages, the Caco-2 epithelial cell line, and neutrophil MAPKs that are activated by the HMGB1-RAGE axis include p38, p42/44 (Erk1/2), and stress-activated protein kinase/c-Jun N-terminal kinase (JNK/SAPK) (175, 193, 194). Smooth muscle cell migration mediated by HMGB1 involves MAPK signaling and an unidentified G-protein-coupled receptor (125).

HMGB1 has also been described, as mentioned above, as a ligand for TLR2 and TLR4. The binding of HMGB1 to TLRs appears to trigger activation of canonical downstream signalling cascades involving MyD88 and IRAK-1 that ultimately promote NFκB activation, its nuclear translocation, and ultimately p50/p65 NFκB-driven transcription of inflammatory genes, as well as maturation of dendritic cells (195).

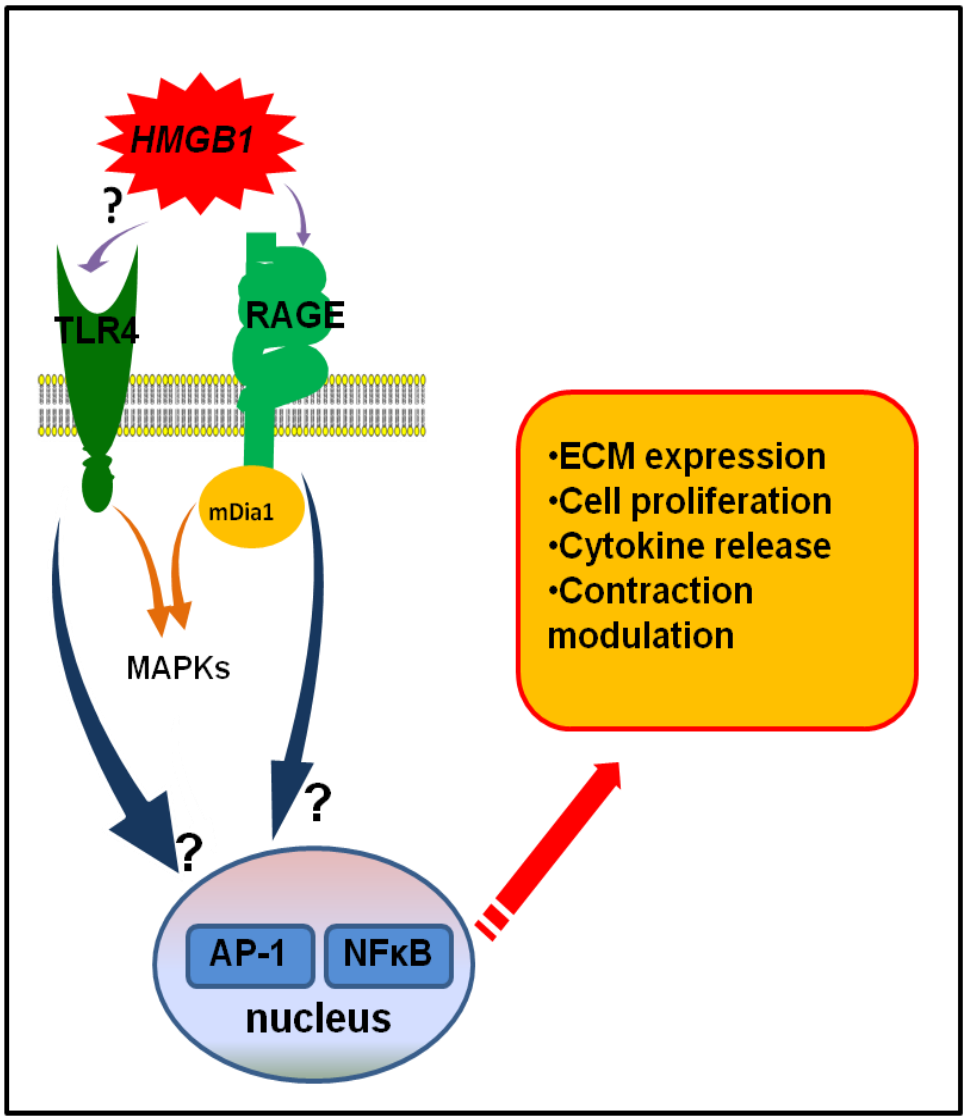


Figure 2.3. *Schematic representation showing HMGB1 signaling pathways.*

HMGB1 can bind to RAGE (through mDia1) and TLR4 (196). HMGB1 activates MAPK pathway, and then sequentially activates AP-1 and NF-κB which could increase cell proliferation, increased ECM expression and inflammation in ASM cells.

2.9. HMGB1 as a pro-inflammatory cytokine

Several pro-inflammatory activities of HMGB1 have been revealed from in vitro studies using cell culture, and a picture has emerged of extracellular HMGB1 as a significant pro-inflammatory signal. The response to HMGB1 includes pathophysiological features of infectious and non-infectious inflammatory diseases, in particular those characterized by a delayed kinetic response relative to TNF and IL-1 levels as could be seen in septic shock or arthritis (197-199). The first description of a specific pro-inflammatory activity for HMGB1 were revealed from studies using cultured monocytes and macrophages in which the addition of purified released HMGB1 significantly stimulated the release of TNF, IL-1β, IL-6 and nitric oxide (121). The activation of macrophages occurred at extremely low concentrations (5-50 ng/mL) of HMGB1, indicating that it is among the most potent endogenous inducers for TNF release. HMGB1 shows pro-inflammatory activity on other cells, such as neutrophils in which the nuclear translocation of NF-κB is induced to stimulate the release of several pro-inflammatory cytokines (175) HMGB1 signal transduction in human neutrophils activates the p38 MAP kinase, phosphatidylinositol 3-kinase/Akt and ERK1/2 pathways (148). The kinetics of neutrophil activation by HMGB1 differs significantly from that induced by endotoxin: neutrophils release TNF within 60 min after addition of HMGB1,

whereas maximal TNF mRNA levels in endotoxin-stimulated neutrophils occurs 4 h after addition of LPS (175). Thus, from a kinetic standpoint, rapid neutrophil responses to HMGB1 contrast with those of monocytes and macrophages.

Adding recombinant HMGB1 to cultured human enterocytic monolayers revealed the pro-inflammatory activity of HMGB1 on epithelial cells (193). HMGB1 mediates an increase in permeability of epithelial monolayers, an effect that is dependent on increased inducible nitric oxide synthase (iNOS) expression (200). Inhibition of RAGE-mediated signaling using RAGE-blocking antibodies abrogates approximately 50% of the HMGB1-mediated barrier dysfunction in epithelial cells. This is consistent with the concept that an HMGB1-RAGE signaling axis contributes partially to the response in epithelial cells (193). Systemic administration of HMGB1 to mice significantly increases mucosal permeability and bacterial translocation to mesenteric lymph nodes, but mice deficient in iNOS are protected from HMGB1-mediated barrier dysfunction, indicating that HMGB1 mediates epithelial gut barrier failure through increased nitric oxide production (201). HMGB1 can also activate cytoskeleton reorganization in rat smooth muscle cells and stimulate chemotaxis and chemokinesis. Indeed, smooth muscle cells exposed to recombinant HMGB1 develop a polarized morphology that is typical of mobile cells. Smooth muscle cell migration mediated by HMGB1 is abrogated by antibodies against RAGE, and is also inhibited by addition of pertussis toxin or the ERK1/2 MAP kinase inhibitor PD9805922. Significant binding of HMGB1 to the surface of smooth muscle cells occurs in association with the expression of RAGE on the plasma

membrane in rat smooth-muscle cells (125). Endothelial cells, which also express RAGE, respond to recombinant HMGB1 and exhibit a time- and dose-dependent increase in expression of vascular cell adhesion molecule-1 (VCAM-1), RAGE and intercellular adhesion molecule-1 (ICAM-1) (154). Other pro-inflammatory cellular endothelial responses to HMGB1 include increased release of TNF, IL-1, IL-8, monocyte chemoattractant protein 1 (MCP-1), plasminogen activator inhibitor-1 and tissue plasminogen activator (154). Interestingly, anti-TNF antibodies partially inhibit the HMGB1-mediated stimulation of endothelial cells (198). HMGB1-mediated signal transduction in these cells is in part dependent on activation of p42/p44 MAPK and JNK (154). When considered with the previously discussed data, HMGB1 is able to activate neutrophils, monocytes and smooth muscle cells, and the release of HMGB1 can drive a pro-inflammatory endothelial immune cell response as well as disrupt epithelial barrier integrity.

2.10. Targeting HMGB1 as a therapy

After reporting the pathogenic role of HMGB1 in sepsis, there were improvements made in targeting HMGB1 as a therapy for different diseases. Mechanisms for the pro-inflammatory effects of HMGB1 have been sought for a possible treatment of inflammatory diseases. So far two different pathways have been targeted:

i) Blocking of extracellular HMGB1 function;

ii) Prevention of HMGB1 secretion.

Blocking extracellular HMGB1 for treatment of inflammatory diseases has generated some success in animal models of sepsis, chronic arthritis and CNS injury (202-205). The pro-inflammatory effects of HMGB1 appear to be mediated by its B-box (206). Therefore, peptide mimics of the HMGB1 A-box, which can act as a ligand for HMGB1 receptors without inducing pro-inflamamtory signaling, holds potential as an HMGB1 antagonist. A-box peptides have been studied in animal models of chronic arthritis, sepsis and pneumonia and stroke (150, 203, 205, 207).

Using HMGB1-specific neutralizing polyclonal antibodies have been shown to protect mice against endotoxin and sepsis lethality. Using these antibodies in rodents has reduced signs and symptoms of arthritis, also attenuated levels of TNF and IL-1β in tissue of joints (118). Administration of anti-HMGB1 antibodies to animals with established sepsis significantly reverses the signs of sepsis and prevents lethality in animals with established infection. Three separate approaches have been reported to inhibit HMGB1 in rodent sepsis and each of these improves survival significantly. Neutralizing anti-HMGB1 antibodies have been produced and are defined based on their ability to inhibit the macrophage stimulating activity of recombinant HMGB1. Neutralizing antibodies significantly improve survival from sepsis even when the first dose of antibodies is administered to animals 24 h after the onset of infection (207). Humanized anti-HMGB1 monoclonal antibodies could therefore find applications in both acute and chronic inflammatory diseases. Blockage of the RAGE signaling pathways could also result in attenuation of the proinflammatory effects of

HMGB1. Several strategies, such as the administration of soluble forms of RAGE or the blocking of the Fab fragment derived from anti-RAGE and/or anti-HMGB1 IgG, have been reported (208-210).

Hmgb1 structure shows us that B box has pro-inflammatory properties, while Box A can act as an antagonist for HMGB1, which makes A box, a potential candidate to treat HMGB1 related diseases (174). Treatment with A box has been successful for shock, sepsis, DIC (174, 207) and hepatitis (211). Using these antibodies in rodents has reduced signs and symtoms of arthritis and attenuated level of TNF and IL-1β in joint tissue (203). Different anti-HMGB1 antibodies have provided promising therapeutic results in chronic arthritis, sepsis, pneumonia and stroke (150, 203, 205, 207).

Considered together with the results of anti-HMGB1 antibodies and the A box protein, it now appears that neutralizing HMGB1 can significantly improve treatment of inflammatory diseases. It remains to be seen whether neutralizing antibodies to HMGB1 will be associated with the development of immunosuppression or toxicity. Also Thrombomodulin has recently been shown to bind to HMGB1 so that thrombin– thrombomodulin complexes can effectively degrade HMGB1 into a less proinflammatory form (212-214). This means that recombinant thrombomodulin can promote the degradation of HMGB1 and suppress the proinflammatory effects of HMGB1 (214).

sRAGE, the soluble isoform of RAGE which prevents binding of HMGB1 to RAGE, can decrese growth of tumors (191) and ameliorate arthritis (215).

Several small-molecule chemical compounds have been used to inhibit HMGB1 proinflammatory activities. These pharmacological agents belong to the class of cytokine-release inhibitory drugs (CRIDs) and include ethyl pyruvate, the cholinergic agonists nicotine and acetylcholine, and steroid-like pigment tanshinone IIA (216-219). These agents were found to interfere specifically with HMGB1 release from the nucleus into the extracellular space, without affecting HMGB1 mRNA or protein levels (218, 220). The HMGB1 inhibiting molecules have shown impressive efficacy in animal models of lethal endotoxemia and sepsis, with protective effects at therapeutically achievable, safe doses, supporting the therapeutic potential of these inhibitors in HMGB1- mediated human inflammatory diseases such as shock, sepsis and DIC (216-219). Another molecule, glycyrrhizin, inhibits the chemotactic and mitogenic activities of HMGB1 (221). Unlike CRIDs, glycyrrhizin does not interfere with the release of HMGB1, but directly inhibits its extracellular cytokine activities (221). This means that glycyrrhizin can inhibit not only actively released HMGB1 but also passively released HMGB1. However, the affinity of glycyrrhizin for HMGB1 is relatively modest and will need to be improved for any therapeutic application (221). Several other commercially available drugs, such as sivelestat, nafamostat, antithrombin III, and γ-globulin, have also been suggested to modulate inflammatory response through HMGB1-related mechanisms (222-225). With respect to blocking HMGB1 release, ethylpyruvate, oxaliplatin, gold sodium thiomalate and gherlin have all been shown to dampen secretion from macrophages (218, 220, 226, 227).

In summary, HMGBl and RAGE expression are correlated with inflammatory diseases, and HMGBl expression is correlated with disease severity. Additionally, these proteins display reduced expression in controlled asthma. Continued research is needed to determine whether/how HMGB1 and RAGE expression promote inflammation to be able to target them for treatment of these diseases.

2.11. HMGB1 and asthma

The role of HMGB1 in asthma pathogenesis is not established but its expression is significantly elevated in the induced sputum samples of asthmatic compared with healthy patients (228). HMGB1 has been implicated directly in some lung diseases. Abraham et al. (150) identified HMGB1 as a late mediator of lethality in sepsis, and suggested it may play a key role in the pathogenesis of acute lung injury. Intratracheal challenge with HMGB1 in mice causes acute lung injury; manifested by neutrophil accumulation, lung edema, and increased pulmonary cytokine levels, including TNF, IL-1β, and MIP-2 (150). Elevated HMGB1 has been found in plasma and lung epithelial lining fluid of patients with acute lung injury and in mice instilled with LPS (229). Treatment with anti-HMGB1 antibodies in mice exposed to intratracheal LPS significantly decreases lung edema and neutrophil accumulation, but does not suppress LPS-induced pulmonary cytokines (150, 230). These observations suggest HMGB1 is a central pathogenic factor in acute lung injury.

A recent report describes findings that damaged airway epithelia induces the chemotactic movement of human eosinophils (231), and that HMGB1 enhances eosinophil survival, also acting as a chemoattractant (232). These reports are of significance because both neutrophils and eosinophils play important roles in the pathogenesis of inflammatory airway diseases such as asthma and chronic bronchitis. Notably, HMGB1 is present in airway smooth muscle cells that encircle the conducting airways and that are local orchestrators of tissue inflammation and remodeling (233). HMGB1 can support proliferation, chemotaxis, and the synthesis of metalloproteinases by stromal fibroblasts (112, 234). HMGB1 also enhances the synthesis of pro-inflammatory cytokines by immune cells and mesenchymal cells (120, 235); This effect could be in part be potentiated by the ability of HMGB1 to bind pro-inflammatory cytokines such as IL-1β and LPS (235, 236).

RAGE, a member of immunoglobulin super family, and a main receptor for HMGB1 (181, 182), is expressed by most human cell types at low to undetectable levels but it can be up-regulataed by HMGB1 binding (233). Lung cells, especially pulmonary endothelial cells, bronchiolar epithelial cells, alveolar macrophages and type I alveolar epithelial cells express RAGE at high levels (237, 238). Of note for a possible role in the airway remodeling process, HMGB1 is released from airway epithelial cells and inflammatory cells. It is significantly elevated in lung lavage from patients with idiopathic pulmonary fibrosis. Also inflammation and fibrosis in bleomycin exposed mice are suppressed by HMGB1 blocking antibodies (239). This suggests an important role for RAGE in lung

homeostasis and potential to modulate lung disease pathogenesis. Consistent with this concept, the binding of HMGB1 to RAGE promotes cell chemotoxis, wound healing and regeneration in a number of tissues (112, 240, 241). This response can be associated with pathological tissue repair; for example, HMGB1 and RAGE ligation in the kidney promote trans-differentiation of tubular epithelial cells to myofibroblast and contributes to tissue fibrosis by mediating synthesis of collagen and TGF-β (242). Taken together, HMGB1 released in the airways during inflammatory processes may contribute to important histopathological features of tissue remodelling.

CHAPTER II. HYPOTHESIS AND OBJECTIVES

Rationale

Over 300 million people worldwide suffer from asthma, including 3 million (8.4%) in Canada with an economic burden near $1 billion annually (243, 244). Manitoba has the highest rate of asthma of all the provinces in Canada (244). Asthma is a chronic intermittent inflammatory airway disorder characterized by paroxysmal dyspnea and airway hyperresponsiveness (4, 245). A key feature of severe asthma is the progressive development of airway wall remodeling, which is thought to occur through repeated rounds of inflammation and tissue repair. These structural changes include increased mass of airway smooth muscle cells (ASM cells) and excessive collagen deposition and accumulation in the airway wall (246). ASM cells from asthmatics are hyper-proliferative and exhibit altered extracellular matrix (ECM) expression as well as increased cytokine and chemokine synthesis (e.g. IL-8 and eotaxin-1) (247-250).

The high-mobility group box-1 (HMGB1) protein, also known as high-mobility group 1 (HMG-1) or amphoterin, is a highly conserved, abundant non-histone nuclear protein expressed in almost all eukaryotic cells (80, 251). Within the nucleus, HMGB1 modulates nucleosomes, bends DNA and binds bent DNA in chromatin structure, facilitating transcription of different genes (252). HMGB1 can also be secreted by necrotic and inflammatory cells in response to stress signals, and thus act as a pro-inflammatory "alarmin" in the extracellular milieu (117, 125, 135). Extracellular HMGB1 modulates tissue injury/repair by triggering infiltration of mononuclear cells to orchestrate the inflammatory response and wound healing processes (93, 117, 125, 135,

253). High levels of HMGB1 are associated with lung inflammation, acute lung injury, cystic fibrosis, adult respiratory distress syndrome, and idiopathic pulmonary fibrosis (IPF) (101, 118, 172, 230, 254, 255).

A principal receptor for HMGB1 is the multi ligand immunoglobin family **r**eceptor for **a**dvanced **g**lycation **e**nd products (172), which is highly expressed in the lung. RAGE increases in response to inflammation, and regulates lung injury and fibrosis, in part via effects on epithelial-mesenchymal transition (256, 257). Activation of RAGE by ligands such as HMGB1 also leads to pro-inflammatory signaling (258), and HMGB1 can cause lung fibrosis by increasing collagen synthesis through RAGE-mediated MAP kinase signaling (242). The short cytoplasmic tail of RAGE has no signaling capacity *per se,* rather it associates with the small GTPase scaffolding protein, mammalian diaphanous 1 (mDia1)(187, 259-261), which enables ligand-induced activation of Rac1 and Ras, thus inducing MAP kinases (ERK1/2, p38 and JNK) and transcription factors such as AP-1 and NFκB that promote cytokine gene expression (242, 262-267). These signaling cascades are also associated with cell proliferation, ECM expression, and turnover, and cytokine release in multiple cell types, including ASM cells (15, 268-270).

Work performed and described in this thesis is designed to test the **Hypothesis** that, HMGB1 induces signaling pathways that controls responses in human ASM cells that have been linked to airway inflammation, remodeling and hyperresponsiveness in obstructive lung disease, such as asthma. To test this hypothesis we formulated five specific objects.

Objectives:

Objective 1. To investigate the role of HMGB1 in promoting ECM synthesis, by treating immortalized human ASM cells with recombinant HMGB1 and assessing collagen A1 expression using immunoblotting;

Objective 2. To assess the effects of HMGB1 exposure on activation of intracellular signaling pathways, specifically MAP kinases and NFκB, by: stimulating human ASM cells with HMGB1 and using immunoblotting to measure phosphorylation of ERK 1/2, p38 and JNK/SAPK, and measuring phosphorylation of IKappa-B (immunoblotting) as well as measuring nuclear translocation of p65 NFκB using a commercial cell-based ELISA assay;

Objective 3. To determine whether HMGB1-induced MAP kinase phosphorylation is mediated via a RAGE-mDia1 axis in human ASM cells, using quantitative (real-time) PCR and immunoblotting to assay the impact of HMGB1 exposure on RAGE expression, and preparing cell lines of primary human ASM cells with stable silencing of mDia1 and measuring impact on HMGB1-induced MAP kinase activation.

Objective 4. To examine whether HMGB1 can induce changes in human ASM cell function, specifically: proliferation, measured by increase in cell number during exposure to HMGB1; and, ECM protein expression and secretion, measured using qRT-

PCR and immunoblotting, in the presence and absence of the pro-fibrotic cytokine, TGF-β1.

Objective 5. To further decipher the role of HMGB-1 in pro-fibrotic function of human ASM cells in response to TGF-β1: we measured HMGB1 release upon TGF-β1 treatment using immunoblotting, and generated ASM cell lines with stably silenced endogenous HMGB1 expression to measure the effect on TGF-β1-induced ECM protein expression to dissect the role of intracellular HMGB1 on ECM expression.

