Investigation of the proteins SPARC and HMGB1 in chronic airways disease

A thesis submitted for the degree of
Doctor of Philosophy

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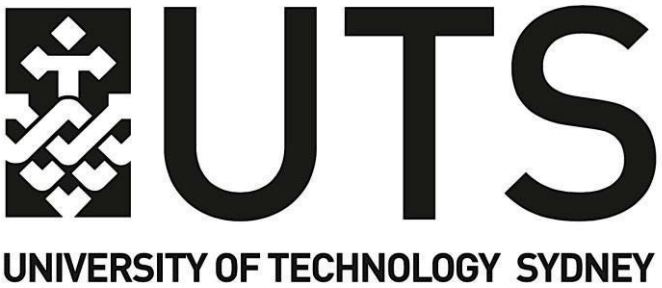

March 2017

CERTIFICATE OF ORIGINAL AUTHORSHIP

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PUBLICATIONS AND PRESENTATIONS DURING CANDIDATURE

Published journal manuscripts

The SPARC protein: an overview of its role in lung cancer and pulmonary fibrosis and its potential role in chronic airways disease

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Presentations

Potential role of SPARC, a downstream mediator of TGF-β in chronic airways disease

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SPARC: a downstream mediator of TGF-β, which may potentially play a role in chronic airways disease

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LIST OF ABBREVIATIONS

4-PBA	4-phenylbutyric acid
AECs	Airway epithelial cells
AHR	Airway hyperresponsiveness
APP	Amyloid precursor protein
ASM	Airway smooth muscle
ATF6	Activating transcription factor 6
Ca^{2+}	Calcium
CAPZA1	F-actin-capping protein subunit alpha-1
COPD	Chronic obstructive pulmonary disease
CRT	Calreticulin
DAMP	Danger-associated molecular pattern
ECM	Extracellular matrix
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid
eIF2α	Eukaryotic translation initiator factor 2α
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
GO	Gene Ontology
GRP78	Glucose-regulated protein 78
GSK-3β	Glycogen-synthase kinase-3 beta
HMGB1	High mobility group box 1
hrCNE	High resolution clear native electrophoresis
IFN-γ	Interferon gamma
ILC2s	Type 2 innate lymphoid cells
ILC3s	Type 3 innate lymphoid cells
IPF	Idiopathic pulmonary fibrosis
IRE1α	Inositol-requiring kinase 1 alpha
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MEFs	Mouse embryonic fibroblasts
MMPs	Matrix metalloproteinases

MWCO	Molecular weight cut-off
NSCLC	Non-small cell lung cancer
ORMDL3	Orosomucoid like 3
PAI-1	Plasminogen activator inhibitor-1
PAMPs	Pathogen-associated molecular patterns
PBST	Phosphate Buffered Saline containing Tween
PDGF	Platelet-derived growth factor
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
RAGE	Receptor for advanced glycation end products
RIDD	IRE1-dependent decay
ROS	Reactive oxygen species
SASP	Senescence-associated secretory phenotype
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
Siglec	Sialic acid-binding immunoglobulin-like lectin
SPARC	Secreted protein acidic and rich in cysteine
TBST	Tris-buffered saline containing Tween
TGF-β	Transforming growth factor-beta
TGFβRII	TGF-β-receptor type II
TLRs	Toll-like receptors
TMAO	Trimethylamine N-oxide dehydrate
TNF-α	Tumor necrosis factor alpha
TSLP	Thymic stromal lymphopoietin
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
XBP-1	X-box binding protein 1
XBP-1s	Spliced XBP-1
ZO-1	Zona occludin -1

ABSTRACT

The matricellular protein, secreted protein acidic and rich in cysteine (SPARC), mediates the interaction between cells and their surrounding extracellular matrix (ECM) but does not contribute structurally to the matrix. It regulates basic cellular functions such as cell adhesion and proliferation, as well as the processing and deposition of ECM proteins. SPARC is overexpressed in many fibrotic tissues including the lung. SPARC also serves as a down-stream mediator of transforming growth factor-beta (TGF-β), a key driver of airway remodeling in chronic airways disease, and demonstrates context-dependent immunoregulatory functions. Although airway inflammation and remodeling are prominent features of asthma and chronic obstructive pulmonary disease (COPD), the role of SPARC in these conditions has not been studied.

In this thesis, we investigated the expression of SPARC in airway structural cells including airway epithelial cells (AECs) and airway smooth muscle (ASM) cells, and also determined if its expression is altered in cells derived from subjects with asthma or COPD. We demonstrated that TGF-β increases SPARC expression and release in AECs and ASM cells, although to a lesser extent in the former. We observed that type 1 and type 2 cytokines tend to suppress basal and TGF-β-mediated SPARC expression in AECs, and showed that TGF-β-induced SPARC expression in ASM cells is regulated by the unfolded protein response (UPR). Notably, we observed distinct abnormalities in SPARC expression in asthma and COPD. Our preliminary studies suggest SPARC is overexpressed in AECs from subjects with asthma. In contrast, there

was a trend for reduced SPARC expression in ASM cells from COPD subjects, compared to those from non-COPD subjects. Functional studies indicate SPARC does not impart immunoregulatory functions or regulate changes in airway epithelial cell phenotype, although this requires further validation.

Our studies herein also explored the potential homeostatic role of extracellular high mobility group box 1 (HMGB1) in AECs. HMGB1 is a danger-associated molecular pattern (DAMP) that normally resides in the intracellular compartment, and is released into the extracellular space upon cellular injury, stress or death to orchestrate inflammatory responses. Although it is implicated as a mediator of the airway inflammatory response, its physiological role in lung homeostasis has received little attention. Interestingly, we detected HMGB1 in the culture supernatant of AECs under basal conditions, and found that it presents exclusively as a constituent of protein complexes. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based proteomic approaches, we generated an unbiased profile of HMGB1-binding proteins in the extracellular space of unstimulated AECs. Protein network analysis of identified binding proteins indicates a role for extracellular HMGB1 in epithelial cell homeostasis and airway mucosal immunity.

In summary, findings in this thesis suggest aberrant regulation of SPARC expression in airway structural cells may be a contributing factor to the pathogenesis of chronic airways disease. Our studies also provide a new understanding of the extracellular functions of HMGB1 in AECs and opens new research directions for its use as a therapeutic target.

Chapter 1

General Introduction

INTRODUCTION

Asthma and chronic obstructive pulmonary disease (COPD) are chronic inflammatory disorders of the lung which pose significant health and economic burden, affecting approximately 1 in 12 people worldwide (1). Asthma and COPD are heterogeneous conditions in terms of clinical presentation and response to therapy, and can overlap, giving rise to the asthma-COPD overlap syndrome (ACOS) (1). Patients with asthma experience intermittent episodes of breathlessness, chest tightness, coughing and wheezing which become more frequent in severe disease, whereas these symptoms occur daily for patients with progressive COPD. Similarly, airflow obstruction is present in both conditions, usually variable and reversible in asthma, but is fixed in COPD and severe asthma (2).

The heterogeneity of asthma and COPD are driven by genetic factors and its complex interplay with diverse environmental exposures such as air pollutants, allergens, cigarette smoke or early-life viral infections (3). The study of disease phenotypes or the observable characteristics such as the inflammatory profiles of patients, has provided enhanced understanding of the disease heterogeneity. There is now increased interest in understanding the specific biological pathways that explain and support the observable properties of a phenotype, termed as endotype (4, 5). About 10% of asthmatic patients have severe asthma and are refractory to the mainstay maintenance therapies of inhaled corticosteroids and long-acting β-agonists (LABAs) (6). Stratification of these patients into phenotypes and endotypes have effectively identified patients with T_H2-high inflammation or eosinophilia to have a more favourable treatment

response to novel biologic agents such as anti-IgE, anti-interleukin (IL)-5, anti-IL-13 antibodies (5, 7). No effective targeted therapy is yet available for T_H2-low patients who tend to have neutrophilia. The use of dual long-acting bronchodilators indicated for severe asthma, LABA and long-acting muscarinic antagonist (LAMA), as well as alternative therapy such as low dose macrolides become important treatment options (8). This means understanding and elucidation of the cellular and molecular mechanisms which drive the specific subtypes of asthma and COPD or endotypes (rather than clinical traits), are imperative for the move towards delivery of personalized medicine to improve treatment outcomes.

PATHOPHYSIOLOGY OF ASTHMA AND COPD

Asthma and COPD are driven by distinct underlying molecular pathways although chronic inflammation and structural abnormalities in the airways are common hallmark features in these diseases. For instance, asthma usually begins in childhood and is associated with atopy or IgE-dependent sensitization to environmental allergens, although adult onset asthma can sometimes occur in the absence of allergy (9). Meanwhile, cigarette smoke is the main risk factor of COPD and in developing countries, indoor air pollution from household use of biomass fuels and exposure to occupational dust and chemicals also contribute to the disease (10). Notably, COPD is also a condition of accelerated ageing, whereby COPD patients commonly have age-related co-morbidities such as cardiovascular diseases and metabolic syndromes. This is thought to be due to an excess of reactive oxygen species (ROS), inducing irreconciliable cellular

damage and causes cells to go into senescence and become growth arrested (11).

The pattern of inflammation and remodeling in the asthmatic airways is also different from that of COPD airways. For instance, COPD has three main components which may coexist or that one component can take predominance: (1) chronic bronchitis or inflammation in the large airways; (2) small airways disease featuring inflammation, peribronchiolar fibrosis and airway obstruction; and (3) emphysema defined as alveolar tissue destruction which causes the loss of lung recoil properties (10). Meanwhile, inflammation and remodeling in asthma are present in both small and large airways (12), as further discussed below.

AIRWAY INFLAMMATION

At the lung mucosal surface, the sentinel airway epithelium along with alveolar macrophages and antigen-presenting dendritic cells (DCs), form the first line host defence against external environmental insults such as allergens, air pollutants, infectious pathogens and cigarette smoke (Figure 1.1) (6). DCs can be activated directly by environmental exposures or indirectly by epithelial-derived inflammatory molecules, and serve as an important link between innate and adaptive immune responses (13). In response to allergens, activated epithelial cells release damage-associated molecular patterns (DAMPs) such as high mobility group box 1 (HMGB1), ATP, uric acid and IL-1α. DAMPs are released or mobilized from cells in response to damage, stress or death to initiate and perpetuate immune responses, the significance of which is further

discussed in the final section of this introduction (13, 14). AECs also produce chemokines such as CC-chemokine ligand 2 (CCL2) and CCL20, as well as innate cytokines such as IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), collectively stimulating the migration of DCs to draining mediastinal lymph nodes (MLN) where they skew differentiation of naive T cells into T_H2 cells (13). The innate cytokines also activate type 2 innate lymphoid cells (ILC2s) which produce type 2 cytokines, IL-4 and IL-13, independently of T_H2 cells (15). These type 2 responses ultimately lead to IgE production by B cells and eosinophilic inflammation important for the development of asthma (13).

While epithelial cell – DC interactions play a major role in allergic inflammation, AECs and macrophages drive the airway immune responses in COPD. Inhaled cigarette smoke and other irritants induce the release of pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α, and chemotactic factors such as CCL2, CXC-chemokine ligand 1 (CXCL1), CXCL8, CXCL9, CXCL10 and CXCL11, from AECs and macrophages (12, 16). These mediators promote the infiltration of many immune cell types to the airway submucosa including monocytes, neutrophils, T_H1 cells and cytotoxic T cells, and induce neutrophilic inflammation (12, 16).

While COPD is characterized by predominantly neutrophilic inflammation, and eosinophilic inflammation is associated with asthma, neutrophilia in asthma and eosinophilia in COPD also exist due to the heterogeneity of these diseases (12). As mentioned earlier, while patients with asthma usually present with T_H2 gene signatures, a subgroup of T_H2-low patients who lack evidence of elevated

type 2 biomarkers exist. These are commonly patients with severe asthma who have airway neutrophilia, but not eosinophilia, and are less responsive to corticosteroids (17). Type 1 and type 17 inflammation, driven by interferon-γ (IFN-γ) and type 17 cytokines, IL-17 and IL-22, is thought to contribute to the neutrophilic response associated with type 2-low asthma (17). Indeed, type 17 cells are shown to be increased in the airways of patients with COPD (16). Finally, airway smooth muscle (ASM) cells also have secretory functions and contribute to the inflammatory milieu in both asthma and COPD by releasing cytokines and chemokines such as IL-6 and IL-8 (18).

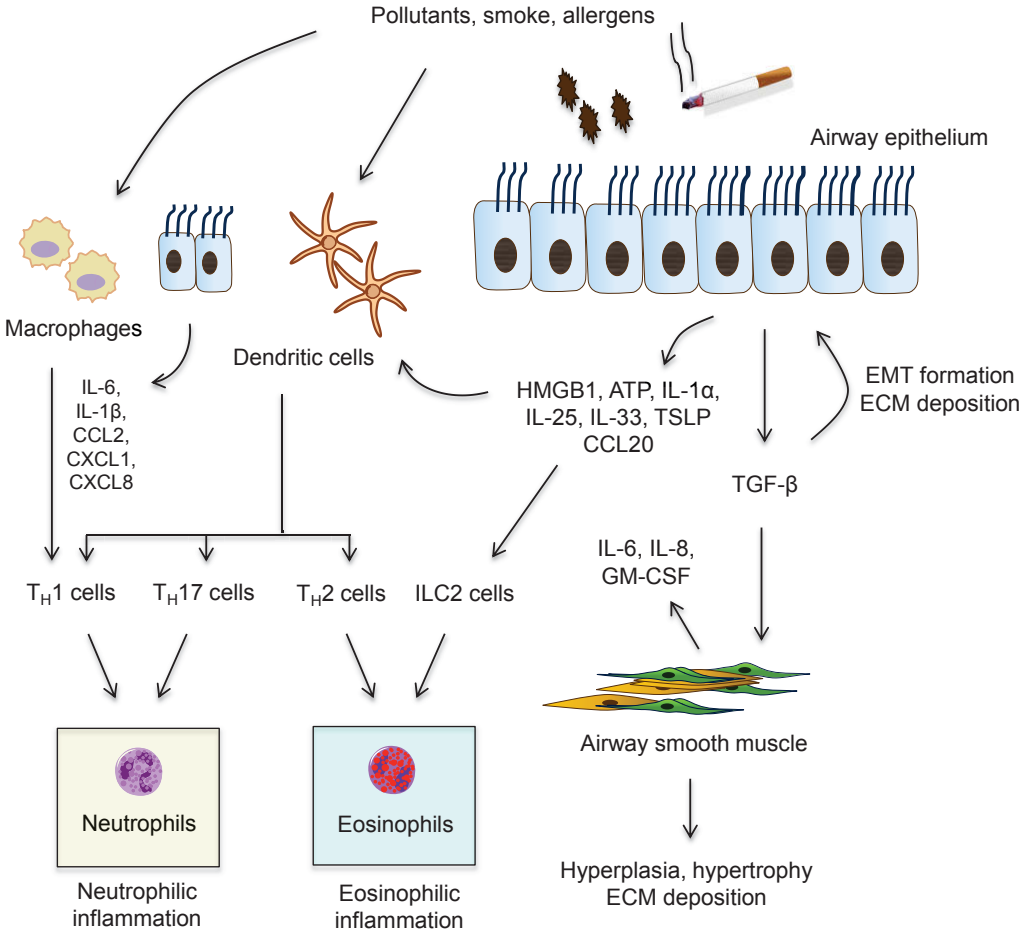


Figure 1.1: Overview of airway inflammation and remodeling in chronic airways disease.
The airway epithelial cells (AECs), alveolar macrophages and dendritic cells (DCs) form the first line barrier against the external environment. Environmental exposures such as cigarette smoke and allergens directly activate DCs and induce the release of pro-inflammatory immune mediators from AECs, which also indirectly stimulate the DCs. Epithelial-derived DAMPs (HMGB1, IL-1α) and innate cytokines (IL-25, IL-33 and TSLP) stimulate DC migration to the draining lymph nodes where they induce differentiation of naïve T cells into T_H2 cells. Innate cytokines also activate type 2 innate lymphoid cells (ILC2s). Together, these type 2 responses promote allergic eosinophilic inflammation, the predominant inflammatory phenotype in asthma. Meanwhile, production of chemotactic factors (CCL2, CXCL1, CXCL8) and inflammatory cytokines (IL-6, IL-1β) by AECs and macrophages lead to infiltration of neutrophils and T_H1 cells to the airway submucosa, resulting in airway neutrophilia in COPD. There is also evidence for a role for T_H17 cells in mediating neutrophilic inflammation in asthma and COPD. In both diseases, airway epithelial damage by external stimuli also leads to the release of growth factors such as TGF-β which promotes airway remodeling by inducing airway smooth muscle hyperplasia (increased number) and hypertrophy (increased size) as well as extracellular matrix protein deposition. TGF-β also drives de-differentiation of the epithelial phenotype by inducing formation of epithelial-mesenchymal transition (EMT) in AECs.

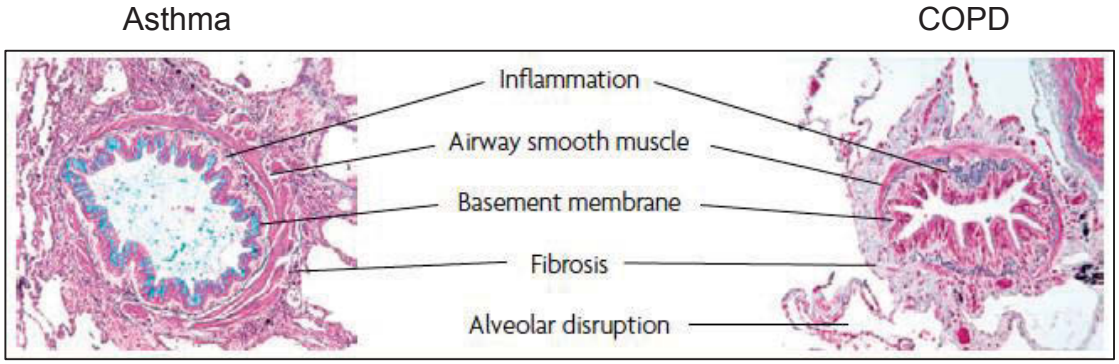
AIRWAY REMODELING

Airway remodeling occurs in the large and small airways in asthma and primarily the latter in COPD (12). It involves the airway epithelium, basement membrane, smooth muscle layer and its surrounding extracellular matrix (ECM) which is made up of a complex network of macromolecules that maintains the structural integrity of airway wall; it also involves the bronchial vasculature (Figure 1.2) (19). The epithelium is chronically injured in both asthma and COPD due to an inherent inability to resolve damage (20). In response to injury, pseudostratified epithelial cells comprising of basal, ciliated and secretory cells de-differentiate and flatten in order to allow migration of cells and closure of the injury or wound (21). Indeed, loss of the ciliated cell layer is evident in asthma and COPD, and the epithelium displays features of de-differentiation towards mesenchymal cells (19, 22). Goblet cell hyperplasia also features in both diseases, resulting in mucus hypersecretion (19). In asthma, the epithelium is typically shedded and denuded while squamous cell metaplasia is increased in COPD. Thickening of the reticular basement membrane (RBM) and subepithelial fibrosis are also only present in asthma but not COPD (19, 22, 23).

The smooth muscle layer is increased in both asthma and COPD as a result of ASM hyperplasia and hypertrophy, an increase in cell number and cell size, respectively (19). The ECM within the smooth muscle layer, lamina propria and adventitia are also thickened in asthma and COPD, but is disproportionately increased in COPD. This is thought to be the reason for the relatively fixed airflow obstruction (cannot dilate nor contract) as opposed to asthma where enhanced contraction of the smooth muscle in the presence of stimuli is

responsible for narrowing of the airway lumen during airway hyperresponsiveness (24). In the asthmatic airways, enhanced deposition of the main constituents of the ECM including collagen I and III, fibronectin, tenascin, hyaluronan and laminin $\alpha 2/\beta 2$ have been reported while collagen IV and elastin are found to be consistently decreased (25). Dysregulated ECM protein expression is also observed in the COPD airways although changes in the specific ECM proteins have been inconsistent (24, 26). For instance, Kranenburg and colleagues showed increased collagen I and III in the basement membrane, lamina propria and bronchial adventitia of bronchial tissues derived from subjects with COPD (27). On the contrary, Annoni and colleagues found collagen I is reduced in the lamina propria of large airways in COPD patients, as well as in all compartments of the small airways including the lamina propria, muscle layer and adventitia. They also reported no differences in collagen III in the large or small airways of COPD patients (28).

The bronchial vascularity is also enhanced in asthma and COPD, contributing to further thickening of the airway wall. Angiogenesis (formation of new vessels) and increased vascular area are observed in both diseases. In addition, parallel increase in vascular size and permeability, as well as edema are evident in asthma (29, 30).



Features	Fatal Asthma	Severe COPD
Inflammation	+++	+++
Airway smooth muscle	+++	+
Basement membrane	++	-
Fibrosis	+ subepithelial	+++ peribronchiolar
Alveolar disruption	-	+++

Figure 1.2: Distinct histopathologies in the asthmatic and chronic obstructive pulmonary disease (COPD) airways.
Small airway tissue sections were isolated from a patient who died from asthma (left panel) and a patient with severe COPD (right panel). Infiltration of inflammatory cells occurs in both asthma and COPD. While the smooth muscle layer is enhanced in both conditions, thickening of the basement membrane and subepithelial fibrosis are present only in asthma, and fibrosis within the COPD airway occurs primarily in the peribronchiolar area. Additionally, the alveolar attachment to the parenchymal tissue is disrupted in COPD due to emphysema but is intact in asthma. The images are reproduced with permission from Nature Reviews Immunology (12).

TGF-β IS A MASTER REGULATOR OF AIRWAY REMODELING

The multifaceted roles of the pro-fibrotic cytokine, TGF-β in airway remodeling in asthma and COPD are well-established. Firstly, TGF-β causes AECs to de-differentiate and lose their epithelial phenotype, and acquire mesenchymal characteristics, termed as epithelial-mesenchymal transition (EMT). This process is thought to contribute to the increased number of fibroblasts which serve as a primary cellular source of ECM proteins and hence, subepithelial

fibrosis (22, 31). TGF-β also induces proliferation and differentiation of fibroblasts into myofibroblasts (32). In addition, TGF-β promotes ASM proliferation and hypertrophy contributing to the thickened smooth muscle layer within the airway wall (33, 34). Finally, TGF-β can directly induce ECM protein synthesis such as fibronectin and collagen by airway structural cells, including AECs, ASM cells and lung fibroblasts (32). The role of AECs and ASM cells as primary effector cells in airway remodeling and inflammation in chronic airways disease, in addition to their structural roles are well-recognized (Table 1.1), and hence form the basis for investigation in these cells.

TGF-β as a therapeutic target in chronic airways disease is not a feasible option given its roles in many physiological processes. Current mainstay therapies including inhaled corticosteroids and the novel biologic agents, primarily target inflammation and there is to date, no therapy that can prevent or reverse airway structural changes (35), pushing for the need to better understand factors that regulate the remodeling process. In addition, emerging evidence indicates that inflammatory phenotypes which form the basis for current disease management, are unstable and that up to 40% patients experience changes in their inflammatory profiles within 1 year irrespective of disease severity (7, 36, 37). A remodeling-centric phenotyping of patients may be superior in light of its relative irreversibility, although it has so far been hindered because tissue analysis from bronchial biopsy remains the gold standard for assessment of airway remodeling (35). Thus, further understanding of novel mediators and/or biological pathways which underlie airway remodeling are compelling and may potentially be useful for patient phenotyping and endotyping.

Table 1.1: Roles of airway epithelial cells and airway smooth muscle cells in airway inflammation and remodeling in chronic airways disease.

Airway epithelial cells (13, 14, 20, 23, 38-41)

• **Dysfunctional epithelial barrier function**
  ○ Loss of epithelial integrity
  ○ EMT formation causing epithelial de-differentiation; and
  ○ Impaired epithelial repair response
• **Secretion of immune mediators** such as HMGB1, IL-1α, IL-25, IL-33 and TSLP
• **Secretion of growth factors** such as epidermal growth factor (EGF), TGF-β1, TGF-β2, insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF)
• **Mucus hypersecretion and goblet cell hyperplasia**
• **Production of ECM proteins** contributes to subepithelial fibrosis and thickening of the basement membrane

Airway smooth muscle cells (18, 42)
• **Secretion of cytokines and chemokines** such as IL-5, IL-6, IL-13, GM-CSF, RANTES (CCL5) and eotaxin (CCL11) • **Secretion of growth factors** such as TGF-β, vascular endothelial growth factor (VEGF) and PDGF which affect cell proliferation, migration and apoptosis • **Expression of cell adhesion molecules** such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1) important for infiltration of immune cells to the airway submucosa • **Production of ECM proteins** may influence cell phenotype and function • **Increased ASM mass** attributed to ASM hypertrophy and hyperplasia • **Enhanced contractile properties** lead to airway hyperresponsiveness (AHR) due to increased bronchoconstriction in response to little provocation (less prominent in COPD)

MATRICELLULAR PROTEINS REGULATE ECM DEPOSITION AND ASSEMBLY

Matricellular proteins are a group of ECM-associated proteins which mediate interaction between cells and the surrounding ECM. These proteins include thrombospondin-1, osteopontin, tenascin-C, periostin and secreted protein acidic and rich in cysteine (SPARC) (43). In contrast to the ECM proteins, matricellular proteins do not have structural roles within the matrix and are not deposited into the ECM. Rather, they critically regulate ECM deposition and assembly, as well as fundamental cell functions such as cell adhesion, proliferation and migration (43, 44). Notably, several matricellular proteins such as osteopontin and SPARC have been shown to be implicated in fibrogenesis and can modulate TGF-β activity (45, 46), suggesting their potential role in chronic airways disease. Matricellular proteins have unrelated primary structures and highly context-dependent physiological and pathological functions (43).

SPARC AS A POTENTIAL PLAYER IN CHRONIC AIRWAYS DISEASE

SPARC is important for normal tissue development and repair. It is usually highly expressed during embryonic development and is diminished in normal adult tissues (44, 47). SPARC expression is increased in cells exhibiting a high turnover rate such as the epithelial cells in gut, skin and glandular tissue, as well as during tissue injury and inflammation, reinforcing its importance in tissue regeneration and repair (48). Interestingly, SPARC-null mice have been shown

to be viable and fertile but these mice exhibit phenotypes of premature ageing such as early onset cataract, osteopenia, accumulation of excessive adipose tissue and intervertebral disc degeneration (43). These mice also exhibit deficiencies in ECM assembly and composition such as smaller and morphologically altered collagen fibrils (49). These observations suggest SPARC may be dispensable for development but defective tissue repair due in part to dysregulated ECM structure may contribute to the accelerated ageing process.

Importantly, dysregulation of SPARC expression is emerging as an important contributor to several chronic conditions including cancer, fibrosis and diabetes (50-52). The altered SPARC expression in cancer tissues is complex and highly contextual as reviewed by Nagaraju and colleagues; it is overexpressed in breast cancer, melanoma and gliomas but is suppressed in others such as lung, ovarian and prostate cancer (51). It is hence perceivable SPARC has opposing actions in tumorigenesis, acting as a tumor suppressor gene in cases where endogenous SPARC expression is low, but leads to a more aggressive phenotype when present in high levels (51). SPARC is also overexpressed in fibrotic diseases of the liver, lung and the Tenon's capsule (thin membrane surrounding the eyeball) (53-55). This is envisioned given that SPARC is responsible for the processing and deposition of mature collagen I fibrils (organized) into the ECM, and is essential for fibronectin matrix assembly (56, 57). In addition, TGF-β is a well-established inducer of SPARC expression, and mediates its pro-fibrotic responses in a SPARC-dependent manner (46, 58). Some evidence suggests SPARC overexpression in fibrotic conditions may be a

consequence of enhanced TGF-β expression or signaling (59). SPARC also plays a part in inflammatory response although this is not well understood. SPARC serves to dampen inflammation in some disease contexts but in SPARC-null mice, it has also been shown to demonstrate reduced inflammation and faster recovery, consistent with the highly contextual nature of SPARC activities (60, 61).

In the lung, SPARC drives pathological responses in non-small cell lung cancer and idiopathic pulmonary fibrosis (IPF) by promoting microvascular remodelling and excessive deposition of ECM proteins (62). Asthma and COPD also involve significant remodelling in both the airway and vascular compartments, and TGF-β is a key driver of airway remodeling in these conditions (19, 32). Thus, it is necessary to explore the potential role of SPARC in asthma and COPD, and hence this is the focus of Chapters 3 and 4 in this thesis.

ENDOPLASMIC RETICULUM STRESS IS IMPLICATED IN CHRONIC AIRWAYS DISEASE

Endoplasmic reticulum (ER) stress and prolonged activation of the ensuing unfolded protein response (UPR), which serves as an important cellular adaptive response to restore homeostasis, are emerging as key causative factors of many diseases including chronic airways disease (63, 64). The ER is an organelle responsible for the biosynthesis and folding of secretory and membrane proteins, which make up one-third of all proteins in cells (65). The ER also maintains a high Ca^{2+} concentration within the ER lumen to support functions of the Ca^{2+}-dependent chaperones and folding enzymes to ensure

proteins are properly folded and assembled before they exit the ER for secretion (66). Conditions such as Ca^{2+} depletion, oxidative stress, microbial infections, ageing and even increased physiological demand for protein secretion such as during inflammation perturb ER function, thus leading to accumulation of unfolded or misfolded proteins in the ER lumen. Subsequent activation of the UPR aims to re-establish homeostasis but prolonged or severe ER stress drives cells into apoptosis, termed 'terminal UPR' (63, 65).

The UPR constitutes a complex cascade of events co-ordinated through three distinct branches following activation of the corresponding ER stress sensors including inositol-requiring kinase 1 (IRE1), protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) (Fig 1.3). The initiation of the UPR is dependent on the key ER chaperone, glucose-regulated protein 78 (GRP78; also known as immunoglobulin heavy chain-binding protein or BiP) which is up-regulated during ER stress, hence its common use as a marker of ER stress (63, 65). Under homeostatic conditions, binding of GRP78 to the luminal domains of three ER stress sensors retains them in an inactive state. Upon accumulation of misfolded proteins in the ER, GRP78 dissociates from the sensors to bind to the hydrophobic domains of these misfolded proteins. This primes IRE1 and PERK for oligomerization and auto-transphosphorylation, and exposes an ER export motif in ATF6, leading to the activation of downstream signaling cascades (Figure 1.3) (63, 65).

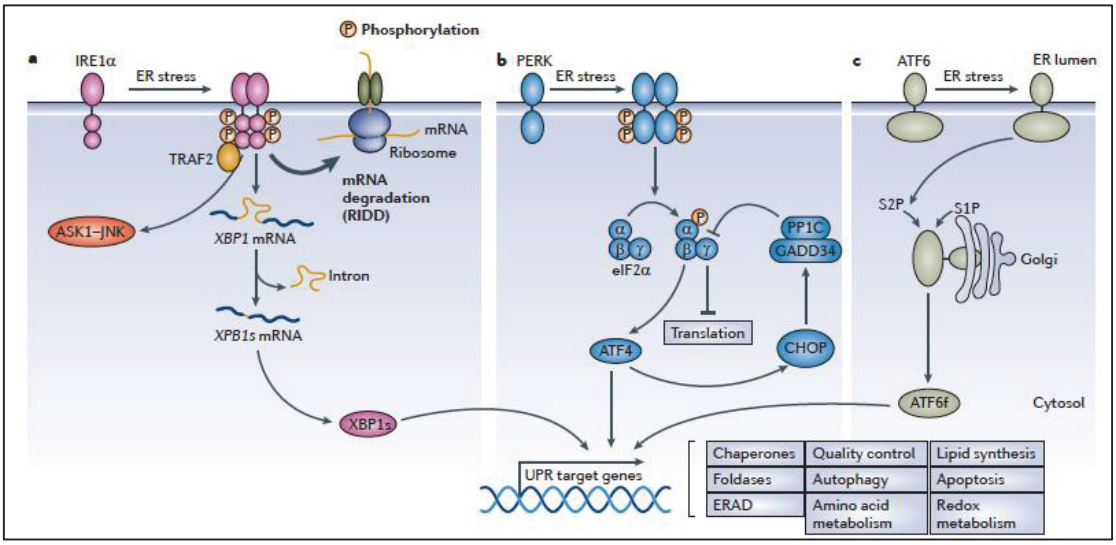


Figure 1.3: The signaling arms of the unfolded protein response (UPR). Endoplasmic reticulum (ER) stress sensors including inositol-requiring kinase 1 alpha (IRE1α), protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6), co-ordinate a complex cascade of events for the UPR, mediated through three different pathways. **a**| Activation of IRE1α leads to its assembly into higher order oligomerization, *trans*-autophosphorylation and the activation of its RNase domain. IRE1α RNase processes the X-box binding protein 1 (XBP-1) mRNA to produce the active transcription factor, spliced XBP-1 (XBP-1s) which upregulates UPR target genes involved in protein folding, ER-associated protein degradation (ERAD) and organelle biogenesis. IRE1α RNase also degrades select mRNAs through regulated IRE1-dependent decay (RIDD), especially mRNAs encoding proteins of the secretory pathway. In addition, IRE1α engages the adaptor protein tumour necrosis factor receptor-associated factor 2 (TRAF2) to activate the JUN N-terminal kinase – apoptosis signal-regulating kinase 1 (JNK-ASK1) pathway, contributing to cell apoptosis. **b**| Activation of PERK is similar to IRE1α, involving its dimerization and *trans*-autophosphorylation. Activation of PERK attenuates global protein synthesis through phosphorylation of the eukaryotic translation initiator factor 2α (eIF2α). Phosphorylation of eIF2α also allows for selective translation of the transcription factor ATF4, which regulates genes involved in antioxidant responses, amino acid metabolism, autophagy as well as genes encoding for ER chaperones. ATF4 also regulates expression of pro-apoptotic molecules, C/EBP-homologous protein (CHOP) and growth arrest and DNA damage-inducible 34 (GADD34). GADD34–protein phosphatase 1 (PP1) complex participates in a feedback loop manner to dephosphorylate eIF2α and subsequently restores protein synthesis, which can be counter-productive if ER stress is unresolved. **c**| In response to ER stress, ATF6 translocates to the Golgi apparatus where it is processed by site 1 protease (S1P) and site 2 protease (S2P) to release its cytosolic fragment, ATF6f (also known as ATF6p50). ATF6f is a transcription factor which regulates genes involved in protein folding and ERAD. The image is reproduced with permission from Nature Reviews Drug Discovery (67).

One of the first responses to re-establish ER homeostasis is the attenuation of global protein translation to reduce entry of newly synthesized proteins into the ER. This is achieved through PERK-mediated phosphorylation of its downstream mediator eukaryotic translation initiator factor 2α (eIF2α) as well as through regulated IRE1-dependent decay (RIDD) of mRNAs encoding proteins of the secretory pathway (Figure 1.3) (63, 65). Another mechanism set in place involves enhanced elimination of misfolded proteins through ER-associated protein degradation (ERAD) and/or autophagy-mediated lysosomal degradation. The UPR also expands the ER protein folding capacity by increasing ER biogenesis and enhances gene transcription of ER chaperones such as calreticulin and calnexin. In addition, amino acid metabolism and antioxidant responses are also enhanced, altogether reducing the cellular stress imposed on the ER and allow for its recovery (63, 65).

Chronic ER stress and the resulting cellular injury are becoming an important contributor to the development and progression of asthma and COPD. The seminal genome-wide association study (GWAS) by Moffatt and colleagues identified the gene encoding ER transmembrane protein orosomucoid like 3 (ORMDL3) to be an asthma susceptibility gene, suggesting ER stress may play a role in asthma pathogenesis (68). A recent study demonstrated that ORMDL3-null mice are protected from Alternaria-induced allergic asthma although how the inhibitory effect of ORMDL3 on sphingolipid synthesis contributes to ER stress is not currently known (69). ER stress is also significantly increased in several experimental mouse models of allergic

asthma, and the use of chemical chaperones such as 4-phenylbutyric acid (4-PBA) and tauroursodeoxycholic acid (TUDCA) have proven effective in reducing ER stress, airway inflammation and fibrosis, and airway hyperreactivity (AHR), indicating ER stress is implicated in the pathogenesis of asthma (70-73). Importantly, the expression of ER stress markers, GRP78 and C/EBP-homologous protein (CHOP) were shown to be significantly higher in the bronchoalveolar lavage (BAL) fluid, as well as peripheral blood mononuclear cells (PBMCs) and lung fibroblasts from asthmatic subjects than from healthy subjects (73, 74).