CHAPTER III. MATERIALS AND METHODS

1. Reagents and antibodies

Cell culture plastic ware was obtained from Corning Costar Co. (Tewksbury, MA). Cell culture media (Dulbecco's Modified Eagle Medium; DMEM), supplements (fetal bovine serum (FBS) and insulin, transferrin and selenium (ITS-A, 1%), trypsin-EDTA and antibiotics (penicillin and streptomycin) were obtained from Invitrogen (Carlsbad, CA, USA). Enhanced chemiluminescence reagent was from Amersham (Oakville, ON, Canada). Mouse IgG anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), goat IgG anti-collagen A1, and rabbit IgG anti-fibronectin antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Recombinant human TGF-β1, mouse IgG anti-β-actin antibody, horseradish peroxidase (HRP)-conjugated goat IgG anti-mouse antibody, and HRP-conjugated goat IgG anti-rabbit antibody were purchased from Sigma (St. Louis, MO). Mouse IgG anti-phospho-Thr202/Tyr204-p42/p44 MAPK monoclonal, and rabbit IgG anti-p42/p44 MAPK polyclonal antibodies were obtained from Cell Signaling Technology (Beverly, MA). FITC-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Platelet-derived growth factor (PDGF) was purchased from Calbiochem (La Jolla, CA).

2. Primary human airway smooth muscle cell cultures

As per our established protocols (271, 272) primary human airway smooth muscle cells were obtained from smooth muscle bundles by enzymatic dissociation from isolated by microdissection of macroscopically healthy segments of second to fourth generation lobar or main bronchus of patients undergoing surgery for lung carcinoma in accordance

with the procedures approved by the Ethics Committee of the University of Manitoba (Winnipeg, Canada). Informed consent for tissue harvesting was obtained from all patients. Cells were plated onto 100 mm culture dishes and grown to ~80% confluent using in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. For all experiments cultures were maintained in a humidified chamber at 37 °C, 5% CO2 and all media contained 100 units/mL of penicillin G and 100 µg/mL of streptomycin sulfate. At 80% confluency, cells were switched to serum-deficient medium supplemented with or without ITS (insulin 5µg/ml, transferrin 5µg/ml and selenium 5µgn/ml).

3. Immortalized human airway smooth muscle cell culture

For some experiments at least three senescence-resistant human ASM cell lines were used. These cell lines were prepared by forcing stable ectopic expression of the human telomerase reverse transcriptase gene (hTERT) as we have previously described (273). Briefly, primary and low-passage cultures were infected with a retrovirus vector harbouring the Moloney murine leukemia virus (MMLV) to drive expression of the hTERT gene. To generate retroviral transducing vectors a plasmid (pGRN145) containing hTERT cDNA expression vector was purchased from ATCC (American Type Culture Collection) (Manassas, VA). The hTERT expression cassette was cloned into pLXIN (Clontech), and replication-incompetent virus was generated in HEK293 cells. Primary and first-passage cultures of human airway smooth muscle cells were infected then selected with 100 mg/mL G418 for 1 week (wk). Expression of hTERT was verified in immortalized cells by reverse transcriptase polymerase chain reaction (RT-PCR) using telomerase-specific primers as we have previously described (273). For all experiments, passages 16–21 of ASM cells were grown on uncoated plastic dishes in DMEM

supplemented with 50 U/mL streptomycin, 50 μg/mL penicillin, and 10% FBS. Before each experiment, unless otherwise specified, cells were grown to 70% sub-confluence and then serum starved for 2 days in DMEM supplemented with 50 U/mL streptomycin, 50 μg/mL penicillin, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL selenium

4. Cell proliferation assay

Cells were grown to sub-confluence (~70%) in 6-well cluster plates, serum-starved for 2 days in DMEM, and then incubated with mitogens (as specified in figure legends) for 4 days in DMEM supplemented with 50 U/mL streptomycin and 50 μg/mL penicillin. Subsequently, at specified time points cells were lifted by trypsinization (with 0.05% Trypsin - EDTA containing phenol red) and then counted manually using a hemocytometer. For all experiments at least 3 replicates were performed for each condition and time point for at least three cell lines.

5. HMGB1 Immunocytochemistry

Human ASM cells were plated onto pre-cleaned glass coverslips in six-well culture dishes. At experimental time points, cells were fixed for 15 min at 4 °C in phosphate buffered saline (PBS) containing 4% paraformaldehyde (PFA). The cells were then rinsed 3x, for 10 min in PBS and permeabilized by incubation for 5 min at 4 °C in Cold Cytoskeletal buffer (CB): (10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM $MgCl^2$ and 5 mM glucose at pH 6.1), containing 3% PFA and 0.3% Triton X-100. For immunofluorescence microscopy, fixed cells were blocked for 2 h at room temperature in cyto-Tris buffered saline (TBS) containing 1% bovine serum albumin (BSA) and 2% normal donkey serum. Cells were then incubated overnight at 4°C with anti-HMGB1 (2μg/mL) diluted in cyto-Tris buffer saline tween (TBST). For negative controls, samples

were incubated with either isotype-matched mouse IgG or rabbit antiserum. All coverslips were then incubated with FITC-conjugated secondary antibody (1:100) for 2 h at room temperature in cyto-TBST. After washing cells nuclei were stained by incubating cells for 30 seconds in PBS containing Hoechst 33342 (10 μg/ml). After coverslips were rinsed in distilled water and were mounted with ProLong Gold antifade reagent from Molecular Probes (Eugene, OR). Fluorescent imaging was performed by capturing a mid-nucleus section of 0.3 μm focal depth with an Olympus LX-70 FluoView confocal laser scanning microscope (Olympus America) equipped with a 40x objective.

6. Characterize and quantify secretion of ECM proteins

Secretion of ECM proteins was measured in supernatant medium. The supernatant was concentrated ~10-fold by centrifugation using <10 kDa MW Amicon Ultra-15 Centrifugal Filter Units (Millipore, Bedford, MA), and centrifuged at 2000 ×g for 30 min.

7. Preparation of protein lysates from human ASM tissue and cells

Intact ASM tissue was isolated from human bronchial specimens by micro dissection at 4 °C. Subsequently, tissues were homogenized in ice cold RIPA buffer (composition: 40 mM Tris, 150 mM NaCl, 1% IgepalCA-630, 1% deoxycholic acid, 1mM NaF, 5mM β-glycerophosphate, 1 mM Na3VO4, 10μg/ml aprotinin, 10 μg/ml leupeptin, 7 μg/ml pepstatin A, 1 mM PMSF, pH 8.0). Lysates were transferred to 1.5 mL plastic tubes and centrifuged at 760 × g for 5 min. The supernatants were stored at -20 °C for subsequent protein assays and immunoblotting. Protein content of samples was determined using the BioRad protein assay kit (based on the “Bradford” method to assess dye binding); BSA was used as a standard to generate standard curves (BioRad, Hercules, CA).

8. Immunoblotting

Immunoblotting was performed by using protocols described in our prior reports (62, 273). Protein content in supernatant samples was determined using the BioRad protein assay with bovine serum albumin as a reference (BioRad, Hercules, CA). Briefly, after reconstituting samples in ice-cold RIPA buffer (composition: 40 mM Tris, 150 mM NaCl, 1% Igepal CA-630, 1% deoxycholic acid, 1 mM NaF, 5 mM β-glycerophosphate, 1 mM Na3VO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 7 μg/ml pepstatin A, 1 mM PMSF, pH 8.0) supplemented with a cocktail of protease inhibitors (Sigma-Aldrich), 10–25 μg of protein was loaded per lane and size-separated electrophoretically under reducing conditions using SDS-polyacrylamide gels. Protein bands were then transferred onto nitrocellulose membranes by electrobloting using reservoir buffer (Trizma-base 0.025 M, Glycine 0.192 M, SDS 0.1% w/v). Thereafter, blots were blocked with TBS (composition: 10 mM Tris HCl, pH8.0, 150 mM NaCl) containing 5% skim milk (w/v) and 0.2% Tween-20 (unless otherwise stated in figure legends). Membranes were then incubated with relevant primary antibodies diluted in TBS containing 1% skim milk (w/v) with or without 0.2% Tween-20. The membranes were incubated in secondary antibody HRP-conjugated goat anti-mouse IgG (1:3000) or HRP-conjugated goat anti-rabbit IgG (1:5000) antibodies prepared in 1% skim milk. All the blots were developed by enhanced chemiluminescence as recommended by the supplier (Amersham Pharmacia, Oakville, ON). β-actin or GAPDH primary antibodies were used to normalize for differences in protein loading using densitometry using the Epson Perfection 4180 Station gel documentation system (AlphaEaseFC, Alpha Innotech Corporation, San Leandro, CA) to assess relative protein abundance.

9. Stable gene silencing: lentiviral delivery of shRNA

In some experiments we purchased pre-made lentiviral particles to express RAGE shRNA or control (scrambled/non-coding) shRNA from Santa Cruz Biotechnology (Santa Cruz, CA). To generate lentiviral transduction particles for other studies we employed three plasmids (for each target gene 3) constructs harboring shRNA for HMGB1, mDia1 or RAGE purchased from Open-Biosystems (Huntsville, AL) distributed by the Biomedical Functionality Resource, University of Manitoba. Plasmids were provided in stably transformed bacterial inoculates grown on agarose plates – to isolate plasmids individual colonies were cultured in 2 mL of LB broth with 100 μg/mL ampicillin (for bacterial selection) for 8 hours at 37 °C, with aeration (280 RPM). The cultures were then added to 200 mL of LB broth with ampicillin (100 μg/mL) and incubated as above overnight. The culture was centrifuged and plasmid purified with a Hi Speed Plasmid Maxi Kit (Qiagen cat# 12663). The plasmids was transfected into 293T cells using Ca^{2+}-phosphate 3 plasmid transfection VSVG (envelope vector), 8.2Δvpr (packaging vector) and expression vector for either HMGB1, mDia1 or RAGE were used for transfections to generate lentiviral particles generated by puromycin resistant colonies, as previously described (274). Viral particles harboring non-coding shRNA (shRNAi non-code) was used as a transduction control. To isolate viral particles transfected 293T cells were incubated for 3 days at 37 ºC, then supernatant containing virus was concentrated by ultracentrifugation. The virus was resuspended in DMEM with 0.5% FBS for 24 hr at 4 °C, aliquoted and stored at - 80 °C. For transduction using viral particles, human primary ASM cells were cultured to 70-80% confluence in twelve-well plates and virus was then added at a multiplicity of infection (275) from 2-10. The

transduction was repeated twice and the cells were then maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin for 48 hours. The viability of the transduced cells was >98%, as assessed by trypan blue dye exclusion after completion of the experiment. After transduction, cells that integrated shRNA expression cassettes were selected by adding 4 μg/mL of puromycin to culture media and cells were maintained for 3 days. In studies using pre-made, commercially avaialable lentiviral particles, primary human ASM cells were infected with virus harboring RAGE shRNA or non-coding shRNA. Stable clones were selected by maintaining cells in puromycin-containing media as described above - all procedures were performed in accordance to manufacturer protocol (Santa Cruz Biotechnology, Santa Cruz, CA).

10. RNA isolation and RT-PCR

Using the Qiagen RNeasy Mini Kit in accordance with the manufacturer's recommendations (Qiagen, Mississauga, ON), total RNA was extracted from human bronchial tissue enriched in ASM that was micro dissected from two different human donors. Total RNA (1 μg) was reverse transcribed using MMLV reverse transcriptase (Promega, Adison, WI) for 2 h at 37°C followed by 5 min incubation at 95°C, and the diluted 1:10 with RNase-free water. The RT-PCR reactions for cDNAs of interest were carried out in a thermal cycler (Mastercycler, Eppendorf, Hamburg, Germany) using primer pairs listed in Table 1. Primers for transcripts of interests were designed using PRIMER-3 and IDT programs available online (http://simgene.com/Primer3 , http://www.idtdna.com/pages/scitools?gclid=COzj_prDs7sCFQISMwodnDoAFA). Cycle parameters were as follows: denaturation (94°C for 45 s), annealing (60°C for 45 s), and extension (72°C for 45 s). The initial denaturation period was 4 min and the final

extension was 5 min. Glyceraldhyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. Amplified products were analyzed by DNA gel electrophoresis in 2% agarose, and visualized by Gelstar staining under ultraviolet illumination using a gel documentation system (AlphaEaseFC, Alpha Innotech Corporation, San Leandro, CA).

11. Real-Time RT-PCR analysis

Total RNA was extracted from human ASM cells using the RNeasy Plus Mini Kit in accordance with the manufacturer's recommendations (Qiagen, Mississauga, ON). The RNA concentration and purity were assessed with optical density measurements (276); a ratio of OD at 260nm:OD at 280nm of 1.8-2.0 was used to identify mRNA of suitable quality for susequnt analysis. For PCR, total RNA (1 μg) was reverse transcribed using the Quantitect Reverse Transcription Kit as recommended by the supplier (Qiagen, Mississauga, ON). Real-Time PCR for the cDNAs of interest were carried out with the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA), using primers indicated in Table. 1. Each reaction contained the following: 2x Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 0.4 μM of each primer and cDNA template in a final volume of 20 μl. After initial denaturation for 10 min at 95°C, the reactions were cycled 40 times for 15 min at 95°C, 1 min at the annealing temperature of 60°C and 30 s at 72°C for extension of both Fibronectin and 18S ribosomal RNA (served as interbal control). Product specificity was determined by melting curve analysis. Relative quantitation of gene expression was performed using the 7500 Sequence Detection software v.1.4 (Applied Biosystems, Foster City, CA, USA).

12. Data analysis

Values reported for all data represent means ± standard error of means (127). For all studies, replicate data (2 or 3) from at least 3-4 different cell cultures were obtained. The statistical significance of differences between means was determined by either an unpaired two-tailed Student's t-test, or one-way ANOVA with either Bonferroni's Multiple Comparison Test or Tukey's multiple range test. Differences were considered to be statistically significant when $p < 0.05$.

Table 1: list of Primers sequence (5'-3') of mRNA for qPCR

Primers	**Forward Sequence**	**Reverse Sequence**
18s RNA	CGCCGCTAGAGGTGAAATTC	TTGGCAAATGCTTTCGCTC
Fibronetn	CGAAATCACAGCCAGTAG	ATCACATCCACACGGTAG
GAPDH	AGCAATGCCTCCTGCACCACCAAC	CCGGAGGGGCCATCCACAGTCT
RAGE	GAAATAATGCTTATGAATCCCAAAG	CACGCTCCTCCTCTTCCTCCTGGTTTTCTG
TLR4	TCCCTCCAGGTTCTTGATTACAGTC	TGCTCAGAAACTGCCAGGTCTG

CHAPTER IV. RESULTS

I. Effect of HMGB1 in activation of MAP Kinase pathway

In order to assess whether human airway smooth muscle cells are capable of responding to exogenous HMGB1, we determined the impact of HMGB1 on activation of MAP kinases. We focused on these effectors based on published reports that HMGB1-mediated signal transduction in cultured human endothelial cells is in part dependent on activation of MAP kinase pathways (154). Primary human ASM cells from three different donors were stimulated with HMGB1 (10 ng/mL) for up to 24 hours and whole cell lysates were collected at various time points during exposure. Using immunoblotting we measured (i) total ERK1 and 2, and phospho-Thr202/Tyr204 ERK1 and ERK2; (ii) total p38 kinase and phospho-Thr180/Tyr182 p38 kinase; and (iii) total JNK/SAPK as well as phospho-Thr185/Tyr185 JNK/SAPK. Phosphorylated protein abundance determined by densitometry and normalized to total ERK1/2, p38, JNK/SAPK as well as being adjusted for differences in loading, as assessed based on GAPDH abundance. HMGB1 stimulation significantly induced ERK1/2 phosphorylation 5 and 45 minutes after HMGB1 exposure ($n=3$, $P<0.05$) (Fig. 1A and 1B). The degree of ERK1/2 was similar to that induced by exposure to PDGF (10 ng/mL), which was used as a positive control (Fig. 1C).

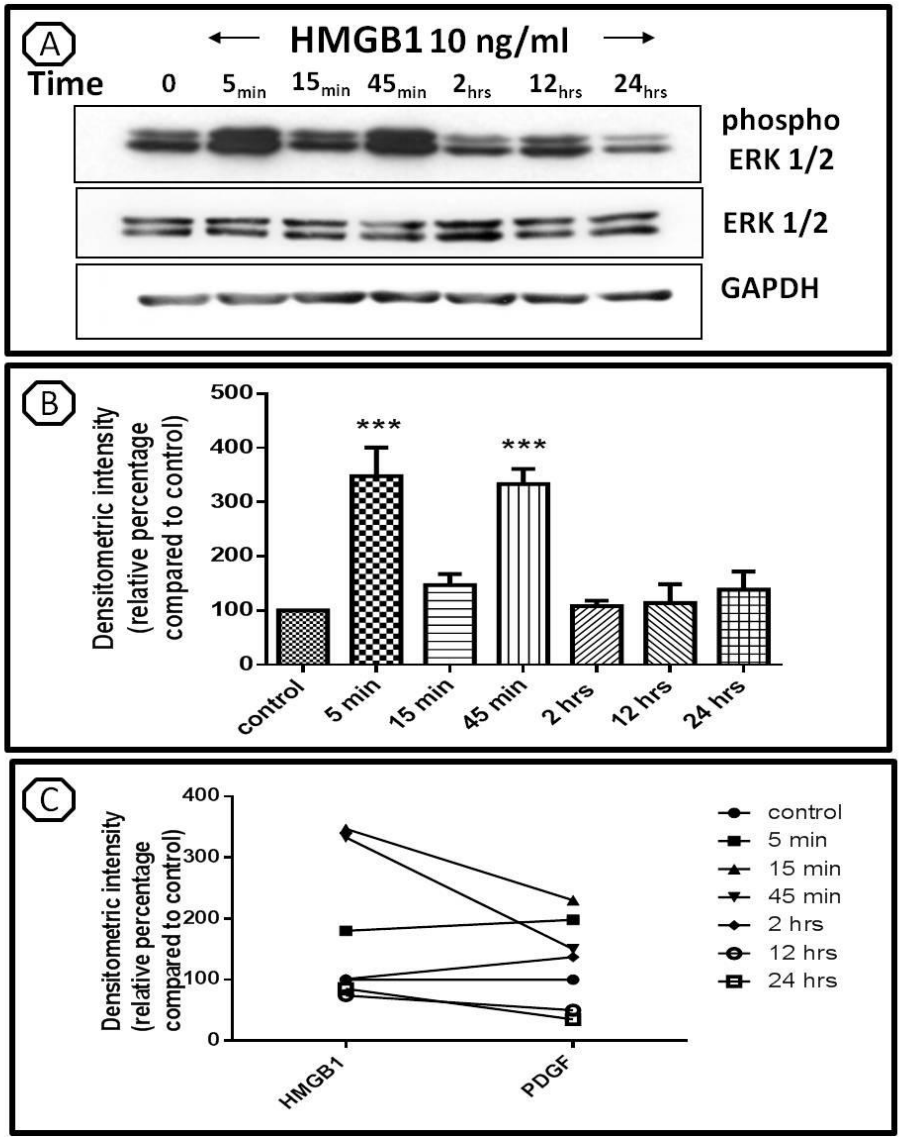


Figure 1: ***Effect of HMGB1 on the phosphorylation of ERK1/2.*** *Representative western blots from experiments carried out using three different primary human ASM cells (each from a different donor) are shown. These cells were stimulated with HMGB1 (10 ng/mL) up to 24 hours. Whole cell lysates were collected and probed with antibodies against total and phosphorylated ERK1/2 (A). For all analyses GAPDH was used as loading control. Panel (B) shows a bar graph summarizing densitometric intensity of blots in which each group was compared to the control group (unstimulated cells) represent mean ± SEM from at least three different experiments. One-way ANOVA was performed to compare data at each time point - P<0.001 (***) compared to control group (unstimulated cells). (C) The relative effects of HMGB1 and PDGF (10ng/mL) on ERK1/2 phosphorylation in individual cell lines are shown. Two-way ANOVA*

was performed to compare mean response to each stimulus – no significant difference was apparent (P=0.176)

HMGB1 also significantly augments JNK/SAPK phosphorylation within 15 and 45 minutes (n=3, $P<0.05$) (Fig. 2A and 2B), to a level similar to that induced by PDGF (10 ng/mL) (Fig. 2C) ($P=0.248$).