Min and colleagues also showed ER stress is augmented in COPD and correlates with disease severity, given CHOP and phospho-eIF2α expression are most abundant in lung tissues derived from Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage III/IV patients when compared to GOLD stage I and II tissues (75). Interestingly however, Korfei and colleagues could not detect the expression of ER stress markers including CHOP, ATF4 and cleaved p50ATF6, as well as the active, spliced XBP-1 mRNA in COPD lung tissues (76). It is noted however that disease severity of these patients was not identified, and could be the factor accounting for the discrepancy observed.

While emerging evidence indicates ER stress is implicated in chronic airways disease, the underlying mechanisms are largely unknown. Of note, emerging studies suggest ER stress plays a part in tissue fibrosis. Indeed, studies from alveolar epithelial cells showed ER stress induces the formation of EMT through activation of the Smad2/3 and Src pathway, although the involvement of TGF-β

was not examined (77, 78). The potential role of ER stress in airway remodeling is further supported by evidence of ER stress mediating TGF-β-induced myofibroblast differentiation and collagen production in lung fibroblasts (74, 79). Since SPARC may be implicated in airway remodeling in chronic airways disease, the question of whether ER stress impacts on SPARC expression needs to be investigated (Chapter 4).

HMGB1 IS EMERGING AS AN IMPORTANT MEDIATOR OF CHRONIC AIRWAYS DISEASE

HMGB1 is a prototypical DAMP which serves as a critical modulator of the innate and adaptive immune responses in the airways, as discussed above (Figure 1.1). Emerging evidence suggests that HMGB1 also plays an important role in lung repair and remodeling of the airways. Of particular relevance to this thesis, a novel link between HMGB1 and SPARC in AECs has recently been identified.

By definition, DAMPs are dual-function proteins that have distinct roles inside or outside cells. They usually reside in the nucleus and exert important physiological functions, but when exposed to the extracellular environment, they become ‘visible’ to the immune system and serve to alert the body about danger, by stimulating the inflammatory response and promoting the regeneration process (80, 81). We and others have identified HMGB1 as an important mediator in the initiation and progression of the airway inflammatory response in mouse models of allergic asthma, and have also shown that inhibition of HMGB1 provides amelioration for the disease (82-84). Increased

levels of HMGB1 have also been detected in the sputum, BAL fluid and airway tissues of patients with asthma and COPD, and were positively correlated with disease severity (83, 85, 86).

Although implicated as a mediator of the inflammatory response, HMGB1 is emerging as a key player in tissue repair and airway remodeling. HMGB1 has been shown to promote wound closure and ECM protein deposition in lung epithelial cells, the latter of which is indispensable for migration of repairing cells (87, 88). Importantly, one of the earliest studies showed HMGB1 is a cell surface-binding protein in 'healthy' cortical neurons and serves to regulate cell motility and neurite outgrowth (89, 90). This evidence of HMGB1 localization at the leading edge of the cells under physiological conditions suggests that HMGB1 potentially has other yet undefined functions in fundamental cellular processes. Consistent with this notion, we detected the presence of HMGB1 in the culture supernatant of AECs under basal conditions, suggesting extracellular HMGB1 may also have homeostatic functions. Notably, HMGB1 was recently shown to induce the expression of SPARC in AECs (91). Similar to SPARC, HMGB1 also induces formation of EMT in multiple cell types and induces differentiation of lung fibroblasts into myofibroblasts (91-93). The potential role of HMGB1 as a pro-repair and pro-remodeling factor in the airways, in addition to its established function as a DAMP, is intriguing, but requires further investigation.

HMGB1 interacts with diverse proteins and receptors. For instance, HMGB1 binds to toll-like receptor (TLR) ligands such as lipopolysaccharide (LPS) and

Pam_3CSK_4, and these complexes lead to enhanced inflammatory responses via activation of TLR signalling (94, 95). HMGB1 also binds CXCL12 and amplifies CXCL12-mediated chemotactic activity through activation of the CXCL12 receptor, CXCR4 (96). In contrast, a small glycosyl-phosphoinositol (GPI)-anchored protein, CD24 interacts with sialic acid-binding Ig-like lectin (Siglec)-10, to form a ternary complex with HMGB1; this complex dampens the immuno-stimulatory activity of HMGB1 (97). It is evident that when HMGB1 is in complex with its binding partners, it signals through the receptor of the partner molecule, suggesting the latter indirectly dictates HMGB1 function (98). Thus, in order to gain insight into the biological functions of extracellular HMGB1 in AECs, we used a proteomic approach to identify its binding partners (Chapter 5).

HYPOTHESES AND AIMS

Although the matricellular protein SPARC is implicated in numerous diseases where inflammation and tissue remodeling play an important role, the role of SPARC in chronic airways disease has not as yet been investigated. Thus, in order to inform this investigation, a literature review was undertaken, specifically focusing on studies which have examined the role of SPARC in other lung diseases such as lung cancer and pulmonary fibrosis (**Chapter 2**).

In light of evidence that SPARC is down-stream effector of TGF-β, an important mediator of tissue remodeling, we hypothesized that airway structural cells such AECs and ASM cells, secrete SPARC when exposed to TGF-β, and that SPARC in turn, acts on these cells to augment the disease process. We also hypothesized that SPARC expression is dysregulated in chronic airways disease, in part due to increased ER stress and activation of the UPR.

Thus, in order to address these hypotheses, the aims of this thesis were to:

1. Determine if TGF-β induces SPARC expression and secretion in AECs (**Chapter 3**) and ASM cells (**Chapter 4**).
2. Determine if TGF-β-induced SPARC secretion is regulated by the UPR; these studies were conducted in ASM cells only as TGF-β had a modest stimulatory effect in AECs (**Chapter 4**)
3. Determine whether SPARC regulates airway epithelial cell inflammatory responses and cellular phenotype (**Chapter 3**)
4. Determine whether SPARC expression is dysregulated in chronic airways disease; we investigated SPARC expression in epithelial cells

from asthmatic subjects (**Chapter 3**), and ASM cells from COPD subjects (**Chapter 4**).

HMGB1 is a classical DAMP which is released during cellular stress and injury to orchestrate immune and inflammatory responses, and tissue repair process. Interestingly, HMGB1 has also been shown to be released under physiological conditions but this and its potential significance as a homeostatic molecule has been largely overlooked (90, 99). Notably, we detected the presence of HMGB1 in the culture supernatant of AECs under basal conditions, suggesting the basal release of HMGB1 in AECs may play a part in cell turnover and/or homeostasis.

To address this hypothesis, the aim was to:

5. Perform a global analysis of HMGB1-binding proteins in the extracellular milieu of AECs under basal conditions, followed by protein network analysis on the identified HMGB1-binding proteins to provide contextual insight into the potential homeostatic function of HMGB1 (**Chapter 5).**

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Chapter 2

The SPARC protein: an overview of its role in lung cancer and pulmonary fibrosis and its potential role in chronic airways disease

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ABSTRACT

The SPARC (secreted protein acidic and rich in cysteine) protein is matricellular molecule regulating interactions between cells and their surrounding extracellular matrix (ECM). This protein thus governs fundamental cellular functions such as cell adhesion, proliferation and differentiation. SPARC also regulates the expression and activity of numerous growth factors and matrix metalloproteinases essential for ECM degradation and turnover. Studies in SPARC-null mice have revealed a critical role for SPARC in tissue development, injury and repair and in the regulation of the immune response. In the lung, SPARC drives pathological responses in non-small cell lung cancer and idiopathic pulmonary fibrosis by promoting microvascular remodelling and excessive deposition of ECM proteins. Remarkably, although chronic airway conditions such as asthma and chronic obstructive pulmonary disease (COPD) involve significant remodelling in both the airway and vascular compartments, the role of SPARC in these conditions has thus far been overlooked. In this review, we discuss the role of SPARC in lung cancer and pulmonary fibrosis, as well as potential mechanisms by which it may contribute to the disease process in asthma and COPD.

INTRODUCTION

The SPARC (secreted protein acidic and rich in cysteine) family of proteins modulate interactions between cells and the extracellular environment. They regulate extracellular matrix (ECM) assembly and deposition and growth factor signalling. SPARC family proteins have in common three domains: an acidic N-terminal domain, a cysteine-rich follistatin-like (FS) domain and an α-helical extracellular (EC) calcium-binding domain with an EF-hand motif. They are classified into four groups based on sequence homology of the EC domain (Table 2.1). The FS and EC domains are conserved, conferring activities common to the SPARC family, while the N-terminal domain varies between the family members (1-3). Given these structural and functional similarities, there may be a level of redundancy with respect to their physiological actions, but this is not yet clearly established.

Table 2.1: Classification of SPARC family proteins.

Group	SPARC family proteins
1	SPARC (osteonectin, BM-40) Hevin (SPARC-like 1)
2	Secreted modular calcium binding protein (SMOC) 1 and 2
3	Testican 1, 2 and 3 (SPOCK, SPARC/osteonectin, CWCV, and Kazal-like domains proteoglycans)
4	Follistatin-like protein 1 (FSTL1, TSC-36/Flik, follistatin related protein (FRP), TGF-β inducible protein)

One of the best studied members of this protein family is SPARC. SPARC is a highly conserved matricellular protein and, although it is most often encountered as a secreted glycoprotein, it is also expressed on the cell surface and within the intracellular compartment. Interestingly, while extracellular SPARC functions as a matricellular protein, intracellular and membrane-associated SPARC regulate cellular apoptotic pathways (4, 5) (Table 2.2). SPARC expression is elevated during embryonic development and is diminished in normal adult tissues. Significantly however, its expression is increased in epithelial cells exhibiting a high turnover rate such as those in the gut, skin and glandular tissue, during tissue injury and inflammation, and under conditions of abnormal tissue growth associated with neoplasia, suggesting its importance in tissue regeneration and repair (1, 3, 6).

SPARC-null mice have yielded significant insight into its biological functions. These mice exhibit several aberrant features, related mainly to dysregulation of ECM structure and composition. For instance, they have less and smaller fibrillar collagen within connective tissues of the heart, in adipose tissue and in the skin (7-10). SPARC-null mice also exhibit early onset cataracts due to altered morphology of collagen IV (the major structural protein of the lens basement membrane) and compromised structural integrity of the lens capsule (11, 12). Other aberrant manifestations include osteopenia, accelerated wound healing and greater deposition of subcutaneous fat (7, 13, 14). Notably, the reported biological effects of SPARC at the tissue and cellular level are quite variable, but given it acts as a ‘communicator’ at the cell-ECM interface, this is thought to be due to differences in the cellular and tissue microenvironment.

Hence, this calls for the need to scrutinize the role of SPARC in a tissue- and cell-specific manner and in specific pathophysiological contexts.

Importantly, there is now a large body of evidence implicating SPARC in several chronic diseases including cancer, fibrosis, glaucoma and diabetes. In the lung, SPARC is heavily implicated in both cancer development and pulmonary fibrosis. Surprisingly, however, little is known about its role in other lung diseases featuring inflammation and tissue remodelling, most notably asthma and chronic obstructive pulmonary disease (COPD). The ECM microenvironment is essential for maintaining lung homeostasis, and perturbation of the ECM usually causes or accompanies chronic lung diseases. Because SPARC dictates crosstalk between cells and the ECM, a comprehensive understanding of its role in the lung and its contribution to pathological inflammation and remodelling are crucial. This review consolidates current understanding of the role of SPARC in lung cancer and idiopathic pulmonary fibrosis (IPF), highlights areas for future research in chronic airway diseases such as asthma and COPD and discusses potential strategies for exploiting and targeting SPARC for therapeutic purposes.

Table 2.2: Role of SPARC in different cellular compartments.

Extracellular SPARC	References
Cell adhesion, proliferation, migration Mediates interactions between cells and their surrounding ECM	(15-17)
Regulates angiogenic activity	(18-22)
Binds to ECM proteins such as collagen I and IV	(23)
Regulates expression and activity of MMPs involved in ECM proteolysis and turnover	(24)
Regulates ECM-associated growth factors and signaling, for example VEGF, basic FGF and TGF-β	(22, 25-29)
Membrane SPARC	
Augments apoptotic signaling via interaction with pro-caspase 8	(4)
Intracellular SPARC	
Enhances cell viability and renders resistance against apoptosis via activation of Fyn/ERK kinase signaling	(5)
bFGF, basic fibroblast growth factor; MMPs, matrix metalloproteinases; TGF-β, transforming growth factor beta; VEGF, vascular endothelial growth factor.	

SPARC AND LUNG CANCER

SPARC expression in NSCLC tissues is associated with disease prognosis

Lung cancer is the leading cause of cancer deaths worldwide, and the 5 year survival rate remains poor at 16% (30, 31). Lung cancer can be classified into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), depending on the type of cells affected. NSCLC is more common, accounting for approximately 85% of all lung cancer cases (30). Although extensive evidence implicates SPARC in NSCLC, there have been no studies that have examined its role in SCLC.

SPARC is expressed heterogeneously in NSCLC tissues. It is predominantly found in the tumour-associated stroma, specifically in the cytoplasm and ECM of stromal fibroblasts. Within the tumour itself, SPARC expression is rare, but when present, it is localized to sites of necrosis (32-34). The tumour-associated stroma, comprising fibroblasts, immune cells, vasculature and ECM, is crucial for supporting and facilitating the tumour. Indeed, the interaction of the tumour with its surrounding stroma is essential for tumour growth, differentiation, progression and metastasis and thus determines the aggressiveness of the cancer (35). The predominant stromal versus tumoral expression of SPARC in NSCLC suggests SPARC is important in supporting the survival and progression of lung tumours, most likely by assisting crosstalk at the tumour–stroma interface.

Interestingly, the localization of SPARC in NSCLC tissues is linked to disease prognosis. High levels of SPARC gene expression within NSCLC tumours, albeit rare, are associated with longer survival, while its absence is a negative prognostic factor (34, 36). On the other hand, patients bearing SPARC-positive stroma have significantly poorer overall post-operative survival. Indeed, the expression of SPARC within the stroma is often associated with extensive tumour necrosis, acidity, hypoxia and oxidative stress, all of which are key features of an aggressive tumour (32). These findings support the idea that stromal SPARC supports tumour growth and tumour–stroma interactions, contributing to a more aggressive malignancy. When expressed within the tumour, SPARC could be protective and possibly buffer the aggressiveness of the tumour itself, highlighting the tissue-specific functions of SPARC.

The expression of SPARC within NSCLC tumours appears to be heavily influenced by epigenetic factors. Shao and colleagues showed that treatment of patient-derived tumour xenografts with the demethylating agent, 5-aza-2′-deoxycytidine (decitabine), leads to increased SPARC expression, suggesting that low or absent expression of SPARC in these tumour xenografts is due to methylation of its gene promoter region (37). Interestingly, SPARC has been identified as a downstream target of the tumour suppressor gene ras-association domain family 1 isoform A (RASSF1A), which is also known to be hypermethylated at its promoter region and down-regulated in lung cancer. Notably, in tumours where RASSF1A is ‘switched-off’, SPARC mRNA expression is also decreased (38). Thus, epigenetic factors that enhance DNA methylation status ultimately affect SPARC expression in the tumour. Given that

tumour, rather than stromal, localization of SPARC within NSCLC tissues is associated with disease prognosis, further studies are needed to establish the factors that regulate differential SPARC expression in NSCLC, and whether SPARC serves different functions in the tumour and in the stroma.

SPARC promotes metastasis in NSCLC by promoting cell invasion and development of the tumoral vasculature network

Metastasis is thought to occur during the early phase of NSCLC. It is the key factor driving the aggressiveness of NSCLC and the primary cause of mortality. The recurrence rate of NSCLC following surgery is remarkably high at 40%, suggesting the presence of underlying micro-metastases which are not readily detected at the time of surgery (39). Metastasis is a multi-step process, involving the initial local invasion of tumour cells into the stroma, followed by dissemination of tumour cells into the vasculature and then colonization at distant organs (40).

SPARC has been identified as a key mediator of cell invasion in NSCLC and thus may play an important role in NSCLC metastasis. Indeed, it has been shown that Snail, a zinc finger transcription factor that is up-regulated in NSCLC tissues, promotes cell invasion in A549 lung epithelial cells in a SPARC-dependent manner (41, 42). It is proposed that Snail enhances cell invasion by up-regulating TGF-β1 and SPARC expression via the activation of MAP kinase, MEK/ERK signalling pathways (42). Consistent with this finding, cell invasion is inhibited when SPARC expression is suppressed by the zinc finger transcription factor Kruppel-like factor 4 (KLF4). This is corroborated by evidence showing

that KLF4-transfected A549 cells recover their invasive ability when SPARC expression is restored (43).

Further to its role in cell invasion, SPARC is also implicated in the development of the tumour vascular network. The vasculature is essential for tumour growth and metastasis it provides oxygen and nutrients essential for tumour survival and facilitates the migration of detached tumour cells to distant organs (40). Interestingly, within the tumour site, SPARC is highly expressed in small immature, but not large, blood vessels, suggesting it is important in blood vessel maturation and, perhaps, that it is no longer required once the vasculature develops. Notably, and consistent with evidence that localization of SPARC to the stroma is associated with poorer prognosis, stromal SPARC expression is associated with a higher density of mature intra-tumour vessels. Interestingly however, it is not associated with overall vascular density or the expression of angiogenic factors such as VEGF and basic FGF, suggesting SPARC has a minimal role in the formation of new vessels (32). Thus, SPARC appears to primarily enhance tumour growth and progression by promoting vessel maturation under unfavourable hypoxic and acidic conditions within the tumour microenvironment.

SPARC AND PULMONARY FIBROSIS

SPARC confers resistance to apoptosis in lung fibroblasts and is a downstream effector of TGF-β-induced fibrosis in IPF

Pulmonary fibrosis is the end stage of parenchymal lung diseases that result in respiratory insufficiency. The most common form of pulmonary fibrosis, IPF,

typically presents with alternating regions of normal lung parenchyma, interstitial inflammation, fibrosis and honeycombing (distorted architecture) and is characterized by excessive deposition of ECM protein and irreversible destruction of lung architecture (44). Notably, SPARC is expressed in lung tissue obtained from IPF patients but is absent in healthy subjects. In IPF lung tissue, SPARC is mainly localized to the cytoplasm of migrating and synthetically active fibroblasts within fibroblastic foci, suggesting SPARC is involved in the initial phase of fibrosis (45). Consistent with this finding, lung fibroblasts isolated from IPF patients constitutively express more SPARC than those derived from subjects without IPF (46). Together, these findings provide strong support for the involvement of SPARC in IPF pathogenesis.

Resistance to apoptosis in lung fibroblasts is a fundamental feature of IPF. In this disease, repeated injury of alveolar type II (ATII) epithelial cells leads to sustained recruitment of repair mediators. In addition, resident fibroblasts proliferate and differentiate into myofibroblasts which deposit ECM proteins. Critically, these myofibroblasts are resistant to apoptosis and thus sustain a non-degradative ECM, leading to the development of fibrotic scars and loss of alveolar function (44, 47). Particularly, SPARC expression in IPF lung fibroblasts leads to downstream activation of β-catenin and increased expression of plasminogen activator inhibitor-1 (PAI-1), which confers resistance to plasminogen-induced apoptosis. Thus, overexpression of SPARC in IPF potentially drives tissue fibrosis via the induction of PAI-1 expression, leading to a pool of myofibroblasts that are resistant to apoptosis and, consequently, a dysregulated ECM milieu (46).

Moreover, TGF-β induces SPARC expression in human lung fibroblasts (48). TGF-β is a key pro-fibrotic mediator secreted by ATII epithelial cells, macrophages and myofibroblasts following injury. It promotes lung fibrosis in IPF by mediating the recruitment of fibroblasts and their differentiation into myofibroblasts, by stimulating the production of ECM proteins such as collagen and fibronectin, and suppressing the activity of matrix metalloproteinases (MMPs), plasminogen activators and elastases involved in ECM turnover (31, 44, 46, 49). This suggests SPARC could be a downstream effector of TGF-β-induced fibrotic responses. Of note, TGF-β and SPARC are both capable of inducing PAI-1 expression, but whether TGF-β induces PAI-1 in a SPARC-dependent manner is yet to be determined (46, 50). In addition, TGF-β also plays a key role in driving the production of ROS and consequently oxidant-induced epithelial injury in IPF (51). Lung fibroblasts derived from IPF patients have an intrinsic capacity to generate greater levels of ROS, compared with those from healthy subjects, and SPARC mediates TGF-β-induced hydrogen peroxide production in these cells (48). Thus, future studies should examine whether TGF-β/SPARC signalling is an important axis that underlies pro-fibrotic and pro-oxidant responses in IPF pathogenesis.

Mouse models of bleomycin-induced IPF reveal a distinct role for SPARC in inflammatory versus fibrotic components of the disease

Consistent with data from human studies, bleomycin-induced pulmonary fibrosis in mice is associated with higher levels of SPARC expression in the lung and, as observed in human IPF tissues, SPARC is predominantly expressed in the cytoplasm of fibroblasts localized to areas of lung injury. As might be expected,

SPARC-null mice develop less fibrosis and express lower levels of collagen following bleomycin exposure (52, 53). Interestingly, however, Savani and colleagues reported increased collagen deposition and fibrosis and increased destruction of the alveolar architecture in bleomycin-treated SPARC null mice (54). The reason for the difference in findings is not immediately clear, although Savani and Strandjord employed the same strain of SPARC-null mice, excluding this as a factor.

In addition to examining the role of SPARC in the fibrotic response, Sangaletti also examined its role in the underlying interstitial inflammation. Intriguingly, they found that while SPARC-null mice were protected against bleomycin-induced fibrosis, they had more intense inflammation, indicating that SPARC acts to depress the inflammatory response. Moreover, using chimeric mice, they showed that fibroblast-derived SPARC induces fibrosis by promoting the assembly of mature, functional collagen fibers, while leukocyte-derived SPARC attenuates fibrosis by reducing the extent of underlying inflammation (53). Interestingly, in the study mentioned above, Savani reported increased fibrosis together with increased inflammation. While this is inconsistent with the findings of Sangaletti, it is possible that the extensive fibrosis observed in this study may have been driven by the inflammatory response, although this was not examined (54). Nevertheless, the net effect of SPARC in pulmonary fibrosis is likely to be due to its temporal expression by distinct cell types at distinct disease stages. Indeed, it is possible that fibroblast-derived SPARC mediates the onset of the fibrotic process following lung injury while leukocyte-derived SPARC acts at later stages to resolve inflammation. Therefore, the cell-specific

and temporal actions of SPARC and how they differentially affect the disease process certainly require further investigation.

SPARC AND CHRONIC AIRWAYS DISEASE

SPARC activity overlaps with TGF-β, a key mediator in asthma and COPD

Asthma and COPD are heterogeneous inflammatory disorders of the respiratory tract characterized by airflow limitation, which is typically variable and reversible in asthma, but progressive and irreversible in COPD. Chronic airway inflammation is a hallmark of both conditions and is thought to drive the structural abnormalities of the airways, collectively termed airway remodelling. There are overlapping features in asthma and COPD, but the pattern of inflammation and remodelling is distinct in each condition. Importantly, however, TGF-β is a well-established mediator of asthma and COPD and is regarded as the 'master switch' that orchestrates remodelling processes in both conditions, albeit via distinct mechanisms (55).

While the expression and function of SPARC in asthma and COPD have not been studied to date, TGF-β induces SPARC expression in many cell types, and it is well established that SPARC is a downstream effector of TGF-β signalling (42, 48, 56). Moreover, SPARC has also been shown to regulate TGF-β expression and activity, indicating the presence of a reciprocal regulatory relationship. SPARC induces phosphorylation of Smad2, a principal mediator of TGF-β signalling in lung epithelial cells (29). Furthermore, the combined stimulation of mesangial cells with SPARC and TGF-β induces a synergistic increase in Smad2 phosphorylation. Consistent with this, mesangial cells

isolated from SPARC-null mice exhibit reduced levels of phosphorylated Smad2, which can be restored with the addition of exogenous SPARC (28). Interestingly, TGF-β neutralizing antibodies inhibit SPARC-induced Smad2/3 nuclear translocation in lung epithelial cells, indicating that regulatory effects of SPARC on Smad signalling may be due to its ability to induce TGF-β expression (29). Furthermore, there is evidence to suggest that SPARC regulates TGF-β/Smad signalling by directly interacting with TGF-β receptors. Specifically, it has been shown that the SPARC binds to the TGF-β-receptor type II (TGFβRII) in a TGF-β1-dependent manner (28). It is possible that SPARC binds to a structural conformation that encompasses TGF-β1 and TGFβRII or that SPARC interacts with TGFβRII following a conformational change induced by TGF-β1.

Potential role of SPARC in airway wall remodelling

In view of the considerable overlap between TGF-β and SPARC with respect to their biological activity, and evidence of their reciprocal regulation and direct interaction, the investigation of SPARC biology in airway wall remodelling in asthma and COPD is an important area of future research. Indeed, SPARC may contribute to changes in airway structure and function via a number of possible mechanisms. In the sections below, we will discuss the potential role of SPARC with regard to three key aspects: changes in the ECM milieu, angiogenesis and epithelial-mesenchymal transition (EMT). Figure 2.1 summarizes potential mechanisms by which SPARC might regulate airway and vascular remodelling in chronic disease of the airways.

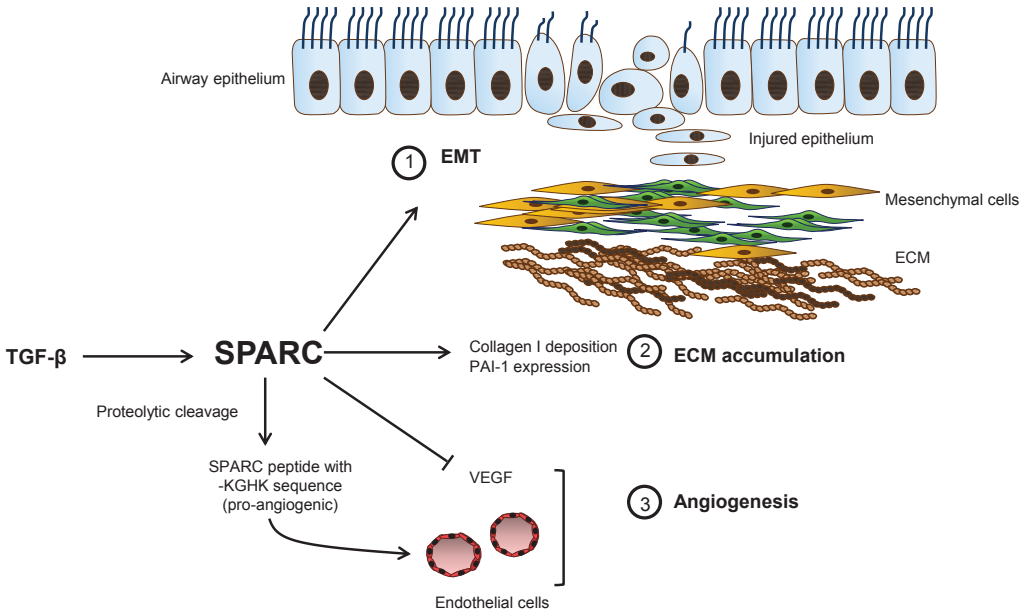

Figure 2.1: Proposed role of SPARC in airway and vascular remodelling in asthma and COPD.
TGF-β induces SPARC mRNA and protein expression in various cell types, including lung cells. We propose that many of the pro-remodelling effects of TGF-β in asthma and COPD are mediated by SPARC. Acting downstream of the transcription factor Snail, SPARC may potentially drive TGF-β-induced EMT (1). Increased deposition of a non-degradative ECM within the airway wall may also be attributed to SPARC's ability to enhance collagen deposition and augment PAI-1 expression (2). SPARC inhibits the expression and secretion of the pro-angiogenic factor VEGF. However, cleavage of SPARC may potentially lead to the generation of SPARC peptide fragments with –KGHK sequence, which may confer pro-angiogenic activity. The balance of these processes may be an important determinant of angiogenic activity in airways disease (3).

As mentioned above, SPARC is an important regulator of the dynamic ECM milieu and has a role in maintaining the structural and functional integrity of the ECM. Altered deposition of ECM proteins is a key feature of airway wall remodelling in asthma and COPD, thus it is likely that any change in the expression and/or activity of SPARC will affect ECM changes in these conditions. In asthma, there is increased deposition of collagen I, III, V, fibronectin, tenascin, hyaluronan, versican, laminin α2/β2, lumican and biglycan while the deposition of collagen IV, decorin and elastin appears to be decreased

(57-60). Similarly in COPD, there is evidence of increased deposition of collagen I, III and IV, fibronectin and laminin (61). As discussed above, there is altered deposition of collagen I and IV, as well as laminin, in SPARC-null mice, and it is well established that SPARC binds to collagen and is essential for the assembly and deposition of mature collagen fibres in the ECM (8, 62) (28). Thus, the extent to which alterations in SPARC expression or activity influence collagen deposition in chronic airways disease is of particular interest for future research. Moreover, SPARC may regulate ECM synthesis via its interplay with the TGF-β/Smad2/3 pathway which is known to regulate the synthesis of several ECM proteins including collagen, versican and biglycan (63). As mentioned, the ability of SPARC to induce PAI-1 expression could also be a downstream effect of TGF-β, resulting in the existence of apoptotic-resistant myofibroblasts that sustain a non-degradative ECM (44). SPARC may also alter ECM synthesis and degradation via its ability to regulate the production and activity of numerous MMPs (24).

Angiogenesis and microvascular remodelling contribute to dysregulation of the vascular network and airway remodelling in asthma and COPD. This is manifested as either an increase in the number (angiogenesis) and size of blood vessels (vasodilatation) and enhanced vascular permeability (64-66). SPARC appears to primarily act as an angiosuppressive molecule as it inhibits the expression and secretion of the key pro-angiogenic mediator VEGF and also acts to inhibit VEGF-induced growth of endothelial cells (20-22). Indeed, dermal fibroblasts derived from SPARC-null mice demonstrate enhanced VEGF production relative to those derived from wild-type mice (67). Another important

consideration is that SPARC has the capacity to directly bind VEGF-A (22, 25, 26). Although it is not clear how this influences its activity, it is known that VEGF-A interacts with distinct receptors that can either promote or inhibit angiogenic processes (68). Thus, it will be important to determine whether direct interactions between SPARC and VEGF favour the anti-angiogenic response of SPARC mentioned above or whether this is altered in disease to induce pro-angiogenic responses instead.

Interestingly, while full-length SPARC is angiosuppressive, structural studies indicate that certain SPARC-derived peptide fragments have pro-angiogenic effects. For instance, MMP3-cleaved peptides containing a KGHK sequence induce angiogenesis in vivo and in vitro (18, 19). On the other hand, however, the EGF-like part of the same follistatin-like domain has anti-angiogenic activity (69, 70). Thus, it appears that the extent and pattern of SPARC proteolysis could determine its overall angiogenic activity. Further studies should investigate whether the inflammatory environment in asthma and COPD favour SPARC proteolysis and the nature of the proteolytic fragments generated, as this may be a factor underlying pro-angiogenic processes in asthma and COPD. Finally, it should also be determined whether SPARC acts downstream of TGF-β to modulate VEGF expression and activity.

Another key factor underlying airway remodelling in chronic airways disease is aberrant wound repair in response to epithelial injury and damage. The EMT is characterized by the loss of epithelial tight junctions and increased mesenchymal markers and is an important component of the tissue repair

response, but when dysregulated, EMT leads to an accumulation of mesenchymal cells and tissue fibrosis (71). Overexpression of SPARC induced fibroblast-like morphology in melanocytes, with concomitant loss of E-cadherin and P-cadherin expression, along with increased expression of mesenchymal markers (72). Moreover, SPARC is a downstream mediator of the transcription factor Snail, a key regulator of TGF-β1-induced EMT formation (42). Indeed, since TGF-β is one of the major factors implicated in dysregulation of EMT in asthma and COPD, it is likely that TGF-β/SPARC signalling is involved in this response.

EVIDENCE FOR SPARC IN THE IMMUNE AND INFLAMMATORY RESPONSE

The role of SPARC in the immune response is less well understood, but this is an area of increasing interest and investigation. SPARC is expressed by various immune cell types, including macrophages, follicular dendritic cells and $CD4^+$ T cells (73, 74). Apart from studies which have examined its role in bleomycin-induced lung inflammation (53, 54), there have been no other investigations of its role in the inflammatory component of lung disease. There is, however, a growing body of literature which suggests it has numerous immunoregulatory properties, and thus, its potential role in the airway inflammatory response in asthma and COPD is worthy of investigation.

Evidence from various disease models suggests that SPARC has pleiotropic roles in the immune and inflammatory response. For instance, thioglycollate-induced peritonitis in SPARC-null mice is associated with impaired leukocyte

recruitment, indicating a role for SPARC in leukocyte trafficking (75). This is supported by evidence that leukocyte-derived SPARC binds vascular cell adhesion protein-1 and mediates leukocyte transmigration in endothelial monolayers in vitro (75). SPARC can also influence immune and inflammatory processes by regulating the availability of TGF-β. Using an experimental model of dextran sodium sulphate-induced colitis, Ng and colleagues showed that SPARC-null mice exhibit attenuated inflammatory responses and faster recovery, compared with their wild-type counterparts. In this case, the dampened inflammatory response was associated with increased expression of TGF-β, which has potent anti-inflammatory activities (76). Similarly, loss of SPARC was associated with increased availability and activation of TGF-β in a model of pancreatic cancer. However, in this case, increased production of TGF-β was associated with a more aggressive malignancy, highlighting the distinct immunoregulatory effects of SPARC in different disease contexts (77).

In contrast to the studies mentioned above, SPARC exerts anti-inflammatory properties in some disease contexts, as discussed earlier in relation to bleomycin-induced lung injury (53, 54). Indeed, loss of SPARC has been associated with enhanced activation of immune and inflammatory responses in a number of experimental models (53, 54, 78-81). This may be attributed to lack of ECM structure and reduced ECM density and consequently a more permissive microenvironment that facilitates cellular trafficking and immune activation (78). Interestingly, it has been shown that SPARC limits bacterial spreading by inducing the formation of acute inflammatory reactions with granuloma-like features. However, at the same time, granuloma-associated

SPARC inhibits dendritic cell migration to the draining lymph nodes and thus increases susceptibility to infection. Consistent with this, protective immunity against Salmonella typhimurium was restored in SPARC-null mice, thus providing further evidence that loss of SPARC may be associated with enhanced immune activation in certain contexts (82).

Of note, studies in SPARC-null mice have also identified an important role for SPARC in lymphopoiesis. SPARC-null mice display an impaired immune response that is associated with abnormal spleen architecture and altered B and T cell populations within the bone marrow (83). Indeed, loss of SPARC is associated with reduced numbers of B cell progenitors within the bone marrow and secondary lymphoid organs, suggesting that SPARC plays a role in the early stage of B cell lymphopoiesis (84, 85). In corroboration with this, loss of SPARC is associated with defective follicular dendritic cell networks in lymph nodes. This severely delays the arrangement of germinal centres where B cells proliferate and differentiate. Such defects result in the delayed development and differentiation of T_H17 cells, indicating SPARC is essential for normal lymphopoietic function (74).

Although there is very limited understanding of the role of SPARC in the immune response, studies to date suggest a highly complex and pleiotropic role. Whether SPARC is implicated in the aberrant and sustained activation of immune and inflammatory responses in chronic airways disease is a compelling question that warrants investigation. It is likely that SPARC will have direct immunomodulatory effects, as well as secondary effects consequent to

alterations in ECM structure and TGF-β signalling. The cellular source of SPARC, in terms of immune cells rather than structural cells, is also likely to differentially influence immune and inflammatory responses in this setting (53).

THERAPEUTIC IMPLICATIONS

With mounting evidence implicating SPARC in respiratory disease, it is also essential to explore strategies by which this molecule may be targeted therapeutically. Pharmacological inhibition of SPARC is not currently possible as small molecule inhibitors and neutralizing antibodies have yet to be developed. However, the discovery of SPARC peptide fragments with distinct biological functions may open the way to new therapeutic opportunities. As mentioned above, full-length SPARC and its peptide fragment that contain an EGF-like module are angiosuppressive, while the SPARC peptide with the KGHK sequence is pro-angiogenic (18, 19, 69, 70). Thus, specific peptide fragments containing the EGF-like module may potentially be developed to selectively inhibit angiogenesis in the absence of other effects attributed to the parent molecule. Rahman and colleagues have also shown that native SPARC enhances the efficacy of chemotherapy drugs via its ability to augment apoptosis in cancer cells. Moreover, they identified the N-terminus as the region responsible for this effect and further showed that a SPARC peptide fragment which spans the N-terminus domain has a greater chemosensitizing capacity than the parent molecule (86). While these findings are encouraging, further understanding of the biological actions of SPARC and that of its peptide fragments is necessary before their therapeutic potential can be fully realized.