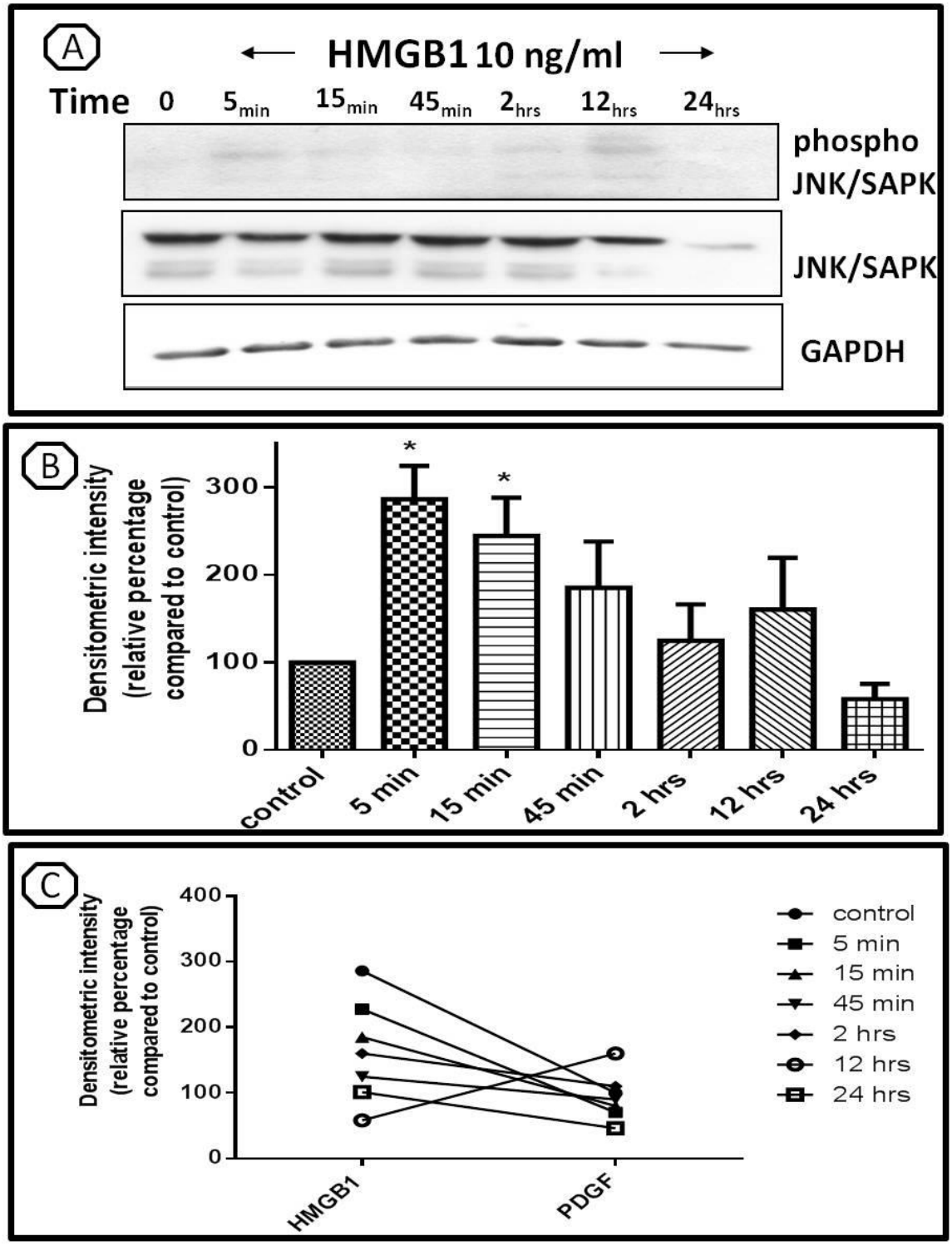


Figure 2: ***Effect of HMGB1 on the phosphorylation of JNK/SAPK.*** *Representative western blots from experiments carried out using three different primary human ASM cells (each from a different donor) are shown. These cells were stimulated with HMGB1 (10 ng/mL) up to 24 hours. Whole cell lysates were*

collected and probed with antibodies against total and phosphorylated JNK/SAPK (D). GAPDH was used as loading control. Panel (B) shows a bar graph representing mean ± SEM of densitometric analyses from at least three different experiments. One-way ANOVA was performed to compare time points, P<0.05 (*) compared to control group (unstimulated cells). Panel C demonstrates relative induction of JNK/SAPK phosphorylation by HMGB1 and PDGF (10 ng/mL) in specific cell lines. Two-way ANOVA was performed to compare mean phosphorylation at induced by each stimulus at each time point – no significant differences were found (P=0.248).

In contrast to the impact on ERK1/2 and JNK/SAPK, we did not see any significant effect of HMGB1 exposure on p38 MAPK phosphorylation (Fig. 3A and B).

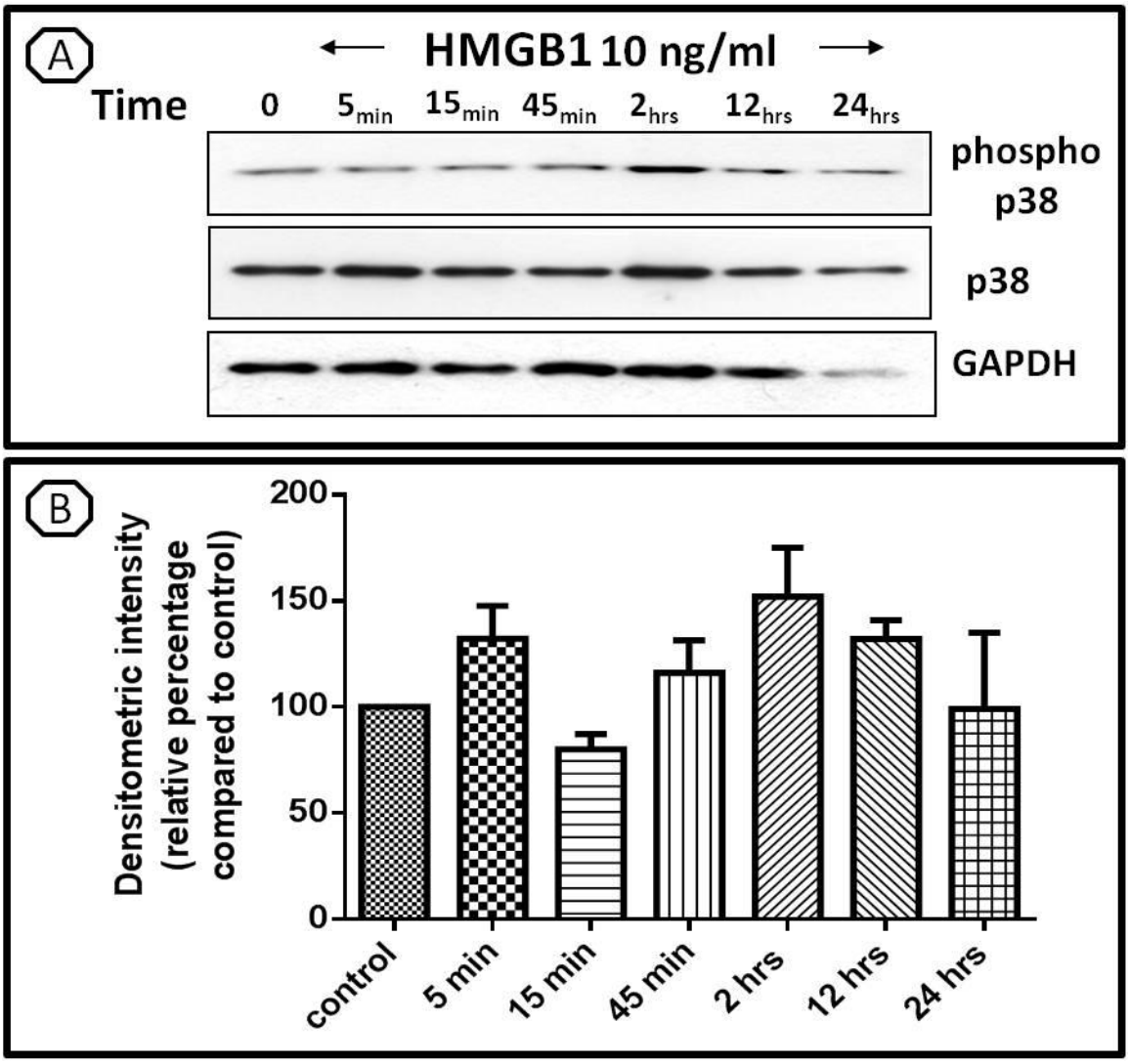


Figure 3: ***Effect of HMGB1 on the phosphorylation of p38 MAP kinase.*** *Representative western blots from experiments carried out using three different primary human ASM cells (each from a different donor) are shown. Cells were stimulated with HMGB1 (10 ng/mL) up to 24 hours. Whole cell lysates were collected and probed with antibodies against total and phosphorylated p38 (A). GAPDH was used as loading control. Panel B shows a bar graph summarizing data from densitometric analysis; mean ± SEM from at least three different experiments. One-way ANOVA was performed to compare means at each time point – no significant differences were identified (P=0.09).*

II. MAP Kinase activation by HMGB1 through RAGE-mDia1 axis

HMGB1 can bind and activate several receptors, including RAGE and TLR4 (175, 179, 277). Our lab had previously reported that human ASM cells express both RAGE and TLR4 (278). To assess whether exposure to recombinant HMGB1 induced any change in the abundance of RAGE and TLR4 we treated primary human ASM cells (HMGB1, 10 ng/mL) for up to 48 hours. RAGE and TLR4 mRNA abundance was measured using quantitative (real-time) PCR at different time points (Fig. 4A and 4B). Recombinant HMGB1 treatment did not significantly change the abundance of mRNA for RAGE (Fig. 4A) or TLR4 (Fig. 4B).

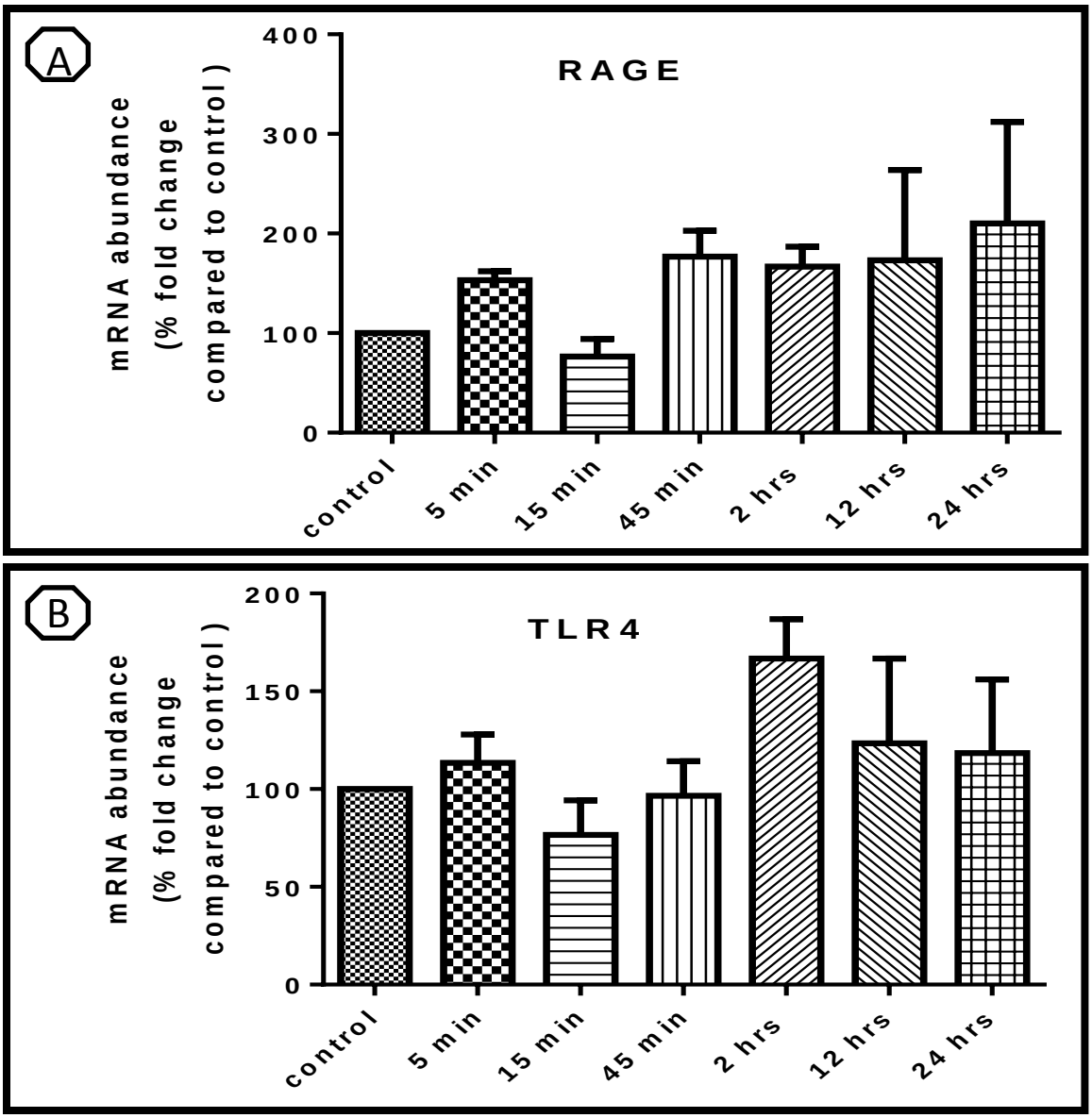


Figure 4: ***Effect of HMGB1 on RAGE and TLR4 mRNA abundance. Histograms show*** *results of quantitative RT-PCR for (A) RAGE and (B) TLR4 mRNA from lysates of primary human ASM cells stimulated with recombinant HMGB1 (10 ng/mL) for up to 48 hours. For all assays 18S RNA was used as an internal control. The data are expressed as abundance of mRNA in each group compared to control group (unstimulated cells); Panel A and B represent mean ± SEM from at least three different experiments respectively for RAGE and TLR4 realtive abundance, respectively. One-way ANOVA was performed to compare mRNA abundance at each time point – no significant differences were seen for either transcript across time points (n=3, P>0.05 for both RAGE and TLR4).*

As the lung is unique in that is expresses RAGE at high levels in healthy tissues, we further assessed the impact of HMGB1 treatment on the abundance of RAGE protein abundance in human airway smooth muscle cells. Cells were treated with HMGB1 (10 ng/mL) and whole cell protein lysates were collected at various time points thereafter, and immunoblotted to assess RAGE abundance (Fig. 5). Immunoblotting confirmed the qPCR analysis, revealing no visible effect of HMGB1 on RAGE abundance in airway smooth muscle cells.

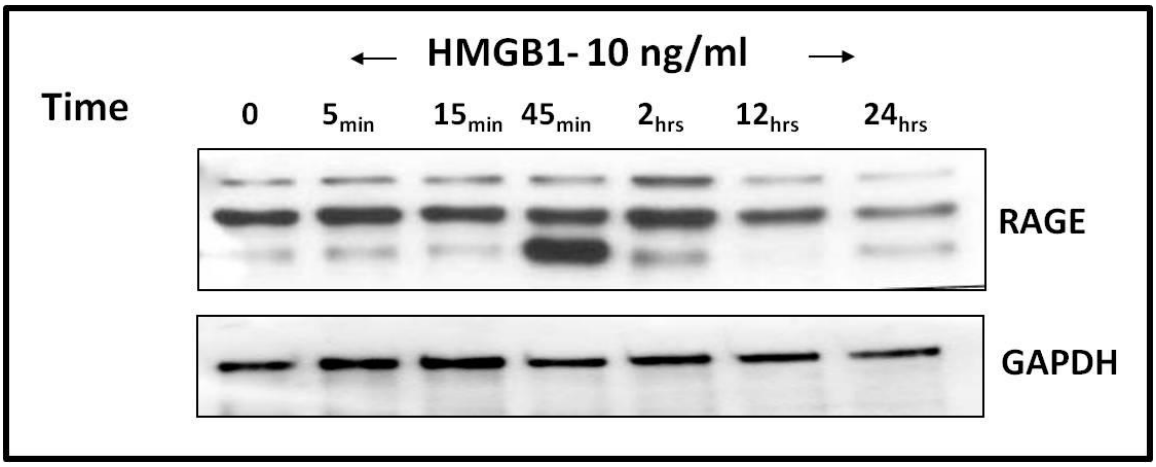


Figure 5: ***Effects of HMGB1 on RAGE protein abundance.*** *Representative western blots from experiments carried out using primary human ASM cell lines stimulated with recombinant HMGB1 (10 ng/mL) for up to 24 hours. Blots were probed with antibodies against human RAGE and GAPDH, the latter serving as a loading control. (min: minutes , hrs: hours)*

Though RAGE mediates multiple intracellular signal transduction pathways, it possesses a short cytoplasmic domain without catalytic capacity per se (187, 188). Recent work has revealed that the signaling protein anchor (scaffold protein), mammalian diaphanous 1 (mDia1), an adaptor protein that associates with and regulates small GTPases, is required for ligand-stimulated signaling through RAGE (187, 188, 259, 261,

279-281). To determine if mDia1 and RAGE are required for HMGB1-induced intracellular signaling in human ASM cells, we generated hTERT immortalized human ASM cell lines in which we stably silenced mDia1 expression with ectopic short hairpin RNA (shRNAi mDia1), expressed stably after lentiviral transduction. Using immunoblotting we observed that shRNA interference resulted in a reduction of mDia1 protein about 80-90% (Fig. 6).

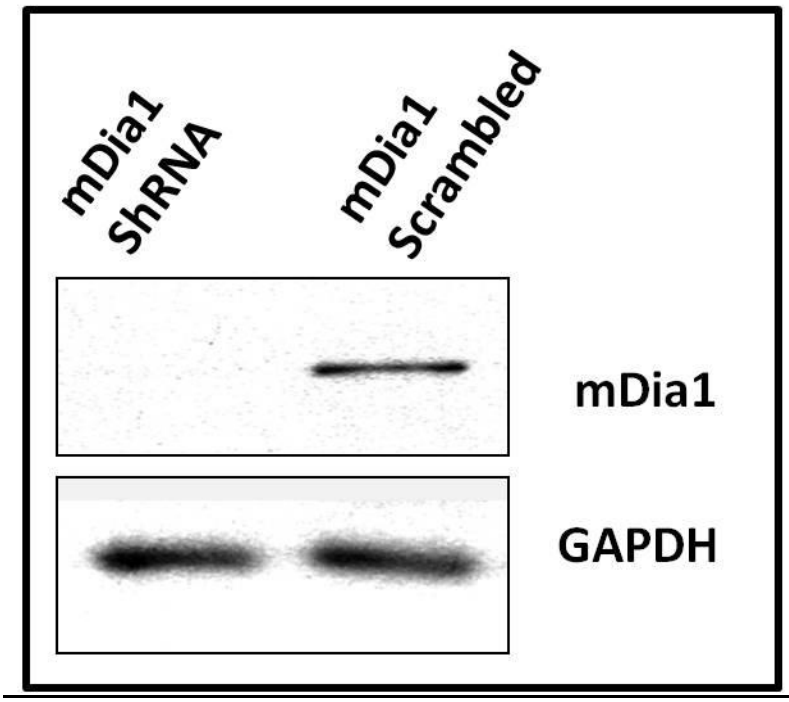


Figure 6: Lentivirus mediated delivery of shRNA to silence mDia1 expression. *A representative western blot is shown for two different hTERT immortalized human ASM cell lines (shRNAi mDia1 and shRNAi non-coding). GAPDH is shown as loading control. The blots shown are representative of those prepared for at least 3 different lines generated using mDia1-selective and non-coding shRNA.*

To determine whether mDia1 is needed for HMGB1-induced activation of MAP kinase pathways, we treated mDia1-silenced human ASM cell lines with HMGB1 (10

ng/mL) for up to 24 hours. As a control we treated human ASM cell lines that stably express a non-coding shRNAi. After HMGB1 exposure, we prepared whole cell lysates at different time points, then performed immunoblotting and probed with antibodies against total and phosphorylated ERK1/2 (Fig. 7A). We observed that in cells lacking mDia1, 5 and 15 minutes after treatment HMGB1 induced markedly less phosphorylation of ERK1/2 compared to ASM cell lines expressing non-coding shRNAi (n=3, $P<0.05$).