Therapeutic modulation of SPARC expression in specific disease contexts may also be possible via strategies that exploit its epigenetic regulation. As mentioned above, reduced intratumoral SPARC is associated with poor survival in NSCLC (34, 36). Thus, the use of demethylating agents that selectively target the tumour site to increase SPARC expression may improve prognosis in these patients. The ability of SPARC to bind albumin may also be potentially exploited for therapeutic purposes. In some cancers, high levels of intratumoral SPARC is associated with a more favourable treatment response which is thought to be due to better localization and retention of albumin-bound chemotherapy drugs at the tumour site (87). Indeed, it has been shown that the efficacy of albumin-bound paclitaxel (nab-paclitaxel, Abraxane) in patients with head and neck tumours correlates with tumoral SPARC expression (88). Consistent with this, tumour SPARC expression was also positively correlated with the inhibitory effects of Cellax on tumour growth in a breast cancer mouse model. Compared with native docetaxel, Cellax, which is a polymer-docetaxel conjugate, has a greater ability to absorb albumin, and better internalization of this drug is observed in tumours in which SPARC is highly expressed (89).

In NSCLC, the level of SPARC expression within the tutor region is not related to treatment response or the overall survival of patients receiving albumin-bound drugs. Thus, the ‘albumin effect’ is likely to be dependent on the type of tumour or other yet to be identified factors (37, 90-92). Further investigation of the mechanisms by which SPARC influences the retention of albumin-bound drugs may lead to improved therapies for lung cancer and other respiratory diseases. Collectively, the emerging evidence suggests that SPARC expression

at pathological sites, whether high or low, may be exploited for therapeutic purposes via a number of strategies. Thus, SPARC may serve as a potential 'biomarker' for targeted therapies in certain patients and disease settings.

CONCLUSION AND FUTURE DIRECTIONS

SPARC is an ECM-associated protein that does not contribute structurally to the ECM but mediates interactions at the cell-matrix interface. This link governs fundamental cellular functions including cell adhesion, proliferation and differentiation. Thus, perturbations in the cellular microenvironment and/or cellular function may be accompanied by altered expression and activity of SPARC, as is evident in respiratory diseases such as lung cancer and IPF.

Interestingly, a recent study demonstrated increased expression of the SPARC family member, follistatin-like protein 1 (FSTL1) in lung macrophages of patients with severe asthma, and further demonstrated that FSTL1 induces airway remodelling in mouse models of asthma (93). Moreover, it has recently been shown that the enigmatic protein high mobility group box 1 (HMGB1) induces SPARC expression in airway epithelial cells (94). This is of relevance, because HMGB1 is implicated in asthma and COPD (95) and has also been identified as a mediator of EMT and ECM synthesis in airway epithelial cells, thus suggesting a potential link between HMGB1 and SPARC signalling in chronic airways disease (94, 96). Together with our discussion above, these studies provide further impetus for investigation of SPARC in asthma and COPD.

As a starting point, further studies should establish whether pathological abnormalities associated with aberrant airway function in asthma and COPD are related to changes in the expression and/or activity of SPARC. Added to this, it is important to determine to what extent SPARC contributes to TGF-β-dependent effects in asthma and COPD and whether SPARC/TGF-β interactions contribute to these. Indeed, while TGF-β is an attractive target for drug development in chronic airways disease, its global inhibition is associated with increased risk of cancer and autoimmune disease (97, 98). Thus, given the close relationship between TGF-β and SPARC, the latter may prove to be a better alternative for therapeutic targeting, although this requires improved understanding of the interplay between TGF-β and SPARC and the role of SPARC in disease pathogenesis.

There is a strong association between COPD and the development of lung cancer; long-term smokers with COPD have 4.5-fold increase in cancer risk (99). Of note, genome-wide association studies have identified overlapping candidate genes, CHRNA3 and CHRNA5 (neuronal acetylcholine receptor subunit α-3/5) for COPD and lung cancer, suggesting common inherent genetic predispositions for these conditions, although oxidative stress and EMT formation in COPD are also thought to be the precursors of malignant transformation (100). Given that SPARC is implicated in NSCLC, the possibility that SPARC provides the molecular link between COPD and lung cancer progression certainly warrants investigation.

In conclusion, SPARC is emerging as a key player in numerous respiratory diseases, and there is a strong basis for its further investigation in chronic inflammatory airways disease. SPARC exhibits heterogeneous expression in certain pathologies, and its actions are not only dependent on the cellular source, but also the cellular and tissue microenvironment. Thus, future studies should examine the expression and activity of SPARC in distinct structural cells implicated in airway remodelling, for example airway epithelial cells, fibroblasts and airway smooth muscle cells, as well as in immune and inflammatory cells involved in the airway inflammatory response. Elucidation of the cell and tissue-specific roles of SPARC in chronic airways disease may certainly open the door to new opportunities for the therapeutic management of these conditions.

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Chapter 3

Expression and function of SPARC in human airway epithelial cells

INTRODUCTION

Airway epithelial cells are key effector cells of airway inflammation and remodelling in asthma (1). They express pattern recognition receptors (PRRs) which detect inhaled pathogens, pollutants and allergens, triggering the release of DAMPs, inflammatory cytokines and chemokines in response to epithelial damage and stress. These lead to the initiation of the innate and adaptive immune responses important for asthma pathogenesis (2). Structural abnormalities of the airway epithelium including epithelial shedding, denudation, thickening of the basement membrane and subepithelial fibrosis are also evident in asthma (3). Diminished expression of epithelial junctional proteins such as E-cadherin and zona occludin-1 (ZO-1), and increased acquisition of mesenchymal cell phenotype, collectively termed as EMT result in the disrupted lining of the respiratory epithelium (4). The loss of epithelial integrity and excessive ECM deposition in fibrosis are consequences of defective epithelial repair in asthma. While the important roles of EMT and ECM deposition in epithelial repair and remodelling processes have been extensively studied (3), the significance of epithelial cell-matrix interaction which critically regulates these processes (5), to epithelial remodelling as well as inflammation in asthma, is less well understood.

SPARC is a matricellular protein which mediates interaction between cells and the matrix, but does not contribute structurally to the ECM. It is highly expressed during normal development and repair, because it is necessary to regulate cell behaviour such as cell adhesion, proliferation and migration (6, 7). It is also important in the regulation of ECM assembly through its ability to facilitate

collagen I processing and its incorporation into the ECM (8). Of note, SPARC regulates TGF-β, the key mediator of airway inflammation and remodelling in asthma (9), in a reciprocal regulatory manner; SPARC is a TGF-β-inducible protein and in turn, SPARC also enhances TGF-β expression (10, 11). TGF-β acts on AECs to promote formation of EMT and ECM protein deposition, and imparts immunoregulatory functions to resolve inflammation following injury (9, 12, 13). Albeit not in the lung, SPARC also induces EMT in melanocytes and elicits distinct pro- and anti-inflammatory activities in a context-dependent manner (14, 15). SPARC overexpression is associated with fibrogenesis and in the lung, it has been shown to be a key contributor to IPF (16). Considering airway inflammation and remodelling are prominent features in asthma, and that the epithelium orchestrates these processes, the expression and function of SPARC in the airway epithelium, and whether this is related to TGF-β, is a compelling area that needs to be explored.

Hence, we determined whether SPARC is expressed and/or released by AECs, under basal conditions and in response to TGF-β stimulation as a first step. We then examined if SPARC expression and secretion are altered in asthmatic AECs, and if it is regulated by type 1 (IFN-γ and TNF-α) and type 2 (IL-4 and IL-13) cytokines, the two main subtypes of cytokines implicated in asthma, in the presence or absence of TGF-β given these cytokines were previously shown to modulate TGF-β-mediated responses (17, 18). We also determined whether SPARC exerts immunoregulatory effects and changes in cellular phenotype in AECs.

MATERIALS AND METHODS

Human airway epithelial cells

The human airway epithelial cell line 16-HBE14o- was obtained from Professor D.C. Gruenert (University of California, San Francisco). 16-HBE14o- cells were cultured in 10% Fetal Bovine Serum (FBS) in Minimal Essential Medium (MEM) supplemented with 2mM L-glutamine, 1mM sodium pyruvate, 1% MEM non-essential amino acids, 100U/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml amphotericin B. All cell culture reagents were purchased from Life Technologies, Australia unless otherwise stated.

Primary AECs were isolated from macroscopically normal bronchial tissue obtained from patients undergoing lung resection or lung transplantation as described previously (19). The epithelial layer was removed from the bronchial tissue by macrodissection and cultured in complete Bronchial Epithelial Cell Growth Medium (BEGM™), consisting of Bronchial Epithelial Basal Medium (BEBM™) supplemented with epidermal growth factor, bovine pituitary extract, hydrocortisone, epinephrine, transferrin, insulin, retinoic acid, triiodothyronine and amphotericin B (Lonza, Australia). Ethics approval for the use of human lung tissue (X14-0045, HREC RPAH) was obtained by The University of Sydney Human Research Ethics Committee and Sydney South West Area Health Service, and informed consent was obtained from all subjects. Asthmatic and non-asthmatic AECs were also purchased from Lonza, Australia and cultured in BEGM™.

Airway epithelial cell stimulation

16-HBE14o- cells were seeded in 12-well culture plates at a density of 2.5 x 10^4cells/cm^2 and grown to 90% confluence in 10% FBS/MEM with supplements as described above. Cells were then serum-deprived in supplemented MEM containing 0.1% BSA for 24 h and then stimulated with recombinant human TGF-β1 in fresh 0.1% BSA/MEM for a further 24 or 72 h.

Primary human AECs were seeded in 12-well culture plates at a density of 2.5 x 10^4 cells/cm^2 and grown to 90% confluence in BEGM™. The media was then replenished and cells were incubated for a further 24 h. Following this, cells were stimulated with recombinant cytokines including TGF-β1, IL-4, IL-13, IFN-γ and TNF-α, or SPARC in fresh BEGM™ for a further 24 or 72 h. Cytokines and SPARC were purchased from R&D Systems, UK.

ELISA

SPARC (#DSP00), IL-6 (#DY206), GM-CSF (#DY215), CCL20 (#DY360) and IL-1α (#DY200) (R&D Systems, UK) and HMGB1 (#IBST51011) (IBL International, Germany) were measured in culture supernatants by ELISA.

Immunoblotting

Whole cell lysates were extracted with RIPA buffer (Sigma-Aldrich, Australia) containing protease and phosphatase inhibitor cocktails, cOmplete™ ULTRA Tablets (Mini) and PhosSTOP™, respectively (Roche Diagnostics, Australia). Protein concentrations were determined using the Pierce BCA Protein Assay Kit according to manufacturer's instructions (Thermo Fisher Scientific, Australia).

10 – 25µg protein was loaded per lane and separated using NuPAGE™ Novex™ 4 – 12% Bis-Tris protein gels. The proteins were electrophoresed at 200V for 50 mins, and protein transfer onto PVDF membranes was performed at 20V for 1 min, 23V for 4 min and 25V for 2 min using iBlot2® Dry Blotting System. PVDF membranes were then incubated with 5% non-fat milk powder in Tris-buffered saline containing 0.1% Tween (TBST) for 1 h at room temperature. Membranes were then incubated with primary antibodies detecting E-cadherin (sc-7870), GAPDH (sc-32233), SPARC (sc-25574) (Santa Cruz Biotechnology, Dallas, Texas), α-smooth muscle actin (ab5694) (Abcam, Australia) and α-tubulin (#2125) (Cell Signalling Technology, Danvers, MA) at 4^0C for 16 h. All antibodies were diluted 1:1000 in 5% non-fat milk powder in 0.1% TBST. PVDF membranes were then washed with 0.1% TBST three times (5 min each) and incubated with anti-rabbit IgG HRP-linked secondary antibody (Cell Signalling Technology, Danvers, MA) diluted 1:1000 in 5% non-fat milk powder in 0.1% TBST for 1 h at room temperature. Membranes were then washed with 0.1% TBST three times and protein bands were visualized using enhanced chemiluminescence (ECL) (GE Healthcare, UK). Densitometric analysis of protein bands was performed using Image J software (v1.47).

For analysis of SPARC protein expression in AECs from Lonza, secondary antibodies IRDye® 800CW anti-Rabbit IgG (#926-32211) or IRDye® 680RD anti-Mouse IgG (#926-68070) (LI-COR Biosciences, Lincoln NE) were used (1:15,000 dilution) and protein bands were visualized using Odyssey CLx Imaging System (LI-COR Biosciences, Lincoln NE). Densitometric analysis of protein bands was performed using Image Studio software (v5.2.5). All pre-cast

gels, buffers, molecular weight markers and iBlot2® Transfer Stacks were purchased from Life Technologies, Australia.

Statistical analysis

Data were expressed as means ± SEM. Mean data for SPARC release were presented as either absolute values or fold change relative to unstimulated cells. Results were analysed using one-way ANOVA, followed by Bonferroni post hoc test to determine differences between treatment groups. A *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

Effect of TGF-β on SPARC protein expression and secretion in human AECs

To examine the effect of TGF-β on SPARC expression in human AECs, we performed initial studies in 16-HBE14o- cells, as this is a readily accessible cell line amenable for extensive investigations. Surprisingly however, basal concentrations of secreted SPARC were ~ 300 ng/ml and ~ 500 ng/ml after 24 h and 72 h culture respectively (Figure 3.1A, B). Moreover, TGF-β at concentrations between 1 – 10 ng/ml did not augment SPARC secretion (Figure 3.1A, B), suggesting that 16-HBE14o- cells were maximally primed for SPARC secretion, and therefore not appropriate for use in these studies. Thus, we performed all subsequent experiments in primary human AECs.

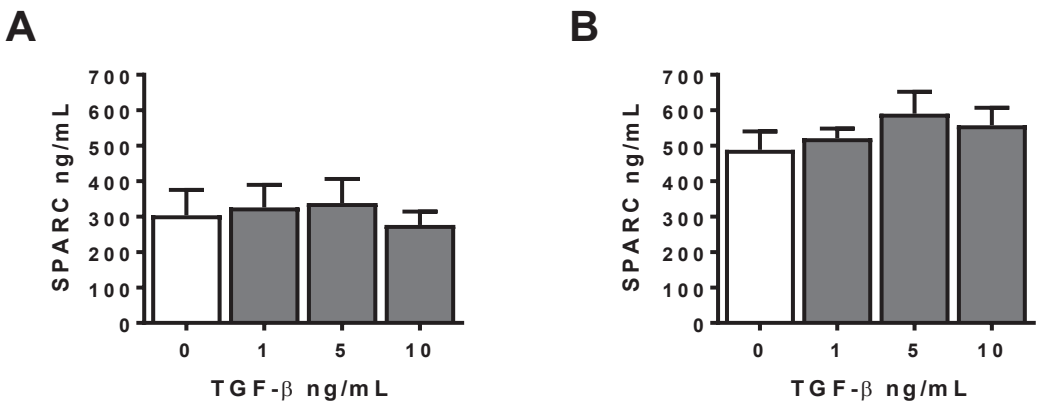
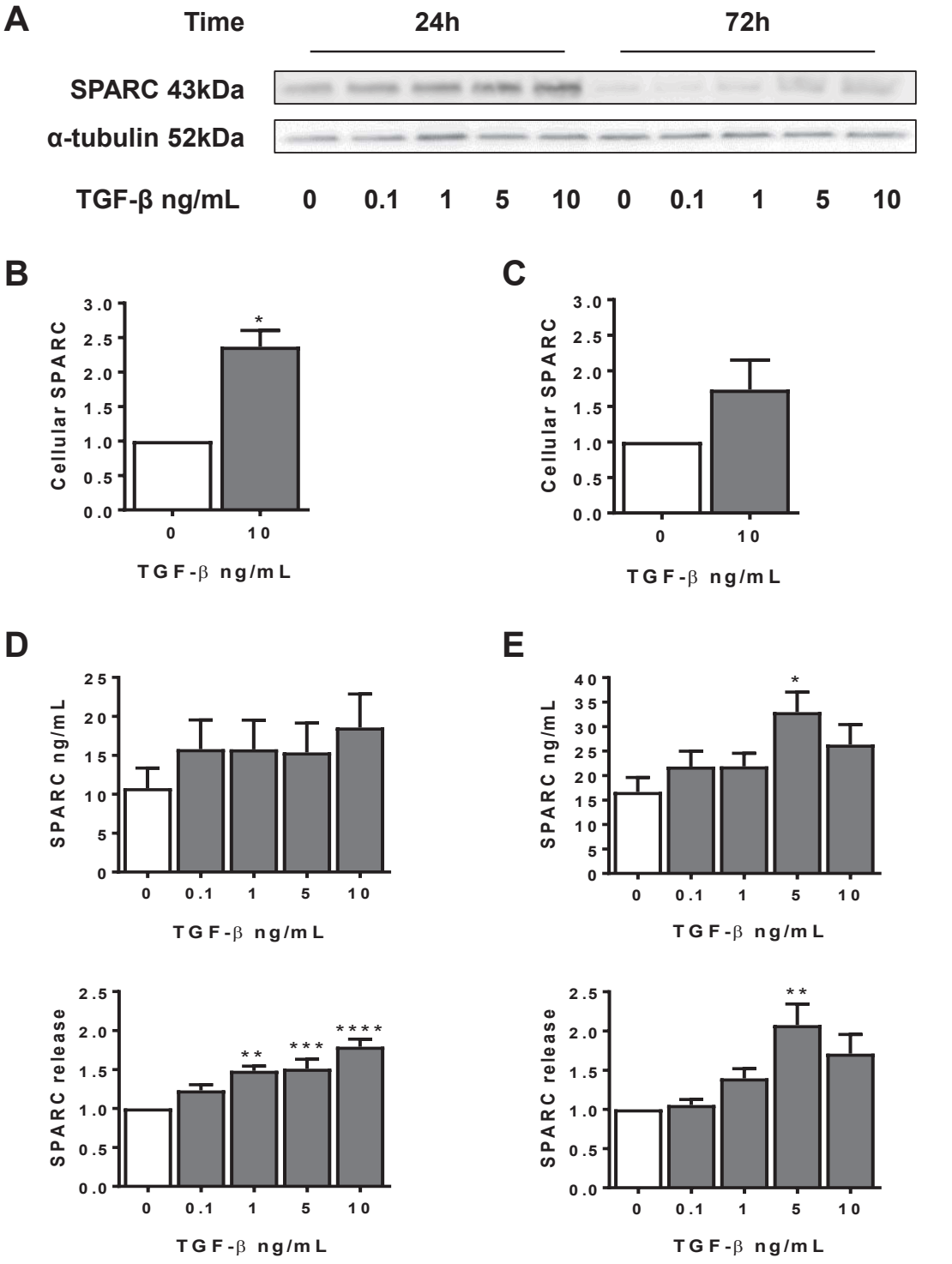


Figure 3.1: Effect of TGF-β on secreted SPARC in human airway epithelial cells 16-HBE14o-.
16-HBE14o- cells were stimulated with TGF-β for 24 h (A) or 72 h (B) (n=3-4), and soluble SPARC was measured in culture supernatants by ELISA. Bars represent mean data (± SEM). One-way ANOVA with Bonferroni's correction was used to determine statistical differences.

In preliminary experiments, we examined cell-associated SPARC expression in AECs following stimulation with TGF-β (0.1 – 10 ng/ml) for either 24 h or 72 h. Interestingly, compared with the 24 h culture period, SPARC expression was markedly reduced at 72 h, both under basal conditions and after stimulation with TGF-β at all concentrations tested (Figure 3.2A, F). In further experiments, we showed that while TGF-β (10 ng/ml) induced ~ 2.5-fold increase in cell-associated SPARC expression at 24 h, it did not have a significant effect on SPARC expression at 72 h (Figure 3.2B, C). Moreover, TGF-β (0.1 – 10 ng/ml) had a modest effect on SPARC secretion (Figure 3.2D, E). The basal concentrations of secreted SPARC were ~ 10 and ~ 20 ng/ml at 24 h and 72 h respectively. Although stimulation with TGF-β (10 ng/ml) induced ~ 2-fold increase in SPARC secretion at 24 h, SPARC concentrations were ~ 10-fold lower than those detected under comparable stimulation conditions in human

ASM cells. Thus, compared to ASM cells, TGF-β has a relatively modest effect on SPARC expression and secretion in AECs.



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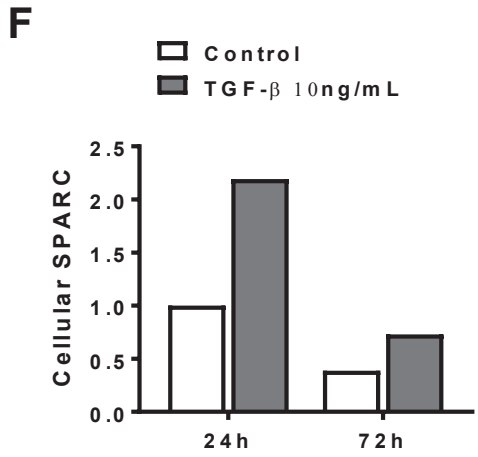


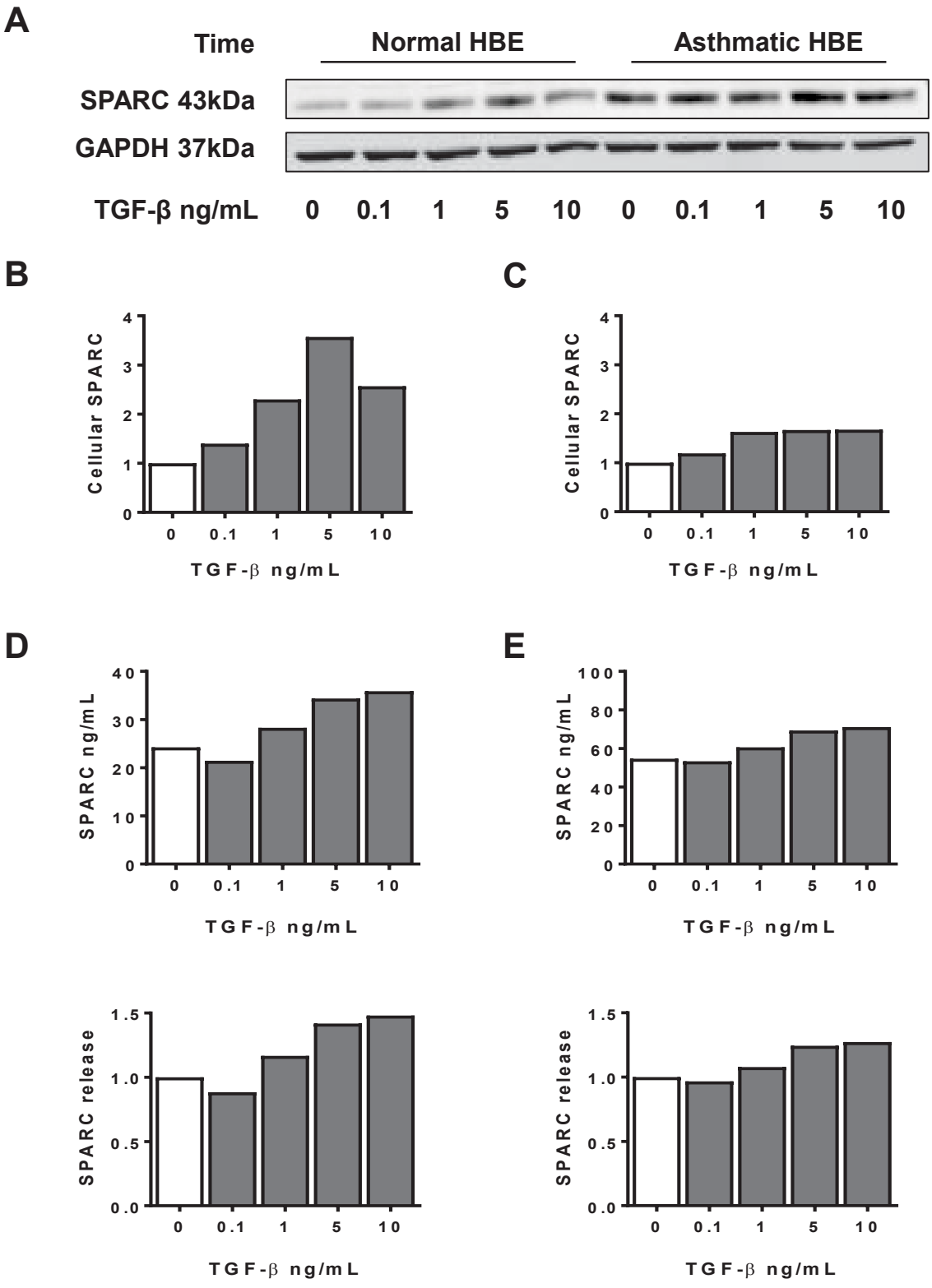
Figure 3.2: Effect of TGF-β on cell-associated and secreted SPARC in primary human airway epithelial cells.
Primary AECs were stimulated with TGF-β for 24 h (A, B, D) or 72 h (A, C, E). SPARC expression in whole cell lysates was determined by immunoblotting and a representative immunoblot is shown in (A). Cell-associated SPARC expression was normalised to α-tubulin and expressed as fold change relative to unstimulated cells (B, C). Soluble SPARC was measured in culture supernatants by ELISA. Data is expressed as absolute values (D, E upper panels) or fold change relative to unstimulated cells (D, E lower panels). Cell-associated SPARC expression, under basal conditions and following stimulation with TGF-β for 24 h and 72 h were compared (F). Bars represent mean data (± SEM) from 4 – 8 cell donors (A – E) or mean data from 2 cell donors (F). One-way ANOVA with Bonferroni's correction was used to determine statistical differences. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 compared to unstimulated cells.

Effect of type 1 and type 2 cytokines on SPARC protein expression and secretion in asthmatic and non-asthmatic AECs

To determine if epithelial-derived SPARC plays a role in the airway inflammatory response in asthma, we extended our studies to determine if basal or TGF-β-induced SPARC expression and secretion are altered in asthmatic AECs, and if it is regulated by type 1 (IFN-γ and TNF-α) and type 2 (IL-4 and IL-13) cytokines. Consistent with findings above, our initial studies in 1 cell donor of non-asthmatic AECs indicate TGF-β induced ~ 2 to 3-fold increase in cell-

associated SPARC expression (Figure 3.3A, B) and had a modest effect on SPARC secretion at 24 h post-stimulation (Figure 3.3D). In asthmatic AECs derived from 2 cell donors, although TGF-β had a similarly modest effect on SPARC secretion (Figure 3.3E), it did not enhance cell-associated SPARC expression. This lack of response may be due to higher basal levels of SPARC in asthmatic cells. Indeed, compared to non-asthmatic cells, basal levels of cell-associated and secreted SPARC were higher in asthmatic cells (Figure 3.3F, G) although this trend needs further validation.

Our preliminary studies indicate stimulation of AECs with type 1 (IFN-γ and/or TNF-α; Figure 3.4) and type 2 (IL-4 and/or IL-13; Figure 3.5) cytokines tended to decrease cell-associated SPARC expression and SPARC secretion in both non-asthmatic and asthmatic cells. The inhibitory effects of type 1 and type 2 cytokines were observed in cells stimulated with each of the cytokines or cytokine combinations alone; and in cells that were stimulated with type 1 and 2 cytokines in the presence of TGF-β. Of note, combined stimulation with IFN-γ and TNF-α had the most pronounced inhibitory effect on cell-associated SPARC expression (Figure 3.4A, B). These findings however, require confirmation in more cell donors.



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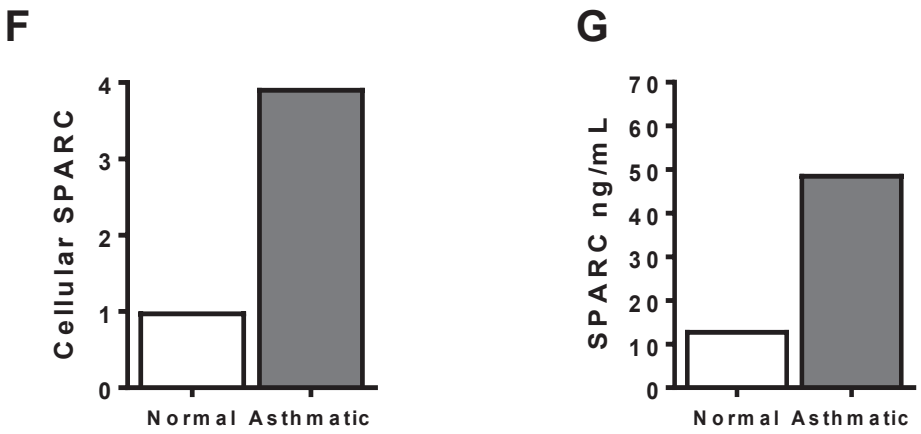


Figure 3.3: Expression of cell-associated and secreted SPARC in asthmatic and non-asthmatic airway epithelial cells under basal conditions and in response to TGF-β.
Asthmatic (A, C, E) and non-asthmatic (A, B, D) AECs were stimulated with TGF-β for 24 h. SPARC expression in whole cell lysates was determined by immunoblotting and a representative immunoblot is shown in (A). Cell-associated SPARC expression was normalised to GAPDH and expressed as fold change relative to unstimulated cells (B, C). Soluble SPARC was measured in culture supernatants by ELISA. Data is expressed as absolute values (D, E upper panels) or fold change relative to unstimulated cells (D, E lower panels). Basal levels of cell-associated (F) and secreted SPARC (G) in asthmatic and non-asthmatic cells following 24 h incubation were compared. Bars represent data from 1 cell donor (B, D, F) or mean data from 2 cell donors (C, E, G).

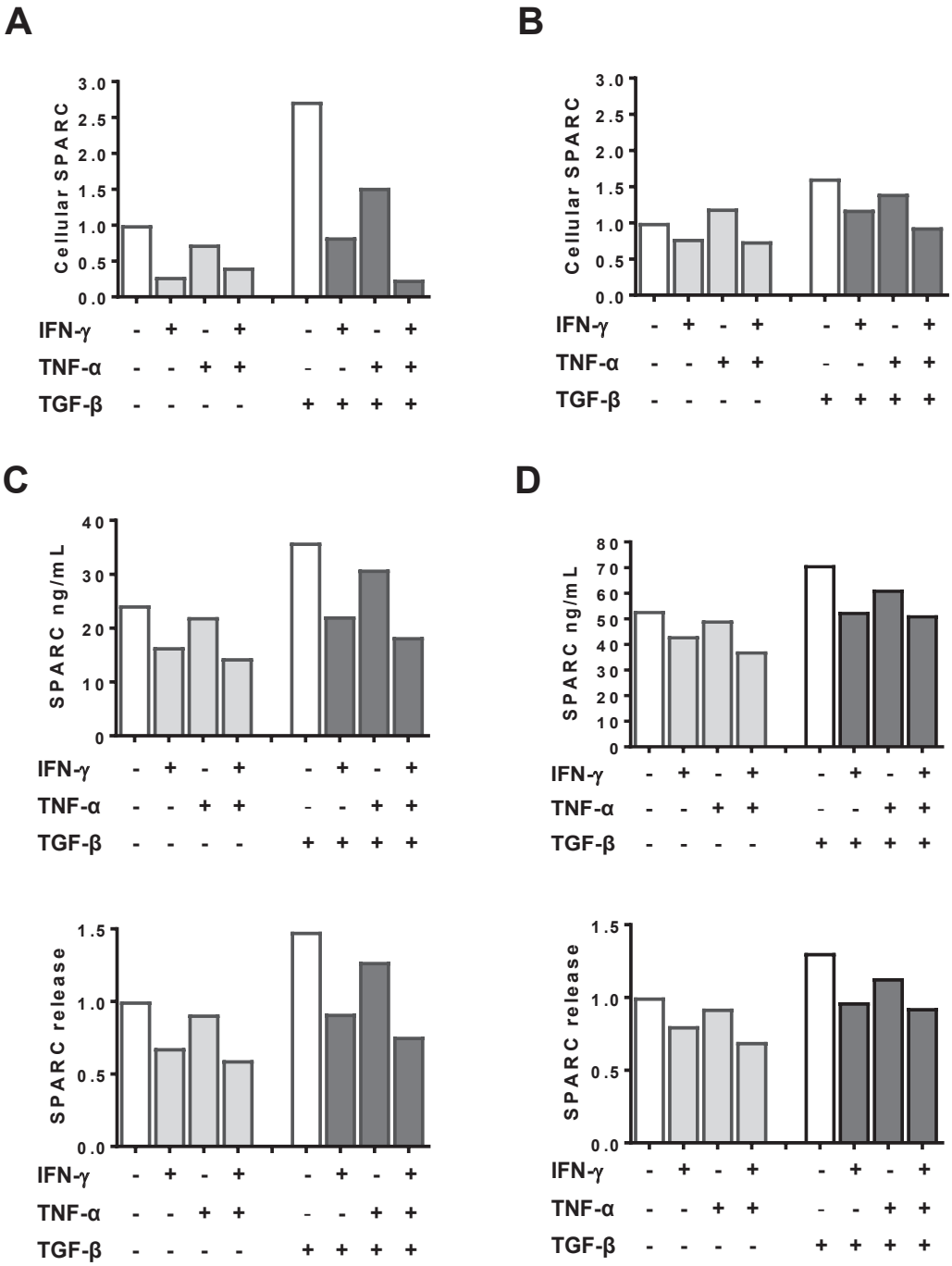


Figure 3.4: Effect of type 1 cytokines on cell-associated and secreted SPARC in asthmatic and non-asthmatic airway epithelial cells.
Asthmatic (B, D) and non-asthmatic (A, C) AECs were stimulated with IFN-γ (10ng/ml) and/or TNF-α (10ng/ml), alone or in combination with TGF-β (10ng/ml) for 24 h. SPARC expression in whole cell lysates was determined by immunoblotting. Cell-associated SPARC expression was normalised to GAPDH and expressed as fold change relative to unstimulated cells (A, B). Soluble SPARC was measured in culture supernatants by ELISA. Data is expressed as absolute values (C, D upper panels) or fold change relative to unstimulated cells (C, D lower panels). Bars represent data from 1 cell donor (A, C) or mean data from 2 cell donors (B, D).