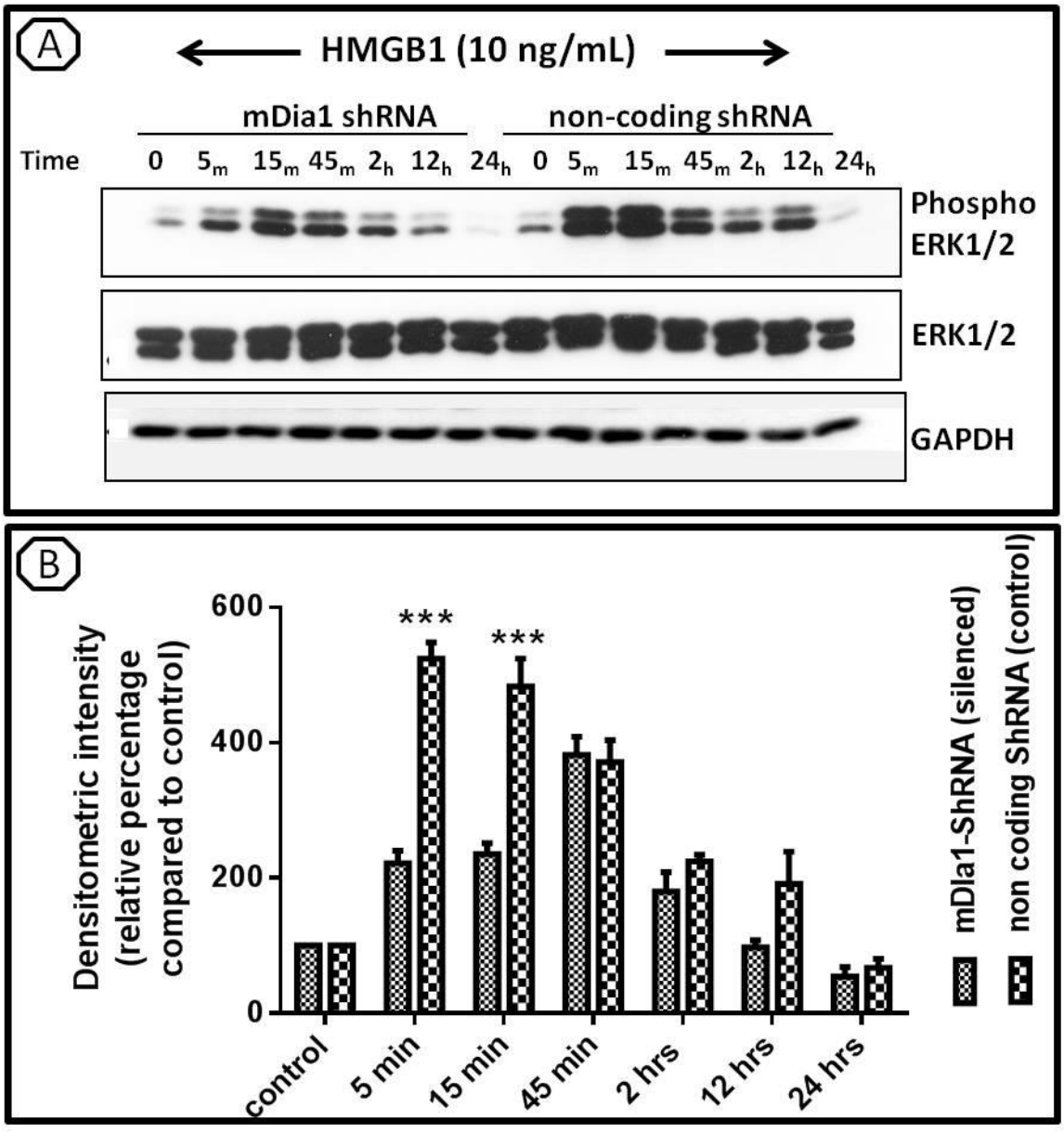


Figure 7: ***Role of mDia1 on HMGB1-induced phosphorylation of ERK1/2.*** *(A) Representative western blots were carried out using two different hTERT immortalized human ASM cell lines (shRNAi HMGB1 and shRNAi non-coding). Cells were stimulated with HMGB1 (10 ng/mL) for up to 24 hours. Blots using whole cell lysates were probed with antibodies against total and phosphorylated ERK1/2. GAPDH was used as a loading control. (B) Bar graph representing mean ± SEM from densitometry of blots from three different experiments. Two-way ANOVA was performed to compare data in different cell lines at each time point, (n=3, P<0.001 (***) compared to time match control group).*

We also assessed the impact of mDia1 silencing on HMGB1-induced JNK/SAPK phosphorylation (Fig. 8A). Consistent with our analysis of ERK1/2, densitometric analysis of blots revealed that HMGB1-induced phosphorylation of JNK/SAPK at15 and 45 minutes after stimulation was markedly suppressed in ASM cells with silenced mDia1 compared to cells expressing non-coding shRNAi (n=3, $P<0.05$) (Fig 8B).

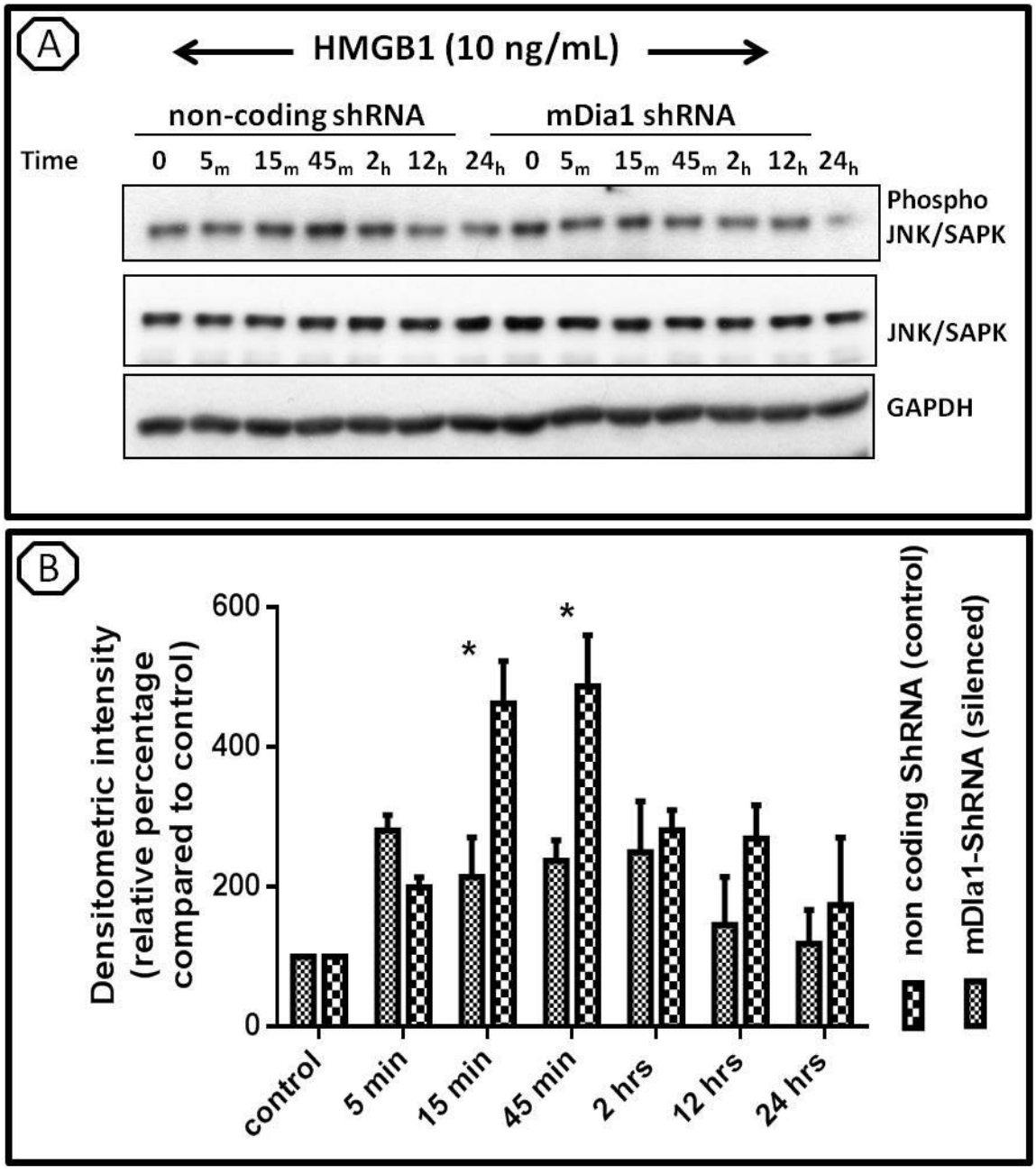


Figure 8: ***Role of mDia1 in HMGB1-induced phosphorylation of JNK/SAPK.*** *(A) A representative western blot for hTERT immortalized human ASM cell lines (shRNAi HMGB1 and shRNAi non-coding) is shown. Cells were stimulated with HMGB1 (10 ng/mL) for up to 24 hours, and whole cell lysates were collected for immunoblotting using antibodies against total and phosphorylated JNK/SAPK. GAPDH was used as a loading control. (B) Bar graph represents mean ± SEM from densitometry of immunoblots from*

three different experiments. Two-way ANOVA was performed to compare data from cell lines at each time point (n=3, P<0.001 (***), and p<0.01 (**) compared to time match control group).

Experiments using novel human ASM cell lines lacking expression of mDia1 indicated that HMGB1-driven induction of JNK/SAPK and ERK1/2 requires mDia1. As mDia1 is a known mediator of signal transduction pathways downstream of RAGE, our data suggest that an HMGB1-RAGE-mDia1 axis may exist in human ASM cells.

III. HMGB1 effects on ASM cells: impact of RAGE inhibition

To determine the role of RAGE in human ASM cells, we used different concentrations of a monoclonal blocking antibody against human RAGE (MAB11451) and assessed impact on responses to exogenously added HMGB1. For these studies, primary human ASM cells were pretreated with RAGE blocking antibody (40 ng/mL, 100 ng/mL, 200 ng/mL, 1 µg/ml, 30 µg/ml and 100 µg/ml) for 2 hours, then stimulated cell cultures with HMGB1 (10ng/mL). Whole cell lysates were collected and immunoblot analysis was performed for phosphorylation of ERK 1/2. GAPDH was used as loading control (Figure 9A and 9B). We found no evidence that this blocking RAGE antibody could block HMGB1 activation of ERK1/2 phosphorylation. Pretreatment of ASM cells with higher concentrations of RAGE blocking antibody (> 1 µg/ml) did not cause change in HMGB-1 induced ERK1/2 phosphorylation (compared to media alone). A limitation to our conclusions for these experiments using RAGE blocking antibody (MAB11451) is that the antibody alone did induce phosphorylation of ERK 1/2 prior to stimulation with HMGB1. To determine whether this effect was due to the selectivity of MAB11451 for RAGE, we completed an additional study using pretreatment with isotype matched monoclonal Mouse IgG2B. Primary human ASM cells pretreated with Mouse IgG2B (1-

100 µg/ml, 2hrs) (Figure 9C). Immunoblotting revealed that this antibody alone also induced ERK1/2 phosphorylation compared to basal level, Importantly, unlike RAGE-blocking MAB11451 used at the same concentrations, this did not affect the capacity of HMGB1 to induce ERK to further increase ERK1/2 activity. Collectively these observations offer evidence that RAGE can mediate HMGB1-induced ERK activation in primary human ASM cells.

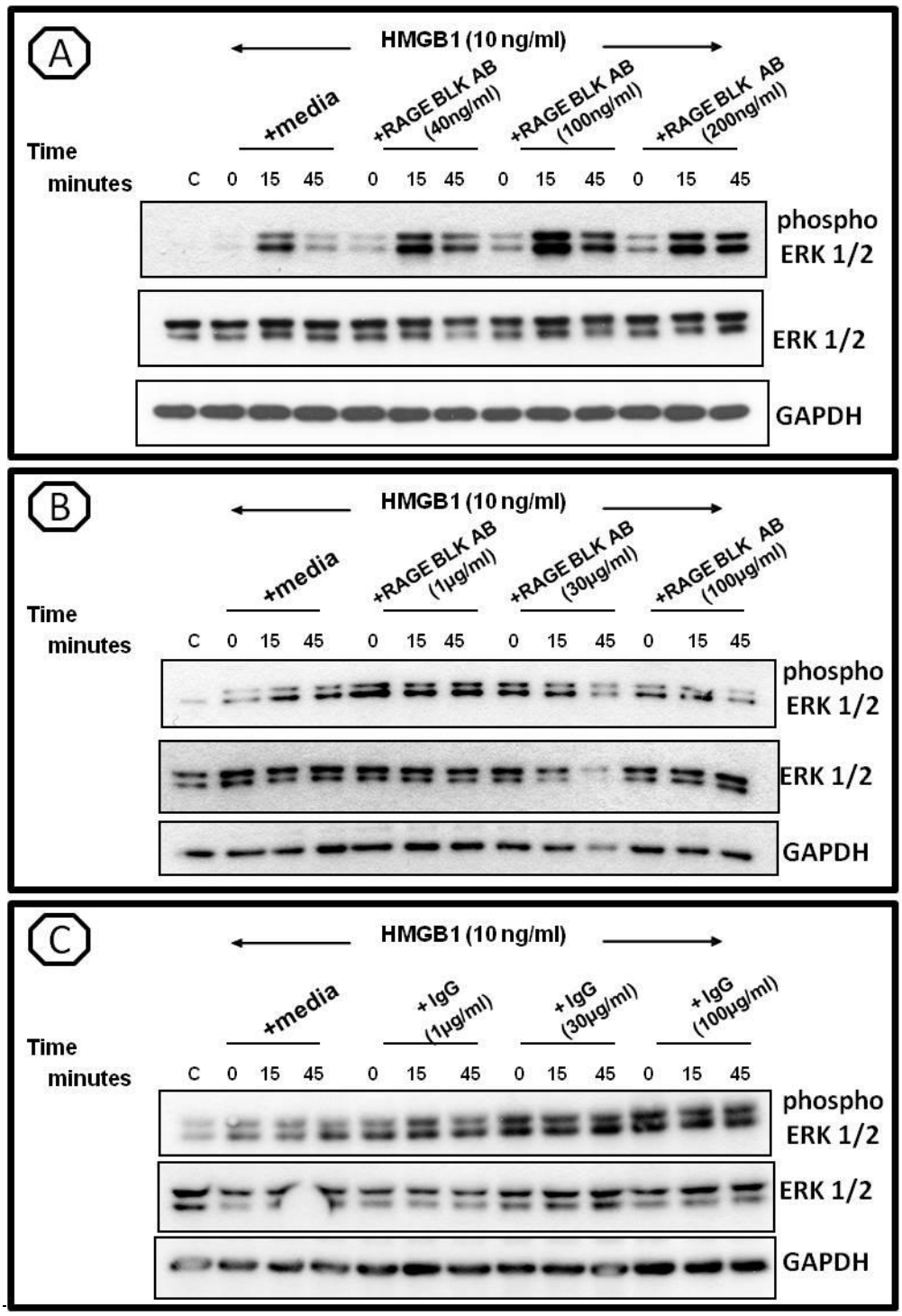


Figure 9: ***Effect of RAGE blocking antibody on the phosphorylation of ERK1/2.*** *Representative Western blots from experiments carried out using primary human ASM cells. (A and B) These cells were treated for two hours with a human RAGE antibody (MAB11451) at various concentrations (40 ng/mL-100 µg/mL). After two hours of incubation with RAGE blocking antibody, recombinant HMGB1 (10 ng/mL) was added*

to the culture medium. Whole cell lysates were collected at varying time points and probed with antibodies against total and phosphorylated ERK1/2. GAPDH was used as a loading control. (C) In order to interpret the results we used similar antibody (Monoclonal Mouse IgG2B). The same procedures followed for this experiment with different concentration of Monoclonal Mouse IgG2B instead of RAGE antibody (MAB11451). (RAGE BLK Ab: RAGE blocking antibody, IgG: Monoclonal Mouse IgG2B)

IV. **Activation of NFκB by HMGB1**

In addition to its effects on MAP kinase signaling, HMGB1 can also promote activation of NFκB, including nuclear translocation of p50/p65 and activation of NFκB-driven gene transcription (195). To assess the effects of HMGB1 on activation of the NFκB pathway, we measured phosphorylation of IκB; this event is associated with dissociation of IκB from p50/p65 subunits, permitting their translocation to the nucleus (282). We stimulated primary human ASM cells with HMGB1 (10 ng/mL), then whole cell lysates were collected and immunoblotting was employed to assess total IκB and phospho-IκB abundance (Figure10A). HMGB1 did not induce detectable phosphorylation of IκB suggesting failure to activate this signaling pathway. To further investigate the potential for HMGB1 to induce NFκB, we next used a commercial ELISA kit to more directly measure nuclear translocation of NFκB (Figure 10B) Activation of NF-κB p50 in 3 different primary human ASM cells stimulated with HMGB1 (10ng/mL) determined using a highly sensitive and specific Transcription Factor ELISA Kit according to the instructions of the manufacturer (catalogue number EK1121; Panomics, Fremont, CA, USA). In fact the activated NFκB p50 (phospho p50) molecules from nuclear extracts of ASM cells bind to an NFκB p50 consensus binding site (NFκB p50 Probe) on a biotinylated oligonucleotide. The activated NFκB p50 (phospho p50), bound to the oligonucleotide, is detected by an antibody directed against NFκB p50 on the

ELISA kit. An additional horseradish peroxidase (HRP)-conjugated secondary antibody reacts with the substrate to provide a sensitive colorimetric readout which has been quantified by spectrophotometry and compared to positive control (nuclear extract or recombinant protein for positive assay control), negative control (specific cold probe for negative assay control) and matched time control with no stimulation; Both positive and negative control is provided by manufacturer. Consistent with immunoblot analysis of IκB phosphorylation, we did not detect any induction of NFκB in HMGB1-treated cells ($n=3$, $P>0.05$). Taken together these data indicate that HMGB1 does not activate NFκB signaling in human ASM cells.

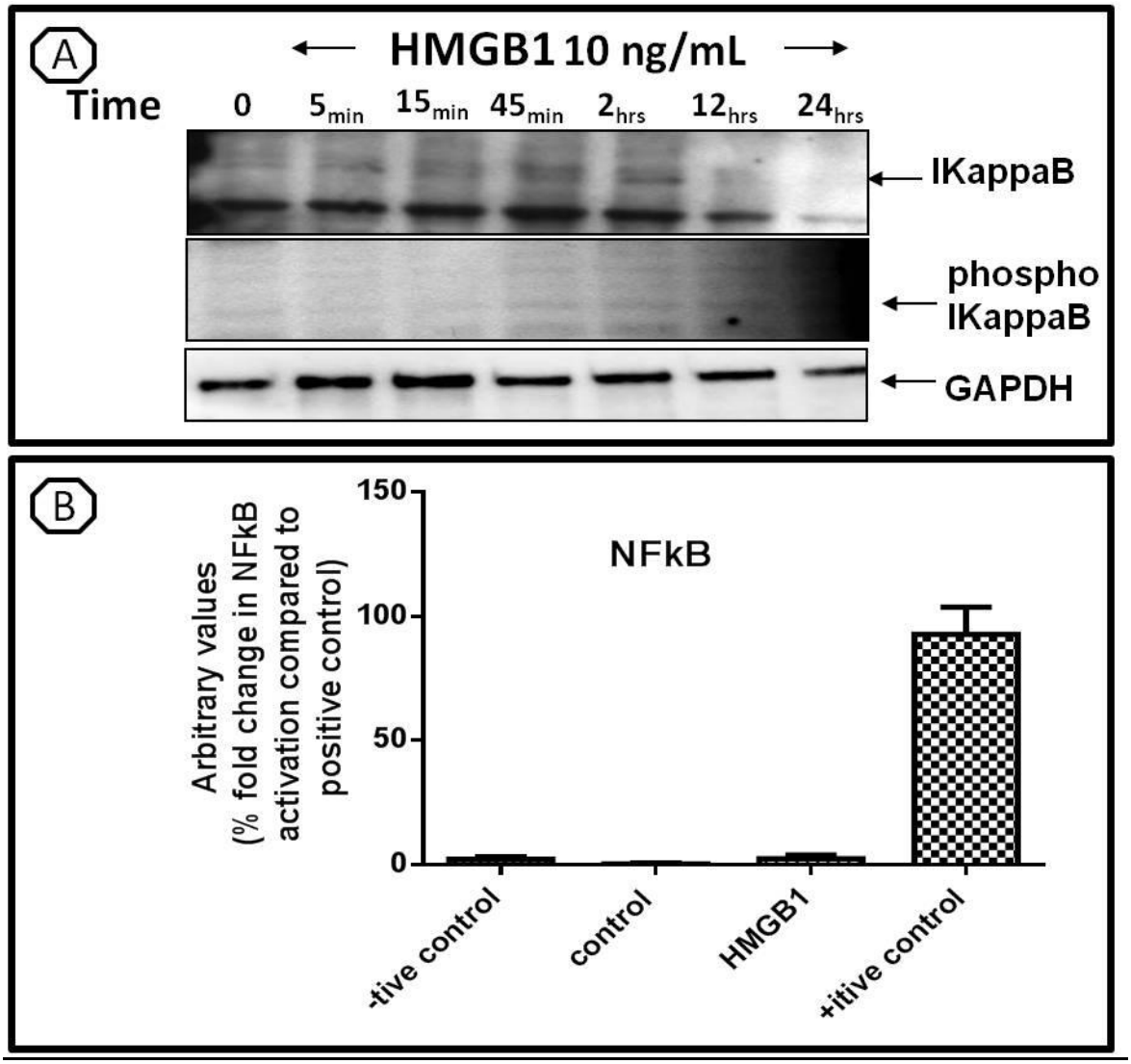


Figure 10: ***Effects of HMGB1 on NFκB pathway activity in human ASM cells.*** *(A) Representative western blots for primary human ASM cells stimulated with HMGB1 (10 ng/mL) for up to 24 hours. Whole cell lysates were collected and immunoblotting with antibodies against total and phosphorylated IκB was performed. GAPDH was used as a loading control. (B) Bar graph summarizing data obtained using an ELISA-based assay for activated p50 nuclear translocation in response to HMGB1 treatment for 30 minutes. The negative and positive controls supplied with the kit were used. One-way ANOVA was performed to compare data at different time points – no differences were seen for HMGB1-treated cultures compared to negative control (n=3, P>0.05). (-tive control (specific cold probe for negative assay control ,control: time-matched control group with no stimulation, +tive control: nuclear extract or recombinant protein for positive assay control)*

V. HMGB1 does not induce proliferation of primary human ASM cells

As we established that HMGB1 could induce MAP kinase signal transduction, we next assessed whether HMGB1 could affect changes in cell function. There are many reports linking the activation of MAP kinase pathways to cell proliferation and survival (283, 284). Thus, we assessed whether HMGB1 induces human ASM proliferation by stimulating 3 different primary human ASM cells (each from a different donor) with HMGB1 (10 ng/mL) for up to 3 days, then performing cell counts and comparing to a negative control (cells in medium alone) and a positive control (medium supplemented with 10% FBS). Figure 11 demonstrates that recombinant HMGB1 alone does not promote cell proliferation, as cell numbers were unchanged compared to media-alone treated cells, and was significantly less than that measured for cells grown in FBS-containing media.