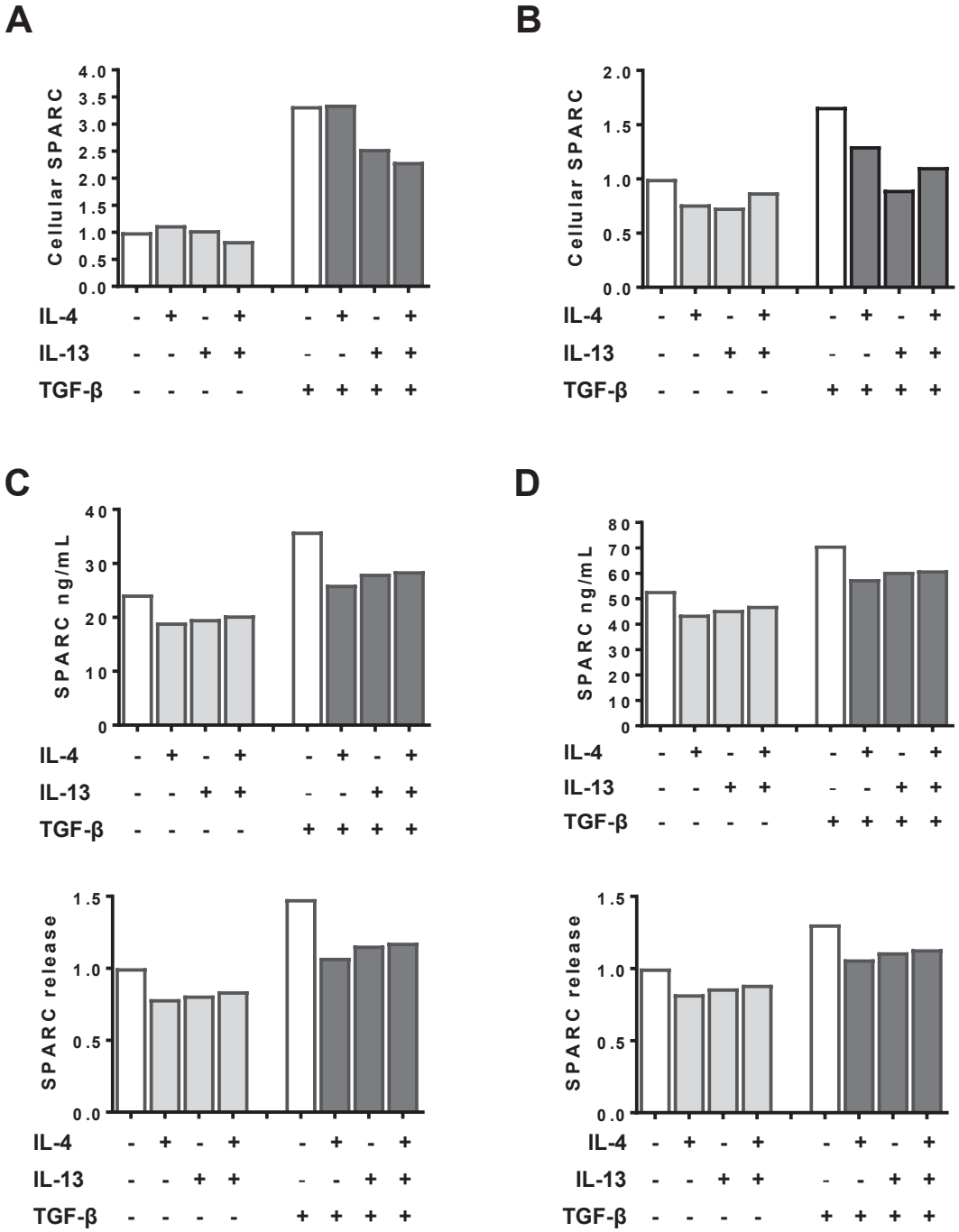
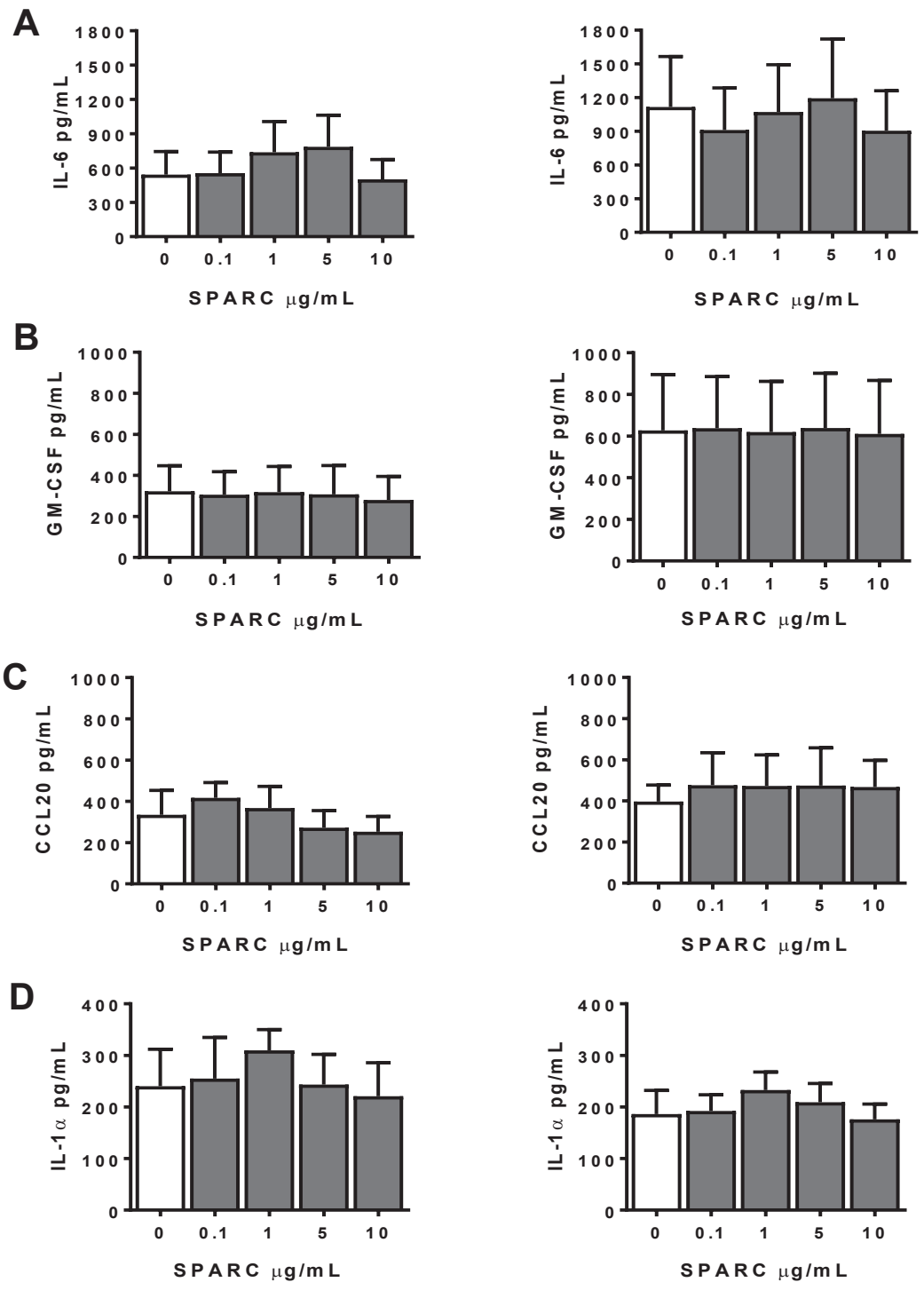


Figure 3.5: Effect of type 2 cytokines on cell-associated and secreted SPARC in asthmatic and non-asthmatic airway epithelial cells.
Asthmatic (B, D) and non-asthmatic (A, C) AECs were stimulated with IL-4 (10ng/ml) and/or IL-13 (10ng/ml), alone or in combination with TGF-β (10ng/ml), for 24 h. SPARC expression in whole cell lysates was determined by immunoblotting. Cell-associated SPARC expression was normalised to GAPDH and expressed as fold change relative to unstimulated cells (A, B). Soluble SPARC was measured in culture supernatants by ELISA. Data is expressed as absolute values (C, D upper panels) or fold change relative to unstimulated cells (C, D lower panels). Bars represent data from 1 cell donor (A, C) or mean data from 2 cell donors (B, D).

Comparative effect of TGF-β and SPARC on cytokine and chemokine secretion in human AECs

AECs have the capacity to secrete a wide array of cytokines and chemokines, and are implicated as crucial mediators of airway inflammatory response in asthma. Moreover, TGF-β has previously been shown to regulate cytokine and chemokine secretion in human AECs (19, 20). Thus, since SPARC is a TGF-β inducible protein, and is known to have immunoregulatory properties (15), we hypothesized that SPARC regulates cytokine and chemokine secretion in human AECs. To test this hypothesis, we stimulated human AECs with TGF-β or SPARC for 24 or 72 h and measured the expression of a panel of cytokines and chemokines including IL-6, GM-CSF, CCL20, IL-1α and HMGB1. As expected, TGF-β induced the secretion of IL-6 and GM-CSF in a dose-dependent manner, although this did not achieve statistical significance (Figure 3.7A, B). Interestingly however, SPARC had no effect on IL-6 or IL-8 secretion (Figure 3.6A, B). Moreover, TGF-β and SPARC had no effect on CCL20 (Figure 3.6C, Figure 3.7C), IL-1α (Figure 3.6D, Figure 3.7D) or HMGB1 secretion (Figure 3.6E, Figure 3.7E).



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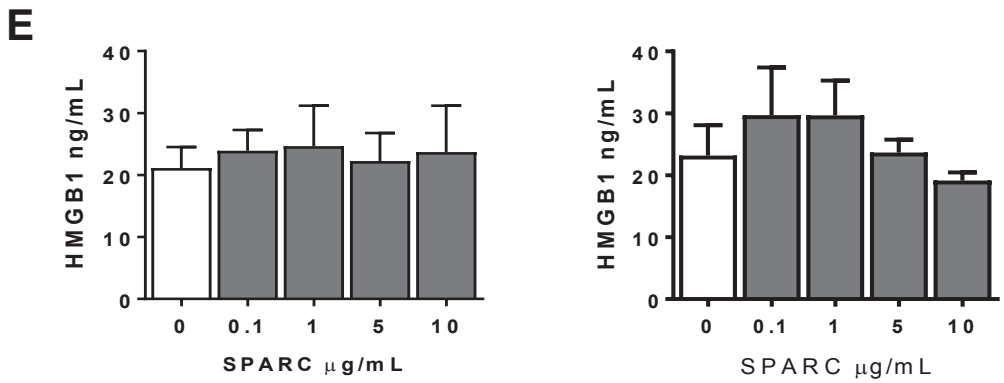
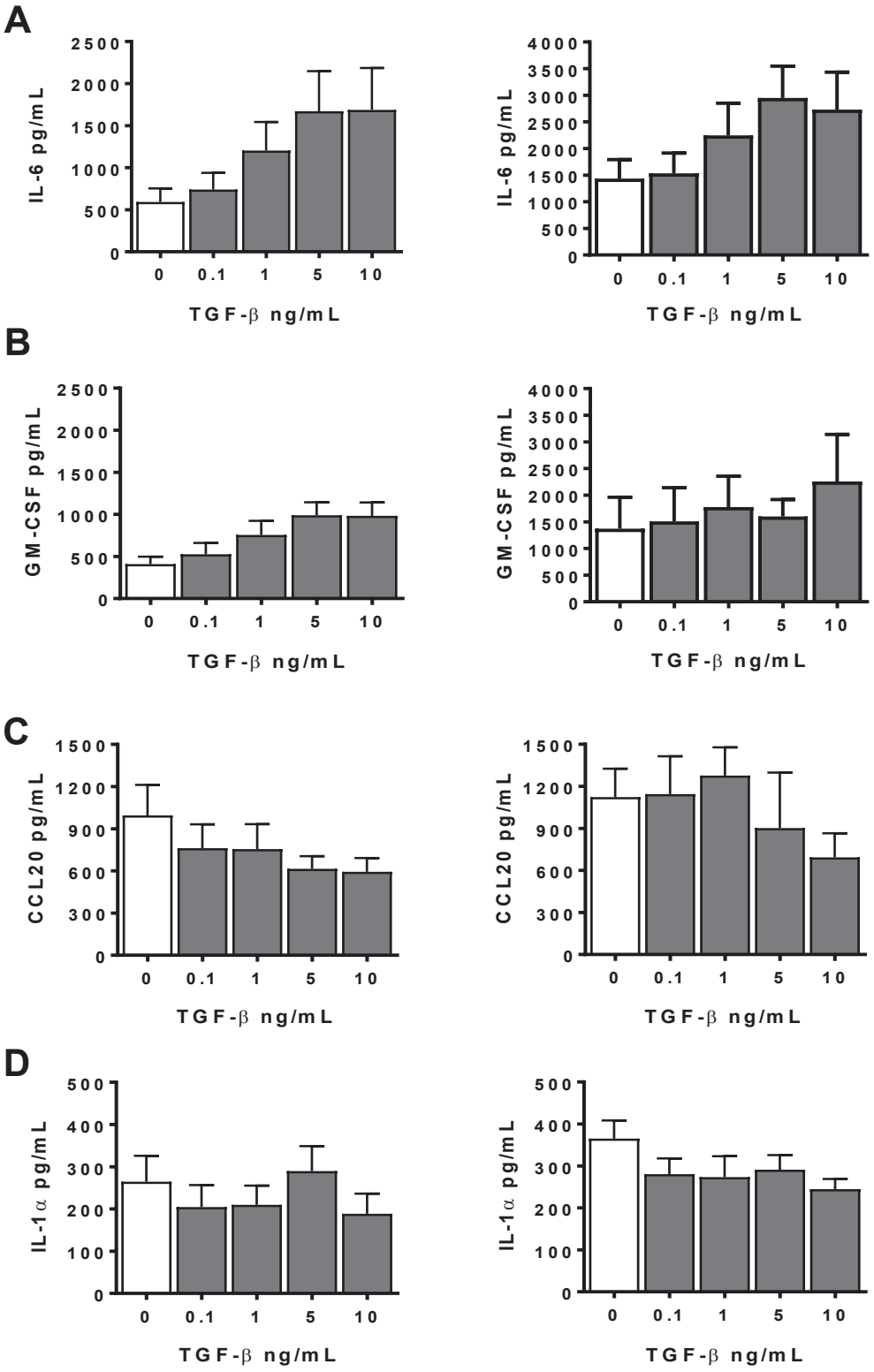


Figure 3.6: Effect of SPARC on cytokine and chemokine secretion in primary human airway epithelial cells.
Primary AECs were stimulated with SPARC for 24 h (left panels) or 72 h (right panels). IL-6 (A), GM-CSF (B), CCL20 (C), IL-1α (D) and HMGB1 (E) release were measured in culture supernatants by ELISA. Bars represent mean data (± SEM) from 3 – 4 cell donors. One-way ANOVA with Bonferroni's correction was used to determine statistical differences.



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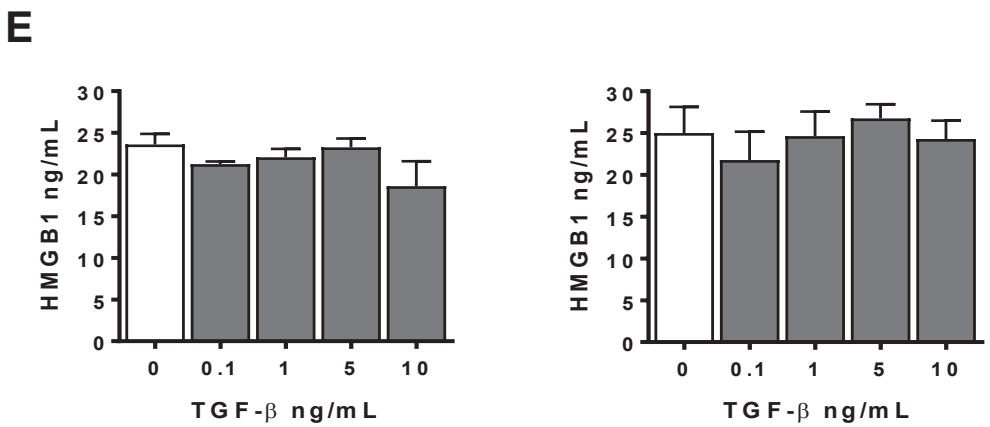

Figure 3.7: Effect of TGF-β on cytokine and chemokine secretion in primary human airway epithelial cells.
Primary AECs were stimulated with TGF-β for 24 h (left panels) or 72 h (right panels). IL-6 (A), GM-CSF (B), CCL20 (C), IL-1α (D) and HMGB1 (E) release were measured in culture supernatants by ELISA. Bars represent mean data (± SEM) from 4 – 8 cell donors. One-way ANOVA with Bonferroni's correction was used to determine statistical differences.

Comparative effect of TGF-β and SPARC on epithelial and mesenchymal markers in human AECs

TGF-β induces EMT in AECs, which is characterized by the loss of epithelial tight junction and adherens junction proteins such as E-cadherin and ZO-1, and increased expression of mesenchymal markers such as vimentin and α-smooth muscle actin (α-SMA) (21). Thus, we examined the comparative effects of TGF-β and SPARC on E-cadherin expression in AECs. Stimulation of AECs with TGF-β or SPARC for 24 h or 72 h had no significant effect on E-cadherin (Figure 3.8A, B). Although preliminary, our studies suggested that SPARC does not induce α-SMA expression in AECs (Figure 3.8C).

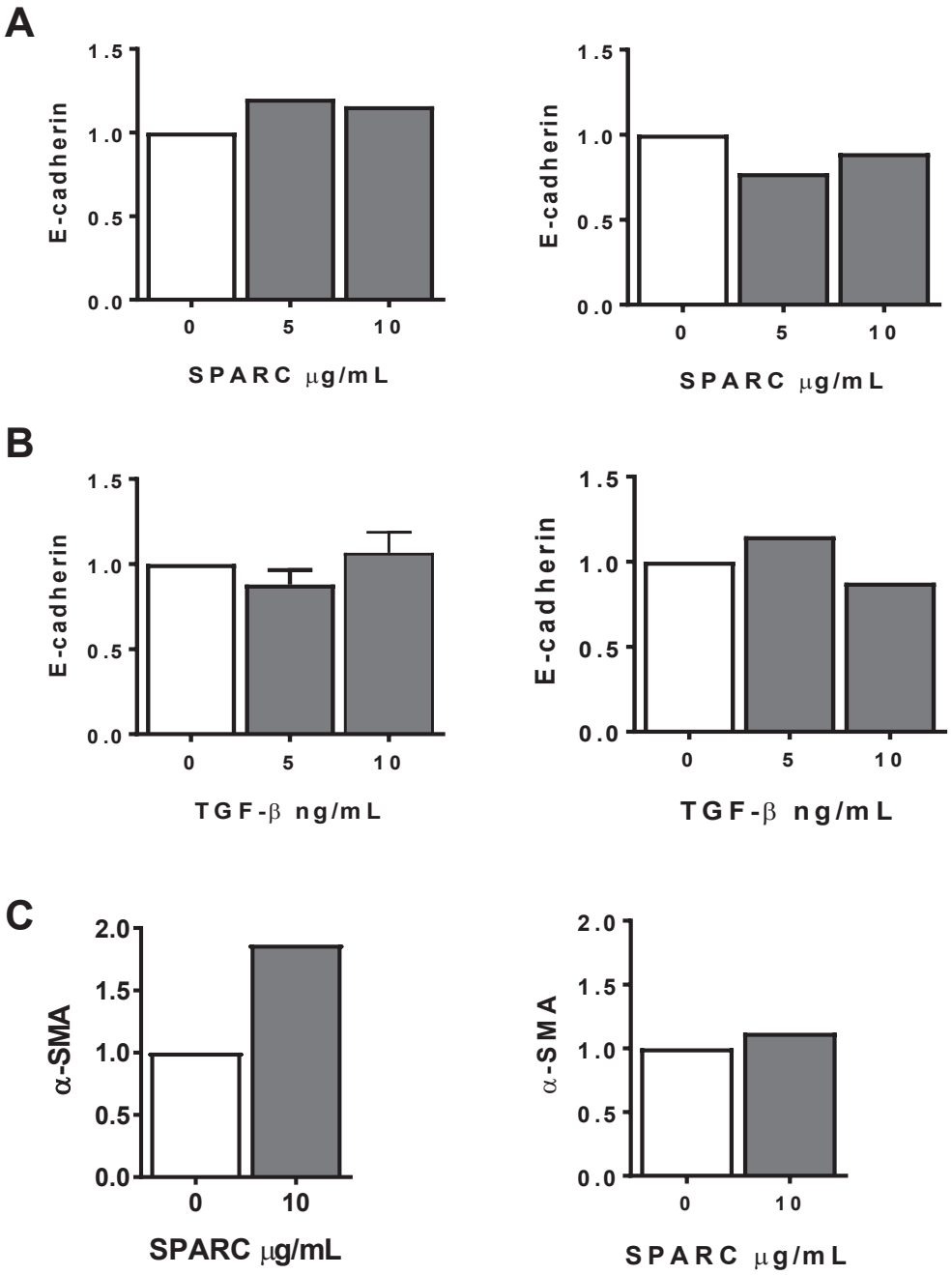

Figure 3.8: Comparative effect of SPARC and TGF-β on epithelial-mesenchymal transition markers in primary human airway epithelial cells. Primary AECs were stimulated with SPARC for 24 h (left panels) or 72 h (right panels), and E-cadherin (A) and α-SMA (C) expression in whole cell lysates were determined by immunoblotting. Primary AECs were stimulated with TGF-β for 24 h (left panels) or 72 h (right panels) and E-cadherin expression in whole cell lysates were determined by immunoblotting (B). E-cadherin and α-SMA protein expression were normalised to α-tubulin and expressed as fold change relative to unstimulated cells. Bars represent mean data for 2 cell donors, except for B (left panel) where bars represent mean data (± SEM) from 3 – 5 cell donors.

DISCUSSION

This is the first study examining SPARC protein expression and function in primary AECs. We showed TGF-β had a modest, but significant stimulatory effect on cell-associated and secreted SPARC in primary AECs. Although preliminary, constitutive expression of cell-associated and secreted SPARC were elevated in asthmatic AECs when compared to non-asthmatic AECs. Interestingly however, preliminary studies showed type 1 (IFN-γ and TNF-α) and type 2 (IL-4 and IL-13) cytokines had inhibitory effects on SPARC expression. Functional studies showed exogenous SPARC did not regulate immunomodulatory function or induce changes in the epithelial phenotype in AECs although these require further validation.

We showed SPARC is a TGF-β-inducible protein in AECs, both cell-associated and secreted SPARC, consistent with findings in other structural cells (11, 22). Interestingly, at the 72 h time-point, TGF-β 5ng/ml significantly induced SPARC secretion, however, a modest but not significant increase was observed at higher concentrations. This is not unusual as the effects of TGF-β on ECM protein synthesis and cell proliferation in other cell types occur at lower but not higher concentrations (23, 24). TGF-β has multifaceted functions and orchestrates its activity via diverse signaling pathways, hence, at any given concentration, the TGF-β responses are potentially due to activation of distinct signaling pathways.

While TGF-β induces SPARC in AECs, it is clear however that this effect is marginal, consistent with the fact that the airway epithelium is an important but

not the major cellular source of ECM proteins in the lung, given SPARC is a critical regulator of ECM protein deposition (25). Indeed, we observed higher levels of secreted SPARC in ASM cells cultured under similar conditions, both basally and in response to TGF-β (Chapter 4). This is in line with the fact that thickening of the smooth muscle layer and its surrounding ECM are key components of airway remodelling in asthma (26).

SPARC does not have structural contribution to the ECM, and immunohistochemistry staining of human tissues confirmed SPARC itself is not deposited into the ECM (27, 28). Thus, the detection of SPARC in cell lysates reflects SPARC that resides in the intracellular compartment. Interestingly, we observed a marked decrease in the expression of cell-associated SPARC at 72 h compared to 24 h, even in cells that were stimulated with TGF-β. However, we did observe a significant increase in secreted SPARC at 72 h, suggesting that the majority of SPARC produced by AECs was released into the extracellular milieu and there was minimal retention of SPARC within the cells by 72 h. This is consistent with the role of SPARC as a matricellular protein, targeted for release to the extracellular space to mediate interaction between cells and the ECM (7).

Importantly, our preliminary studies showed SPARC is overexpressed in asthmatic AECs. This may be the consequence of aberrant epithelial repair mechanisms intrinsic to the diseased cells. The failure of injured epithelium to re-epithelialize following injury is a common feature in asthma, leading to repeated cycles of reparative responses and ultimately, a chronic wound

scenario characterized by persistent recruitment of repair mediators and excessive deposition of ECM proteins (5, 29). SPARC is highly expressed in the course of tissue repair. It is necessary for regulating cell adhesion, migration and proliferation for wound closure, and also for the assembly and incorporation of new ECM proteins especially collagen I (30). Heguy et al. characterised the human airway epithelial repair transcriptome by obtaining populations of the airway epithelium before and at day 7 and 14 following mechanical injury induced by brushing in healthy subjects. They found SPARC gene expression was significantly up-regulated at 7 days post injury but this level diminished by day 14 as wound closure took place (31). The sustained basal SPARC levels in asthmatic AECs, although subjected to interrogation in more cell donors, is consistent with the idea that the asthmatic epithelium is inherently abnormal and remain under chronic repair mode even at physiological conditions.

Although TGF-β is an important repair mediator and is usually induced during tissue injury, the trend of elevated SPARC expression in asthmatic AECs is unlikely to be due to epithelial expression of TGF-β expression. Investigation of TGF-β expression and release in asthmatic AECs have provided inconsistent findings to date, due to different forms of TGF-β measured (intracellular, latent or active) and the techniques used (32). Hastie and colleagues demonstrated enhanced TGF-β levels in asthmatic AECs, but the significance of the small increase detected, on SPARC expression is questionable (33). Asthmatic AECs maintained under air-liquid interphase conditions showed no change in TGF-β release while reduced TGF-β release have been reported in AECs from asthmatic children (34, 35). Notably, our initial findings indicate maximal

SPARC release in non-asthmatic AECs in the presence of exogenous TGF-β was less than basal SPARC levels in asthmatic AECs (30 vs. 50 ng/ml, respectively). This suggests aberrant expression and/or activity of repair factors other than TGF-β accounts for the SPARC overexpression observed.

Interestingly, type 1 and type 2 cytokines showed inhibitory effects on basal and TGF-β-induced SPARC expression in both asthmatic and non-asthmatic AECs although these preliminary observations require validation in more cell donors. The similar regulatory pattern showed by type 1 and type 2 cytokines is unanticipated as they are known to have opposing effects in gene regulation in AECs (36, 37). For instance, the anti-fibrotic cytokine IFN-γ inhibited TGF-β2 expression induced by IL-4 and IL-13 in AECs (37). While IFN-γ and TNF-α were previously shown to inhibit SPARC expression in periodontal ligament cells and chondrocytes, supporting our studies herein (38-41), the mechanism involved was not examined. Notably however, SPARC is known to be susceptible to proteolytic cleavage by MMPs, and can also be internalized and targeted for degradation by the scavenger receptor stabilin-1 as well as integrin-α5 (42-44). Stabilin-1 is commonly expressed on professional scavenging cells such as macrophages, and is activated by IL-4 in the presence of dexamethasone, but not IL-4 alone. While the expression of stabilin-1 in AECs remains to be determined, AECs are capable of performing endocytosis (45, 46). Moreover, type 1 and type 2 cytokines induce the expression of various MMPs and SPARC has been shown to be degraded by MMP-3 (47-50). Thus, the trend of reduced SPARC expression in the presence of type 1 and type 2 cytokines may be attributed to its degradation by MMPs and/or endocytosis.

The loss of SPARC in the presence of cytokines in AECs may be biologically relevant as it is associated with a more permissive ECM microenvironment important for facilitating cellular trafficking during immune and inflammatory responses (51). ECM proteins are also degraded by MMPs during the acute inflammatory phase following injury, and their production are reinstated and enhanced in the subsequent proliferative/repair phase. SPARC may be regulated in the same manner as ECM proteins given it is necessary for mediating cell-matrix interactions and ECM synthesis during this phase.

Given SPARC expression may potentially be elevated in asthmatic AEC and was induced by TGF-β, we hypothesized that SPARC plays a role in asthma by imparting immunoregulatory effect on AECs. Thus, we determined if SPARC directly modulates the expression of several epithelial derived mediators including IL-1α, HMGB1, GM-CSF, CCL20 and IL-6 (19, 20, 52-54). Interestingly, SPARC had no effect on the secretion of these mediators. This is unlikely to be attributed to the SPARC concentrations used as the same range of concentrations have been shown to be biologically active in other cell types, in regulating endothelial cell barrier function and MMPs expression (41, 55). However, it is possible that stimulation of cells with SPARC alone was not sufficient to activate AEC inflammatory responses. For instance, SPARC alone has no effect on Smad2 phosphorylation but co-stimulation of SPARC and TGF-β has been shown to amplify TGF-β-induced activation of Smad2. SPARC and TGF-β were shown to converge on the TGF-β receptor II in a TGF-β-dependent manner (10). Notably, no specific receptors for SPARC have been identified to

date; rather SPARC mediates its biological activities through 'borrowed' receptors such as integrins and TGF-β receptors (56, 57). SPARC has been shown to orchestrate distinct pro- and anti-inflammatory properties in a context-dependent manner, thus even though SPARC appears not to be a positive regulator of inflammation in AECs, the possibility of SPARC serving as an anti-inflammatory molecule in the presence of stimuli remains to be examined (15).

SPARC did not appear to regulate epithelial junction proteins and mesenchymal markers in AECs although these observations need to be verified. This is unanticipated given SPARC is an important player in tissue repair and changes in epithelial phenotype through EMT is essential for wound closure (7). It is notable that we also did not observe downregulation of E-cadherin expression in response to TGF-β, as has been demonstrated by Hackett and colleagues (21). We suspect the use of complete BEGM during cell stimulation in our study, instead of BEBM, accounts for the lack of TGF-β-mediated loss of epithelial phenotype. Several components in the supplements such as epidermal growth factor (EGF), retinoic acid and triiodothyronine could inhibit TGF-β-mediated responses and signalling (58-60). The impact of these supplements on SPARC activity is not known, and hence further studies need to be performed in cells rendered quiescent in BEBM only. It is also notable that we have examined the effect of SPARC on E-cadherin and α-SMA only in this study. To validate this preliminary analysis, the effect of SPARC on additional EMT markers is warranted. Additionally, interrogation of these EMT markers in AECs overexpressing SPARC and the use of air-liquid interface cultures, which

portray more closely the physiological conditions of the airway epithelium, is an essential next step to verify the function of SPARC in AECs.

In conclusion, we demonstrated the SPARC protein is expressed by primary AECs and is enhanced following TGF-β stimulation. The preliminary observation of augmented SPARC expression in asthmatic AECs when compared to non-asthmatic AECs is an exciting finding that needs to be verified. Similarly, the inhibitory trend of type 1 and type 2 cytokines on basal and TGF-β-induced SPARC expression also warrants further interrogation. While SPARC did not appear to regulate the inflammatory profile or impact on the epithelial phenotype in AECs, its potential overexpression in asthmatic AECs compels further understanding of its role, and how it relates to TGF-β in asthma.

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Chapter 4

SPARC expression in airway smooth muscle is regulated by the unfolded protein response and is diminished in chronic obstructive pulmonary disease

INTRODUCTION

Increased thickness of the smooth muscle layer is a prominent feature of airway remodelling in COPD. Although the increase in ASM mass is attributed to a number of factors, accumulation of ECM components within and around the muscle layer is a key pathological factor that underlies this response (1). It is well recognised that ASM cells regulate their surrounding ECM, and that changes in both the rate of ECM deposition and the composition of the ECM impact ASM structure and function (2, 3). Chen and colleagues have recently shown that ASM cells from people with COPD display intrinsic differences in their capacity to produce ECM components and MMPs involved in ECM turnover, suggesting that abnormalities at the level of the smooth muscle contribute to perturbations of the ECM in COPD (4).

It is increasingly recognised that COPD is a condition of accelerated lung aging, and that changes to the ECM may reflect age-related biochemical changes in ECM biosynthesis, assembly and turnover (5). SPARC is a matricellular protein that co-ordinates various biological processes such as cellular adhesion, proliferation, and migration by regulating cell-matrix interactions, growth factor signalling and ECM assembly (6). Of note, SPARC binds collagen and participates in the coordination of post-synthetic procollagen processing and the formation and assembly of mature cross-linked insoluble collagen fibrils (7). Interestingly, SPARC expression is altered in ageing tissues; it is increased in the hearts of aged mice but also declines with age in human dermal fibroblasts and retinal pigment epithelial cells. Moreover, studies using SPARC-deficient mice showed that it is an important mediator of age-related cardiac fibrosis,

remodelling and inflammation (8-12), suggesting aberrant regulation of SPARC expression is implicated in progressive ageing.

The accumulation of unfolded, misfolded or aggregated proteins is a hallmark of many age-related diseases such as COPD (5). Emerging evidence suggests that the unfolded protein response (UPR), which is activated in response to endoplasmic reticulum (ER) stress caused by the accumulation of unfolded proteins in the ER, is of potential importance in COPD pathogenesis (13). Min and colleagues demonstrated increased expression of UPR proteins in the lungs of COPD subjects (14). Moreover, cigarette smoke exposure, a major causative factor of COPD induces protein misfolding and UPR in the lung and in isolated lung cells (13). ER stress has been linked to the development of tissue fibrosis and remodelling in various organs including the lung (15). In addition, TGF-β, a key mediator of airway remodelling in COPD, has been shown to induce ER stress in lung fibroblasts (16, 17), suggesting TGF-β-mediated activation of the UPR may contribute to its pro-fibrotic effects in COPD.

Although SPARC is implicated in tissue fibrosis and remodelling in a number of disease settings including idiopathic pulmonary fibrosis (12, 18, 19), its potential role as a mediator of airway remodelling in COPD has so far been overlooked. Importantly, SPARC expression is induced by TGF-β in many cell types, and it is a well-established down-stream effector of TGF-β signalling (19). In light of evidence implicating SPARC in age-related ECM changes and tissue remodelling, we hypothesized that SPARC is a mediator of airway remodelling in COPD and that its expression is increased down-stream of TGF-β/UPR

signalling. To explore this hypothesis, we determined whether TGF-β induces SPARC expression in human ASM cells, and whether this response is regulated by the UPR. In addition, we determined whether ASM cells from COPD subjects exhibit intrinsic differences in SPARC expression.

MATERIALS AND METHODS

Human ASM cell culture and stimulation

All studies in Figures 4.1 through to 4.9 were performed in primary ASM cells isolated from macroscopically normal bronchial tissue obtained from carcinoma patients undergoing lung resection or lung transplant patients as described previously (20). To determine if SPARC secretion is altered in COPD (Figure 4.10), ASM cells were isolated from bronchial biopsy tissue or lung resection tissue, as indicated in Table 4.1. In each case, COPD diagnosis was established according to GOLD guidelines (21). COPD subjects had an FEV_1/FVC ratio of less than 0.7, while non-COPD subjects had an FEV_1/FVC ratio and FEV_1 of more than 0.7 and 80%, respectively. Ethics approval for the use of human lung tissue (X14-0045, HREC RPAH) was obtained from the University of Sydney Human Research Ethics Committee and Sydney South West Area Health Service.

Following isolation from lung or biopsy tissue, ASM cells were cultured in 10% fetal bovine serum (FBS) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 0.25µg/ml amphotericin B and 25mM HEPES. All cultures were tested for the presence of mycoplasma. ASM cells were identified by the characteristic 'hill and valley'

morphology when visualised under a light microscope. For experimental studies, cells between passages 3 and 8 were seeded in 6-well culture plates at a density of 1 x 10^4 cells per cm^2 in 10% FBS/DMEM and grown to 90% confluence. Cells were then incubated in DMEM supplemented with 0.1% FBS, 100U/ml penicillin, 100µg/ml streptomycin, 0.25µg/ml amphotericin B and 25mM HEPES for 24 hours. The media was then replenished, and cells were stimulated with recombinant human TGF-β1 (R&D Systems, UK). Where indicated, cells were pre-treated with chemical inhibitors 2 h prior to stimulation with TGF-β. Thapsigargin, ionomycin, ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 4-phenylbutyric acid (4-PBA) and trimethylamine N-oxide dehydrate (TMAO) were purchased from Sigma-Aldrich, Australia. SD208 and APY29 were purchased from Tocris Bioscience, UK and 4μ8C was purchased from Merck Millipore, Australia. All cell culture reagents were purchased from Life Technologies, Australia. The chemical inducers and inhibitors of ER stress were used at concentrations reported in published studies (Table 4.2).

Table 4.1: COPD and non-COPD ASM cell donors in which SPARC secretion was assessed (Fig. 4.10).

Donor	Diagnosis	Biopsy/ Resection	Age	Sex	Smoking status	FEV_1 (L)	FEV_1 (% predicted)	FVC (L)	FVC (% predicted)	FEV_1/FVC ratio (%)
Non-COPD										
1	Healthy	B	29	M	Non-smoker	3.27	77	4.4	86	75
2	Healthy	B	22	F	-	-	-	-	-	-
3	NSCCa	R	65	M	Current smoker	3.11	91	3.98	95	78
4	Ca	R	61	M	Ex-smoker	3.43	100	4.57	106	75
5	NSCCa	R	72	M	Ex-smoker	2.11	75	2.9	83	73
6	PF	R	64	M	-	-	82	-	87	-
COPD										
1	COPD/ NSCCa	R	58	M	Ex-smoker	2.21	-	-	-	-
2	Emphysema	R	53	M	Ex-smoker	0.84	22	2.46	52	33
3	Emphysema	R	54	M	Ex-smoker	0.56	16	2.15	44	28
4	COPD/Ca	R	68	F	Ex-smoker	1.31	50	2.56	78	51
5	COPD/ SCCa	R	71	M	Ex-smoker	1.43	41	2.61	61	54

Abbreviations: B, biopsy; Ca, carcinoma; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; NSCCa, non-small cell carcinoma; PF, pulmonary fibrosis; R, resection; SCCa, small cell carcinoma

Table 4.2: List of chemical inducers and inhibitors of ER stress used for ASM cell treatment.

Chemical/inhibitor	Activity	Reported concentration used	Supplier	Reference
4-PBA	Chemical chaperone	1 – 20mM	Calbiochem	(22)
4μ8C	IRE1α RNase inhibitor	100μM	-	(23)
APY29	IRE1α kinase inhibitor	1μM	-	(24)
Ionomycin	ER stress inducer	1 – 5μM	Calbiochem	(25)
Thapsigargin	ER stress inducer	1 – 10μM	Calbiochem	(26)
TMAO	Chemical chaperone	1M	Sigma	(27)

ELISA

The concentration of SPARC in ASM cell culture supernatants was measured using SPARC Quantikine ELISA (#DSP00) (R&D Systems, UK) according to manufacturer's instructions.

Immunoblotting

Whole cell lysates were extracted with RIPA buffer (Sigma-Aldrich, Australia) containing protease and phosphatase inhibitor cocktails, cOmplete™ ULTRA Tablets (Mini) and PhosSTOP™, respectively (Roche Diagnostics, Australia). Protein concentrations were determined using the Pierce BCA Protein Assay Kit according to the manufacturer's instructions (Thermo Fisher Scientific, Australia). 10 – 25µg of protein was loaded per lane and separated using NuPAGE™ Novex™ 4 – 12% Bis-Tris protein gels. The proteins were electrophoresed at 200V for 50 mins. Protein transfer onto PVDF membranes was performed at 20V for 1 min, 23V for 4 min and 25V for 2 min using iBlot2® Dry Blotting System. PVDF membranes were then incubated with 5% non-fat milk powder in Tris-buffered saline containing 0.1% Tween (TBST) for 1 h at room temperature, and then incubated with primary antibodies detecting GRP78 (#3177), IRE1α (#3294) (Cell Signalling Technology, Danvers, MA); GAPDH (sc-32233), SPARC (sc-25574) (Santa Cruz Biotechnology, Dallas, Texas); or XBP-1 (ab198999) (Abcam, Australia) for 16 h at 4^0C. All primary antibodies were diluted 1:1000 in 5% non-fat milk powder in 0.1% TBST. PVDF membranes were then washed with 0.1% TBST three times (5 min each) and then incubated with IRDye® 800CW anti-Rabbit IgG (#926-32211) or IRDye® 680RD anti-Mouse IgG (#926-68070) (LI-COR Biosciences, Lincoln NE) at

room temperature for 1 h. Secondary antibodies were diluted 1:15,000 in 5% non-fat milk powder in 0.1% TBST. PVDF membranes were then washed with 0.1% TBST three times. For detection of phosphorylated IRE1α using anti–phospho–Ser724–IRE1α antibody (NB100-2323) (Novus Biologicals, Littleton, CO), blocking and antibody incubation steps were performed in 5% bovine serum albumin in 0.1% TBST. Protein bands were visualised using Odyssey CLx Imaging System (LI-COR Biosciences, Lincoln NE) and densitometric analysis was performed using Image Studio software (v5.2.5). Pre-cast gels, electrophoresis reagents, molecular weight markers and iBlot2 Transfer Stacks were purchased from Life Technologies, Australia.

Statistical analysis

Data in Figure 4.1 through to 4.9 are expressed as means ± SEM, and results were analysed using one-way ANOVA followed by Bonferroni post hoc test to determine differences between treatment groups. Data in Figure 4.10 are not normally distributed as determined using Shapiro-Wilk test, hence data are presented as a dot plot graph with median ± interquartile range. The Wilcoxon paired t-test was used to determine statistical differences between control and TGF-β-stimulated cells within each disease group, while the Mann-Whitney test was used for comparison between disease groups. A *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

TGF-β augments cell-associated SPARC expression and induces SPARC secretion in human ASM cells

We examined SPARC expression in human ASM cells that were exposed to TGF-β for 24 or 72 h. At low concentrations (0.1, 1 ng/ml), TGF-β had no effect on cell-associated SPARC expression and did not induce SPARC release. At higher concentrations (5, 10 ng/ml), TGF-β induced approximately 4-fold increase in cell-associated SPARC expression which was evident 24 h post stimulation, and sustained for up to 72 h (Figure 4.1A, B). In parallel, TGF-β had a significant stimulatory effect on SPARC secretion (Figure 4.1C, D). Induction of SPARC release was completely abrogated in the presence of the TGF-βRI inhibitor SD208, confirming this response was mediated down-stream of TGF-β receptor signalling (Figure 4.1E).

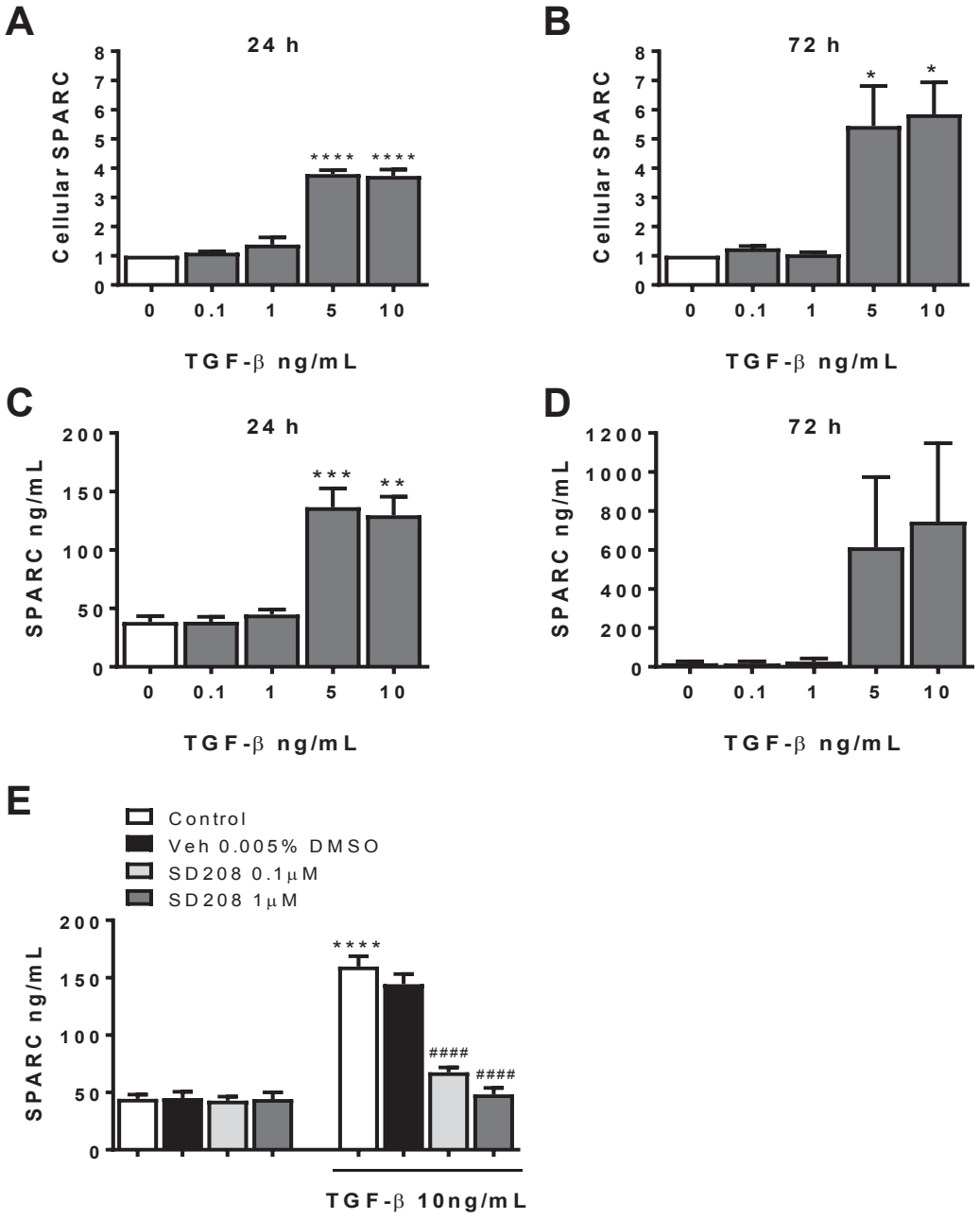

Figure 4.1: TGF-β induces cell-associated and secreted SPARC in human airway smooth muscle cells.

ASM cells were stimulated with TGF-β for 24 h (A, C) or 72 h (B, D). SPARC expression in whole cell lysates was determined by immunoblotting, and cell-associated SPARC expression was normalised to GAPDH and expressed as fold change relative to unstimulated cells (A, B). Soluble SPARC was measured in culture supernatants by ELISA (C, D). ASM cells were stimulated with TGF-β for 24 h, in the presence or absence of TGF-βRI inhibitor SD208 and soluble SPARC was measured in culture supernatants by ELISA (E). Bars represent mean data (± SEM) from 3 cell donors. One-way ANOVA with Bonferroni's correction was used to determine statistical differences. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 compared to unstimulated cells; ####P<0.0001 compared to TGF-β-stimulated cells. Veh, vehicle control.

TGF-β-induced expression of cell-associated and secreted SPARC in human ASM cells is reversed in the presence of chemical chaperones

Chemical chaperones such 4-phenylbutyric acid (4-PBA) and trimethylamine N-oxide (TMAO) alleviate ER stress by improving ER folding capacity and stabilising protein conformation (28). We found that treatment of ASM cells with 4-PBA (Figure 4.2A, B) or TMAO (Figure 4.2C, D) completely inhibited cell-associated SPARC expression, and largely suppressed SPARC secretion in response to TGF-β, although the latter did not achieve statistical significance. These findings suggest that SPARC expression is regulated by the UPR.

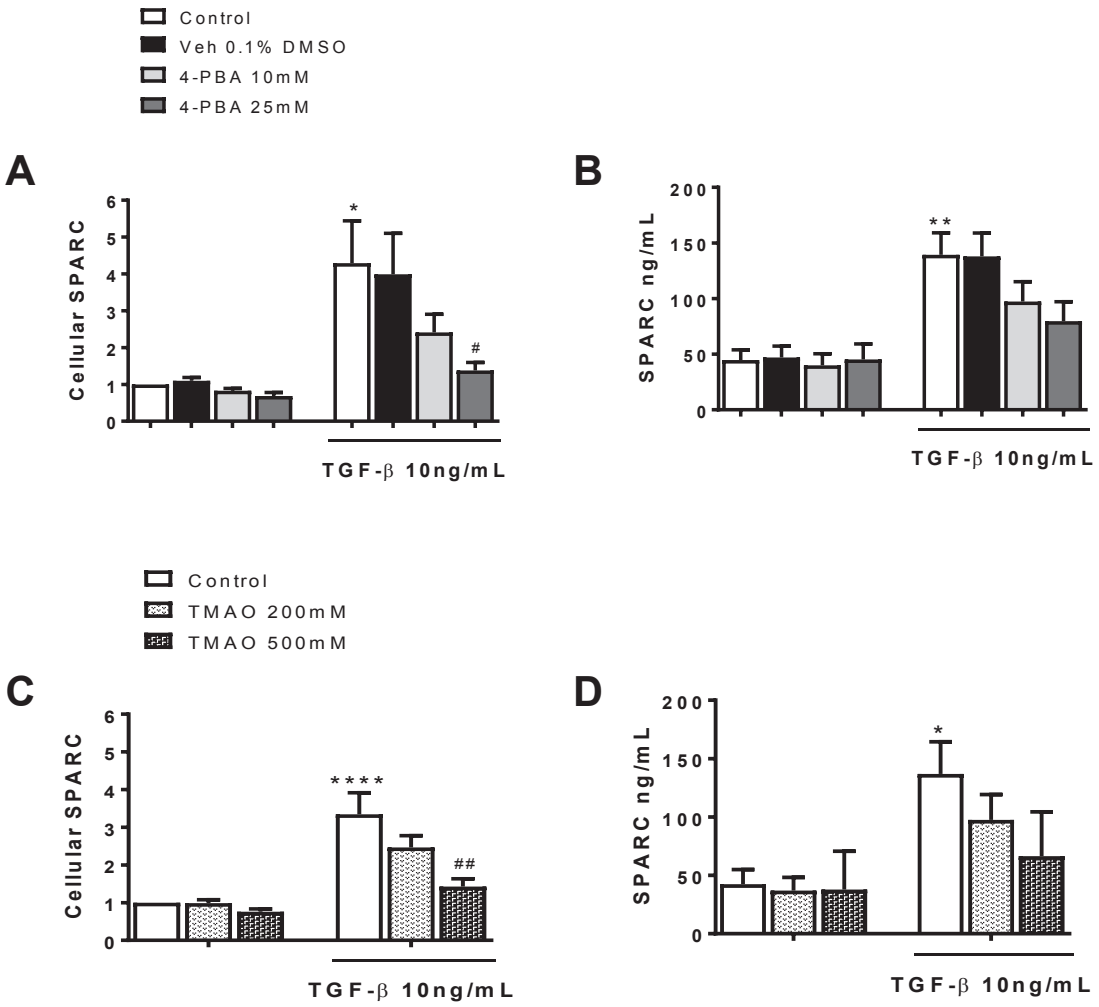

Figure 4.2: Chemical chaperones reverse TGF-β-induced cell-associated and secreted SPARC in human airway smooth muscle cells.

ASM cells were stimulated with TGF-β for 24 h, in the presence or absence of chemical chaperones 4-phenylbutyric acid (4-PBA) (A, B) or trimethylamine N-oxide (TMAO) (C, D). SPARC expression in whole cell lysates was determined by immunoblotting, and cell-associated SPARC expression was normalised to GAPDH and expressed as fold change relative to unstimulated cells (A, C). Soluble SPARC was measured in culture supernatants by ELISA (B, D). Bars represent mean data (± SEM) from 5 – 6 cell donors. One-way ANOVA with Bonferroni's correction was used to determine statistical differences. *P<0.05, **P<0.01 and ****P<0.0001 compared to unstimulated cells; #P<0.05, ##P<0.01 compared to TGF-β-stimulated cells. Veh, vehicle control.

Chemical inducers of ER stress inhibit basal SPARC secretion, but do not modulate cell-associated SPARC expression in human ASM cells

TGF-β signals via several intracellular pathways which may potentially impact SPARC expression in ASM cells (29, 30). Thus, in order to tease out the effects of TGF-β and the UPR on SPARC expression, we determined if induction of ER stress with chemical agents augments SPARC expression. Unexpectedly, we found that thapsigargin, an irreversible sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor that induces ER stress by depleting ER Ca^{2+} stores (31), completely abrogated basal levels of secreted SPARC (Figure 4.3B). This response was not specific to thapsigargin, as the calcium ionophore, ionomycin which also induces ER stress by depleting Ca^{2+} stores had a similar effect (Figure 4.3D) (32). Of note, these responses were not due to cell death as routine inspection of cells following treatment with thapsigargin and ionomycin using bright field microscopy indicated no compromise in cell viability. Moreover, while both thapsigargin and ionomycin had a marked inhibitory effect on SPARC secretion, they did not modulate cell-associated SPARC expression (Figure 4.3A, C).

Depletion of ER Ca^{2+} by thapsigargin and ionomycin is associated with an influx of Ca^{2+} from the extracellular space (33). SPARC is a Ca^{2+} binding glycoprotein (34), thus it is possible that the observed reduction in SPARC secretion was attributed to changes in the extracellular Ca^{2+} concentration. However, incubation of ASM cells with the cell-impermeable Ca^{2+}chelator EGTA at concentrations sufficient to deplete extracellular Ca^{2+} (35), had no effect on basal SPARC secretion, suggesting this was not the case (Figure 4.3E).

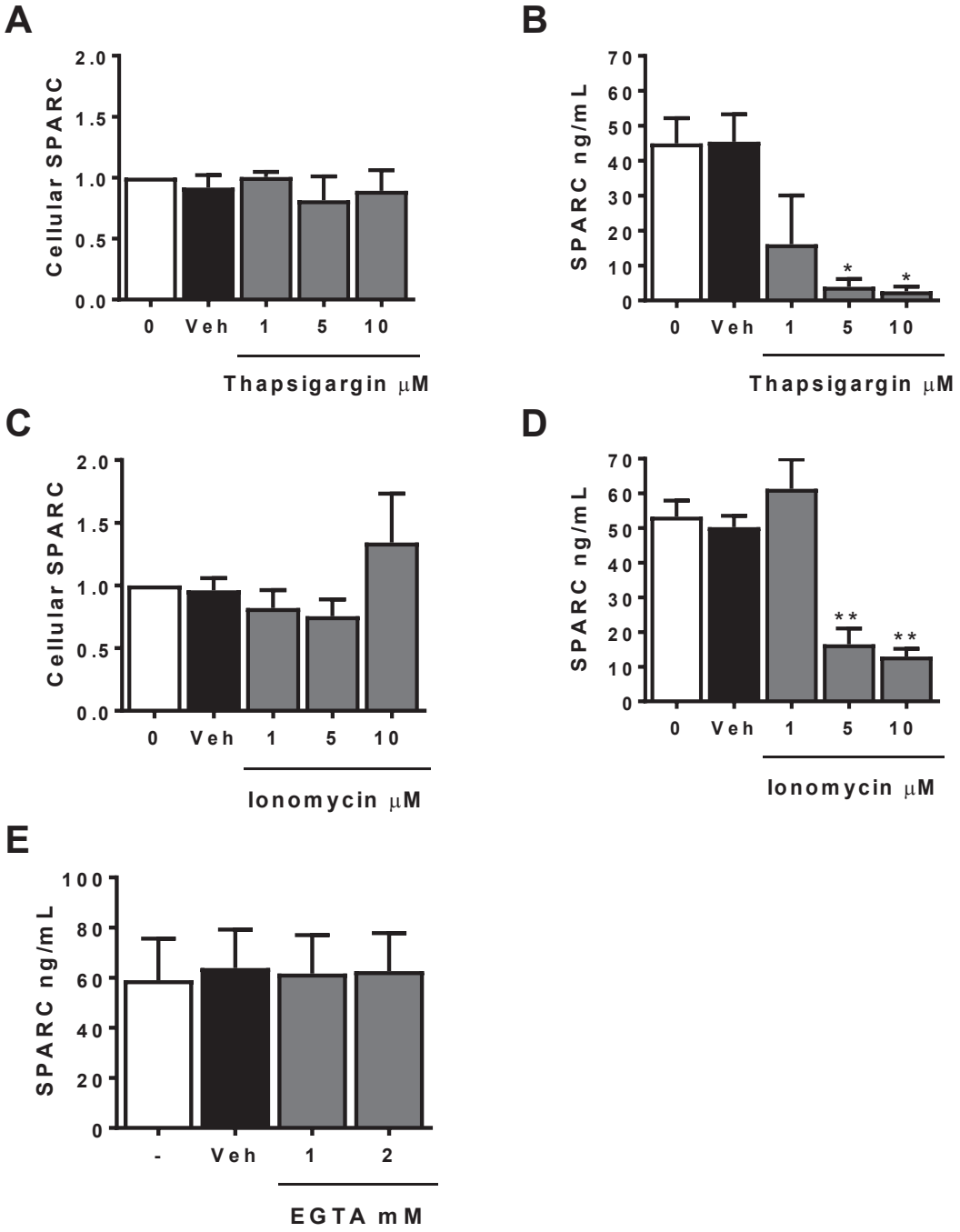

Figure 4.3: Effect of ER stress inducers and calcium chelator on cell-associated and secreted SPARC in human airway smooth muscle cells. ASM cells were stimulated with irreversible SERCA inhibitor, thapsigargin (A, B) or Ca^{2+} ionophore, ionomycin (C, D) for 24 h. SPARC expression in whole cell lysates was determined by immunoblotting, and cell-associated SPARC expression was normalised to GAPDH and expressed as fold change relative to unstimulated cells (A, C). Soluble SPARC was measured in culture supernatants by ELISA (B, D). ASM cells were stimulated with cell-impermeable Ca^{2+} chelator, EGTA for 24 h and soluble SPARC was measured in culture supernatants by ELISA (E). Bars represent mean data (± SEM) from 3 – 4 cell donors. One-way ANOVA with Bonferroni's correction was used to determine

statistical differences. *P<0.05 and **P<0.01 compared to unstimulated cells. Veh, vehicle control. Vehicle controls for thapsigargin and ionomycin are 0.05% and 0.07% DMSO, respectively. Vehicle control for EGTA is 4mM NaOH.

TGF-β and thapsigargin induce different levels of ER stress in ASM cells, but this does not explain their differential effect on SPARC secretion

Although TGF-β has previously been shown to induce ER stress in human lung fibroblasts, and thapsigargin is a well-established and potent inducer of ER stress, the capacity for these different stimuli to induce ER stress in human ASM cells have not been studied (17, 31). GRP78 is an ER chaperone that is up-regulated down-stream of UPR activation, and is a commonly used marker of cellular ER stress (31). Thus, we determined the extent to which TGF-β and thapsigargin induce GRP78 expression in human ASM cells, and the extent to which this could be reversed by chemical chaperones.

Thapsigargin induced an approximately 25-fold increase in GRP78 expression, while in comparison, TGF-β induced an approximately 2.5-fold increase in GRP78 expression (Figure 4.4A). Despite the marked difference in the level of GRP78 expression induced by each of these stimuli, the chemical chaperone 4-PBA reduced GRP78 expression by approximately 50% in each case (Figure 4.4B, C). TMAO, on the other hand, had no effect on thapsigargin or TGF-β induced GRP78 expression (Figure 4.4D, E). Thus, although TGF-β and thapsigargin induced different levels of ER stress in ASM cells, this is unlikely to explain their differential impact on SPARC secretion, because irrespective of the extent of ER stress, 4-PBA was able to similarly impact this response.

Furthermore, unlike the stimulatory effect of TGF-β on SPARC secretion (Figure 4.2B, D), the inhibitory effect of thapsigargin on SPARC secretion could not be reversed in the presence of 4-PBA (Figure 4.5A) or TMAO (Figure 4.5B). This suggests that thapsigargin-mediated inhibition of SPARC secretion may be unrelated to its ER-stress inducing action.

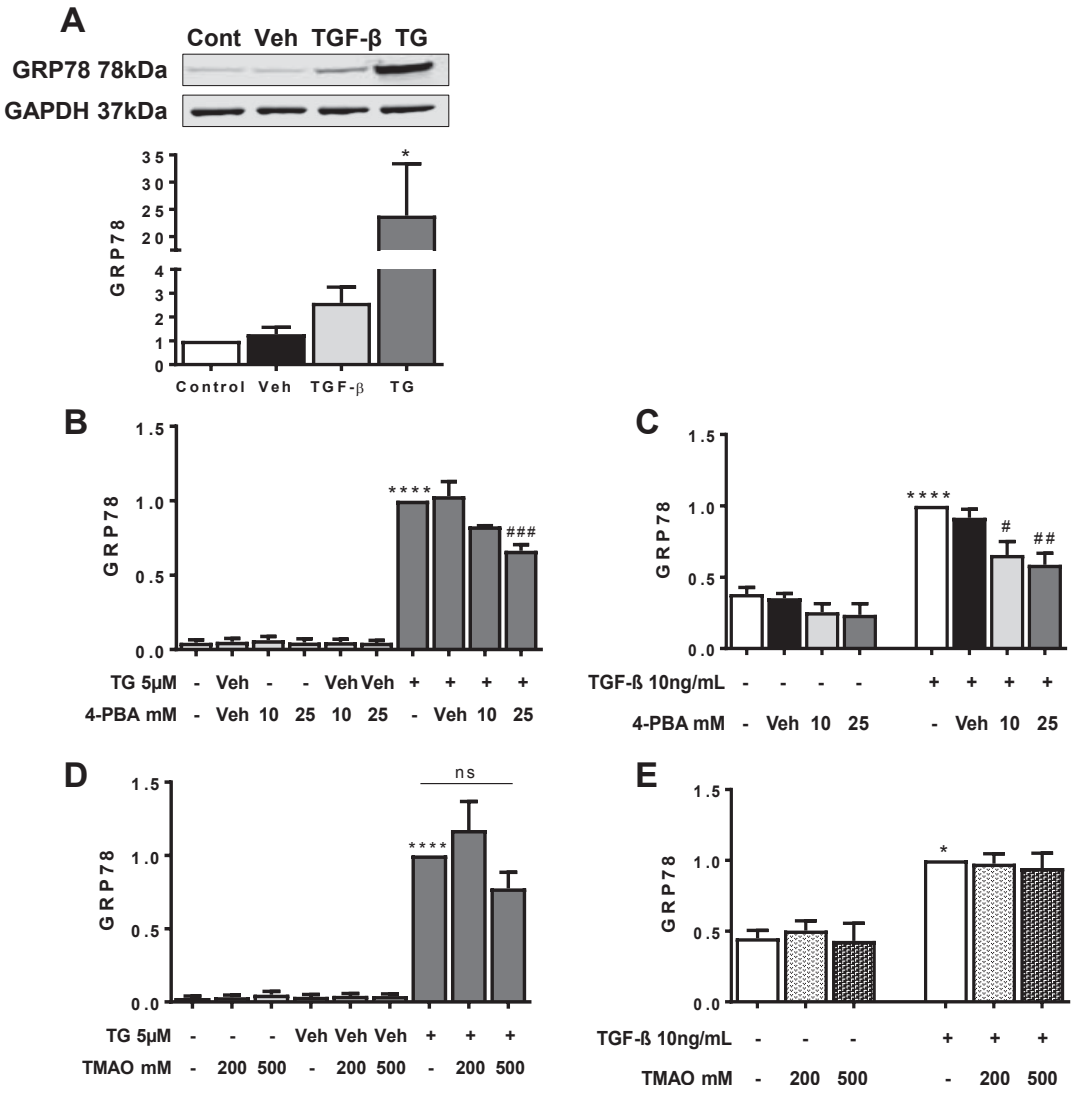

Figure 4.4: Thapsigargin induces greater ER stress than TGF-β in human airway smooth muscle cells.

ASM cells were stimulated with TGF-β (10ng/ml) or thapsigargin (5μM) for 24 h and GRP78 expression in whole cell lysates was determined by immunoblotting, normalised to GAPDH and expressed as fold change relative to unstimulated cells (A). ASM cells were stimulated with thapsigargin (5μM) for 24 h, in the presence or absence of chemical chaperones, 4-PBA (B) or TMAO (D). ASM cells were stimulated with TGF-β (10ng/ml) for 24 h, in the presence or absence of chemical chaperones, 4-PBA (C) or TMAO (E). GRP78 expression in whole cell lysates was determined by immunoblotting, normalised to GAPDH and expressed as fold change relative to thapsigargin-stimulated cells (B, D) or TGF-β-stimulated cells (C, E). Bars represent mean data (± SEM) from 3 – 6 cell donors. One-way ANOVA with Bonferroni's correction was used to determine statistical differences. *P<0.05 and ****P<0.0001 compared to unstimulated cells; #P<0.05, ##P<0.01 and ###P<0.001 compared to thapsigargin-stimulated or TGF-β-stimulated cells. ns, non-significant; 4-PBA, 4-phenylbutyric acid; TG, thapsigargin; TMAO, trimethylamine N-oxide; Veh, vehicle control. Vehicle controls for thapsigargin and 4-PBA are 0.025% and 0.1% DMSO, respectively.

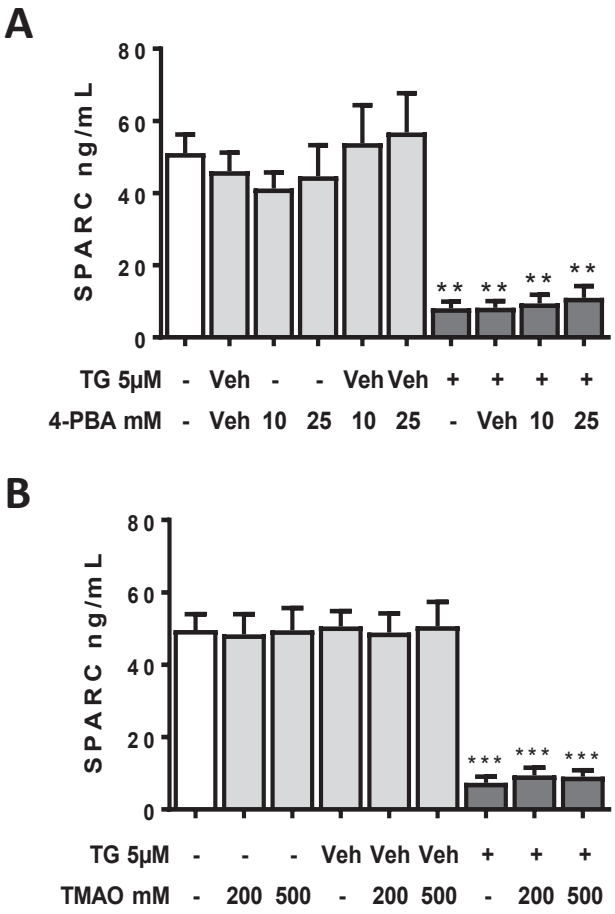

Figure 4.5: Loss of SPARC under conditions of severe ER stress could not be reversed by chemical chaperones.

ASM cells were stimulated with thapsigargin (5μM) for 24 h, in the presence or absence of chemical chaperones, 4-PBA (A) or TMAO (B). Soluble SPARC was measured in culture supernatants by ELISA. Bars represent mean data (± SEM) from 3 cell donors. One-way ANOVA with Bonferroni's correction was used to determine statistical differences. **P<0.01 and ***P<0.001 compared to unstimulated cells. 4-PBA, 4-phenylbutyric acid; TG, thapsigargin; TMAO, trimethylamine N-oxide; Veh, vehicle control. Vehicle controls for thapsigargin and 4-PBA are 0.025% and 0.1% DMSO, respectively.

Thapsigargin mediated inhibition of SPARC secretion is not due to IRE1α/RIDD dependent signalling

As an ER transmembrane protein, IRE1α monitors ER homeostasis through an ER luminal stress-sensing domain and triggers the UPR through a cytoplasmic kinase domain and an RNase domain. Upon ER stress, IRE1α is activated through conformational change, auto-phosphorylation and higher-order oligomerisation. It initiates down-stream signalling of the UPR through unconventional splicing of the transcription factor X-box binding protein 1 (XBP-1) and/or via a mechanism termed regulated IRE1α-dependent decay (RIDD) which cleaves multiple mRNA substrates (36, 37). Because SPARC has been identified as a substrate of the IRE1α/RIDD pathway, we extended our studies to specifically determine if thapsigargin-mediated inhibition of SPARC secretion is due to activation of the RIDD pathway (36).

To explore this, we examined the effect of thapsigargin on IRE1α protein expression and activation in human ASM cells, and for comparison, we also stimulated the cells with TGF-β. Interestingly, while TGF-β increased total IRE1α protein expression by approximately 5-fold (Figure 4.6A, B), TGF-β did not induce IRE1α phosphorylation and had an inconsistent effect on XBP-1s expression (Figure 4.6C, D). Taken together, these data suggest that TGF-β does not induce activation of IRE1α signalling.

In contrast, thapsigargin induced approximately 25-fold increase in total IRE1α protein expression and also led to down-stream activation of the IRE1α

pathway, indicated by approximately 10- and 20-fold increase in phospho-IRE1α and XBP-1s, respectively (Figure 4.6B, C, D). Of note however, treatment of ASM cells with APY29 and 4μ8C which inhibit the kinase and RNase activity of IRE1α, respectively, did not reverse the inhibitory effects of thapsigargin on SPARC secretion (Figure 4.7A, B). This was not due to a lack of activity of the inhibitors. As expected, APY29 suppressed thapsigargin-induced expression and phosphorylation of IRE1α (Figure 4.8A, B), while 4μ8C suppressed thapsigargin-induced XBP-1 splicing (Figure 4.9C). Consistent with this, 4μ8C had no effect on thapsigargin-induced expression and phosphorylation of IRE1α (Figure 4.9A, B) and APY29 did not inhibit XBP-1 splicing at the lower concentration tested (Figure 4.8C). Thus, although thapsigargin is a potent inducer of IRE1α signalling, thapsigargin-mediated inhibition of SPARC secretion does not appear to be due to RIDD-mediated degradation of SPARC mRNA. This data is consistent with findings above, and further suggests that thapsigargin-mediated inhibition of SPARC secretion may not be due to its ER stress inducing action.

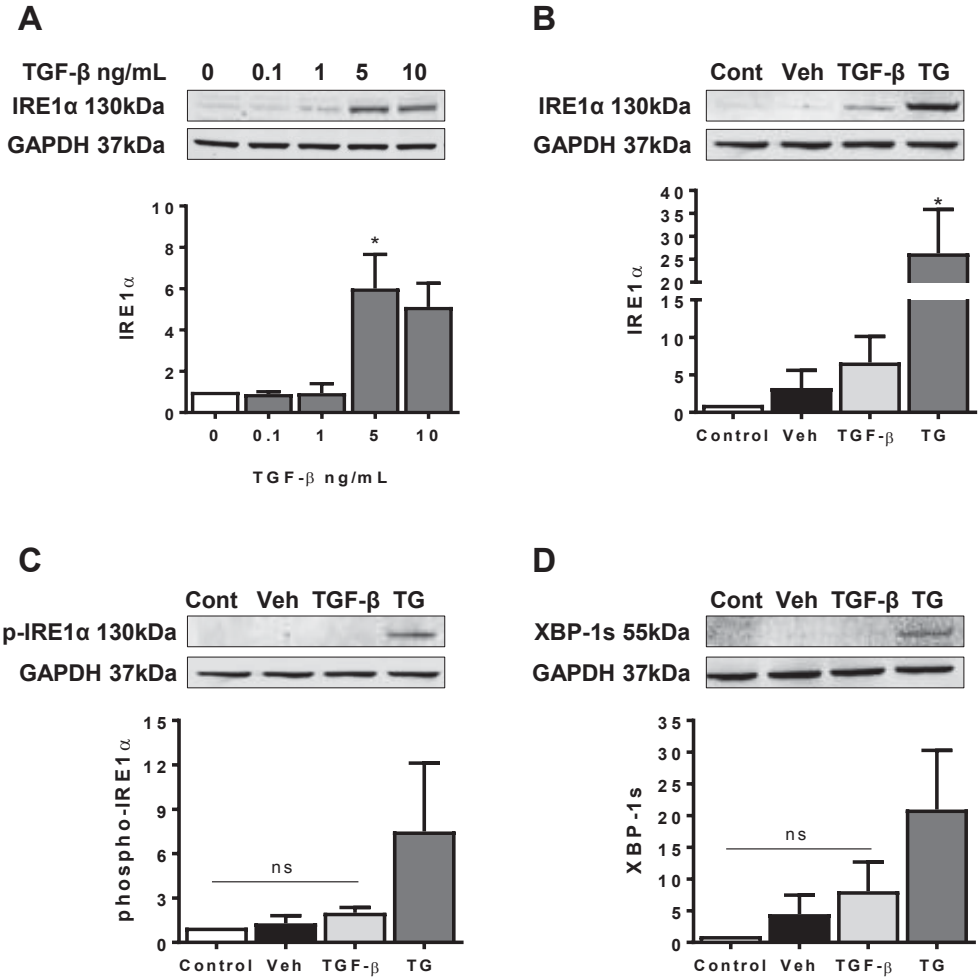

Figure 4.6: Thapsigargin, but not TGF-β activates the IRE1α/XBP-1 pathway in human airway smooth muscle cells.
ASM cells were stimulated with TGF-β for 24 h and total IRE1α in whole cell lysates were determined by immunoblotting (A). ASM cells were stimulated with TGF-β (10ng/ml) or thapsigargin (5μM) for 24 h. Total IRE1α (B), phospho-IRE1α (C), and XBP-1s (D) in whole cell lysates were determined by immunoblotting. Data were normalised to GAPDH and expressed as fold change relative to unstimulated cells. Bars represent mean data (± SEM) from 3 – 5 cell donors. One-way ANOVA with Bonferroni's correction was used to determine statistical differences. *P<0.05 compared to unstimulated cells. ns, non-significant; TG, thapsigargin; Veh, vehicle control. Vehicle control for thapsigargin is 0.025% DMSO.