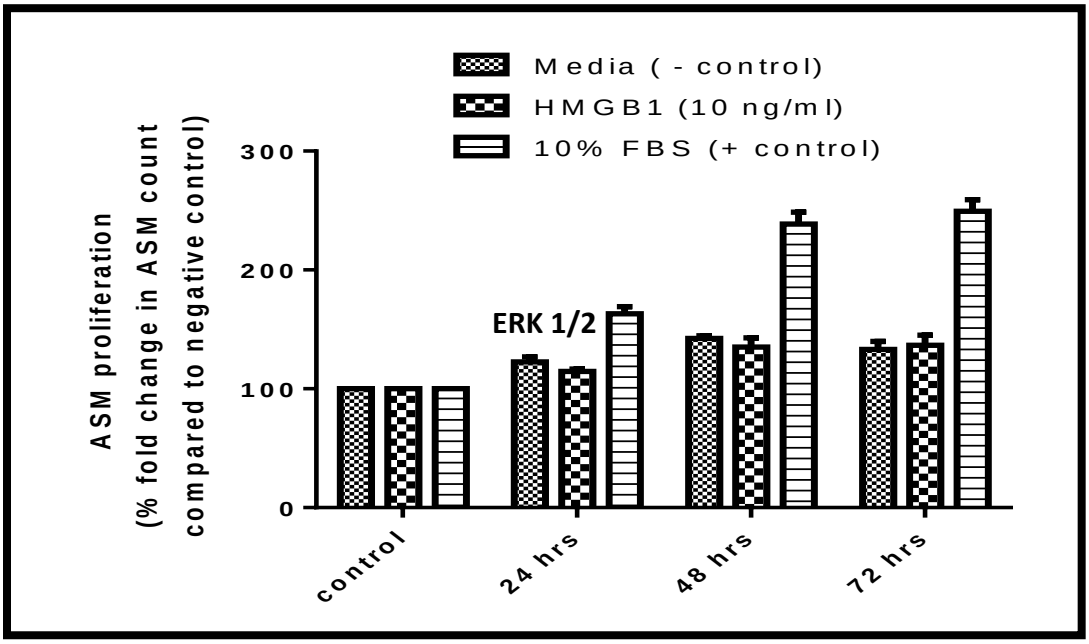


Figure 11: Effects of HMGB1 on ASM cell proliferation. *Bar graph showing results from*

cell counting (performed using a hemocytometer) of primary human ASM cells stimulated with recombinant HMGB1 (10 ng/mL) for up to 72 hours. Data for cells in media alone and media supplemented with 10% FBS are also shown. Bar graph represents mean ± SEM from at least three different experiments on ASM cells obtained from three different donors. (FBS: Fetal Bovine Serum which used as positive control). Two-way ANOVA was performed to determine the significance of data, (n=3, P>0.05).

VI. Role of HMGB1 and RAGE in secretion of ECM proteins in response to TGF-β1

As ASM cells are capable of significant expression of extracellular matrix (ECM) proteins, we next tested whether HMGB1 modulates this response. To this end we confirmed the magnitude of the effect of TGF-β1, the principal pro-fibrotic cytokine associated with asthma (285), on ECM protein expression. Serum deprived, immortalized human ASM cells were stimulated with TGF-β1 (or media-alone as a negative control) for up to 120 hours, and then we performed immunoblotting on whole cell lysates, using collage 1a-selective primary antibodies. . As shown in Fig.12, in contrast to negative control (media alone), TGF-β1 stimulation markedly induced collagen abundance, with peak induction being attained after 48 hours.

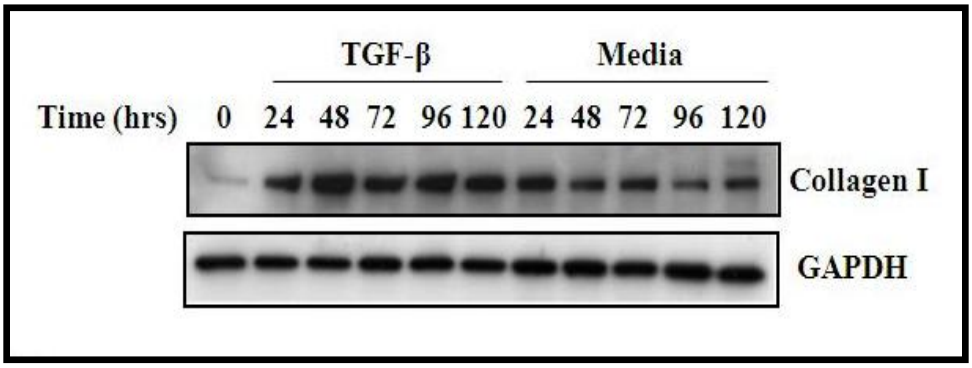
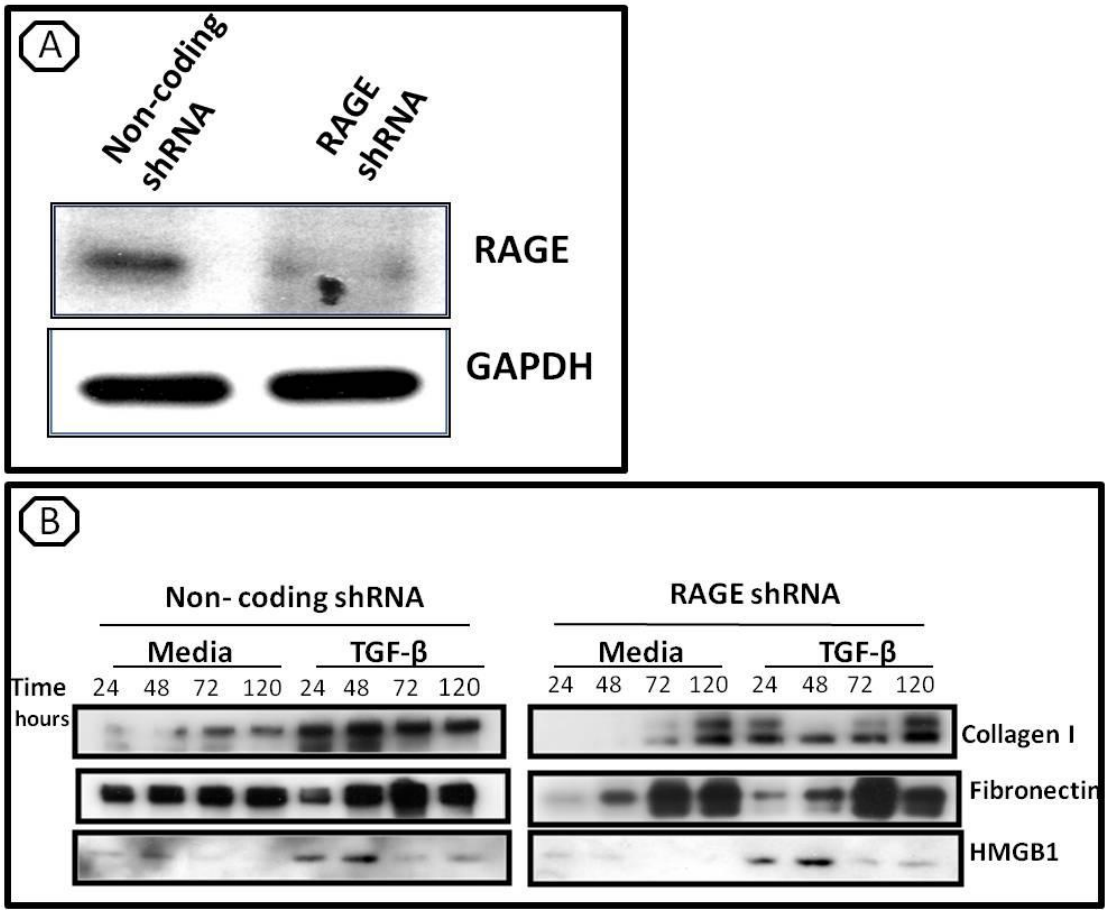


Figure 12: ***Role of TGF-β1 in collagen synthesis and secretion from ASM cells.*** *Representative western blot demonstrating results from 3 different hTERT immortalized human ASM cell lines that were stimulated with TGF-β1 (2.5 ng/mL) for up to 5 days. Whole cell lysates were collected and immunoblots were probed with antibodies against Collagen A1. GAPDH was used as a loading control.*

In another set of experiments, we prepared hTERT cell lines in which we stably silenced expression of RAGE through ectopic expression of selective shRNA. For control cells we also prepared hTERT ASM cell lines that stably express non-coding shRNA ("non-coding-shRNA in Fig. 13A). To confirm silencing of RAGE expression we prepared whoile cell lysates and performed immunoblotting. Figure 13A demonstrates that RAGE was virtually abolished in the cell lines we prepared.

We next assessed RAGE-silenced and non-coding-shRNA cell lines by exposing both groups of the cells to TGF-β1 (2.5 ng/mL) for up to 5 days. We also prepared time-matched negative controls (media exposure without TGF-β1). We collected cell culture media every 24 hours during the treatment period. For subsequent immunoblot analysis, media was concentrated ~10-fold by microcentrifugation (2000 ×g, 30 min) through a <10 kDa MW cutoff filter insert (Amicon Ultra-15 Centrifugal Filter Units). Concentrated cell culture media samples were subjected to immunoblotting to assess relative abundance of secreted HMGB1, collagen A1 and fibronectin (Figure 13B). We observed that TGF-β1-induced release of collagen, fibronectin and HMGB1 did not appear to be affected in RAGE-silenced cell lines compared to non-coding-shRNA

expressing hTERT cells.



<u>Figure 13:</u> Differential expression of ECM proteins in RAGE knock-down and control ASM cells after TGF-β1 treatment*. (A) Representative western blot demonstrating impact of ectopic expression of RAGE shRNA compared to non-coding-shRNA in hTERT cell lines (shRNAi RAGE and shRNAi non-coding, respectively). GAPDH is shown as loading control. (B) Representative Western blots of concentrated cell culture medium from experiments using RAGE shRNA compared to non-coding-shRNA in hTERT cell lines. Cells were stimulated with TGF-β1 (2.5 ng/mL) up to 5 days. Culture medium was collected at various time points and concentrated prior to analysis. For all lanes 20 μg of total protein was loaded.*

We also investigated whether an autocrine loop might exist involving secreted HMGB1 in TGF-β1-induced effects in human ASM cells. We stimulated primary human ASM cells from 3 different donors with TGF-β1 (2.5 ng/mL) for up to 72 hours, and

collected culture media to determine whether HMGB1 was being released. In media that was concentrated using <10kDa MW cut off filters, immunoblot analysis revealed no evidence for HMGB1 accumulation, indicating that TGF-β1 alone is not sufficient to directly induce release secretion of HMGB1 (Figure 14). This is consistent with the fact that RAGE-silencing had no impact on TGF-β1-induced ECM expression, and that HMGB1 release is most typically associated with cell stress and damage (but not as a component of cytokine-induced inflammation per se) (196).

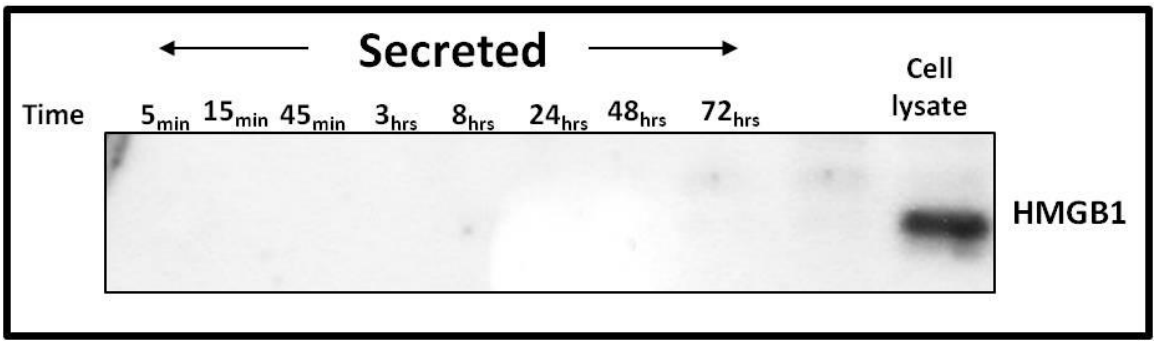


***Figure 14*: TGF-β1 does not induce HMGB1 secretion from ASM cells.** *Representative western blots are show for cell culture media that was collected for up to 72 hours after stimulating three different primary cultured human ASM cell lines with TGF-β1 (2.5 ng/mL). Prior to immunoblotting for HMGB-1, culture medium was concentrated by ~30 times by micro-centrifugation through <10kDa MW cut-off filters. As a positive control whole cell lysates from the cultures understudy were also subjected to immunoblotting for HMGB1 (far right lane).*

We next assessed whether exogenously added HMGB1 is capable of inducing ECM expression in human ASM cells. In our first experiment, we treated hTERT immortalized human ASM cells with recombinant HMGB1 at three different concentrations (1, 10 and 50 ng/mL) for up to 72 hours (Figure 15A). We performed immunblot analysis for collagen 1a using whole cell lysates and found that both 10 and 50 ng/mL HMGB1 is

sufficient to increase expression of collagen compared to time matched negative control cultures.

We further compared the capacity of recombinant HMGB1 (10 ng/mL) and TGF-β1 (2.5 ng/mL) to induce expression of collagen 1a and fibronectin in primary human ASM cells. Cultures were treated with HMGB1 or TGF-β1 for up to 72 hours, and we collected both culture medium and whole cell lysates at different time points. Figure 15B shows immunoblot analyses of whole cell lysates that reveals TGF-β1, but not HMGB1, was sufficient to notably increase cellular ECM protein content. Similarly, the abundance of secreted collagen and fibronectin in cell culture medium concentrates, did not suggest that HMGB1 augmented basal release of either protein (Figure 15C). These data contrast with that seen in our prliminay study in hTERT ASM cells. Though these data could suggest a fundamental difference in these cell lines, they more likely reflect a change in oxidation state of the recombinant HMGB1 used in our experiments. Work with hTERT cells was performed early, whereas experiments with primary cell lines used HMGB1 that had be stored for a number of months. Emerging evidence suggests that oxidation of recombinant HMGB1 changes biological activity (286, 287). This issue will be assessed in more detail in subsequent Discussion sections. Our work with primary human ASM cells suggests that exogenous HMGB1 is not a potent inducer of ECM protein synthesis or secretion, as compared to TGF-β1.

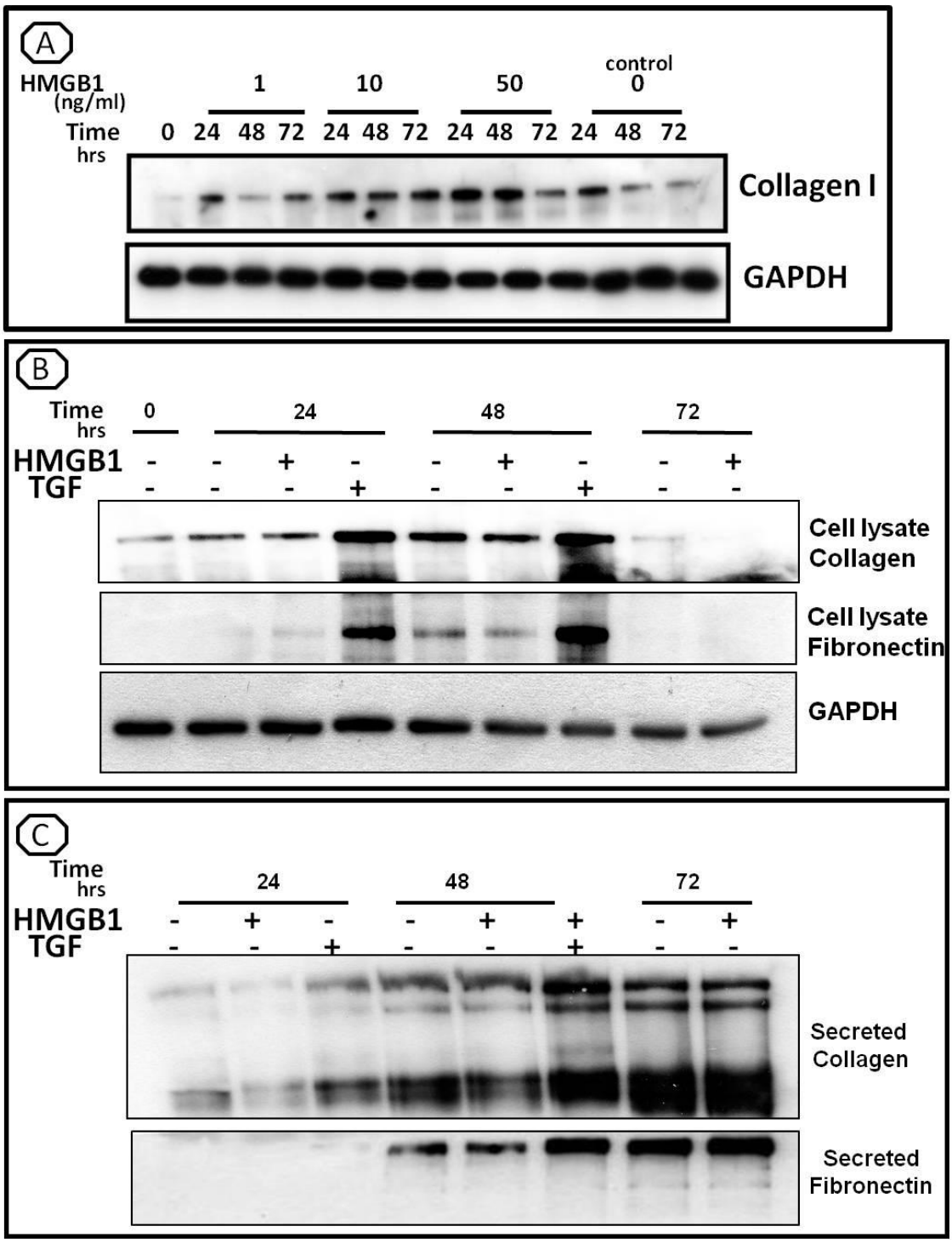


Figure 15: HMGB1 and ECM protein expression. *(A) Representative immunoblots of whole cell lysates prepared from an hTERT human ASM cell line exposed to different concentrations of recombinant HMGB1 (10, 50 and 100 ng/mL) for up to 72 hours. Blots were probed with antibodies for collagen 1a. In each lane*

20 μg of protein from cell lysates was loaded. GAPDH was used as a loading control. Blot is representative of 3 experiments. (B) Representative immunoblot for collagen 1a and fibronectin in whole cell lysates obtained from primary human ASM cell lines that were exposed to recombinant HMGB1 (10 ng/mL) or TGF-β1 (2.5 ng/mL) for up to 72 hours. For each lane 20 μg of total protein was loaded. Blots are representative of 3 experiments using 3 different primary cell lines. GAPDH was used as a loading control. (C)) Representative immunoblot for collagen 1a and fibronectin in concentrated culture medium from primary and human ASM cell lines that were exposed to recombinant HMGB1 (10 ng/mL) or TGF-β1 (2.5 ng/mL for up to 72 hours. For each lane 20 μg of total protein was loaded. Blots are representative of 3 experiments using 3different primary cell lines.

After finding that exogenous HMGB1 alone appears not to be sufficient to induce collagen or fibronectin expression in primary human ASM cells, we tested if combining HMGB1 with TGF-β1 might augment ECM expression. We added recombinant HMGB1 (10 ng/mL), or TGF-β1 (2.5 ng/mL), or HMGB1 (10 ng/mL) and TGF-β1 (2.5 ng/mL) together to serum deprived primary human ASM cells for up to 72 hours, collecting both supernatant and whole cell lysates at different time points. We used immunoblotting to measure the expression of cell-associated and secreted collagen 1a and fibronectin and found that HMGB1 did not increase TGF-β1 –induced expression or secretion (Figs 16A and 16B).