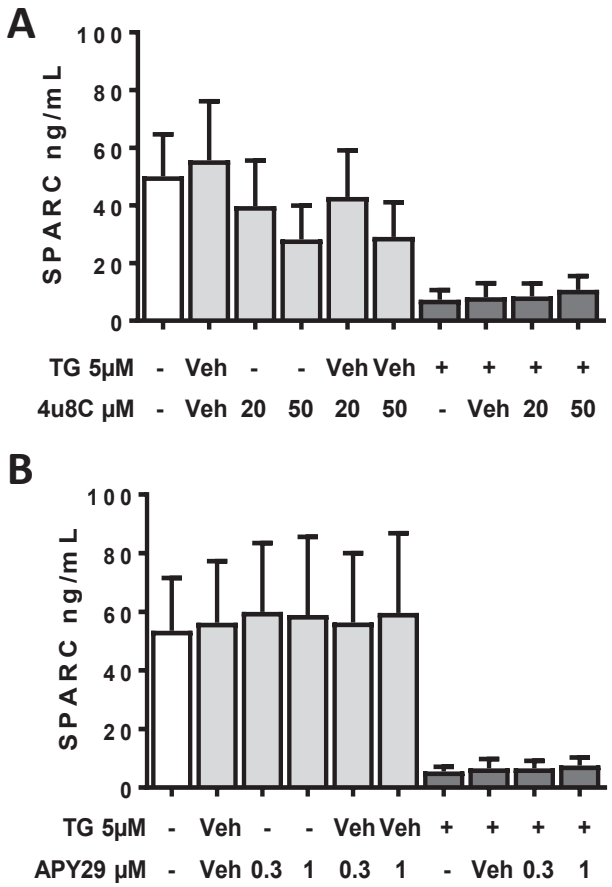

Figure 4.7: Loss of SPARC under conditions of severe ER stress is not due to IRE1α/RIDD dependent signalling.
ASM cells were stimulated with thapsigargin (5μM) for 24 h, in the presence or absence of IRE1α RNase inhibitor, 4μ8C (A) or IRE1α kinase inhibitor, APY29 (B). Soluble SPARC was measured in culture supernatants by ELISA. Bars represent mean data (± SEM) from 3 cell donors. One-way ANOVA with Bonferroni's correction was used to determine statistical differences. TG, thapsigargin; Veh, vehicle control. Vehicle controls for thapsigargin, 4μ8C and APY29 are 0.025%, 0.041% and 0.001% DMSO, respectively.

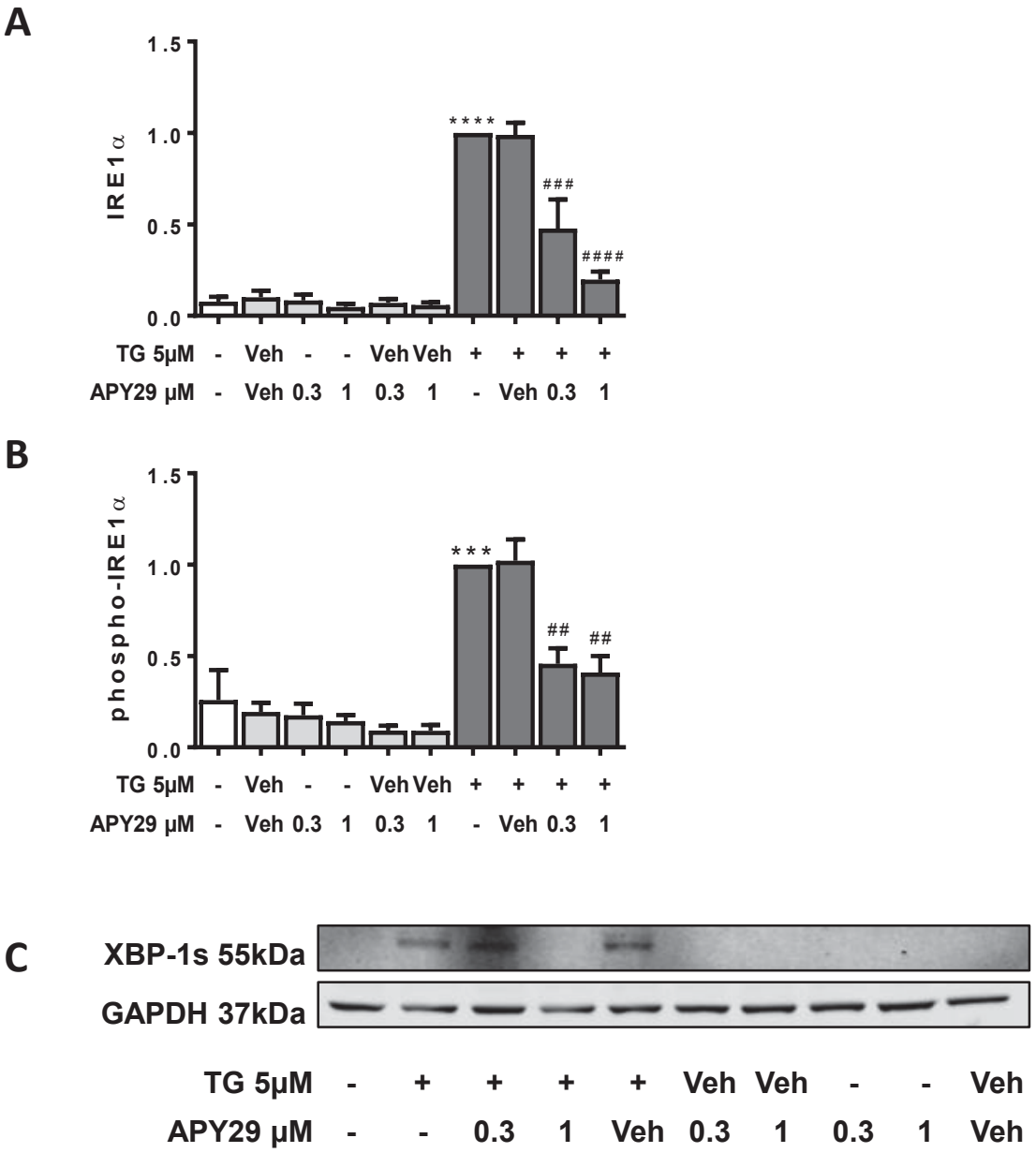

Figure 4.8: Effect of IRE1α kinase inhibitors on IRE1α/XBP-1 signalling. ASM cells were stimulated with thapsigargin (5μM) for 24 h, in the presence or absence of IRE1α kinase inhibitor, APY29, and XBP-1s, total IRE1α and phospho-IRE1α in whole cell lysates were determined by immunoblotting. Total IRE1α (A) and phospho-IRE1α (B) were normalised to GAPDH and expressed as fold change relative to thapsigargin-stimulated cells. A representative immunoblot for XBP-1s was shown in C. Bars represent mean data (± SEM) from 3 cell donors. One-way ANOVA with Bonferroni's correction was used to determine statistical differences. ***P<0.001 and ****P<0.0001 compared to unstimulated cells; ##P<0.01, ###P<0.001 and ####P<0.0001 compared to thapsigargin-stimulated cells. TG, thapsigargin; Veh, vehicle control. Vehicle controls for thapsigargin and APY29 are 0.025% and 0.001% DMSO, respectively.

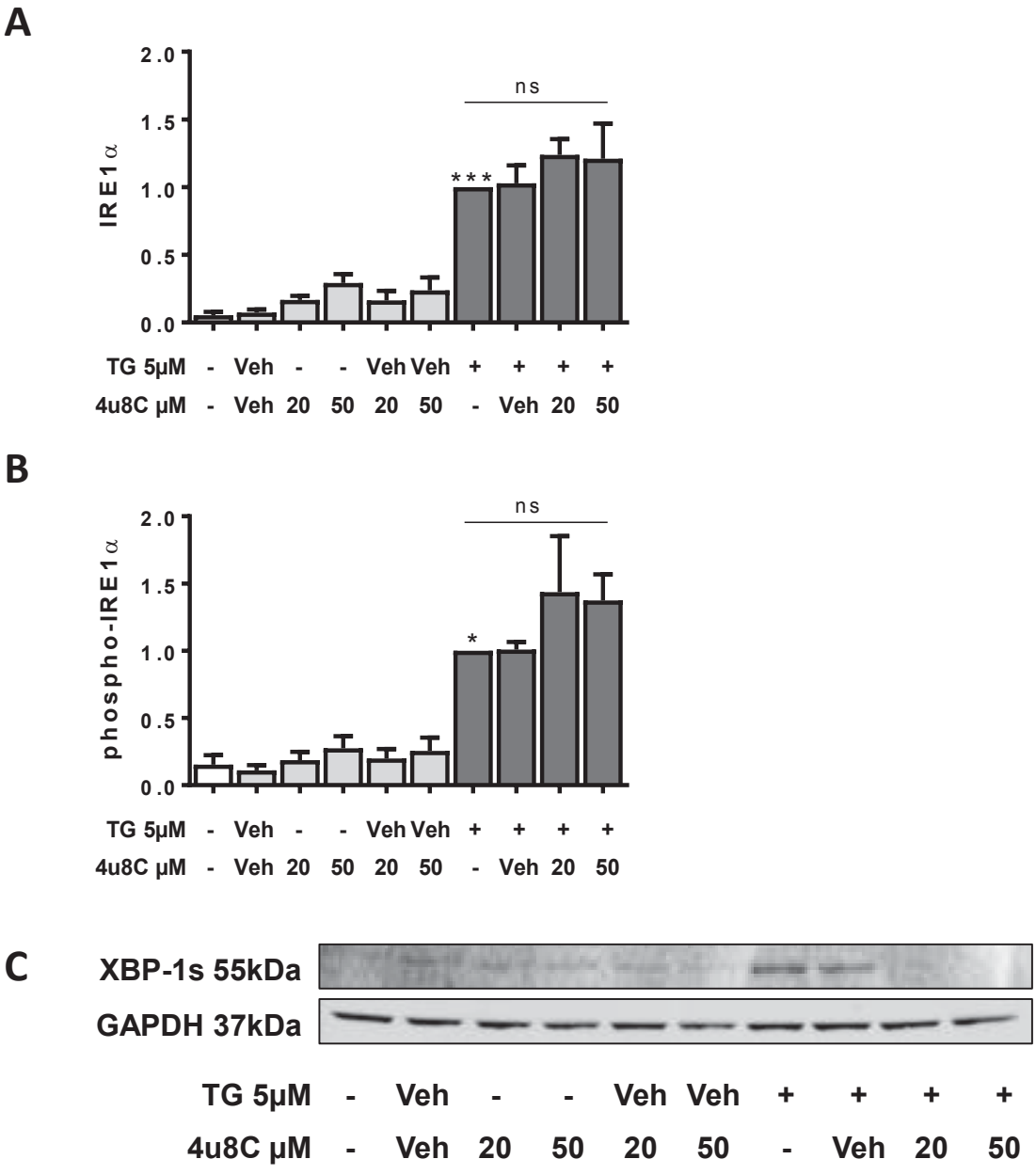

Figure 4.9: Effect of IRE1α RNase inhibitors on IRE1α/XBP-1 signalling. ASM cells were stimulated with thapsigargin (5μM) for 24 h, in the presence or absence of IRE1α RNase inhibitor, 4μ8C, and XBP-1s, total IRE1α and phospho-IRE1α in whole cell lysates were determined by immunoblotting. Total IRE1α (A) and phospho-IRE1α (B) were normalised to GAPDH and expressed as fold change relative to thapsigargin-stimulated cells. A representative immunoblot for XBP-1s was shown in C. Bars represent mean data (± SEM) from 3 cell donors. One-way ANOVA with Bonferroni's correction was used to determine statistical differences. *P<0.05 and ***P<0.001 compared to unstimulated cells. ns, non-significant; TG, thapsigargin; Veh, vehicle control. Vehicle controls for thapsigargin and 4μ8C are 0.025% and 0.041% DMSO, respectively.

SPARC secretion is reduced in ASM cells from subjects with COPD

We extended our studies to determine if SPARC secretion is altered in ASM cells from subjects with COPD. SPARC secretion in cells from subjects with COPD, both under basal conditions and following stimulation with TGF-β, were reduced when compared to cells from subjects without COPD although statistical significance was not achieved (Figure 4.10).

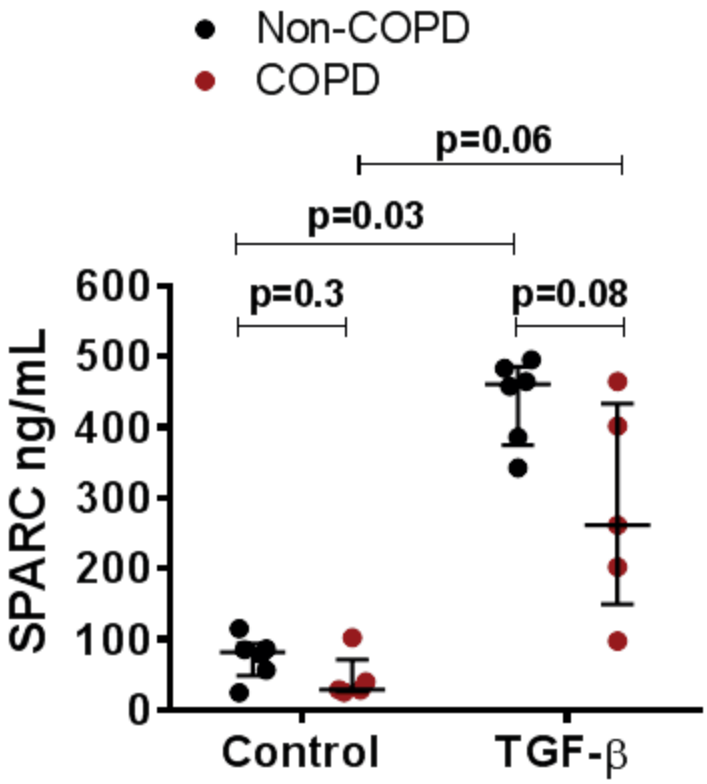

Figure 4.10: TGF-β-induced SPARC expression is attenuated in COPD airway smooth muscle cells.
COPD and non-COPD ASM cells were stimulated with TGF-β for 72 h, and soluble SPARC was measured in culture supernatants by ELISA. Data from 5 – 6 cell donors are presented with median ± interquartile range. The Wilcoxon paired t-test was used to determine statistical differences between control and TGF-β-treated cells within each disease group. The Mann-Whitney test was used for comparison between disease groups.

DISCUSSION

This is the first study to demonstrate that TGF-β and the UPR regulate SPARC expression and secretion in human ASM cells, and that ASM cells from people with COPD potentially have a reduced capacity to secrete SPARC, although the latter requires further validation. We showed that TGF-β increases the expression of UPR proteins, IRE1α and GRP78 and that chemical chaperones which attenuate ER stress and UPR signalling also suppress TGF-β-induced SPARC expression and secretion. Paradoxically, thapsigargin, a well-established inducer of ER stress attenuated, rather than enhanced SPARC secretion in ASM cells. However, this response was not amenable to reversal by chemical chaperones, suggesting it may not be an ER stress-dependent effect. Together, our findings provide the first evidence for a role for UPR signalling in ASM secretory responses, and suggest that altered SPARC expression may be a factor in COPD pathogenesis.

Our study is the first to evaluate the expression of cell-associated SPARC and its secretion in parallel, in any cell type. Consistent with studies in other structural cell types (29, 30, 38), we have shown that TGF-β stimulates SPARC protein synthesis in ASM cells and promotes its release into the extracellular space. TGF-β-induced SPARC expression is mediated through activation of TGF-βRI/II receptors, and depending on the cell type, can involve the activation of Smad-dependent and Smad-independent pathways such as PI3K and p38 MAPK (29, 30). We have previously demonstrated that TGF-β induces activation of the oxidant enzyme NADPH oxidase 4 (Nox4) and an overall increase in intracellular reactive oxygen species (ROS) in ASM cells (39).

Increased oxidant burden is often associated with ER stress and subsequent activation of the UPR (40). Of note, Baek and colleagues demonstrated concomitant production of intracellular ROS and induction of ER stress in TGF-β stimulated lung fibroblasts, and they further showed that TGF-β-induced expression of GRP78, a well-established marker of ER stress, is reversed by anti-oxidants (17). Our studies suggest that TGF-β induced SPARC secretion is mediated by the UPR as this response was reversed by chemical chaperones that alleviate ER stress. Thus, it is possible that TGF-β induced SPARC expression occurs down-stream of Nox4/ROS/UPR signalling in ASM cells, although this needs to be formally proven.

Shibata and colleagues demonstrated that specific siRNA knockdown of SPARC in human lung fibroblasts attenuates TGF-β-induced Nox4 expression and ROS production, suggesting that SPARC mediates the pro-oxidant effects of TGF-β (29). Interestingly, the presence of an autocrine feedback loop between TGF-β and SPARC has also been suggested, as SPARC binds type II TGF-β receptors and has been shown to augment the activation of TGF-β signalling (19). Thus, feedforward signalling by TGF-β and SPARC may potentially drive an amplification loop that adversely affects ASM function by causing an overall increase in cellular ROS and ER stress. Certainly, this is an interesting hypothesis that warrants future investigation.

The ER is a complex and dynamic organelle responsible for the biosynthesis, folding and maturation of secretory proteins. Changes in calcium homeostasis, redox imbalances, altered protein glycosylation or protein folding all perturb ER

function. When ER function is perturbed, unfolded or misfolded proteins accumulate in the ER lumen and this triggers ER stress. The UPR is one of several adaptive pathways by which cells respond to and manage ER stress (41). It is mediated by three transmembrane ER sensors, namely IRE1α, protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6). Although these sensors activate distinct transcriptional programmes, they act in a co-ordinated manner to re-establish cellular proteostasis by reducing the quantity of proteins that enter the ER, eliminating unfolded proteins from the ER, and enhancing the protein-folding capacity of the ER (41).

TGF-β has been shown to induce ER stress and activation of the UPR in other cell types (17, 23, 42-45). However, the relationship between TGF-β and the UPR in ASM cells has not previously been studied. As mentioned above, IRE1α is an ER transmembrane protein with kinase and RNase activities associated with its cytoplasmic tail. In response to luminal activation, activation of the IRE1α RNase catalyses the excision of a 26-nucleotide intron from XBP-1 mRNA and this generates the XBP-1s which has potent transcriptional activity (41). TGF-β has previously been shown to induce IRE1α activation and XBP-1 splicing in epithelial cells and fibroblasts (17, 23, 44). Interestingly however, although TGF-β increased IRE1α expression in ASM cells, it did not induce phosphorylation of IRE1α and did not consistently induce XBP-1 splicing, as we observed evidence of this in only 2 of 5 ASM donors examined. Thus, although TGF-β augments IRE1α expression, our findings suggest that it does not activate canonical IRE1α–XBP-1 signalling. The lack of effect of TGF-β on

IRE1α activation in our study is unlikely to be related to methodological issues, as we detected clear expression of IRE1α, phospho-IRE1α, and XBP-1s in cells that were treated with the ER stress inducer thapsigargin.

A recent study by Kim and colleagues showed that ER stress-induced activation of IRE1α in macrophages leads to the activation of glycogen-synthase kinase-3 beta (GSK)-3β, which in turn induces secretion of the cytokine IL-1β. They also showed that IRE1α-dependent activation of GSK-3β inhibits XBP-1 signalling. Thus, their studies suggest that IRE1α-GSK-3β signalling promotes cytokine release independent of XBP-1 activation (46). It has previously been shown that pharmacological inhibition or siRNA knockdown of GSK-3β attenuates TGF-β-induced pro-fibrotic responses in lung fibroblasts, indicating that TGF-β activates GSK-3β signalling in lung mesenchymal cells (47). Thus, TGF-β induced SPARC expression in ASM cells may be mediated by a mechanism that involves ER stress-dependent activation of GSK-3β and concomitant inhibition of IRE1α RNase activity. Due to time constraint, we did not investigate whether TGF-β-induced SPARC expression is reversed by pharmacological inhibitors or gene knockdown of ER stress sensors including IRE1α. These studies are essential to identify the specific UPR pathways by which TGF-β and the UPR regulate SPARC expression in ASM cells.

The ER membrane contains SERCA pumps that maintain a high Ca^{2+} concentration within the ER lumen. Thapsigargin inhibits SERCA pumps and depletes ER Ca^{2+} stores, hence it compromises the protein folding functions of

Ca^{2+}-dependent ER chaperones and induces ER stress (48). Given that TGF-β induced SPARC secretion was reversed in the presence of chemical chaperones, we expected that thapsigargin would induce SPARC secretion, and thus were surprised to find that it had an inhibitory effect on basal SPARC secretion. It is unlikely that reduced SPARC secretion was due to ASM cell death, as thapsigargin treated cells appeared morphologically normal under brightfield microscopy. However, cell death needs to be definitively excluded using quantitative methods. Of note, Graham and colleagues showed that thapsigargin inhibits collagen I expression in mouse embryonic fibroblasts (MEFs), and that this effect was related to a decrease in ER Ca^{2+} stores (49). In further studies, they showed that collagen I expression is regulated by calreticulin (CRT), one of the main Ca^{2+}buffers in the ER (49). SPARC is a collagen chaperone and is responsible for the processing and deposition of collagen I into the ECM (50, 51), thus loss of ER Ca^{2+} may possibly explain reduced SPARC secretion in thapsigargin-treated cells.

Interestingly, TGF-β enhances CRT expression in various cell types, and CRT has been shown to mediate pro-fibrotic effects of TGF-β, such as collagen I expression (52, 53). Thus, although TGF-β induces ER stress, this effect is unlikely related to loss of ER Ca^{2+}, as TGF-β expands ER Ca^{2+} storage as a consequence of its effect on CRT expression. Hence, the differential impact on ER Ca^{2+} may potentially explain the differential effects of TGF-β and thapsigargin on SPARC secretion in ASM cells. It may also explain why the chemical chaperone 4-PBA did not reverse thapsigargin-mediated inhibition of SPARC secretion, as 4-PBA attenuates ER stress by stabilizing and assisting

with protein folding, but has no documented role in the regulation of ER Ca^{2+} stores.

SPARC release appeared to be reduced in COPD ASM cells when compared to non-COPD ASM cells, under basal conditions and in response to TGF-β stimulation, although validation in more cell donors is required. This observation was unanticipated because ASM cells from COPD subjects exhibit enhanced Smad2/3 activation in the presence of TGF-β, compared to those from non-COPD subjects (54, 55). This suggests SPARC expression in ASM cells may be regulated by non-canonical TGF-β signaling or that the diseased cells are intrinsically defective in SPARC production and release, although this remains to be formally tested. Notably, COPD is a condition of accelerated ageing and SPARC expression has been shown to largely decline with age (56). This is evident in dermal fibroblasts, retinal pigment epithelial cells and intervertebral disc obtained from aged human donors, although SPARC is found to be increased in the myocardium of aged mice, the discrepancy of which is likely due to the distinct cardiac ageing process which involves enhanced collagen deposition and cardiac fibrosis (8, 57-59).

The reason(s) for the loss of SPARC with age have not been scrutinized. It could be linked to cellular senescence, a hallmark feature of ageing where cells become growth arrested and lose the ability to replicate due to irreconciliable DNA damage (56). Senescent cells are however, metabolically active and display the senescence-associated secretory phenotype (SASP) which culminates in enhanced secretion of cytokines, chemokines and endogenous

proteases (56). The enhanced protease secretion may be responsible for the trend of diminished SPARC release in ASM cells derived from COPD subjects given SPARC has been shown to be susceptible to proteolytic degradation by MMP-3 and cathepsin K (60, 61). In support of this, Chen and colleagues have previously demonstrated COPD ASM cells release more MMP-1, MMP-3 and MMP-10 than non-COPD ASM cells (4). The trend of diminished SPARC release in COPD ASM cells in our studies are intriguing and warrant further studies to investigate the presence of endogenous SPARC fragments in the extracellular milieu of COPD ASM cells. Determination of the corresponding cell-associated SPARC expression in ASM cells from COPD subjects will shed light on whether total SPARC protein synthesis is dysregulated in COPD or whether this is limited to SPARC release.

In conclusion, our studies show that TGF-β induces the UPR in ASM cells, and stimulates SPARC production and release in consequent. We provide the first evidence that COPD ASM cells may have impaired capacity to secrete SPARC when compared to non-COPD ASM cells although further interrogation is necessary. The question of whether the trend of suppressed SPARC expression in COPD ASM cells is associated with ageing and how this deficiency impacts on the ECM dynamics and contributes to COPD pathogenesis need further investigation.

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Chapter 5

Proteomic analysis of extracellular HMGB1 identifies binding partners and exposes its potential role in airway epithelial cell homeostasis

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ABSTRACT

The release of damage-associated molecular patterns (DAMPs) by airway epithelial cells (AECs) is believed to play a crucial role in the initiation and development of chronic airway conditions such as asthma and chronic obstructive pulmonary disease (COPD). Intriguingly, we have observed significant levels of the classic DAMP high-mobility group box-1 (HMGB1) in the culture supernatant of AECs under basal conditions indicating a role for HMGB1 in the regulation of epithelial cellular and immune homeostasis. In order to gain contextual insight into the potential role of HMGB1 in airway epithelial cell homeostasis, we used the orthogonal and complimentary methods of high resolution clear native electrophoresis, immunoprecipitation and pull-downs, coupled to liquid chromatography-tandem mass spectrometry (LC-MS/MS) to profile HMGB1 and its binding partners in the culture supernatant of unstimulated AECs. We found that HMGB1 presents exclusively as a protein complex under basal conditions. Moreover, protein network analysis performed on 180 binding proteins revealed 14 that directly associate with HMGB1: amyloid precursor protein, F-actin-capping protein subunit alpha-1 (CAPZA1), glyceraldehyde-3 phosphate dehydrogenase (GAPDH), ubiquitin, several members of the heat shock protein family (HSPA8, HSP90B1, HSP90AA1), XRCC5 and XRCC6, histone 3 (H3F3B), the FACT (facilitates chromatin transcription) complex constituents SUPT1H and SSRP1 and heterogeneous ribonucleoprotein K (HNRNPK). These studies provide a new understanding of the extracellular functions of HMGB1 in cellular and immune homeostasis at the airway mucosal surface and could have implications for therapeutic targeting.

INTRODUCTION

The airway epithelium plays a fundamental role in protecting the lung against the external environment (1, 2). By forming a physical barrier, AECs prevent microbial organisms and other noxious substances from traversing the airway lumen. They also express innate immune receptors which allow them to detect inhaled allergens and pathogens. Ligation of innate immune receptors or physical injury to the epithelial barrier caused by allergens, pathogens and pollutants induce the release of DAMPs or 'alarmins' which then act on local immune cells to elicit the activation of down-stream innate and adaptive immune responses. It is becoming increasingly evident that DAMPs are key upstream mediators of the airway inflammatory response in chronic airway diseases such as asthma and chronic obstructive pulmonary disease (COPD) and are emerging as important therapeutic targets (1-3).

HMGB1 is a nuclear protein that functions as a classical DAMP. Under conditions of tissue injury, infection or inflammation, HMGB1 translocates from the nucleus to the cytoplasm, and is then released into the extracellular space where it regulates immune and inflammatory responses (4-9). When exposed to allergens, viral dsRNA or particulate matter such as silica, AECs mobilize and/or release HMGB1 (10-12). We have identified a crucial up-stream role for HMGB1 in the initiation and progression of the airway inflammatory response in mouse models of allergic asthma (10). Moreover, several studies have reported increased levels of HMGB1 in the sputum and bronchoalveolar lavage fluid of people with asthma and COPD, and within the epithelial layer in airway biopsies from people with COPD (13-15). Interestingly, we have also observed

significant levels of HMGB1 in the culture supernatant of human AECs under basal conditions, raising the intriguing possibility that extracellular HMGB1 is not only released under conditions of tissue damage and inflammation, but is involved in epithelial cell turnover and homeostasis. It is therefore important to gain a better understanding of the extracellular functions of HMGB1, as this will have implications for therapeutic targeting of this protein in disease.

The biological function of HMGB1 in any cellular or tissue compartment is regulated by several factors, including post-translational modifications, its redox state and its interaction with partner molecules (4-9). Using a proteomic approach, Lee and colleagues identified numerous HMGB1 binding partners within the nuclear and cytoplasmic compartments in colon cancer cells (16). While several investigators have identified a number of HMGB1 binding partners in the extracellular space (4-9), there has been no global analysis of HMGB1 interacting proteins in the extracellular space in any cell type to date. Thus, as a first step in determining its potential function in epithelial cell homeostasis, we used high-throughput mass-spectrometry based techniques to build a robust and unbiased profile of HMGB1 binding proteins in the extracellular milieu of unstimulated human AECs. To do this, we profiled HMGB1-binding proteins in culture supernatant using the orthogonal and complimentary methods of high resolution clear native electrophoresis (hrCNE), immunoprecipitation and pull-downs, coupled to liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Figure 5.1). Finally, we performed protein network analysis on the identified HMGB1-binding proteins to provide contextual insight into the role of extracellular HMGB1 in airway epithelial cell

homeostasis. We have identified 14 proteins that directly associate with HMGB1 and thus provide a new understanding of the extracellular functions of HMGB1 in epithelial cell homeostasis at the airway mucosal surface and a new starting point for novel therapeutic targeting.

MATERIALS AND METHODS

Generation of airway epithelial cell culture supernatants

The human airway epithelial cell line 16-HBE14o- was obtained from Professor D.C. Gruenert (University of California, San Francisco). Cells were cultured in 10% fetal bovine serum (FBS) in Minimal Essential Medium (MEM) supplemented with 2mM L-glutamine, 1mM sodium pyruvate, 1% MEM non-essential amino acids, 100U/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml amphotericin B. For experiments, cells were seeded at 1.75 x 10^4 cells per cm^2 in T175 flasks and grown to 90% confluence. Once cells were confluent, the growth media was removed and cells were incubated in a commercially available serum free media, Opti-MEM™ Reduced Serum Medium supplemented with GlutaMAX™ for 24 hours. The media was then replenished and cells were incubated for a further 24 hours. Following this, culture supernatants were collected and stored at -20°C for analysis. All cell culture reagents were purchased from Life Technologies, Australia.

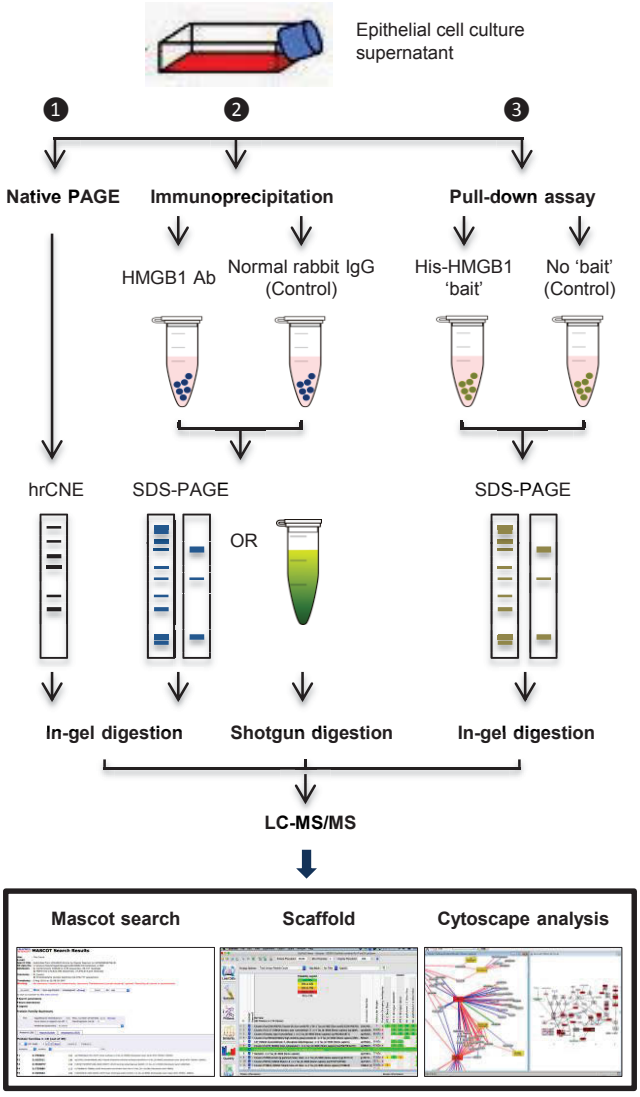

Figure 5.1: Experimental approach used to identify HMGB1-binding proteins in airway epithelial cell culture supernatants.
AECs were grown to confluence and cell culture supernatants were collected for analysis by high resolution clear native electrophoresis (hrCNE), immunoprecipitation or pull-down assay. Outputs were subjected to LC-MS/MS to identify HMGB1-binding proteins. Data was searched using the Mascot server and validated with Scaffold software. Protein network analysis was performed using Cytoscape software.

Separation of protein complexes using 1D hrCNE and 2D hrCNE/SDS-PAGE

Cell culture supernatants were concentrated 20 fold using Vivaspin 3 kDa molecular weight cut-off (MWCO) centrifugal concentrators (Sartorius,

Germany). Protein concentrations were determined using the Pierce BCA Protein Assay Kit according to the manufacturer's instructions (Thermo Fisher Scientific, Australia). For 1D high resolution clear native electrophoresis (hrCNE), 70 – 100μg of protein was separated using NativePAGE™ Novex™ 4 – 16% Bis-Tris gels at 4°C. Electrophoresis was performed using NativePAGE™ Running Buffer at 50V for 30 minutes, followed by 100V for 1 hour, and then 150V until separation was complete. NativePAGE™ Running Buffer added to the cathode compartment was supplemented with 0.05% sodium deoxycholate. For 2D SDS-PAGE, 1D hrCNE gel lanes were excised and equilibrated in SDS equilibration solution, consisting of 2% SDS, 6M urea, 250mM Tris-HCl pH 8.5, and 0.0025% w/v bromophenol blue. After 30 minutes, 1D hrCNE gel lanes were loaded horizontally and separated on Criterion™ XT 4 – 12% Bis-Tris gels (Bio-Rad, Hercules, CA). Separate 1D hrCNE gel lanes, and 2D hrCNE/SDS-PAGE gels were fixed with 40% methanol/10% acetic acid and stained with colloidal Coomassie blue overnight for visualization, for subsequent trypsin digestion and MS analysis, or were subjected to immunoblotting, as described below. All pre-cast gels, buffers and molecular weight markers were purchased from Invitrogen (Thermo Fisher Scientific, Australia) unless otherwise stated.

Detection of HMGB1 using immunoblotting

Proteins were transferred onto PVDF membranes using an iBlot2® Dry Blotting System (Life Technologies, Australia). For 1D hrCNE gels, protein transfer was performed at 20V for 6 minutes, while for 2D hrCNE/SDS-PAGE gels, protein transfer was performed at 20V for 1 min, 23V for 4 min and 25V for 2 min.

PVDF membranes were then incubated with 5% non-fat milk powder in Tris-buffered Saline containing 0.1% Tween (TBST) for 1 hour at room temperature. Following this, PVDF membranes were incubated with anti-HMGB1 antibody (#ab18256; Abcam, Australia) diluted 1:1000 in 5% non-fat milk powder in 0.1% TBST at 4°C for 16 hours. PVDF membranes were then washed with 0.1% TBST three times (5 min each) and then incubated with HRP-linked anti-rabbit IgG (#7074S; Cell Signalling Technology, Danvers, MA) diluted 1:1000 in 5% non-fat milk powder in 0.1% TBST for 1 hour at room temperature. PVDF membranes were then washed with 0.1% TBST three times and protein bands were visualised using enhanced chemiluminescence (ECL) (GE Healthcare, UK).

Isolation of HMGB1-binding proteins using immunoprecipitation

Cell culture supernatants were concentrated using Vivaspin 3kDa MWCO centrifugal concentrators, and protein concentrations were determined as described above. 1.5mg of protein was then incubated with 10µg rabbit anti-HMGB1 antibody (#ab18256; Abcam, Australia) or 10µg normal rabbit IgG (#12-370; Merck Millipore, Australia) at 4°C overnight with rotation. Following this, protein samples were incubated with 1.5mg Dynabeads Protein G (Life Technologies, Australia) for 4 hours at 4°C and then washed five times in 1ml Phosphate Buffered Saline containing 0.05% Tween (PBST). To elute binding proteins, Dynabeads were incubated sequentially with 0.5M NaCl in PBS, 1.5M NaCl in PBS, and 0.1M glycine (pH 2.5) and then heated to 70°C for 10 minutes in NuPAGE® LDS Sample Buffer. Eluents collected from each step of the IP were separated using SDS-PAGE and HMGB1 was identified by

immunoblotting. To identify binding proteins using LC-MS/MS analysis, all elutions were separated using SDS-PAGE and visible protein bands were subjected to in-gel trypsin digestion in biological replicate 1. For biological replicate 2, shotgun digestions were performed for all elutions (except for LDS Sample Buffer) and peptides were desalted using Oasis® HLB solid-phase extraction (SPE) 1ml cartridges (Waters, Australia) as per the manufacturer's instructions.