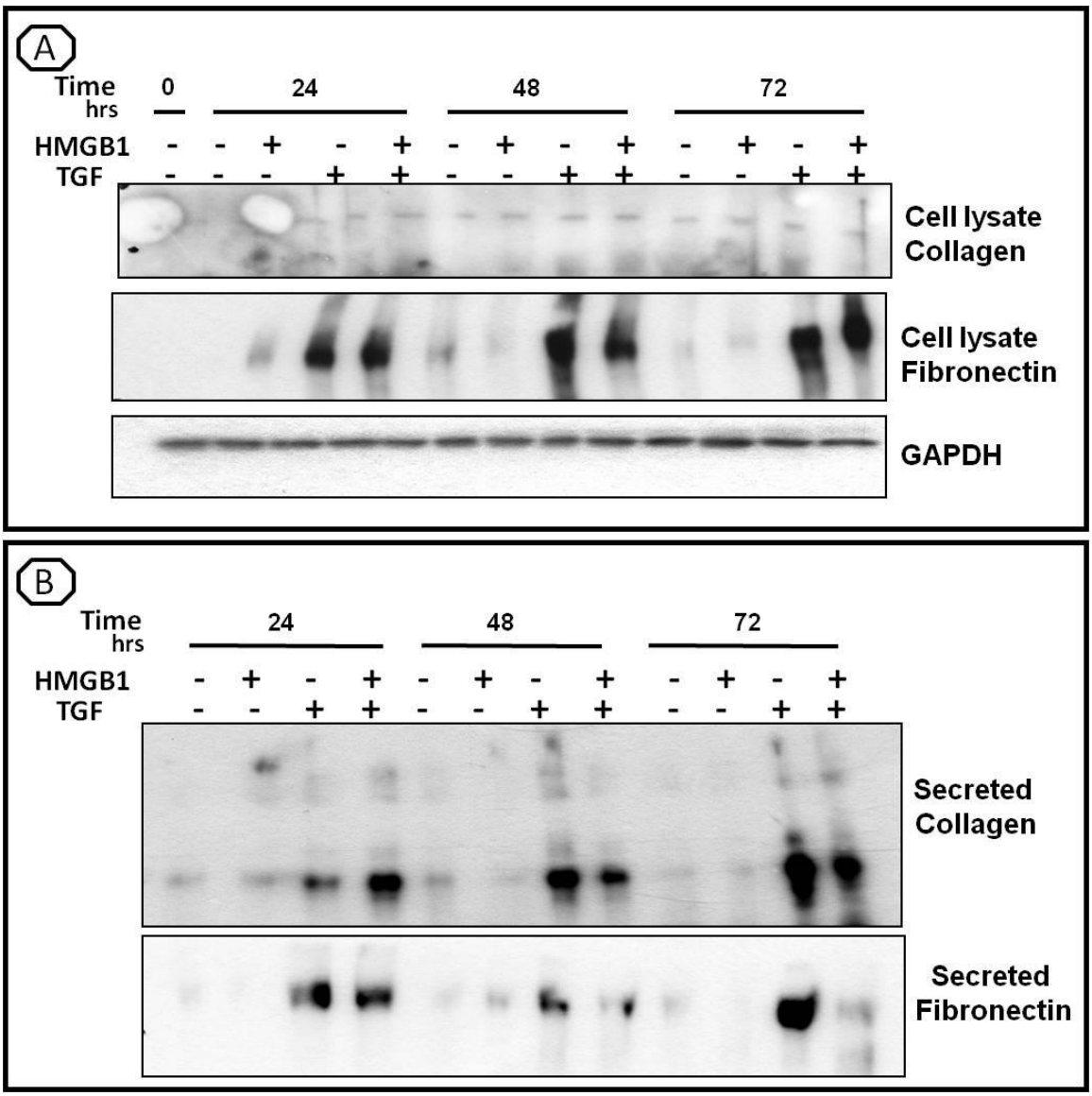


Figure 16: Impact of combining HMGB1 with TGF-β1 on ECM protein expression by primary human ASM cells. *(A) Representative immunoblots for collagen 1a and fibronectin from whole cell lysates from primary human ASM cell lines treated with HMGB1 (10 ng/mL), TGF-β1 (2.5 ng/mL) or HMGB1 (10 ng/mL) and TGF-β1 (2.5 ng/mL) combined for up to 72 hours. Samples from cultures that were maintained in medium alone used as time matched controls. Each lane was loaded with 20 μg of total protein lysate. GAPDH was used as a loading control. The blot shown is representative of 3 experiments using 3 different primary cell lines.*

We next tested whether the apparent lack of effect of HMGB1 on ECM expression and secretion induced by 2.5 ng/mL TGF-β1 might be due to our use of a concentration of TGF-β1 that we determined to induce a near-maximum response. To test this hypothesis we stimulated primary human ASM cells with HMGB1 (10 ng/mL), sub-optimal concentrations of TGF-β1 (0.1, 1 and 2.5 ng/mL) or HMGB1 (10 ng/mL) combined with TGF-β1 (0.1, 1 and 2.5 ng/mL). Whole cell lysates were collected 48 hours post stimulation and assessed by immunoblotting for cell collagen 1a and fibronectin abundance (Fig. 17). We observed that TGF-β1 at concentrations as low as 0.1 ng/mL was able to induce ECM expression. However, despite combining HMGB with low concentrations of TGF-β1 we did not detect any increase in collagen 1a or fibronectin protein compared to that induced by TGF-β1 alone. Taken together, these experiments show that exogenous HMGB1 alone, or in combination with TGF-β1 does induce ECM expression in human ASM cells.

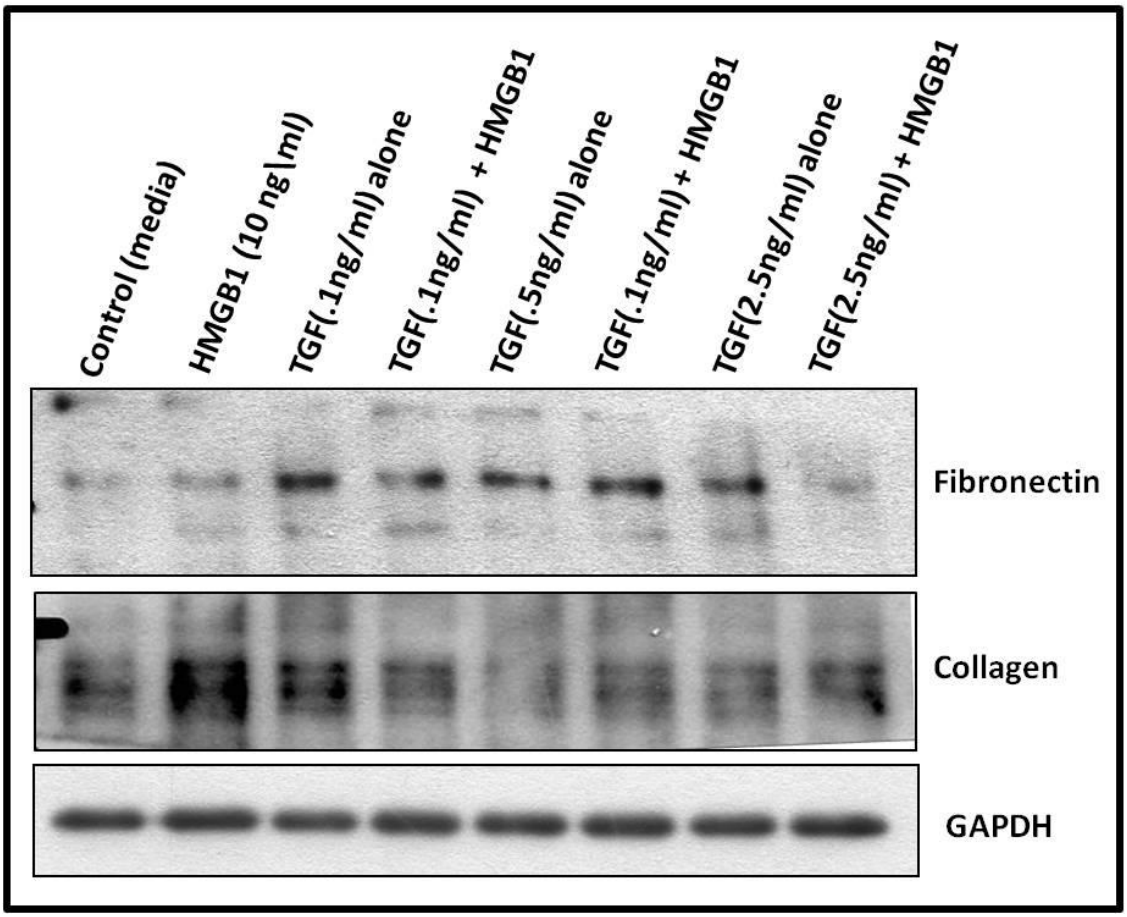


Figure 17: Impact of combining HMGB1 with different concentrations of TGF-β1 on ECM expression by primary human ASM cellsTGF-β1. *Representative immunoblots are shown for primary human ASM cells treated with HMGB1 (10 ng/mL), TGF-β1 (0.1, 1 and 2.5 ng/mL) or HMGB1 (10 ng/mL) and TGF-β1 (0.1, 1 and 2.5 ng/mL). Whole cell lysates were collected 48 hours post stimulation. The samples were probed with antibodies for collagen 1a or fibronectin. GAPDH was used as a loading control. For each lane, 20 μg of total protein lysate was loaded. The blot is representative of 3 experiments using 3 different cell lines.*

VII. Silencing endogenous HMGB1 impairs TGF-β1-induced ECM protein expression

Our experiments indicate that ECM protein expression by human ASM cells is refractory to exogenous HMGB1. To assess the role for endogenous HMGB1, in hTERT ASM cell lines we generated HMGB1 deficient cells by stable ectopic expression of selective shRNAi using lentivirus transduction and subsequent antibiotic selection. For control cells, in the same hTERT cell lines we used lentiviral transfer to generate cells that stably express non-coding scrambled-shRNA We suceeded to generate two separate HMGB1 silenced hTERT cell lines in which endogenous expression levels of HMGB1 protein was diminished by over 95% compared to the initial hTERT cells as well as in non-coding shRNA control lines (Fig. 18A). Using these cell lines we investigated the impact of endogenous HMGB1 suppression on TGF-β1-induced collagen 1 and fibronectin protein. For these experiments, after 48 hours of serum deprivation cells were stimulated with TGF-β1 (2.5 ng/mL) for up to 48 hours, using cells in TGF-β1-deficient culture media as a negative control group. As shown in Fig. 18B, in whole cell lysates we observed that silencing HMGB1 expression markedly blunted TGF-β1-induced ECM protein synthesis. These data suggest an enhancer role for endogenous HMGB1 in TGF-β1 induced ECM expression.

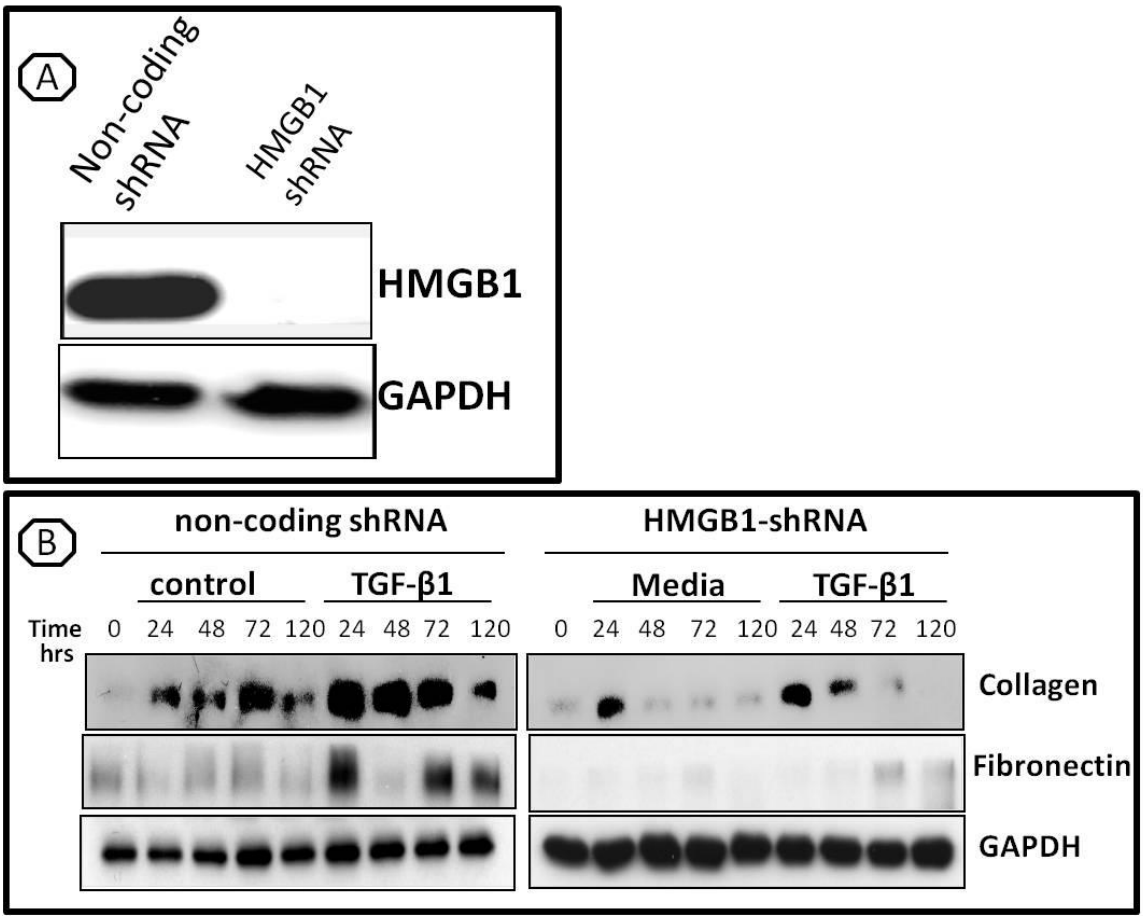


Figure 18: Impact of silencing HMGB1 on TGF-β1 induced ECM expression. *(A) Representative western blots showing HMGB1 expression in hTERT cell lines generated having with stable ectopic expression of shRNA for HMGB1 or non-coding scrambled-shRNA. GAPDH is shown as loading control. For the lanes shown, 20 μg of total protein from whole cell lysates was loaded. The blot is representative of lysates obtained from 3 different cell lines. (B) Western blots showing impact of TGF-β1 exposure (2.5ng/mL, up to 5 days) on the expression of collagen and fibronectin protein in hTERT ASM HMGB1 knock-down cells (HMGB1 shRNA) and matching hTERT ASM non-coding shRNA expressing control ASM cells. For each lane, 20 μg of total protein from whole cell lysates was loaded, and blots were probed with the antibodies for the proteins indicated. GAPDH was used as a loading control. The blots shown are representative of 3 experiments conducted using 3 different cell lines.*

We next determined if the blunted TGF-β1-induced ECM protein expression in HMGB1 silenced cells could be rescued by adding exogenous HMGB1. For these studies we stimulated hTERT ASM HMGB1 shRNA cells and hTERT ASM non-coding shRNA cells with HMGB1 (10ng/mL), TGF-β1 (2.5 ng/mL) or both. We collected cell culture media after 48 hours thereafter as well as preparing whole cell lysates for subsequent immunoblot analysis of cellular collagen 1a and fibronectin abundance at basla levels (Figure 19A and B). We noted that basal levels of cell-associated collagen 1a and fibronectin are significantly greater in HMGB1-deficient cells compared to cells expressing non-coding shRNA.

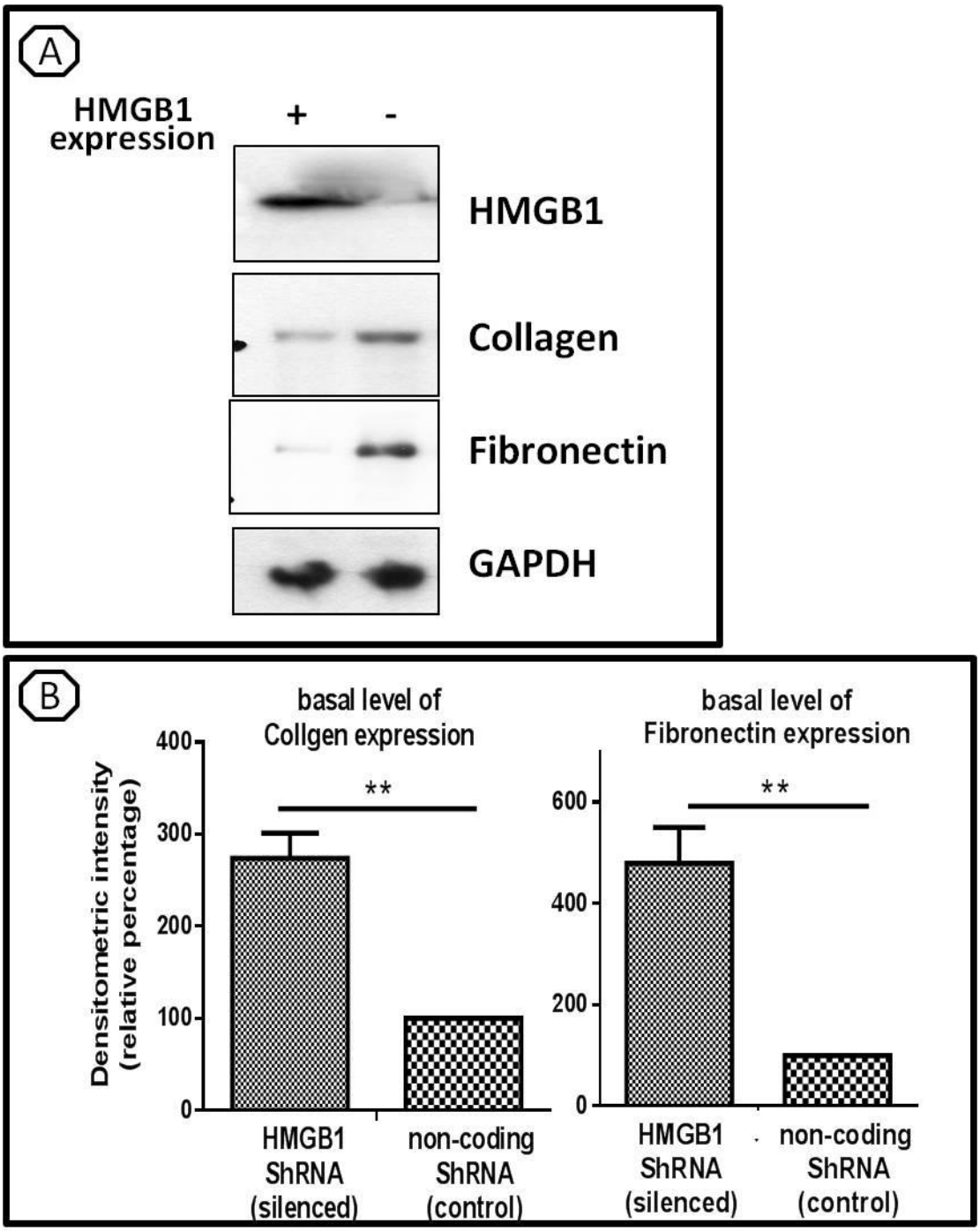
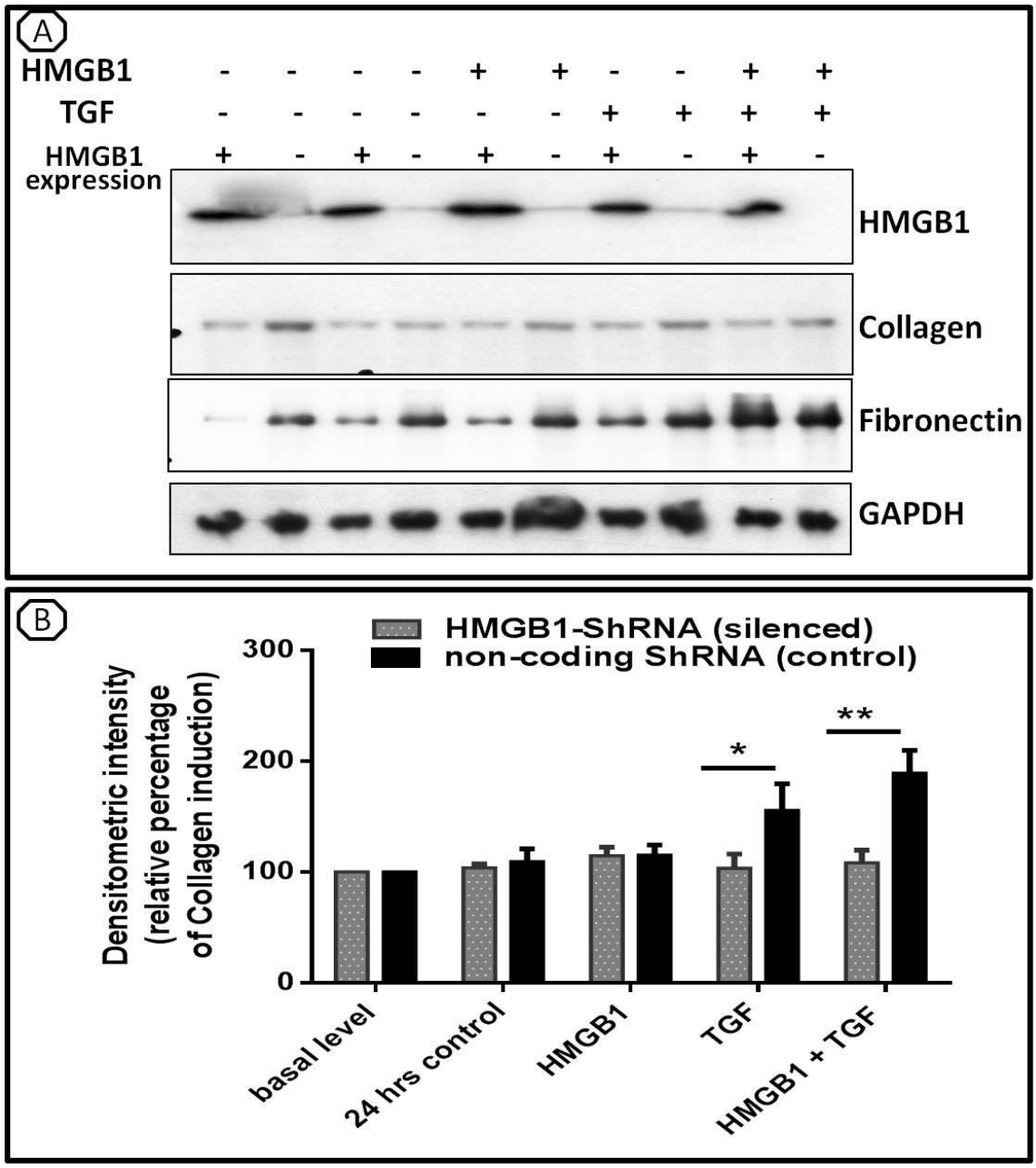


Figure 19: Effect of HMGB1 silencing on amount of Collagen and Fibronectin in whole cell lysate samples *(A) Representative western blots carried out using two different hTERT immortalized human ASM cell lines (shRNAi HMGB1 and shRNAi non-coding). Whole cell lysate samples were collected 48 hours with no stimulation. The samples were probed with antibodies against collagen and fibronectin. Panel (B) shows a bar graph representing mean ± SEM of densitometric analyses from at least three different*

experiments. Unpaired student's t-test was performed to compare basal amount of collagen and fibronectin between shRNAi HMGB1 (HMGB1 silenced) and shRNAi non-coding (control group), P<0.01 (**).

Since basal levels of collagen and fibronectin were different between HMGB1 silenced and control ASM cells, in order to assess the response to exogenous HMGB1 we normalized protein abundance at each time after treatment with the corresponding basal level. In this way we were able to assess the capacity of HMGB1 to induce new ECM protein expression in the different cell lines we studied (Figure 20). For these studies we found that exogenous recombinant HMGB1 was not sufficient to induce ECM protein expression in HMGB1-expressing and HMGB1-silenced cells – this is paradoxical in light of our prior observations (see Figure 15A), and likely reflects loss of biological activity of HMGB1 due to oxidation during storage (discussed in detail in Discussion). Nonetheless, our analyses also revealed that in HMGB1-deficient ASM cells, TGFβ-1-induced ECM protein expression was markedly reduced compared to cells that express HMGB1. Our data further suggest that addition of extracellular HMGB1 does not compensate for the effects of endogenous HMGB1 depletion on TGF-β1 induced collagen and fibronectin expression. Taken together these observations suggest that the inhibitory effect of HMGB1 silencing on ECM protein expression likely stems from intracellular regulatory mechanisms rather than from a role for extracellular HMGB1 in pathways that support ECM protein expression.


A
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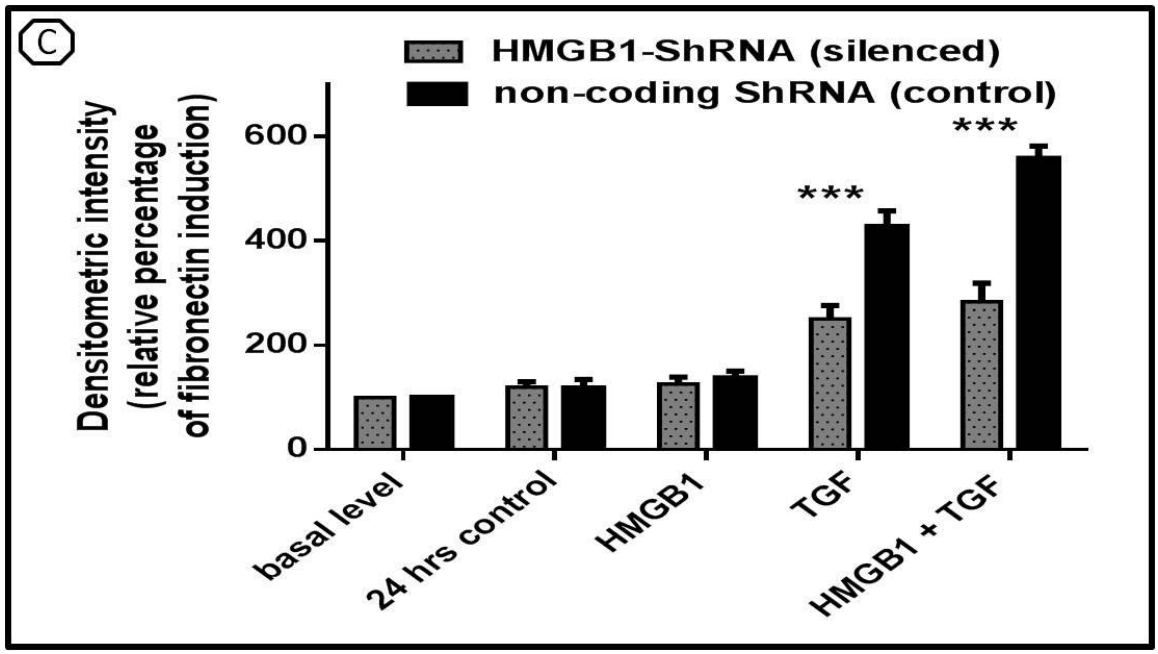

Figure 20: Effect of HMGB1 silencing in TGF-β1-induced ECM protein synthesis *(A) Representative Western blots carried out using two different hTERT immortalized human ASM cell lines (shRNAi HMGB1 and shRNAi non-coding), which were stimulated with HMGB1 (10 ng/mL), TGF-β1 (2.5 ng/mL) or HMGB1 (10 ng/mL) + TGF-β1 (2.5 ng/mL). Whole cell lysate samples were collected 48 hours post stimulation (Panel A). The samples were probed with antibodies against collagen and fibronectin. GAPDH was used as a loading control. Panels (B) and (C) each shows a bar graph summarizing densitometric intensity of blots in which expression of collagen (B) and fibronectin (C). Amount of proteins in each group have been normalized according to the amount of basal protein expression of each protein and also GAPDH. Corresponding bar graph representing mean ± SEM from at least three different experiments on ASM cells obtained from three different cell lines. Two-way ANOVA was performed to determine the significance of data, (n=3, P<0.001(***), P<0.01(**), P<0.05(*)).*

To complement experiments that examine the impact of silencing HMGB1 on cellular accumulation of ECM proteins, we next measured whether there were effects on collagen and fibronectin secreted into the culture medium. For these studies we stimulated hTERT ASM HMGB1 shRNA cells and hTERT ASM non-coding shRNA cells with HMGB1

(10ng/mL), TGF-β1 (2.5 ng/mL) or both TGF-β1 (2.5 ng/mL) and HMGB1 (10ng/mL) for 48 hours before collecting and concentrating culture medium using <10 kDa MW cutoff Amicon Ultra-15 Centrifugal Filter Units. Immunoblotting and densitometry revealed that secreted collagen 1a and fibronectin was reduced in HMGB1 knockdown cells in unstimulated conditions, as well as in cultures treated with HMGB1, TGF-β1 or both HMGB1 and TGF-β1 (Figure 21). It seems that ASM cells need "nuclear HMGB1" to be able to respond to TGF-β1 induced ECM expression (intracellular and secretory), which means TGF-β1 treatment does not increase intracellular or extracellular ECM in HMGB1 deficient cells. It seems HMGB1 silencing leads to increased abundance of cellular ECM at basal and reduced extracellular ECM which means ASM cells need "cytoplasmic HMGB1" to let the ECM to be secreted (based on lower extracellular ECM compared to intracellular one in HMGB1 silenced in basal level (non-treated cells).

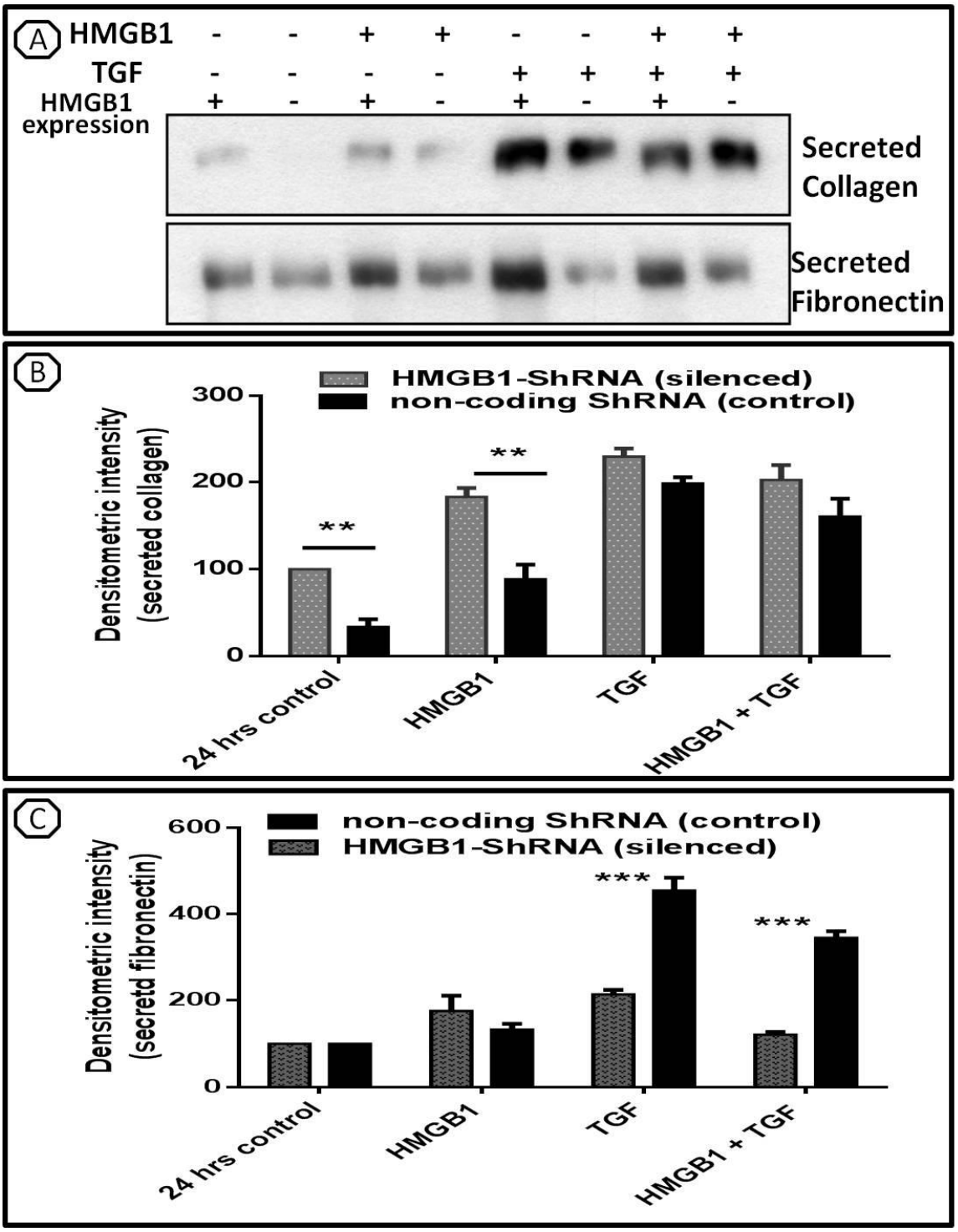

Figure 21: Effects of silencing HMGB1 expression on ECM protein secretion *(A) Representative immunoblots of concentrated cell culture medium for collagen 1a and fibronectin for hTERT ASM HMGB1 shRNAi and hTERT ASM non-coding shRNAi cells that were stimulated with HMGB1 (10 ng/mL), TGF-β1 (2.5 ng/mL) or HMGB1 (10 ng/mL) + TGF-β1 (2.5 ng/mL) for 48 hours. For each lane 20 µg of total*

protein was loaded. The blots shown are representative of 3 experiments performed using two different hTERT ASM HMGB1 shRNAi and hTERT ASM non-coding shRNAi cell lines. Panels (B) and (C) each shows a bar graph summarizing densitometric intensity of blots in which expression of collagen (B) and fibronectin. Corresponding bar graph representing mean ± SEM from at least three different experiments on ASM cells obtained from three different cell lines. Two-way ANOVA was performed to determine the significance of data, (n=3, P<0.001(***), P<0.01(**)).

VIII. HMGB1 localization in primary human ASM cultured cells

HMGB1 is abundantly expressed in the nucleus by nearly all cell types (87). Though it is primarily a nuclear protein, its location can be variable, for example lymphoid cells contain significant HMGB1 stores in both the cytoplasm and nucleus (88). Furthermore, cells in liver and brain tissues contain HMGB1 predominantly in the cytoplasm (89), and HMGB1 is present on the cell surface of neurons and platelets (90, 91). Since our data point to a significant intracellular role for HMGB1in expression and release of collagen 1a and fibronectin, we surveyed the intracellular distribution of endogenous HMGB1 in human ASM cells. Fluorescence immunocytochemistry revealed that HMGB1 is abundantly expressed through the nucleus, with foci of intense labeling. HMGB1 was also readily detected throughout the cytoplasm, though specific organelle staining was not obvious (Figure 22). These observations support the possibility that that both nuclear and cytoplasmic HMGB1 could be involved with TGFB1-induced ECM protein expression and release.

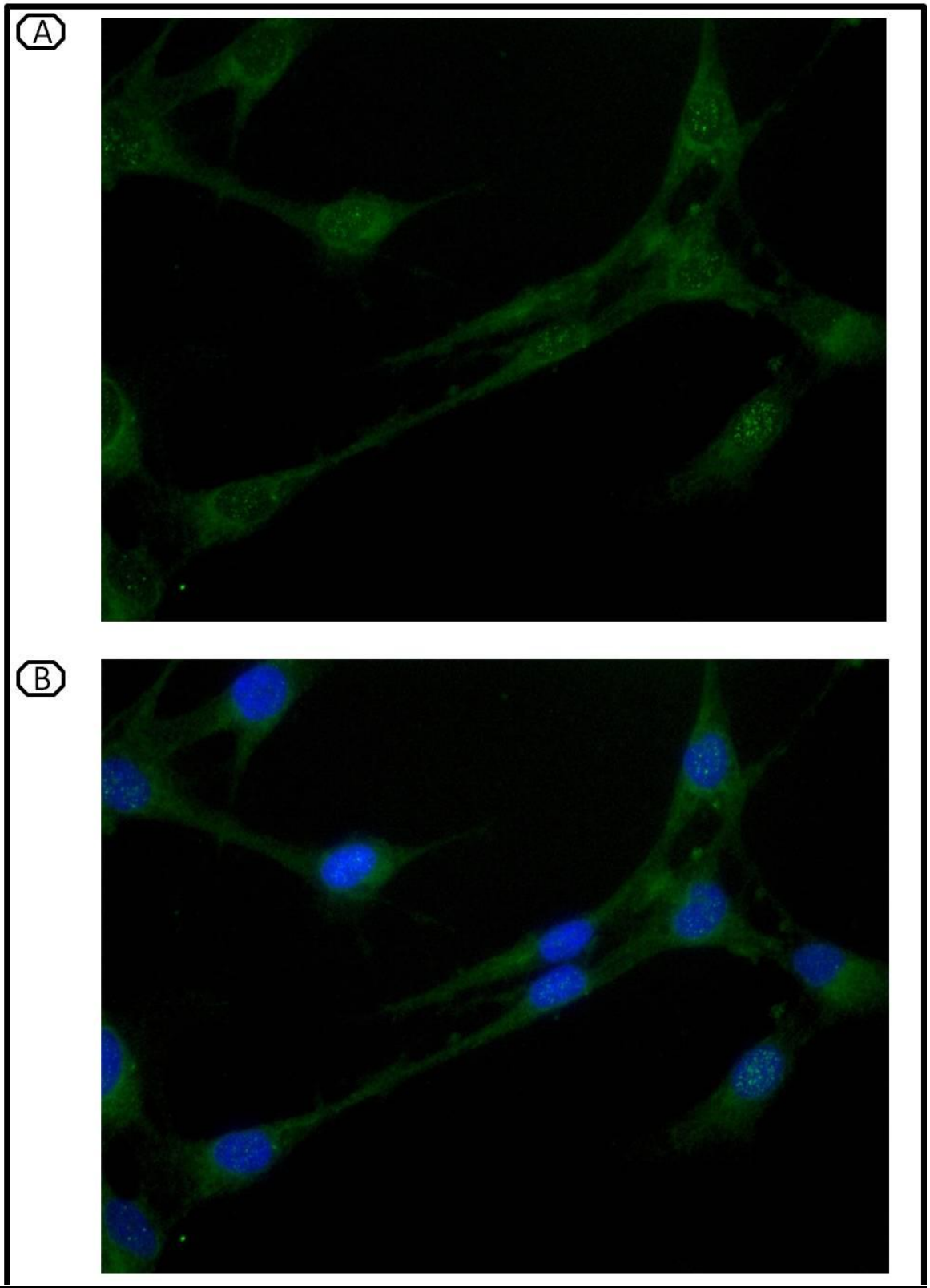

Figure 22: ***Expression of HMGB1 in human ASM cells.*** *Primary cultured human airway smooth muscle cells were grown to 50 % confluence on glass coverslips and subjected to culture in DMEM with 10% FBS*

conditions. Thereafter ASM cells were fixed and labeled for HMGB1. HMGB1 Isotype-matched rabbit IgG anti-serum was used for negative controls (not shown). Secondary antibodies conjugated with FITC were used to label HMGB1 (green (Panel A). Nuclei appear blue and are counterstained with Hoechst 33342 (10 μg/mL) (Panel B). Images were obtained by using a Fluorescencent microscope.

CHAPTER V: DISCUSSION

Airway inflammation and remodeling is the main characteristic of asthmatic patients. The combined action of various inflammatory cells is essential for the occurrence, progression, and persistence of asthma, causing the hyper-responsiveness and airflow limitation exhibited by patients with the disease. Under the effects of epithelial cell- and mesenchymal cell-derived growth factors, such as transforming growth factor (TGF)-β, mesenchymal cells, including ASM cells, produce excess amounts of collagen, elastin, as well as proteoglycans and glycoproteins of the extracellular matrix (ECM) (46). Accumulation of these components contributes to thickening and remodeling of the walls of airways in asthmatic subjects, an effect that is concomitant with increasing mass of ASM cells (48, 50). ASM cells have the capacity to synthesize and release pro-inflammatory cytokines and growth factors such as (TGF)-β in response to a variety of stimuli, thus exhibiting a synthetic/proliferative phenotype that contributes to their role in airway remodeling (15, 59).

It is well established that airway epithelial cells and immune cells can secrete HMGB1 in responses to a variety of stimuli, including LPS, IL-1 and TNF-α (100, 132, 133). In this study, we show that human ASM cells can express HMGB1 and may have capacity to release HMGB1 upon exposure to TGF-β1, and that endogenous intracellular HMGB1 modulates pro-fibrotic function in response to TGF-β1, also perhaps by modulating expression and/or secretion of some ECM proteins. Extracellular HMGB1 promotes activation of inflammatory cells and stimulates production and secretion of inflammation-promoting factors and has been identified recently as a lethal mediator of inflammatory cytokines (135, 219). It is evident that HMGB1 activates cheomtaxis of neutrophils (150,

151) and differentiation of T-cells to a Th1 phenotype (152). HMGB1 plays a critical role in the tissue repair process by promoting the proliferation, differentiation and migration of stem cells and smooth muscle cells (112, 125, 155). Several pro-inflammatory activities of HMGB1 have been revealed from the studies of isolated cell cultures.

Prior to our study, a role for intracellular (nuclear) HMGB1 had not been considered, in particular as a modulator of ASM cell response to cytokines. We began our studies by testing the effects of exogenous recombinant HMGB1on primary cultured human ASM cells. In cultured human endothelial cells, HMGB1-mediated signal transduction is in part dependent on the activation of MAP kinases, extracellular signal related kinases and Jun N-terminal kinases (154). Thus, we investigated effects of recombinant HMGB1 on signal transduction pathways in human ASM cells. We showed that HMGB1 activates ERK1/2, and to lesser extent JNK/STAT. MAP kinases play a crucial role in modulating cellular functions, such as: cell proliferation, survival, muscle contraction and cell migration (283, 284). Our data, in concert with prior studies, indicate that HMGB1 can activate MAP kinase signaling in multiple cell types, including neutrophils, monocytes, endothelial cells and smooth-muscle cells. (154, 288-290). Recent findings indicate that signaling processes associated with intracellular (endogenous) HMGB1 are vital in transitioning septic mice between states of inflammation and immunosuppression during endotoxin tolerance (291). Collectively, these observations suggest that when released, HMGB1 in the extracellular milieu can drive pro-inflammatory responses.

Accumulating evidence shows that HMGB1 may signal through different receptors, including RAGE, TLR2 and TLR4. (173, 175, 177, 179). Indeed, via these receptors HMGB1 can support the secretion of inflammatory cytokines such as TNF-α, IL-8 and

IL-10 (121, 175). HMGB1 can mediate signal transduction pathways via RAGE in Normal Human Bronchial Epithelial (NHBE) cells that drive an inflammatory response (292). RAGE is a multi-ligand cell surface macromolecule, and RAGE-ligand interaction evokes central changes in key biological properties of cells, including proliferation, generation of inflammatory mediators, and migration receptor (125, 182, 187, 188).

The ligation of HMGB1 to RAGE leads to the activation of MAP kinases and the activation of the NFκB pathway. A downstream target of activated MAP kinase is MAPK-activated protein kinase 2, which is believed to be amongst one of the key regulators of pro-inflammatory responses (293).

MAPKs play a crucial role in modulating cellular functions, such as: cell proliferation, cell survival, muscle contraction and cell migration (283, 284). In our study, we showed that HMGB1 activates ERK1/2 and to lesser extent JNK/STAT. Our study suggests that HMGB1 can activate ERK MAPKs through the mDia1/RAGE axis; however, it appears that HMGB1 has no effect on human ASM cell proliferation. HMGB1 promotes the proliferation and migration of HSCs via TLR4-dependent signaling pathways, indicating a functional role of HMGB1 in the development of liver fibrosis (294). Although HMGB1 has been shown to increase NIH/3T3 cell proliferation (4), other scientists have shown that recombinant HMGB1, (over a wide range of doses), has no effect on dermal fibroblasts (295). HMGB1 also promotes the proliferation and differentiation of cardiac cells (155), skeletal muscle cells (296) and 3T3 mouse fibroblasts (297) Furthermore, recent studies have revealed that HMGB1 induces cell proliferation, cell migration and scratch wound closure via RAGE-dependent activation of the ERK1/2 signaling cascade (297).