Isolation of HMGB1-binding proteins using pull-down assay

HMGB1 pull-down assays were performed using a His-tagged recombinant human HMGB1, with truncated acidic C-tail (His-tagged rHMGB1) as the 'bait' while control pull-downs were performed in the absence of 'bait'. The gene encoding HMGB1 (1-185, Figure 5.2) was optimized for expression in *E. coli*, synthesized to include a His-Tag at the C-terminal and cloned into the pZL18-1b plasmid vector (GenScript, Piscataway, NJ). Transformed bacteria cells (*E. coli* strain C41(DE3)) were grown in terrific broth (TB) media to an OD600 greater than 1.0 and expression of the recombinant protein was induced by the addition of 1mM IPTG. Cell pellets were collected by centrifugation at 4000 *x g* for 20 min at 4°C and then resuspended in lysis buffer containing 300mM KCl, 50mM KH_2PO_4 and 5mM imidazole. Cleared lysate was obtained by centrifugation at 10000 *x g* for 30 min at 4°C. Soluble recombinant His-tagged HMGB1 protein was purified by affinity chromatography on nickel-nitrilotriacetic acid (Ni-NTA) agarose. Residual bacterial endotoxin was removed by phase separation with Triton X-114. Final endotoxin levels were measured using the Chromo-LAL assay kit (Associates of Cape Cod, East Falmouth, MA). To

perform the pull-down assays, 0.5ml Ni-charged resin (Bio-Rad, Hercules, CA) was incubated without (control pull-down) or with 0.5mg of His-tagged rHMGB1 at 4°C for 16 hours. Following this, cell culture supernatants containing 5mg total protein were added to the resin/HMGB1 protein mixture and allowed to incubate at 4°C for a further 16 hours. The mixture was then packed into a column and washed four times with 5ml 0.05% PBST. To elute binding proteins, 0.25M NaCl in PBS was added to the column. Elutions were separated using SDS-PAGE and in-gel trypsin digestion was performed for LC-MS/MS analysis.

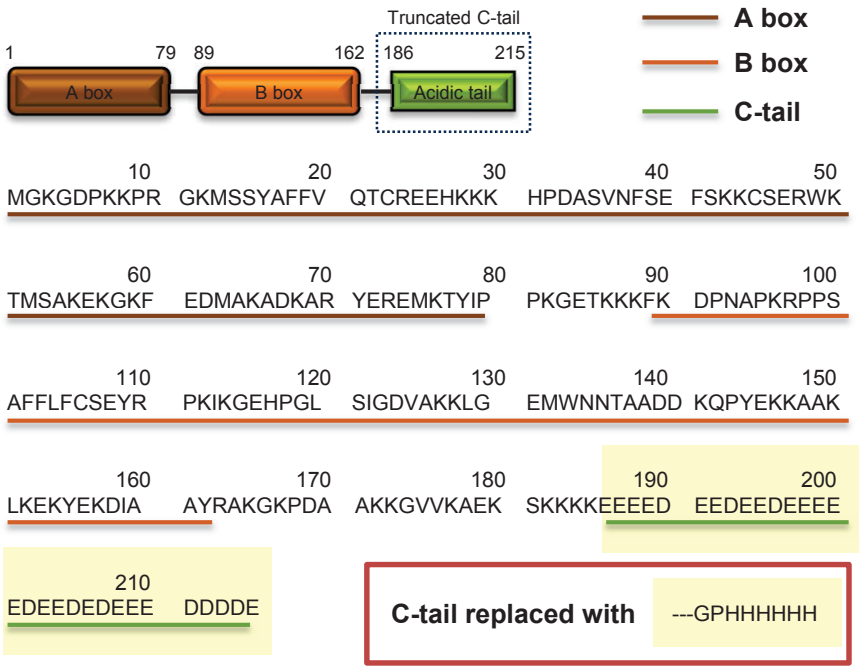

Figure 5.2: Structure and protein sequence of His-tagged recombinant human HMGB1.
The human HMGB1 protein consists of 215 amino acids. It contains two DNA binding domains: HMG A box (1-79) and HMG B Box (89-162), and a C-terminal acidic tail (186-215). A His-tagged recombinant human HMGB1 with truncated acidic C-tail (sequence highlighted yellow) was used as 'bait' in HMGB1 pull-down assay.

Identification of HMGB1-binding proteins using LC-MS/MS

Nanoflow liquid chromatography separation of peptides were performed using Tempo nanoLC system, followed by mass spectrometry analysis using a QSTAR Elite Quadrupole-Time-Of-Flight mass spectrometer as described previously (17). MS/MS data files were searched using Mascot software (hosted by the Walter and Eliza Hall Institute for Medical Research Systems Biology Mascot Server) against the LudwigNR database, composed of the UniProt, plasmoDB and Ensembl databases (vQ312. 19 375 804 sequences; 6 797 271 065 residues) with the following parameter settings: taxonomy: *Homo sapiens;* fixed modifications: none; variable modifications: propionamide, oxidized methionine; enzyme: semitrypsin; number of allowed missed cleavages: 3; peptide mass tolerance: 100 ppm; MS/MS mass tolerance: 0.2 Da; charge state: 2+ and 3+. Scaffold (v4.5.1, Proteome Software, Portland, OR) was used to validate and compare MS/MS-based peptide and protein identifications. An additional search was performed using X! Tandem to match spectra not assigned by Mascot. Peptide identifications made by Mascot and X! Tandem were accepted if their calculated probability assigned by the Protein Prophet algorithm was >95.0% and protein identifications were accepted if their calculated probability was >80.0%. All semitryptic and cleavage-defining peptide identifications were manually inspected for quality of spectra. For protein entries where the unique peptide(s) identified belong to more than 1 specific protein, all proteins identified are listed.

Protein-protein interaction network and Gene Ontology term enrichment analysis

Proteins identified by at least 2 peptides with confidence intervals of greater than 95% were analysed using Cytoscape (v3.4.0 Java version 1.8.0_91) (18), which directly connects to external public databases for annotated data such as Pathway Commons, IntAct, BioMart and NCBI Entrez Gene. Utilising this platform, the protein interaction network of HMGB1 was assembled to generate capture technique-specific networks. Gene Ontology (GO) term enrichment analysis was performed on proteins identified in at least 2 of the 3 techniques performed using BinGo (v3.0.3) (19) and ClueGO (v2.2.5) (20) to provide insight into the represented biological processes, molecular functions and cellular components for the dataset. These proteins were also analysed against the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway repositories utilizing CytoKegg (v1.0.1) for the presence of functionally enriched pathways.

RESULTS

Extracellular HMGB1 exists in a multimeric state in unstimulated human airway epithelial cell cultures

We performed hrCNE to examine the native state of HMGB1 in epithelial cell culture supernatants under basal conditions. Interestingly, HMGB1 was present exclusively as a multimer, as no monomeric HMGB1 was detected at 30kDa in this experiment. Rather, we detected complexes of 242 – 480 kDa (Figure 5.3A), indicating that extracellular HMGB1 either self-associates (21) or forms complexes with other proteins. Our findings suggest that extracellular HMGB1 is a constituent protein of at least 2 multimeric complexes, as the HMGB1 specific

antibody identified two distinct complexes within the 242 – 480 kDa region, annotated IB1 and IB2 in Figure 5.3A. A corresponding gel visualized with coomassie blue revealed multiple proteins of between 242 and 480kDa; these are annotated CB1, CB2, CB3 and CB4 in Figure 5.3B. Proteins CB2 to CB4 likely reflect HMGB1 complexes at the lower molecular weight range (i.e. band IB2, Figure 5.3A), although the presence of co-migrating protein complexes within the same molecular weight cannot be excluded. It is likely that HMGB1 complex(es) detected at the higher molecular weight range (i.e. IB1, Figure 5.3A) were not highly expressed as a corresponding pattern could not be detected using coomassie blue (region designated CB0, Figure 5.3B).

To confirm these observations 2D hrCNE/SDS-PAGE was also performed. This allows all protein constituents of a single complex to be separated vertically in a single profile according to their monomeric molecular weight. Immunoblotting using anti-HMGB1 antibody identified a protein with the molecular weight of 30kDa (Figure 5.3C). The location of this protein aligned with the 242-480 kDa region of the 1D gel confirming that extracellular HMGB1 exists as a complex(s) in the culture supernatant of unstimulated AECs. Consistent with findings above, no proteins of 30kDa were visualised using coomassie blue staining (arrow, Figure 5.3D), thus we did not proceed with LC-MS/MS analysis to identify HMGB1 binding partners. Instead, we performed in-gel digestion and LC-MS/MS analysis on the annotated region CB0 to CB4. Given the low abundance of HMGB1 and the presence of 43 lysines (cleavage site of trypsin) within its length of 215 amino acids, it is not surprising that we did not detect HMGB1 in the LC-MS/MS analysis, as the short peptide fragments generated

are likely to either fall below the lower limit of MS scan range (350-1500 Da) and thus not selected for MS/MS, or be scored against by the search algorithms. Nevertheless, we identified 29 potential HMGB1-binding proteins that were detected with 2 or more peptides in at least 1 biological replicate, or at least 1 peptide in each biological replicate as listed in Appendix I. Of these, 16 proteins were reproducibly detected in both replicates.

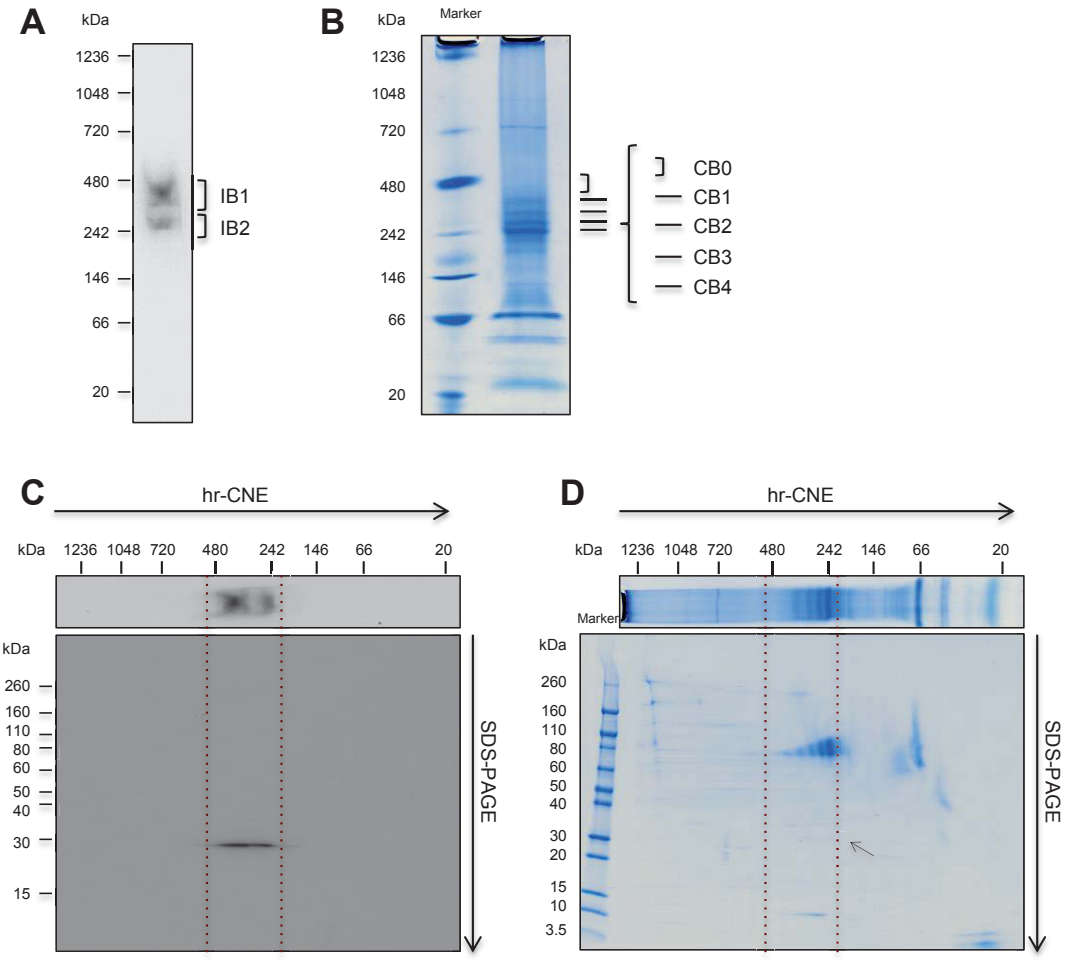

Figure 5.3: Analysis of extracellular HMGB1 using high resolution clear native electrophoresis.
To examine whether extracellular HMGB1 exists as a complex(es), concentrated airway epithelial cell culture supernatants were separated using 1D hrCNE and HMGB1 protein expression was determined by immunoblotting (A). To identify constituent protein(s), corresponding hrCNE gels were stained with coomassie blue. The region corresponding to HMGB1 protein bands IB1 and IB2 in (A), and annotated CB0 to CB4 in (B), was subjected to trypsin digestion and LC-MS/MS analysis. Identified proteins are listed in Appendix I. To confirm HMGB1 was a constituent protein of the complex(es), concentrated airway epithelial cell culture supernatants were subjected to denaturing 2D hrCNE/SDS-PAGE. Although a 30 kDa HMGB1 band was detected by immunoblotting in the corresponding molecular weight region (C), it could not be visualized using coomassie blue staining (arrow, D). The images are representative of two biological replicates.

Enrichment of extracellular HMGB1 and identification of its binding proteins using immunoprecipitation

While we could clearly establish the presence of HMGB1 complexes in our studies above, the apparently low level of HMGB1 expression in culture supernatants presented a challenge for the identification of HMGB1 and its potential binding partners using unfractionated and non-enriched samples in LC-MS/MS analysis. Thus to overcome this issue, we performed immunoprecipitation (IP) to enrich HMGB1 and its binding proteins in the sample. To validate our method, we examined HMGB1 protein expression in samples that were incubated with an anti-HMGB1 antibody or a control IgG antibody at each step of the IP procedure. Comparison of HMGB1 expression in culture supernatants before (S1) and after incubation with the anti-HMGB1 antibody but not the IgG control antibody (S2) was associated with a marked reduction in HMGB1 abundance, indicating that HMGB1 was specifically bound to the HMGB1 antibody (Figure 5.4A). We also detected HMGB1 in samples that were incubated with anti-HMGB1, but not the control antibody, following sequential elutions with high salt (0.5M NaCl in PBS; E1, E2), low pH (0.1M glycine pH 2.5; E5) and a denaturing buffer (NuPAGE® LDS Sample Buffer; E7). Of note, incubation with 0.1M glycine and LDS Sample Buffer led to elution of both HMGB1 and IgG antibodies from respective samples (Figure 5.4A). As visualized by coomassie blue staining, eluents from samples that were incubated with the anti-HMGB1, but not the IgG control antibody, contained co-immunoprecipitated proteins. The majority of co-immunoprecipitated proteins were eluted following the first elution step with 0.5M NaCl in PBS (E1, Figure 5.4B), while subsequent elutions with 0.1M glycine and NuPAGE® LDS Sample

Buffer did not yield many residual proteins. The presence of a 30kDa HMGB1 band in multiple elutions (arrows, Figure 5.4B), at the same apparent molecular weight as the recombinant HMGB1 (R&D, #1690-HMB), were visible by coomassie blue staining, indicating that IP is a specific and robust technique for enriching HMGB1 and its binding partners in culture supernatants.

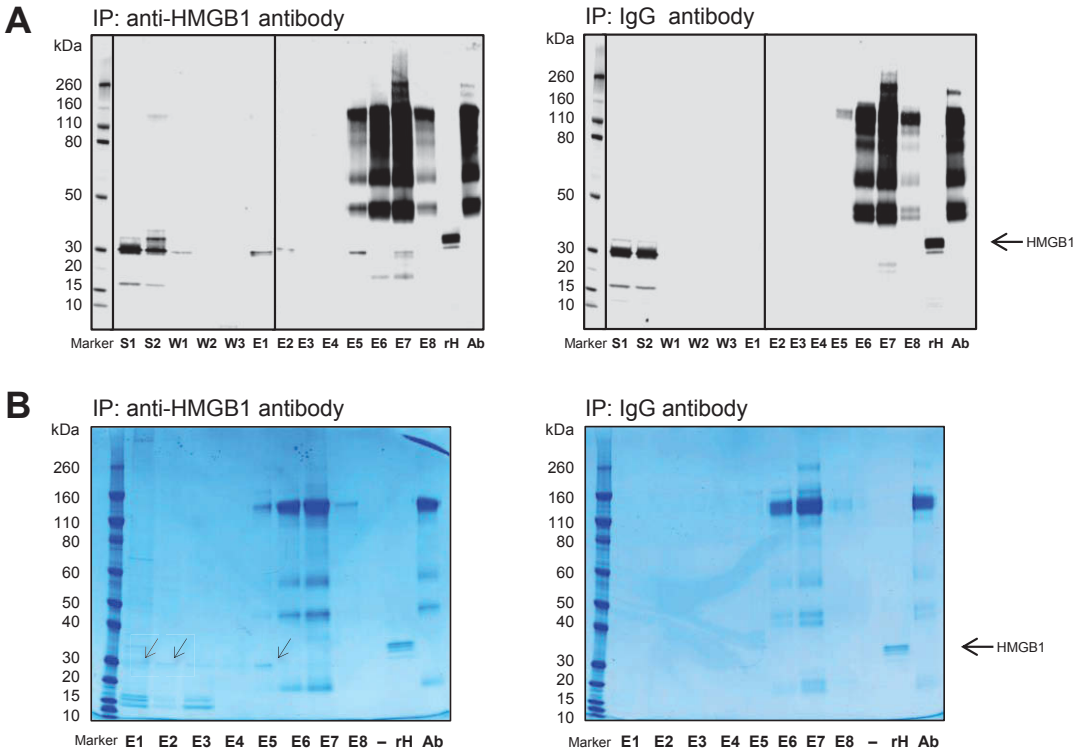

Figure 5.4: Analysis of extracellular HMGB1 using immunoprecipitation. Immunoprecipitation (IP) was used to identify HMGB1 binding proteins. Concentrated airway epithelial cell culture supernatants were incubated with anti-HMGB1 or a control IgG antibody. HMGB1 enrichment at each step of the IP procedure was assessed. Labels for each lane indicate the following: 'Input' culture supernatants (S1); 'Unbound' culture supernatants (S2); Washes with 0.05% PBST (W1, W2, W3), Elution with 0.5M NaCl in PBS (E1, E2); 1.5M NaCl in PBS (E3, E4), 0.1M Glycine (pH 2.5) (E5, E6) and NuPage® LDS sample buffer (E7, E8). Recombinant human HMGB1 (rH) and the specific antibodies used for IP (Ab) were loaded on the same gel for comparison (A). The protein bands from the IP elutions (E1 – E8) were visualised by coomassie blue staining (B). Visible bands were subjected to trypsin digestion and LC-MS/MS analysis. Identified proteins are listed in Appendix II. The images shown are representative of two biological replicates.

To identify potential HMGB1 binding proteins, in the first biological replicate, all proteins visualised by coomassie blue staining were excised and trypsin digested for analysis by LC-MS/MS. However, given that not many protein bands were visible with coomassie blue stain, to increase sensitivity in the second biological replicate, all proteins from the high salt and low pH elutions were pooled and subjected to shotgun digestion for analysis by LC-MS/MS. In each case, samples incubated with IgG control antibody were processed in the same way as those incubated with the anti-HMGB1 antibody. Of note, HMGB1 was reproducibly detected in both biological replicates. In addition, a further 106 proteins were identified with 2 or more peptides in at least 1 biological replicate, or at least 1 peptide in each biological replicate (Appendix II). Of these 18 were reproducibly detected in each replicate. Proteins that were detected with 2 or more peptides in control samples were classified as non-specific contaminants and excluded, while proteins with 1 manually-validated spectrum were considered as flow-through contaminants.

Identification of HMGB1-binding proteins via pull-down assay

We performed a pull-down assay in the absence (control) or presence of a His-tagged recombinant human HMGB1 as 'bait'. Following optimization of the elution buffer, we found that 0.25M NaCl in PBS yielded the most HMGB1-binding proteins with minimal co-elution of the 'bait' (data not shown). Eluents from the HMGB1 pull-down assay and the control pull-down assay were separated using SDS-PAGE and visualized using coomassie blue staining. As depicted in Fig 5.5, an extensive number of proteins were detected in the HMGB1, but not the control pull-down. Moreover, the profile of the His-tagged

recombinant human HMGB1 alone was distinct from that of the HMGB1 pull-down eluent, indicating that the eluted proteins were specifically derived from the culture supernatant.

To identify HMGB1 binding proteins, we excised and trypsin digested the entire gel lane of the HMGB1 and control eluents, and analyzed these with LC-MS/MS. HMGB1 was reproducibly detected in biological replicates of the HMGB1-bait, but not control pull-down. In addition, a further 81 proteins were detected with 2 or more peptides in at least 1 biological replicate, or 1 peptide in each biological replicate (Appendix III). Of these, 37 were reproducibly detected in each replicate. Protein contaminants in control samples were excluded as described for IP studies above. The recombinant HMGB1 used as 'bait' in the pull-down assays was a truncated protein in which the acidic C-tail was deleted. Although this is a limitation, it is important to note that the majority of HMGB1 protein/receptor interactions are attributed to the HMG Box A and Box B regions of the molecule (see Figure 5.2)(5).

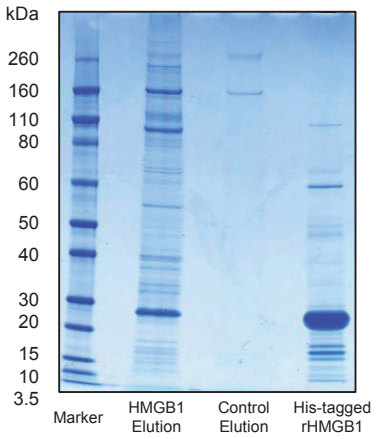

Figure 5.5: Analysis of extracellular HMGB1 using pull-down assays. Pull-down assays were used to identify HMGB1 binding proteins. Airway epithelial cell culture supernatants were incubated without (control) or with a His-tagged recombinant human HMGB1. The protein bands from the HMGB1 (Lane 2) and control (Lane 3) pull-down elutions were visualized by coomassie blue staining. Visible bands were subjected to trypsin digestion and LC-MS/MS analysis. Identified proteins are listed in Appendix III. The His-tagged recombinant HMGB1 were loaded on the same gel for comparison (Lane 4). The gel image is representative of two biological replicates.

Combined profile of HMGB1-binding proteins identified using clear native electrophoresis, immunoprecipitation and pull-down assays

Using three complementary approaches coupled to LC-MS/MS, we identified a total of 185 potential HMGB1-binding proteins (Figure 5.6). Of these, 24 proteins were identified using 2 or more of the techniques used, and were detected with 2 or more peptides in at least 1 biological replicate, or at least 1 peptide in each replicate. In addition, there were 13 proteins that were detected in 2 techniques; but with only 1 peptide in one of the techniques (marked *, Table 5.1). Thus, altogether, this gives rise to a total of 37 proteins that were reproducibly detected using 2 or more techniques, inclusive of 7 proteins detected across all 3 techniques used (Table 5.1). The majority of these 37

HMGB1-binding proteins appeared to be ubiquitous nuclear and cytosolic proteins such as histones, ribonucleo- and ribosomal proteins, heat shock proteins as well as metabolic enzymes and cytoskeletal proteins. In addition to these proteins, 77 other proteins were found exclusively using IP, 56 proteins were found exclusively using pull-down assays, and 15 proteins were found exclusively using clear native electrophoresis, as listed in Appendices I, II and III. The greatest number of HMGB1-binding proteins was identified using IP, consistent with the fact that this approach involves sample enrichment.

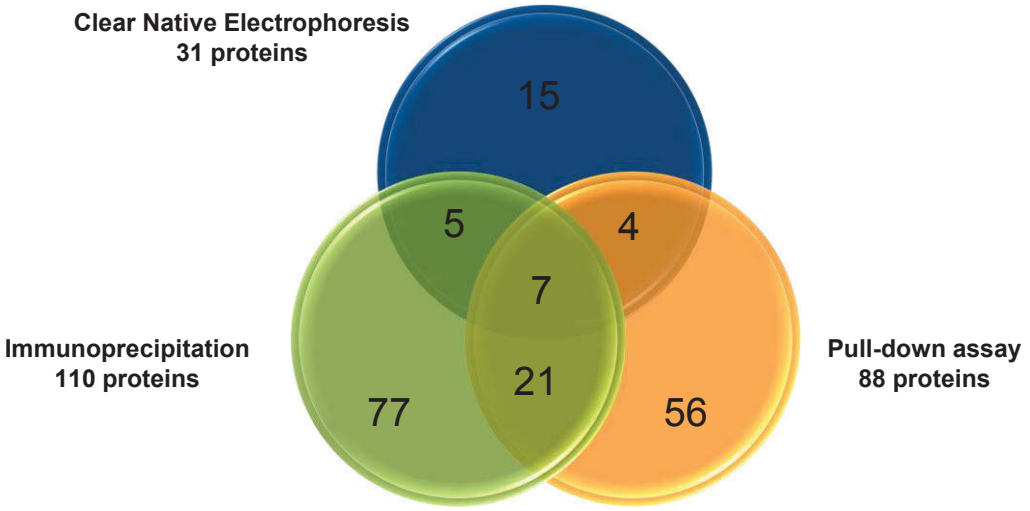

Figure 5.6: HMGB1-binding proteins detected using different approaches. The number of HMGB1-binding proteins identified using high resolution clear native electrophoresis, immunoprecipitation and pull-down assays are shown. A total of 185 proteins were identified. 37 proteins were detected using 2 or more techniques, while 7 proteins were detected by all three techniques.

Table 5.1: HMGB1-binding proteins identified using at least 2 techniques of either high resolution clear native electrophoresis, immunoprecipitation or pull-down assay, coupled to LC-MS/MS

#	Identified Proteins	Gene	Accession Number	MW (kDa)	Total unique peptide									
					hrCNE		IP				Pulldown			
							HMGB1		Control		HMGB1		Control	
					Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
	Nuclear structural proteins													
1	Histone H1.2	HIST1H1C	P16403	21										
	Histone H1.3	HIST1H1D	P16402	22			1	5			12	2		
2	Histone H1.5[a]	HIST1H1B	P16401	23			1				7			
3	Histone H3.3	H3F3A/B	P84243	15										
	Histone H3.1t	HIST3H3	Q16695	16				2			2			
4	Nucleophosmin[a, b]	NPM1	P06748	33	1		2				5	4		
	Gene transcription													
5	High mobility group protein HMG-I/HMG-Y[a]	HMGA1	P17096	12				1			2			
	Cell cycle and DNA repair													
6	X-ray repair cross-complementing protein 6	XRCC6	P12956	70				3			25	9		
	precursor mRNA processing spliceosome													
	Heterogeneous nuclear ribonucleoproteins C1/C2	HNRNPC	P07910	34										
7	Heterogeneous nuclear ribonucleoprotein C-like 1	HNRNPCL1	O60812	32			5	2			9	2		
	Heterogeneous nuclear ribonucleoprotein C-like 4	HNRNPCL4	P0DMR1	32										
	Ribosomal proteins													
8	60S ribosomal protein L12	RPL12	P30050	18			2				5	3		
9	60S ribosomal protein L27[a]	RPL27	P61353	16			2				1			
10	60S acidic ribosomal protein P0	RPLP0	P05388	34			6				11	2		
	Protein folding and ubiquitination													
	Polyubiquitin-B[a]	UBB	P0CG47	26										
	Polyubiquitin-C[a]	UBC	P0CG48	77										
11	Ubiquitin-60S ribosomal protein L40[a]	UBA52	P62987	15			2				1			
	Ubiquitin-40S ribosomal protein S27a[a]	RPS27A	P62979	18										
	Heat shock proteins and chaperones													
12	60 kDa heat shock protein, mitochondrial[a]	HSPD1	P10809	61			8	2			1			
13	78 kDa glucose-regulated protein[b]	HSPA5	P11021	72		2	1	3		1	8			
14	Endoplasmin[a, b]	HSP90B1	P14625	92	4	4	1				33	23		
15	Heat shock protein HSP 90-alpha[b]	HSP90AA1	P07900	85	6	14	6	1			1	1		
16	Heat shock protein HSP 90-beta[b]	HSP90AB1	P08238	83	5	6	14	2			2	2		
17	Heat shock 70 kDa protein 1A	HSPA1A	P0DMV8	70		8	5	2						
	Heat shock 70 kDa protein 6	HSPA6	P17066	71										
18	Heat shock cognate 71 kDa protein[b]	HSPA8	P11142	71	1	6	6	3		1	2			
	Immunity													
19	Beta-2-microglobulin[a]	B2M	P61769	14	2	2					1			
20	High mobility group protein B1	HMGB1	P09429	25			2	8			34	12		
	Enzymes													
21	Protein-glutamine gamma-glutamyltransferase 2[a]	TGM2	P21980	77			3					1		
	Carbohydrate and lipid metabolism													

22	Acetyl-CoA acetyltransferase, cytosolic (Isoform 2)[a]	ACAT2	Q9BWD1-2	45	1		2						
23	Alpha-enolase	ENO1	P06733	47		6	6						
24	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	P04406	36			25	8			2		
25	L-lactate dehydrogenase A chain	LDHA	P00338	37		3	2						
	Cytoskeletal and actin-binding proteins												
26	Alpha-actinin-1[a]	ACTN1	P12814	103	13	23						1	
27	Alpha-actinin-4[a]	ACTN4	O43707	105	15	21						1	
28	Cofilin-1	CFL1	P23528	19	2	2	2						
29	Myosin-9	MYH9	P35579	227			24	7			45		
	Myosin-14	MYH14	Q7Z406	228									
30	Myosin light polypeptide 6	MYL6	P60660	17			2				6		
31	Tubulin alpha-1B chain	TUBA1B	P68363	50			11	2		1	4	5	
32	Tubulin beta-2A chain	Q13885	TUBB2A	50			21	8			3	6	
	Tubulin beta-8 chain	Q3ZCM7	TUBB8	50									
	cDNA FLJ11352 fis, clone HEMBA1000020, highly similar to Tubulin beta-2C chain		B3KML9	45									
	cDNA FLJ56903, highly similar to Tubulin beta-7 chain		B4DY90	52									
	Cell shape and adhesion												
33	Annexin A2	ANXA2	P07355	39				3			12	2	
34	Lectin galactosidase-binding soluble 3 binding protein[a]	LGALS3BP	Q08380	65			1				10	9	
	Extracellular matrix and membrane proteins												
35	Fibronectin	FN1	P02751	263			14					21	
36	Protein CYR61	CYR61	O00622	42				2			8	4	
37	Tyrosine-protein kinase receptor[b]	SDC4-ROS1_S4;R32	M1VKI3	83		2		2			14	4	
		SDC4-ROS1_S4;R34	M1VE83	72									
	Miscellaneous												
38	Amyloid beta A4 protein	APP	P05067	87	3	3					25	14	

For protein entries where the unique peptide(s) identified belong to more than 1 specific protein, all proteins identified are listed.

[a]Proteins were identified with 1 unique peptide in one of the techniques used

[b]Proteins were identified in all 3 techniques used

Exp 1, Experiment 1; Exp 2, Experiment 2

Bioinformatic analysis reveals novel HMGB1-binding proteins and predicts homeostatic functions of extracellular HMGB1

We analysed and visualized the protein network of the 185 HMGB1-binding proteins using Cytoscape. 14 of these proteins were identified as primary interactors of HMGB1, including APP, CAPZA1, GAPDH, H3F3B, HMGA1, HNRNPK, HSPA8, HSP90AA1, HSP90B1, SSRP1, SUPT16H, UBC, XRCC5 and XRCC6 (Figure 5.7). 10 of these proteins have been experimentally reported to have physical protein-protein interactions (PPIs) with HMGB1 (green nodes). 1 protein is reported to be co-expressed with HMGB1 (orange node) while the remaining 3 proteins were predicted to interact based on evidence of genetic, functional or structural homology with HMGB1 or through database mining (purple nodes). The confidence scores and evidence of interactions for the 14 primary interactors of HMGB1 are shown in Appendix IV. Importantly, 9 out of these 14 primary interactors of HMGB1 were reproducibly identified in at least 2 of the 3 techniques used (Table 5.1). Of note, extension of the protein network analysis to indirect, secondary interactions indicate 171 of 185 the identified HMGB1-binding proteins were included in this network (data not shown).

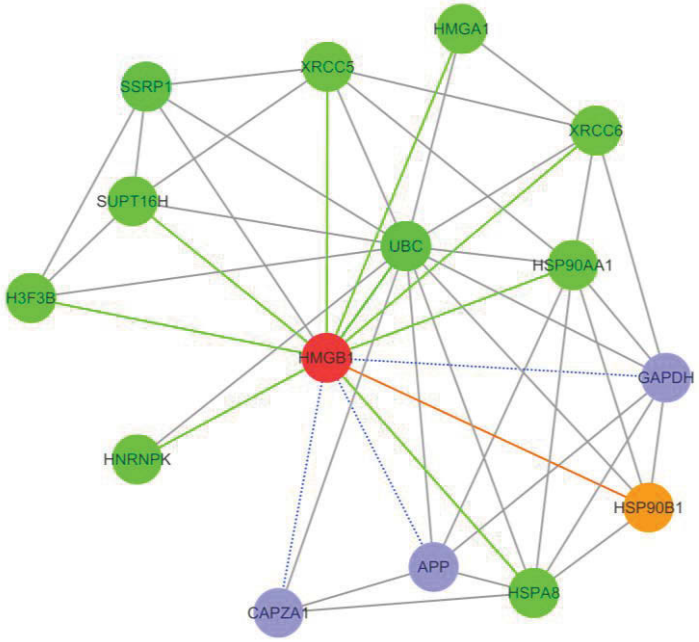

Figure 5.7: Protein network analysis of HMGB1-binding proteins.
Of the total 185 HMGB1-binding proteins identified using three different techniques, 14 proteins were identified as primary interactors of HMGB1. Proteins for which there is experimental evidence of physical interaction (green nodes) or co-expression (orange nodes) are shown. Three proteins (purple nodes) were predicted to directly associate with HMGB1 through database mining.

We also performed GO term enrichment analysis to shed light on the potential functions of extracellular HMGB1 in AECs. We scrutinized only the 37 HMGB1-binding proteins identified in at least 2 techniques used given the robustness of this dataset (Table 5.1). The top 15 enriched terms for cellular component, molecular function and biological process are summarized in Table 5.2, while the comprehensive list of enriched GO terms are presented in Appendices V, VI and VII, respectively. As mentioned, the majority of the 37 HMGB1-binding proteins are canonical intracellular proteins. Interestingly however, both *extracellular region* and *extracellular exosome* topped the enriched terms for

cellular component, with 37 and 35 observed gene count, respectively. This result validates our observation of these proteins in the cell culture media, and also suggests they are not released via the conventional ER-Golgi secretory pathway, but through membrane-bound exosomes. As expected, *nucleus* and *cytoplasmic part* were also enriched with 32 and 26 observed gene counts, respectively. HMGB1-binding proteins seemed to have affinities for a diverse range of ligands, exemplified by the enriched molecular function terms *protein binding, nucleic acid binding, enzyme binding, macromolecular complex binding, carbohydrate derivate binding, ion binding and small molecule binding*, suggesting a pleiotropic role for HMGB1. Of note, the terms *cellular component organization or biogenesis, cellular metabolic process, regulation of cellular process* and *cell communication* were among the top 15 enriched terms for biological process, indicating HMGB1 in association with its binding protein(s), potentially regulate diverse basic cellular processes. The terms *cellular response to stimulus* and *response to stress* were also highly enriched, providing evidence that HMGB1 in the extracellular milieu is essential for cell surveillance and host defense under basal conditions. Consistent with this, the KEGG pathways *antigen processing* and *presentation*, r*egulation of actin cytoskeleton* as well as *protein processing in endoplasmic reticulum* were also enriched (Figure 5.8).

Table 5.2: Top 15 Gene Ontology terms enriched in HMGB1-binding proteins identified using 2 or more techniques.