Although RAGE-dependent signal transduction is dependent on its short cytoplasmic domain, which lacks catalytic activity, all ligand-stimulated RAGE signaling requires association of the cytoplasmic tail with mDia1 (diaphanous1) (185, 186). In fact, RAGE induces activation of ERK1/2 through a signaling cascade mediated via mDia1, which serves as a molecular scaffold (187, 188). There are also reports that cytoplasmic HMGB1 can interact with the cytosolic domain of RAGE to mediate ERK1/2 phosphorylation (187, 188). Thus, the interaction of the RAGE cytoplasmic domain with mDia1 may be required to transduce extracellular environmental cues such as HMGB1 and other RAGE ligands. As RAGE and mDia1 are implicated in the regulation of inflammation and transformed cell migration, we set out to elucidate the roles that RAGE and mDia1 play in HMGB1 induced activation of the MAP kinase pathways. To that end, we generated mDia1 knockdown human ASM cell lines. These cells were refractory to ERK1/2 activation in response to HMGB1 treatment. This observation confirms that a RAGE/mDia1 signaling axis mediates HMGB1-induced MAP kinase activation in human ASM cells. Our studies did not directly determine the requirement of RAGE in HMGB1-induced cell signaling. We attempted experiments using blocking antibodies, but these were compromised because the antibody alone was able to activate MAP kinase signaling. Thus, though not included in this thesis, we launched effort to generate hTERT HASM cell lines with stable silencing of RAGE by shRNA. During preparation of this thesis RAGE knockdown cell lines were completed, thus I was able to contribute to making a tool that is necessary for studies that test whether RAGE (via mDia1) mediates HMGB1-induced signaling.

Binding of HMGB1 to the surface of smooth-muscle cells occurs in association with expression of RAGE on the plasma membrane (233). Endothelial cells, which also express RAGE, respond to recombinant HMGB1 by activating MAP kinases, ERK, JNK, and p38, and in nuclear translocation of transcription factors NF-κB and Sp1 (154). HMGBl can induce receptor-mediated activation of multiple pathways, including those involving the transcription factor NF-κB, which stimulates cytokine production and inflammatory cell aggregation (298). Our data shows that HMGB1 can activate MAP kinase pathway through RAGE-mDia1 axis. We did not observe whether HMGB1 can activate the NF-κB pathway. A study by Ogawa et al (255) suggests that HMGB1, via RAGE activated pathways, is involved in oxidative stress linked to diseases such as rheumatoid arthritis, as well as lung diseases such as ventilation-related lung injury. Little is known about roles of HMGBl and RAGE in the pathogenesis of asthma, but one group has studied protein expression in bronchoalveolar lavage fluid and found that HMGBl was increased (299). Furthermore, HMGB1 activates the nuclear translocation of NF-κB in neutrophils by a mechanism involving MAP kinase pathways, to stimulate the release of pro-inflammatory cytokines (175). It has been shown that recombinant HMGB1 causes the development of a polarized morphology, typical of motile cells in rat vascular smooth-muscle cells (300). Smooth-muscle cell migration induced by HMGB1 is diminished by RAGE blocking antibodies, and can be inhibited by pertussis toxin or the MAP kinase inhibitors (301). All in all, we can say exogenous (recombinant) HMGB1 can activate MAP Kinase pathway through RAGE/mDia axis.

HMGB1 plays a variety of roles in different cell types and disease pathologies. It is both a nuclear DNA binding factor and a secreted protein that modulates cell death and

survival (44). In tissue repair processes HMGB1 promotes proliferation, differentiation and migration of stem cells and smooth muscle cells (112, 125, 155). In stromal fibroblasts, HMGB1 supports proliferation, chemotaxis, and the synthesis of metalloproteinases (112, 234), whereas, in damaged brain tissue, HMGB1 can mediate plasticity, as well as enhance stem cell and progenitor cell recruitment, proliferation, and differentiation (302). HMGB1 has been shown to promote the proliferation and odontoblastic differentiation of human dental pulp cells (303) and regulates the proliferation and invasion of cancer cells in the lung (adenocarcinoma) by modulating the activation of the ERK1/2 and p38 MAP kinases (304). Paradoxically, several studies have also shown that HMBG1 can inhibit cell proliferation. For example, HMGB1 impairs hepatocyte regeneration, and inhibition of HMGB1 increases the proliferation of hepatocyte satellite cells to enhance liver recovery (305). Meng et al (306) discovered that HMGB1 does not affect mesenchymal stem cell proliferation, a result that is consistent with our own findings. Hamada et al (307) demonstrated that intraperitoneal injection of anti-HMGB1 antibody significantly attenuates lung inflammation and lung remodeling in bleomycin-induced pulmonary fibrosis in mice. Moreover, Pittet et al reported that HMGB1 significantly induces proliferation, but did not have an effect to either compromise cell viability nor did it induce collagen synthesis in cultured lung fibroblasts (308).

At the outset of this project a primary focus was to assess the effects that extracellular HMGB1 might have on human ASM cells in culture, in particular the induction of ECM protein synthesis. To establish a benchmark for ECM protein synthesis by cultured human ASM cells, we measured synthesis and release of collagen 1a and fibronectin in

response to treatment with recombinant TGF-β1. As expected, we confirmed this cytokine to be an efficacious inductive signal, markedly increasing intracellular and extracellular (secreted) collagen 1a and fibronectin. These observations also verified the capacity of the cell lines used in our studies to express ECM proteins. Thus, our findings that stimulation with exogenous recombinant HMGB1 was not sufficient to induce ECM protein expression or secretion indicate that HMGB1 does not promote fibrosis associated with airway remodelling. Notably, there are paradoxical reports from other groups regarding the effects of HMGB1 on collagen formation and maintenance. For example, He et al (309) observed that intra-myocardium injection of HMGB1 led to a significant decrease in the expression of collagen I and collagen III. The same group also established that injection of HMGB1 hydrogel in rat hearts reduced collagen content and improved cardiac function (310). Other studies have shown that exogenous HMGB1 can impair collagen synthesis in cultured human and mouse synovial fibroblasts (311, 312), and that by reducing HMGB1 levels in wounds, increased collagen synthesis and tensile strength can be attained (295). Collectively, these different observations might suggest that exogenous HMGB1 can affect fibrosis process in two different ways. While limited evident indicates that HMGB1 can increase fibrosis (307, 313, 314), other works indicate that HMGB1 does not appear to possess pro-fibrotic capacity (315, 316).

Posttranslational modifications that can include acetylation, ADP-ribosylation, phosphorylation, and thiol oxidation greatly affect its function (4, 41, 45, 46). The redox status of HMGB1 determines whether it will behave as cytokine inducer or have chemokine activity (287). For instance, it appears that HMGB1 must be in a reduced state of cysteine in order to bind TLR4 and stimulate cytokine release and inflammation (124).

This appears to be related to a need for disulfide bonds between two cysteines (C23 and C45) residues to confer pro-inflammatory ("cytokine-like") activity (317). In summary, HMGB1 requires both reduced C106 and the formation of an intramolecular disulfide bond between C23 and C45.

HMGB1 activity is highly dependent on its redox status; and even small changes in redox status will affect its cytokine or chemotactic activity (287). As redox modifications are crucial for HMGB1 function, experiments in which recombinant or purified protein is used to stimulate cells require that appropriate storage conditions, using inert gas in vacuum tubes, is necessary. The importance of this issue has only recently been appreciated (317-320), and this has led to generation of commercially available recombinant forms of HMGB1 that are resistant to reduction (320). This issue is highly relevant to our work, as an early study performed at the outset of my program had suggested that exogenous HMGB1 did possess collagen1a-inducing capacity. These results were not reproducible 10 months later using HMGB1 stock that had not been stored under inert gas. Alhough we were not able to determine whether a change in redox state of the stored HMGB1 led to loss of its biological activity (and thus no induction of collagen 1a and fibronectin in ASM cells), future studies using redox-resistant HMGB1 should be performed to ensure that our inability to detect pro-fibrotic capacity was not due to a technical issue.

In patients suffering from asthma, increased expression of HMGBl and RAGE is correlated with greater airway inflammation (321). Moreover, higher levels of HMGBl in sputum of asthmatic patients are associated with increased disease severity, and both HMGB1 and RAGE are reduced in sputum of these asthmatic patients with well-

controlled asthma (322). Sukkar et al has shown patients with obstructive lung diseases (asthma and COPD) have lower amount of sRAGE compared to normal population and also lung sRAGE could be used as an independent predictor of airway inflammation (323). Though these findings suggest that levels of HMGBl and RAGE in induced sputum of asthma patients may be an index of treatment response, their contribution to asthma pathogenesis has not yet been determined. In this regard we performed experiments to determine whether human ASM cells express HMGB1 and if its release can be induced by exposure to the pro-asthma cytokine, TGF-β1. We confirmed that HMGB1 is abundant in cultured human ASM cells, as confirmed by immunoblotting of whole cell lysates. In contrast, we were not able to detect induction of secreted HMGB1 in culture medium, even when cells were treated with TGF-β1. Our inability to detect TGF-β1-induced HMGB1 release does not preclude the possibility that other asthma-associated cytokines (or combinations of cytokines) might be sufficient to induce its release. Thus future studies to test this possibility are needed.

Intranuclear HMGB1 acts as an architectural chromatin-binding factor that bends DNA and promotes protein assembly at specific DNA sites (84). In the nucleus, HMGB1 binds to nucleosomes at the dyad axis and promotes nucleosome sliding. It also relaxes the nucleosome structure and bends DNA, making chromatin more accessible to transcription factors and DNA replication (146). HMGB1 knockout mice show a defect in the transcriptional enhancement activity associated with the glucocorticoid receptor and die shortly after birth (147). Endogenous HMGB1 interacts with several transcription factors, including p53 (324-326), p73 (327, 328), retinoblastoma (Rb) protein (329, 330), members of the Rel/NF-κB family (104, 107), and nuclear hormone receptors such as the

estrogen receptor (331, 332). HMGB1 is also present in the cell cytoplasm, an observation confirmed by our own immunocytochemical analyses of human ASM cells, but its role is not fully elucidated. Cytoplasmic HMGB1 can also integrate in a number of processes, including secretory pathways related to myosin IC isoform A and Ras-related protein Rab-10 (333). Myosin IC is a member of the unconventional myosin protein family, which are actin-based molecular motors. The mouse ortholog of this protein also functions in intracellular vesicle transport to the plasma membrane (334). Ras-related protein Rab 10 belongs to the RAS superfamily of small GTPases and RAB proteins, which localize to exocytic and endocytic compartments and regulate intracellular vesicle trafficking (335, 336). Recent reports indicate that HMGB1 can localize to endosomes and this is linked with secretion of pro-inflammatory mediators, as well as contributing to the membrane distribution of TLR9 (337). There is evidence that HMGB1 associates with the endoplasmic reticulum (ER) and has been described as a regulator of the ER-stress response (338). Additionally, Lotze and his group has described HMGB1 binding to *cytoplasmic* p53 and the role this plays as a determinant of autophagy in cells (339, 340). On this basis we developed human cell lines in which endogenous HMGB1 expression was silenced to investigate its potential regulatory role in TGF-β1-induced ECM protein expression and release.

Our data indicates that the depletion of endogenous HMGB1 inhibits the expression and/or release of collagen 1a and fibronectin by human ASM cells exposed to TGF-β1. We show that under basal conditions, *cellular* collagen 1a and fibronectin abundance is increased, whereas *secreted* collagen 1a and fibronectin abundance was reduced in HMGB1-silenced human ASM cells. Moreover, our analysis of changes in

cellular ECM protein abundance and its release into culture medium reveals that HMGB1-deficient AMS cells do not repond to TGF-β1 stimulation as much as control ASM cells. An early premise of our work was that HMGB1 might be released as an endogenous signal in ASM cells stimulated with TGF-β1, a scenario that would mimic its role as an alarmin. We did find that ablation of HMGB1 diminished TGF-β1 induced expression of collagen and fibronectin. Subsequent to these studies, we tested whether addition of exogenous recombinant HMGB1 to HMGB1-silenced ASM cell lines was able to rescue ECM protein expression and release in response to TGF-β1. Our findings reveal that exogenous recombinant HMGB1 is not sufficient to rescue ECM protein expression in ASM cell lacking HMGB-1. This indicates that *intracellular* HMGB1 is a primary determinant of TGF-β1 induced ECM protein expression by human ASM cells.

Our observations are novel in that our own originally-planned experiments, as well as the vast majority in the field with respect to inflammation have been designed to decipher the role of extracellular HMGB1 (118, 341-344). Indeed, prior to our studies the majority of research concerning HMGB1 as a modulator of fibrosis has focused on its extracellular role, in particular with respect to spatial and temporal effects on inflammation (345-348). Our current findings confirm a primary role for intracellular HMGB1 in the regulation of fibrosis, through involvement in the expression and/or release of ECM proteins by ASM cells. Notably the intracellular localization of HMGB1 is modulated by posttranslational modifications, an affect that determines its role in the nucleus and/or cytoplasm (4, 41, 45, 46). Recent studies also underscore the importance of redox modification in the regulation of HMGB1 translocation, release, and activity in disease (349). This is of relevance as it raises the potential that HMGB1, as well as

mechanisms that determine its post-translational modification, could be intracellular and/or extracellular targets for new therapies targeting inflammation and tissue repair into the future.

Summary and Future Directions

Our initial aim was to determine if exogenous recombinant HMGB1 was sufficient to induce the expression of ECM proteins in human ASM cells. Despite observations that indicated the phosphorylation of ERK1/2 MAP kinase was induced by HMGB1, and that this could be quenched if the RAGE-mDia1 signaling axis was disrupted (Fig. 5.1), we did not observe reproducible evidence that HMGB1 alone could induce collagen 1a and fibronectin synthesis and release. These observations seem reproducible, notwithstanding the possibility that the redox state of stored HMGB1 became altered, thereby compromising the cytokine-like function of the protein. More work related to different effects of different redox states of stored HMGB1 on ASM function such as ECM expression, could be fundamental in understanding the effect of HMGB1 on ASM function in the matter of inflammation and fibrosis. Also, further studies are required to fully elucidate the receptors and activating pathways by HMGB1 in ASM cells.

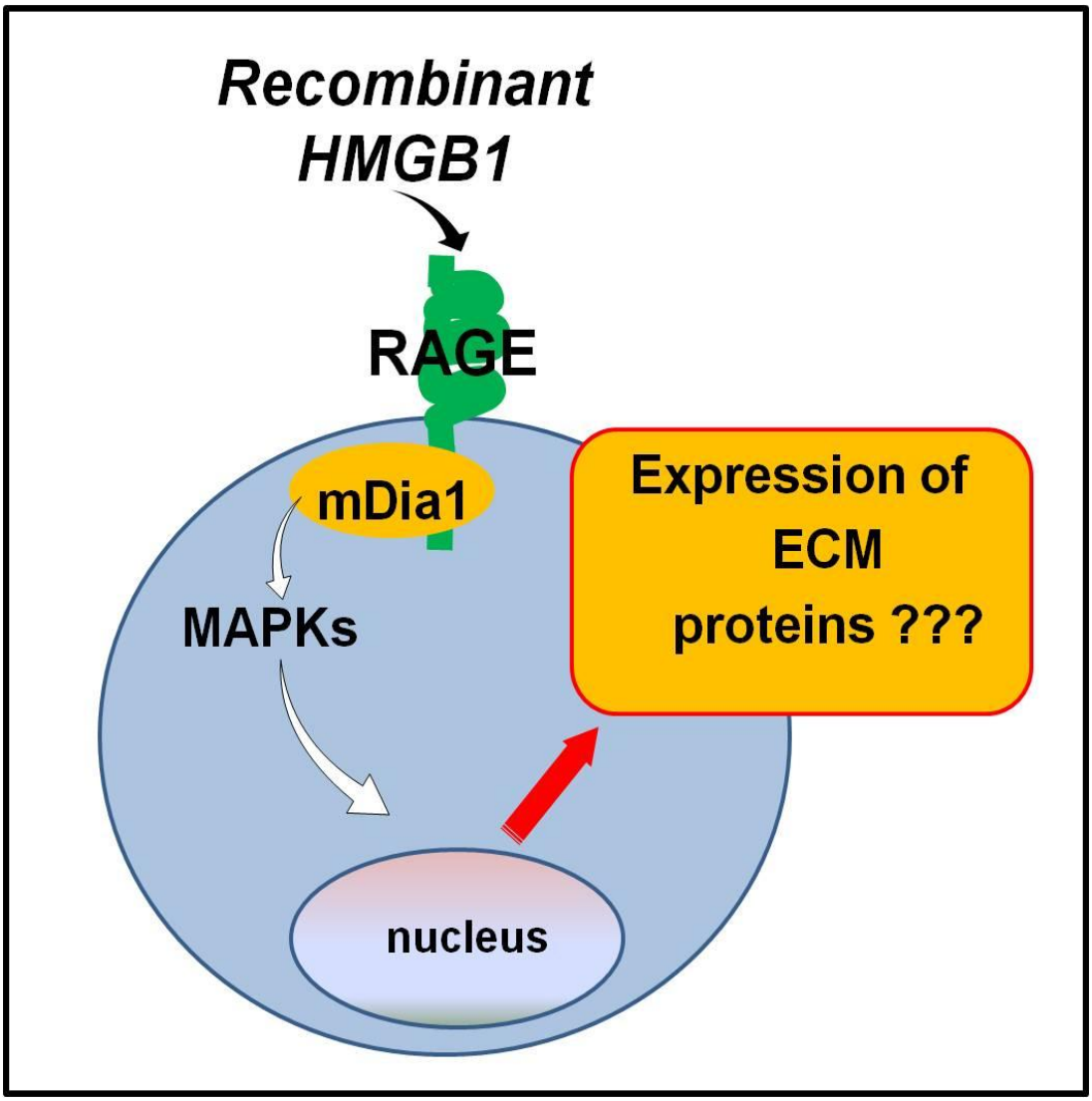

Figure 5.1. Schematic representation showing HMGB1 signaling pathways. *HMGB1 can bind to RAGE, which, through intracellular mDia1, activates MAPK pathways, which could modulate other pathways leading to an integrated regulatory cascade to control expression of ECM proteins.*

In the course of our work we generated novel HMGB1-deficient human ASM cell lines, and in studies using them we observed that TGF-β1-induced ECM synthesis and release were suppressed. This implicates intracellular HMGB1 as a controller of TGF-β1 induced ECM expression and secretion, a conclusion supported by our observation that this response was refractory to exogenously added HMGB1 (Fig. 5.2).

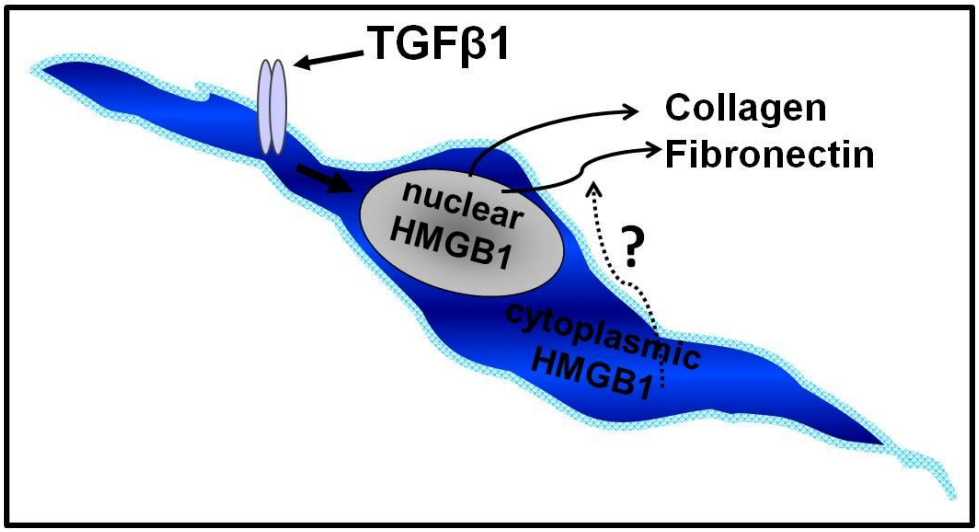

Figure 5.2. Schematic representation showing effect of intracellular HMGB1on ECM expression. *We propose that nuclear HMGB1 can control TGF-β1-induced ECM synthesis and cytoplasmic HMGB1 regulates its release.*

Our experiments do not allow definitive discrimination of a role for nuclear or cytoplasmic HMGB1 in ECM protein generation and secretion. Future work is needed for high throughput survey of the genomic sites that HMGB1 interrogates in cells stimulated with TGF-β1. Moreover, future studies are necessary to dissect the endosome, ER and golgi apparatus compartments that HMGB1 associates with during ECM secretion. Moreover, the identification of the proteins that it might associate with in these cytoplasmic organelles is needed to appreciate the mechanisms involved.

Based on the work reported here and previous findings, (118, 135, 333, 348, 350-352) we propose a model for pro-fibrotic function of human ASM cells that involves nuclear and cytoplasmic HMGB1. Moreover, further investigation is needed to more fully appreciate the role that extracellular HMGB1 may have in inflammation-associated responses. Based on published work, but not fully supported by our own data herein, we

suggest that endogenous HMGB1 could play a significant role in tissue remodeling and fibrosis in the airway wall in chronic inflammatory disorders such as asthma or chronic obstructive lung disease. More work in this area, especially the role of intracellular HMGB1 (nuclear and cytoplasmic) could be instrumental in guiding the development of more effective therapies for chronic inflammatory diseases that involve the release of damage-associated molecular pattern proteins such as HMGB1.

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CHAPTER VI: REFERENCES

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