GO term ID	GO terms	Observed gene count	False discovery rate
CELLULAR COMPONENT			
GO:0043227	membrane-bounded organelle	39	7.16E-05
GO:0005576	extracellular region	37	6.04E-16
GO:0044421	extracellular region part	37	1.79E-18
GO:0070062	extracellular exosome	35	8.27E-20
GO:0031988	membrane-bounded vesicle	34	6.93E-16
GO:0043231	intracellular membrane-bounded organelle	32	0.0216
GO:0005634	nucleus	32	3.78E-06
GO:0044422	organelle part	32	7.04E-05
GO:0044446	intracellular organelle part	30	0.000458
GO:0032991	macromolecular complex	27	1.20E-06
GO:0043233	organelle lumen	27	2.16E-07
GO:0044444	cytoplasmic part	26	0.0156
GO:0005829	cytosol	24	2.47E-07
GO:0043232	intracellular non-membrane-bounded organelle	24	1.20E-06
GO:0070013	intracellular organelle lumen	24	1.42E-05
GO:0043234	protein complex	24	6.68E-06
MOLECULAR FUNCTION			
GO:0005488	binding	37	0.00037
GO:1901363	heterocyclic compound binding	35	7.12E-10
GO:0097159	organic cyclic compound binding	35	8.04E-10
GO:0005515	protein binding	31	8.14E-09
GO:0003676	nucleic acid binding	29	4.06E-09
GO:0003723	RNA binding	26	8.51E-15
GO:0043167	ion binding	24	0.0316
GO:0044822	poly(A) RNA binding	23	3.75E-14

GO:0036094	small molecule binding	20	2.37E-05
GO:0043168	anion binding	19	0.000133
GO:0097367	carbohydrate derivative binding	19	1.08E-05
GO:0019899	enzyme binding	19	4.72E-09
GO:0000166	nucleotide binding	18	0.000116
GO:0044877	macromolecular complex binding	17	1.46E-08
GO:0001882	nucleoside binding	15	0.000577
BIOLOGICAL PROCESS			
GO:0051716	cellular response to stimulus	29	2.57E-05
GO:0016043	cellular component organization	28	3.04E-06
GO:0044237	cellular metabolic process	28	0.0151
GO:0071704	organic substance metabolic process	28	0.0272
GO:0044238	primary metabolic process	28	0.0187
GO:0050794	regulation of cellular process	28	0.0478
GO:0071840	cellular component organization or biogenesis	27	1.37E-05
GO:0043170	macromolecule metabolic process	27	0.00504
GO:0044260	cellular macromolecule metabolic process	26	0.00375
GO:0050896	response to stimulus	26	0.00526
GO:0007154	cell communication	24	0.000533
GO:0043933	macromolecular complex subunit organization	24	1.30E-09
GO:0031323	regulation of cellular metabolic process	24	0.00184
GO:0019222	regulation of metabolic process	24	0.0101
GO:0071822	protein complex subunit organization	23	7.36E-12
GO:0060255	regulation of macromolecule metabolic process	23	0.00274
GO:0080090	regulation of primary metabolic process	23	0.00311
GO:0006950	response to stress	23	1.24E-05
GO:0044700	single organism signaling	23	0.00105

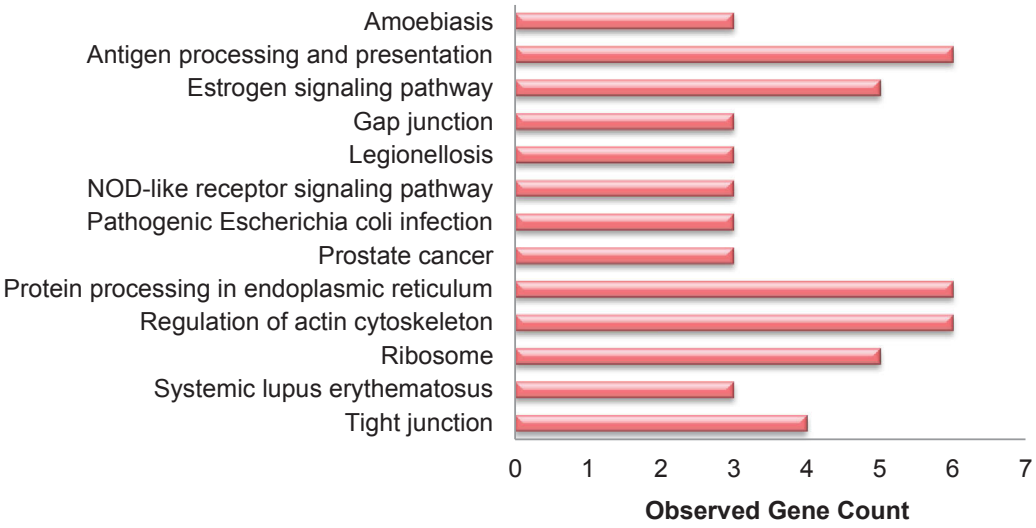

Figure 5.8: KEGG pathway analysis of identified HMGB1-binding proteins. KEGG pathway analysis was performed on the 37 HMGB1-binding proteins detected using at least 2 techniques (Table 5.1).

DISCUSSION

We have undertaken the first unbiased global analysis of extracellular HMGB1 and its binding partners. We found that HMGB1 presents exclusively as a protein complex in the culture supernatant of unstimulated AECs, and using several complementary proteomic approaches, we identified 37 HMGB1 binding proteins (Table 5.1). Bioinformatic analysis revealed 14 proteins which directly associate with HMGB1 (Figure 5.7). Three of these, namely amyloid precursor protein (APP), F-actin-capping protein subunit alpha-1 (CAPZA1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have not previously been identified as HMGB1 binding proteins. Together, our findings suggest that HMGB1 is likely to have a fundamental role in the regulation of epithelial cell homeostasis and airway mucosal immunity.

Secretion of HMGB1 into the extracellular space is largely thought to occur during cellular inflammation, injury or death. However, studies by Rauvala and others (22, 23) have provided clear evidence of physiologically regulated release of HMGB1 that occurs in the absence of inflammation and cell death. Indeed, these investigators first identified HMGB1 as a heparin-binding protein that regulates neurite outgrowth, a key process that occurs during neuronal migration and differentiation. Moreover, they showed that it localizes to the advancing plasma membrane of the filopodia and at the leading edge in motile cells, suggesting that extracellular HMGB1 has a role in cell motility (22, 23). Numerous phenomena related to cell shape and movement, such as migration, adhesion and protrusion are regulated by the dynamic assembly of actin filament networks. The capping protein CAPZA1 inhibits actin polymerization by binding and 'capping' the barbed ends of growing actin filaments. It plays a pivotal role in enabling leading edge protrusion and net migration of cells, and is also involved in the formation of epithelial cell-cell junctions (24-26). Previous studies have shown that HMGB1 is secreted by damaged AECs and acts via its major signaling receptors, receptor for advanced glycation endproducts (RAGE) and toll-like receptor 4 (TLR4), to promote epithelial cell migration and wound closure *in vitro* (27, 28). Identification of CAPZA1 as a primary HMGB1 interacting protein suggests that physiological secretion of HMGB1 may also be required for epithelial cell motility and adhesion under basal conditions. Interestingly, the formin protein diaphanous-1 is an intracellular signaling adaptor of RAGE, and mediates RAGE-dependent migratory responses via activation of the Rho-family small GTPases Cdc42 and Rac1 (29). Formins are a class of proteins that work in concert with capping proteins to regulate cell

migration (30), thus we speculate that HMGB1/RAGE co-operate with capping proteins and formins to regulate epithelial cell motility and barrier integrity under physiological conditions. Certainly this is an interesting concept that warrants future investigation.

The amyloid precursor protein (APP) is a type I transmembrane glycoprotein expressed in many cell types. Although it has been extensively studied in the context of Alzheimer's disease, its normal physiological function in the brain and other organs is not well understood (31). Very few studies have investigated its expression and/or function in AECs; however one study provides evidence of APP protein expression in the bronchial epithelium of aged dogs (32) and another study has demonstrated its expression in A549 cells, an alveolar epithelial cell line (33). APP undergoes extensive proteolysis and, interestingly, while previous studies have shown that HMGB1 binds certain proteolytic fragments of APP, such as sAPPα and the Aβ peptide (34-36), this study is the first to identify its interaction with the full-length molecule. Similar to HMGB1, APP is a heparin-binding protein implicated in cellular adhesion and motility, and has also been shown to mediate neurite outgrowth (31). Moreover, the Aβ peptide has been shown to signal via RAGE (37). It will be interesting to determine whether APP-HMGB1 interaction governs airway epithelial cell growth and turnover via RAGE-dependent mechanisms.

HMGB1 occupies a fundamental role in host defense as it acts as both a sentinel and mediator of the innate immune response (4-9). Our finding that HMGB1 interacts with CAPZA1, and other cytoskeletal proteins (Table 5.1),

suggests that HMGB1-cytoskeletal interactions may play an important regulatory role in cell-autonomous immunity and host defense. Cytoskeletal components integrate with the host immune response via a number of mechanisms. For example, during invasion of host cells, certain bacteria induce modifications in Rho GTPases involved in the formation and organization of actin filaments. Bacterial modifications of Rho GTPases such as Cdc42, Rac1 and others are detected by intracellular immune receptors such as nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and pyrin, and hence in this way, bacterial modification of cytoskeletal targets serves to facilitate their detection by the host (38). A recent study by Li and colleagues showed that capping protein plays an essential role in the innate immune response to bacterial and fungal pathogens in plants (39). Interestingly, Chen and colleagues showed that allergic airway inflammation induced by the protease allergen Pen c 13 is associated with increased lung expression of several cytoskeletal proteins that we have also identified as HMGB1 interacting proteins, namely CAPZA1, cofilin-1 and annexin A2 (40). It is probable that extracellular HMGB1 acts to maintain immune homeostasis at the airway epithelial surface via its interaction with cytoskeletal components, and other immune-regulatory molecules, as discussed below.

HMGB1 interacts with a diverse repertoire of endogenous and exogenous molecules to regulate the immune response (4-9). In a seminal study, Chen and colleagues showed that HMGB1 forms a complex with the membrane protein CD24 and the sialic acid-binding immunoglobulin-like lectin (Siglec)-10 (41). Siglec-10 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM),

and negatively regulates immune/inflammatory responses via the recruitment of tyrosine phosphatases such as SHP1 and SHP2; thus interaction between HMGB1/CD24 and Siglec-10 prevents activation of HMGB1-dependent inflammatory responses (41, 42). Interestingly, lectin galactosidase-binding soluble 3 binding protein (LGALS3BP), which we have identified as a HMGB1-interacting protein (Table 5.1), is also a Siglec-10 ligand (43). Human AECs express Siglec ligands under basal conditions (44), thus it is conceivable that HMGB1 forms complexes with Siglec ligands such as LGALS3BP at the airway epithelial cell surface and that LGALS3BP/HMGB1 complexes maintain immune homeostasis by engaging Siglec-10 on local innate immune cells (42, 44). Pathogen-associated molecular patterns such as LPS increase Siglec ligand expression in human AECs (44), and are also able to directly bind HMGB1. HMGB1 facilitates the activation of immune and inflammatory responses by acting as a 'chaperone' for the delivery of pathogen-associated molecular patterns (PAMPs) to their cognate sensing receptors (4-9, 45). Thus, the presence of PAMPs/allergens at the airway surface may potentially modify homeostatic interactions between HMGB1 and LGALS3BP, cytoskeletal proteins and other extracellular immune regulatory molecules, thereby releasing the break on inhibitory signals to trigger the activation of innate immune responses at the airway mucosal surface.

Indeed, this concept is further supported by our finding that HMGB1 associates with extracellular ubiquitin (Table 5.1, Appendix IV). Ubiquitin is a highly conserved protein that is covalently attached to other proteins via an enzymatic cascade. Post-translational modification of intracellular proteins by

ubiquitinylation is used in the regulation of many biological responses, and has been the subject of much investigation (46). Surprisingly, though, little is known about the role of ubiquitin as an extracellular molecule (47). Relevant to this discussion, however, is the study by Majetschak and colleagues which showed that serum levels of extracellular ubiquitin are markedly up-regulated in sepsis and trauma patients, and that extracellular ubiquitin acts to suppress immune activation, as evidenced by its capacity to inhibit lipopolysaccharide (LPS)-induced TNF-α secretion in peripheral blood mononuclear cells (48). Extracellular HMGB1 is a well-established mediator of sepsis (4-9, 49), and hence the study by Majetschak provides biological context under which HMGB1 and extracellular ubiquitin are co-regulated. Indeed, Majetschak and colleagues proposed that extracellular ubiquitin inhibits immune and inflammatory responses by acting as an “endogenous opponent of DAMPs” (47). Of note, however, Majetschak further showed that extracellular ubiquitin signals via the chemokine receptor CXCR4, which is a HMGB1 signaling receptor (50, 51). Thus, rather than acting as an endogenous opponent of DAMPs, we suggest that ubiquitin associates with HMGB1 and that HMGB1/ubiquitin complexes signal via CXCR4 to maintain immune homeostasis under basal conditions. As suggested above, the presence of PAMPs/allergens may potentially alter the homeostatic interaction between HMGB1 and immune-regulatory molecules (ubiquitin in this case) to initiate activation of immune and inflammatory responses. This is a highly plausible concept as previous studies have demonstrated CXCR4 expression in human AECs, and CXCR4 is implicated in the allergic asthmatic response (52, 53).

In addition to ubiquitin, we found that extracellular HMGB1 associates with numerous other proteins that are classically known for their intracellular functions, but for which extracellular functions are increasingly recognized. These include GAPDH, members of the HSPA (HSP70), HSPC (HSP90) and HSPD (HSP60) heat shock protein families, nucleophosmin, several histone variants and Ku70/Ku86 (XRCC6/XRCC5) complex (54-61). These proteins (including HMGB1) belong to a growing list of 'moonlighting' proteins i.e. proteins with multiple functions that are not due to gene fusions, splice variants or pleiotropic effects (62). Moonlighting proteins display distinct biological functions due to changes in cellular location, redox and oligomeric state of the protein, interaction with binding partners, or a change in the ligand endogenous concentrations. It is worth noting that extracellular heat shock proteins, such as HSPA8 (63-65), HSPD1 (66, 67) and HSP90AA1(68) and extracellular histones including the H1 and H3 variants identified here (69, 70), mediate immune-regulatory and tissue remodeling effects via TLR2 and/or TLR4, both of which are HMGB1 signaling receptors. Moreover, there are a few studies which provide evidence that HMGB1 and histones form biologically active complexes in the extracellular space (71). Although it is becoming increasingly apparent that extracellular heat shock proteins and histones exert pathological effects in the context of the airway inflammatory response (72, 73), evidence of their interaction with HMGB1 under basal conditions suggests a homeostatic role, and certainly this is an important question for future research.

Ku is a highly abundant protein found *in vivo* as a stable heterodimer consisting of two subunits, Ku70 (XRCC6) and Ku86 (XRCC5) (60). Lee and colleagues

have previously identified Ku70 and Ku86 as HMGB1 binding partners within the nuclear/cytoplasmic compartment (16); thus we extend their findings to show that HMGB1 also associates with Ku proteins in the extracellular space. Although the Ku heterodimer is best known for its role in DNA double strand break recognition and repair (60), it has extracellular functions in cell adhesion and migration (74-76). Cell-surface Ku associates with MMP-9, a key enzyme involved in the degradation of ECM components. Mueller and colleagues showed that MMP-9 and Ku co-localize at the periphery of leading edge cells, suggesting a role for Ku/MMP-9 in cellular migration (74, 75). Relevant to this, HMGB1 has been shown to induce MMP-9 expression in primary human AECs (77), and is a substrate of MMP-9 activity (78). Interestingly, Ku86 has been identified as a COPD susceptibility gene (79), and decreased expression of Ku86 is observed in the bronchial epithelium of patients with COPD (80). In COPD, the airway epithelium displays features of de-differentiation towards mesenchymal cells, and this appears to relate to the extent of airway remodeling and impaired lung function (81). It is possible that HMGB1/Ku/MMP-9 might interact in a dynamic manner to modulate airway epithelial cell adhesion and motility in COPD and certainly this is a very important question for future research.

A number of proteins identified as HMGB1-interacting proteins in our study have not previously been described as extracellular proteins; these include SSRP1, SUPT16H and HMGA1 (Table 5.1, Appendix IV). SSRP1 and SUPT16H are constituent subunits of the histone chaperone known as FACT (facilitates chromatin transcription) complex, which regulates nucleosome reorganization

during replication, transcription and DNA repair. SSRP1 contains a C-terminal HMG-1 domain, and interacts with HMG family proteins, including HMGB1 and HMGA1 (82). Thus, detection of this protein cluster in the extracellular space is biologically plausible, and if validated, would be the first evidence of an extracellular role for FACT complex.

In summary, we have generated the first unbiased profile of HMGB1 interacting proteins in the extracellular space. Our findings provide unique insights into the potential role of HMGB1 in maintaining epithelial barrier integrity and immune homeostasis at the airway mucosal surface, however, a number of limitations must be acknowledged. First, it is possible that necrosis in a small proportion of cells may contribute to the overall concentration of HMGB1 in the culture supernatant of serum-deprived epithelial cell lines in submerged culture. Indeed, this is a difficult issue to untangle, as HMGB1 is released by dead cells and low levels of cell death are an inevitable part of *in vitro* cell culture. Further studies are needed to confirm findings in primary human airway epithelial cells grown at air-liquid interface and in epithelial lining fluid from human subjects. Second, we have not validated binding partners using specific approaches, and we have not provided evidence of functional significance, thus targeted validation of identified binding partners and functional studies are essential next steps. Alterations in HMBG1 molecular interactions in the extracellular space may indicate the presence of cellular and tissue dysfunction. Investigation of HMGB1 interactions with its extracellular binding partners, in both health and disease, is therefore an important area of future research.

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Chapter 6

General Discussion

Asthma and COPD are heterogenous conditions comprising a number of subtypes, which may be caused by different underlying pathophysiologic mechanisms (or endotypes). The studies in this thesis examine the biology of the proteins SPARC and HMGB1, which are emerging as important players in chronic airways disease. We provide the first insight into expression of the matricellular protein SPARC in airway structural cells including AECs and ASM cells, and showed that SPARC expression is altered in cells derived from subjects with asthma and COPD although our observation in these diseased cells requires validation (**Chapter 3 and 4**). Our studies herein also propose extracellular HMGB1 orchestrates important physiological and homeostatic functions in AECs, in addition to its established roles in airway inflammatory responses and tissue repair (**Chapter 5**). Together, these studies enhance our understanding of the cellular and molecular mechanisms that underlie chronic airways disease.

We have shown SPARC is an inducible protein by TGF-β in AECs, consistent with studies in other structural cell types (**Chapter 3**) (1-3). TGF-β also enhances synthesis and deposition of ECM proteins such as fibronectin in AECs (4, 5). Given SPARC regulates processing and deposition of mature collagen I fibrils (organized) into the ECM and is essential for fibronectin matrix assembly (6, 7), it is possible that TGF-β stimulates deposition of ECM proteins in AECs in a SPARC-dependent manner. In support of this, TGF-β has been shown to have a modest but significant stimulatory effect on fibronectin deposition in AECs (5), similar to its effect on SPARC expression in our studies. In addition, our preliminary observation of SPARC overexpression in asthmatic

AECs, in the absence of any stimuli, is also in corroboration with findings by Ge and colleagues showing basally enhanced fibronectin deposition in the asthmatic AECs (5). While it remains to be investigated, it is entirely plausible that SPARC levels are elevated in the asthmatic epithelium and serves to positively regulate ECM protein deposition, driving subepithelial fibrosis and thickening of the basement membrane. SPARC siRNA experiments in AECs in the presence or absence of TGF-β is warranted to elucidate the biological significance of SPARC on ECM protein deposition. It will be interesting to elucidate whether SPARC is overexpressed in AECs from subjects with COPD given aberrant epithelial repair is also a feature of the disease (8).

Interestingly, our preliminary studies showed that the basal secreted SPARC levels in asthmatic AECs are greater than the maximal SPARC release induced by TGF-β in non-asthmatic AECs. While this finding needs to be verified, it suggests factors apart from TGF-β may be driving SPARC overexpression in the diseased cells (**Chapter 3**). Growth factors such as platelet-derived growth factor (PDGF) and insulin-like growth factor 1 (IGF-1) have been shown to enhance SPARC protein synthesis in chondrocyte cultures (9). Like TGF-β, PDGF and IGF-1 are important for epithelial repair and regeneration, and are inducible in AECs (10-13). Although the expression of PDGF and IGF-1 in asthmatic AECs have not been studied, PDGF increases TGF-β2 production in AECs while IGF-1 leads to thickening of the basement membrane, indicative of their involvement in ECM protein production (14, 15). In addition, HMGB1 has also been shown to induce SPARC gene expression in AECs and as discussed in Chapter 5, HMGB1 expression is enhanced in asthma and plays a role in

epithelial wound repair and ECM protein synthesis (16, 17). The induction of SPARC expression by growth factors and repair mediators in AECs indicates its importance in epithelial repair, consistent with its potential role in the regulation of ECM assembly in AECs as mentioned above.

We also demonstrated TGF-β and the UPR up-regulate SPARC expression and secretion in ASM cells (**Chapter 4**). ER stress and the UPR have been shown to be implicated in the pathogenesis of asthma and COPD but how ER stress contributes to these conditions has not been defined (18-20). A series of studies by Delmotte and colleagues showed that the inflammatory cytokines, TNF-α and IL-13, disrupt intracellular Ca^{2+} buffering and induce ER stress which then drives ASM cell proliferation (21-23). Since TGF-β is a well-established mediator of ASM proliferation and SPARC also regulates cell proliferation in other cell types, it is possible that the TGF-β/UPR/SPARC axis stimulates ASM proliferation (24, 25). In addition, the UPR has also been shown to mediate TGF-β-induced collagen production in lung fibroblasts (26, 27). Taken together, this suggests that the proposed axis may be one possible mechanism by which the UPR drives airway remodeling in chronic airways disease, although this remains to be investigated.

The reason for the trend of reduced SPARC release in ASM cells cultured from COPD subjects is not clear, given TGF-β/UPR axis induces SPARC expression and has been shown to drive tissue remodeling, an evident feature in COPD (**Chapter 4**) (27, 28). Notably however, SPARC-null mice exhibit signs of premature ageing such as early onset cataract, osteopenia, increased adipose

tissue and accelerated disc degeneration, and COPD is an age-related disorder (29-34). This suggests the trend of reduced SPARC may perhaps drive progressive ageing in COPD although the exact mechanism involved has not been examined. SPARC-null mice also demonstrate deficiencies in ECM assembly and composition such as smaller and morphologically altered collagen fibrils, and age-related diseases are usually associated with dysregulated ECM dynamics (35, 36). Given SPARC is important for normal tissue development and repair which requires ECM protein deposition (36), it is possible that down-regulated SPARC expression results in aberrant tissue repair due in part to dysregulated ECM structure. This may impact on cellular behavior and function, and cause cellular senescence and abnormal ageing process in consequent. The possibility of SPARC serving as an anti-ageing factor is exciting, hence SPARC secretion in COPD ASM needs to be validated. If the trend of diminished SPARC in diseased cells holds true, the impact on ASM cellular function needs to be explored.

Alternatively, as discussed in **Chapter 4,** the trend of reduced SPARC levels in COPD ASM cells may be due to degradation by endogenous proteases given that MMP-3, which has been shown to cleave SPARC, is up-regulated in ASM cells from COPD subjects (37). Of note, cleaved SPARC fragments demonstrate distinct biological activity from the parent molecule. For instance, full-length SPARC is angiosuppressive while SPARC fragments containing –KGHK sequence elicit pro-angiogenic effects (37, 38). This raises the possibility that SPARC fragments may serve as bioactive molecules to drive COPD pathogenesis, akin to the matrikines, which are ECM fragments with biological

activities independent of their structural role within the ECM (39). Although SPARC fragments are known to only regulate angiogenic activity to date, it is possible they contribute to COPD via functions that have yet been defined. Future studies to inhibit endogenous SPARC expression and/or activity to mimic COPD ASM cells may shed light on possible mechanisms by which reduced SPARC levels contribute to COPD, Investigation of SPARC expression in asthmatic ASM cells is also warranted and will provide further insight into the function of SPARC in chronic airways disease. It could be differentially regulated in asthma as the regulation of SPARC expression and function are known to be highly contextual (40).

In addition to structural cells, various immune cells such as macrophages, follicular dendritic cells and $CD4^+$ T cells express SPARC. Evidence from chimeric mice indicates that SPARC derived from different cellular source have distinct functions; resident lung fibroblast-derived SPARC promotes ECM assembly and fibrosis, while leukocyte-derived SPARC attenuates lung inflammation (41-43). While the reason for this differential function has not been examined, given that SPARC is a glycoprotein and that tissue specific glycosylation in human tissues has been demonstrated, it is possible that the extent of SPARC processing varies depending on the cellular origin and subsequently impacts on its downstream activity (44, 45). This is of particular relevance to asthma and COPD as these conditions implicate the complex interplay between numerous cell types including AECs, ASM cells, macrophages, eosinophils, neutrophils and T lymphocytes (46, 47). Our studies herein demonstrate AECs and ASM cells both express SPARC, and that ASM

cells are a primary cellular source of SPARC when compared to AECs, although whether this is the case in the airways in vivo needs to be confirmed (**Chapter 3 and 4**). Our preliminary findings also suggest AECs may not be the main target cells of SPARC in the airways given exogenous SPARC did not have immunoregulatory effect or induce changes in the epithelial phenotype in AECs (**Chapter 3**). Since the cellular origin of SPARC is a critical determinant of its function, whether innate immune cells especially macrophages, the key SPARC-expressing immune cells (48), express SPARC and if this is dysregulated in chronic airways disease are important questions that need to be addressed. Given ASM cells produce robust amount of SPARC, the autocrine and paracrine function of ASM-derived SPARC also needs to be elucidated. Co-culture experiments involving the main cell types involved in chronic airways disease such as ASM cells, AECs and airway macrophages will delineate the distinct role of SPARC from these cells, how they interact and together contribute to the disease pathogenesis.

Although HMGB1 has been shown to induce SPARC expression, and orchestrates pro-inflammatory and pro-remodeling activities in AECs, our findings that HMGB1 is released by AECs under basal conditions, together with global proteomic analysis of its binding partners, suggest extracellular HMGB1 has physiological functions in AECs (**Chapter 5**) (16, 49, 50). An essential next step is to validate the identified binding partners using co-immunoprecipitation. Also, inhibition of HMGB1 activity in the extracellular space of unstimulated AECs using anti-HMGB1 neutralizing antibody is essential to delineate whether any phenotypic or functional changes ensue.

More importantly, the unbiased dataset that we have built here serves as a robust baseline reference of HMGB1-binding proteins in AECs. It is conceivable that this profile of binding partners will be altered qualitatively and quantitatively in diseased AECs. For instance, there could be gain or loss of HMGB1-ligand complexes, or alternatively, a change in the abundance of HMGB1-ligand complexes may follow. Future comparison of our baseline dataset with that of AECs obtained from subjects with asthma or COPD is crucial as changes in the profile of HMGB1-binding proteins in disease may indicate loss or aberrant regulation of specific basic cellular function. Elucidation of the particular binding proteins involved in disease is powerful and critical because HMGB1 signaling can be selectively targeted through inhibition of the partner molecule or receptor, rather than targeting HMGB1 per se, as HMGB1 usually signals through the receptor of the partner molecule when present as a complex (51). This means other important biological functions of HMGB1 will not be compromised as a consequence.

The function of HMGB1 is dictated by its redox state (52, 53). Three different redox forms of HMGB1 have been identified and each has been shown to elicit distinct functions. These include fully reduced HMGB1 which promotes chemotaxis and leukocyte infiltration, pro-inflammatory disulfide HMGB1 involved in stimulating cytokine production and terminally oxidized HMGB1 which has no documented immunoregulatory activity to date (54-56). It is hence important to identify the redox form of HMGB1 released by AECs under physiological conditions and whether a different form predominates in AECs

derived from subjects with chronic airways disease or in AECs exposed to environmental stressors such as allergens, which can be attained using mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy (55, 57). The question of whether the HMGB1 redox state influences its binding to ligands and/or receptors also needs to be addressed.

One limitation of our studies is the lack of *in vivo* evidence to confirm our novel observation of SPARC expression in AECs and ASM cells. While SPARC has previously been detected in the lung tissue of IPF patients, SPARC immunostaining in airway tissue from subjects with and without asthma or COPD will be important validation to our findings, and will provide insight into the cellular source of SPARC within the airway (58). Another limitation of the studies is the lack of insight into the differences between the two forms of SPARC detected in AECs and ASM cells, cellular and secreted. SPARC is a matricellular protein targeted for secretion into the extracellular space (36). This indicates cellular SPARC detected in our studies is potentially the precursor protein of secreted SPARC. There is however, some evidence demonstrating SPARC resides within intracellular compartments to orchestrate distinct functions (Table 2.2). While TGF-β regulated cellular and secreted SPARC expression in the same manner in ASM cells, the disparity between the two forms of SPARC is evident in cells stimulated with thapsigargin. SPARC immunostaining performed over a series of time-points following TGF-β and thapsigargin stimulation, will inform the subcellular localization of SPARC and the relationship between the cellular and secreted forms of the protein.

In conclusion, we provide the first evidence of SPARC expression in airway structural cells including AECs and ASM cells. Our studies also suggest SPARC may play a part in airway remodeling by acting downstream of the TGF-β/UPR axis. Our findings indicate SPARC expression is dysregulated in airway structural cells from subjects with asthma and COPD, although our observation in these diseased cells requires validation. Further scrutiny into the function of SPARC in these conditions is certainly warranted. Additionally, the studies herein provide a new perspective on HMGB1 biology by showing extracellular HMGB1 has potential physiological functions in AECs, in addition to its role in airway inflammatory responses and tissue repair. Together, findings in this thesis highlight the complexity and multifactorial nature of chronic airways disease, compelling further understanding of the cellular and molecular pathways underlying these conditions.

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Appendices

Appendix I. HMGB1-binding proteins identified using high resolution clear native electrophoresis (hrCNE) coupled to LC-MS/MS.

Appendix I: HMGB1-binding proteins identified using high resolution clear native electrophoresis (hrCNE) coupled to LC-MS/MS.

#	Identified Proteins	Gene	Accession Number	MW (kDa)	Total unique peptide		Protein score		Sequence coverage (%)	
					Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
	Nuclear structural proteins									
1	Nucleophosmin[a]	NPM1	P06748	33	1		84		5	
	Heat shock proteins and chaperones									
2	78 kDa glucose-regulated protein	HSPA5	P11021	72		2		101		5
3	Endoplasmin	HSP90B1	P14625	92	4	4	216	99	7	6
4	Heat shock protein HSP 90-alpha	HSP90AA1	P07900	85	6	14	374	585	9	22
5	Heat shock protein HSP 90-beta	HSP90AB1	P08238	83	5	6	347	404	7	9
6	Heat shock 70 kDa protein 1A	HSPA1A	P0DMV8	70		8		617		9
	Heat shock 70 kDa protein 6	HSPA6	P17066	71						
7	Heat shock 70 kDa protein 4	HSPA4	P34932	94		4		94		7
8	Heat shock cognate 71 kDa protein	HSPA8	P11142	71	1	6	88	270	7	20
	Immunity									
9	Beta-2-microglobulin	B2M	P61769	14	2	2	492	130	28	28
	Enzymes									
10	Adenosylhomocysteinase	AHCY	P23526	48		3		181		10
11	Dipeptidyl peptidase 1	CTSC	P53634	52	1	3	59	90	5	13
12	Nucleoside diphosphate kinase A	NME1	P15531	17		2		44		33
	Nucleoside diphosphate kinase B	NME2	P22392	17						
13	Phosphoserine aminotransferase	PSAT1	Q9Y617	40		2		42		11
14	Purine nucleoside phosphorylase	PNP	P00491	32		3		102		15
	Carbohydrate and lipid metabolism									
15	Acetyl-CoA acetyltransferase, cytosolic (Isoform 2)[a]	ACAT2	Q9BWD1-2	45	1		41		3	
16	Alpha-enolase	ENO1	P06733	47		6		173		19
17	Fructose-bisphosphate aldolase A	ALDOA	P04075	39		2		82		7
18	L-lactate dehydrogenase A chain	LDHA	P00338	37		3		92		10
19	L-lactate dehydrogenase B chain	LDHB	P07195	37	2	6	121	208	8	23
20	Malate dehydrogenase, cytoplasmic	MDH1	P40925	36		2		224		8
	Cytoskeletal and actin-binding proteins									
21	Alpha-actinin-1	ACTN1	P12814	103	13	23	475	604	17	31
22	Alpha-actinin-4	ACTN4	O43707	105	15	21	609	651	20	27
23	Cofilin-1	CFL1	P23528	19	2	2	37	60	14	15
	Cell shape and adhesion									
24	Protocadherin-1	PCDH1	Q08174	115		6		172		7
25	Transforming growth factor-beta-induced protein ig-h3	TGFBI	Q15582	75	3	2	105	44	6	4
	ECM and membrane proteins									
26	CD109 antigen	CD109	Q6YHK3	162	8	9	214	143	8	8
27	Metalloproteinase inhibitor 1	TIMP1	P01033	23	1	2	80	84	8	13
28	Tyrosine-protein kinase receptor	SDC4-ROS1_S4;R32	M1VKI3	83		2		32		4

	Miscellaneous									
29	Abnormal spindle-like microcephaly-associated protein	ASPM	Q8IZT6	410	1	1	48	55	1	1
30	Amyloid beta A4 protein	APP	P05067	87	3	3	116	187	4	5
31	Serotransferrin	TF	P02787	77	50	59	13622	11649	61	68

For protein entries where the unique peptide(s) identified belong to more than 1 specific protein, all proteins identified are listed.

[a]Proteins identified with 1 unique peptide using hrCNE but were also detected in either IP or pull-down assay with at least 2 unique peptides

Exp 1, Experiment 1; Exp 2, Experiment 2

Appendix II. HMGB1-binding proteins identified using immunoprecipitation (IP) coupled to LC-MS/MS.

Appendix II: HMGB1-binding proteins identified using immunoprecipitation (IP) coupled to LC-MS/MS.

#	Identified Proteins	Gene	Accession Number	MW (kDa)	Total unique peptide				Protein score		Sequence coverage (%)	
					HMGB1		Control					
					Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exo 2
	Nuclear structural proteins											
1	Histone H1.2	HIST1H1C	P16403	21	1	5			34	79	6	16
	Histone H1.3	HIST1H1D	P16402	22								
2	Histone H3.3	H3F3A/B	P84243	15		2				36		12
	Histone H3.1t	HIST3H3	Q16695	16								
3	Histone H1.5[a]	HIST1H1B	P16401	23	1				34		6	
4	Nucleophosmin	NPM1	P06748	33	2				90		8	
	Gene transcription											
5	High mobility group protein HMG-I/HMG-Y[a]	HMGA1	P17096	12		1				58		15
	Cell cycle and DNA repair											
6	Proliferating cell nuclear antigen	PCNA	P12004	29	3				207		12	
7	X-ray repair cross-complementing protein 6	XRCC6	P12956	70		3				107		8
	precursor mRNA processing spliceosome											
8	ATP-dependent RNA helicase DDX39A	DDX39A	O00148	49	4				60		11	
	Spliceosome RNA helicase DDX39B	DDX39B	Q13838	49								
9	Heterogeneous nuclear ribonucleoprotein A1	HNRNPA1	P09651	39	5				167		20	
10	Heterogeneous nuclear ribonucleoproteins A2/B1	HNRNPA2B1	P22626	37	4				150		14	
	Heterogeneous nuclear ribonucleoproteins C1/C2	HNRNPC	P07910	34								
11	Heterogeneous nuclear ribonucleoprotein C-like 1	HNRNPCL1	O60812	32	5	2			120	38	14	10
	Heterogeneous nuclear ribonucleoprotein C-like 4	HNRNPCL4	P0DMR1	32								
12	Heterogeneous nuclear ribonucleoprotein K	HNRNPK	P61978	51	7	1			186	42	26	4
13	Heterogeneous nuclear ribonucleoprotein U	HNRNPU	Q00839	91	5				119		10	
14	Probable ATP-dependent RNA helicase DDX5	DDX5	P17844	69	3				40		6	
	Ribosomal proteins											
15	40S ribosomal protein S2	RPS2	P15880	31	2				52		16	
16	40S ribosomal protein S3	RPS3	P23396	27	2				32		17	
17	40S ribosomal protein S13	RPS13	P62277	17	2				83		18	
18	40S ribosomal protein S18	RPS18	P62269	18	3				80		25	
19	40S ribosomal protein S20	RPS20	P60866	13	2				64		16	
20	40S ribosomal protein S24	RPS24	P62847	15	2				117		24	
21	60S ribosomal protein L4	RPL4	P36578	48	3				68		8	
22	60S ribosomal protein L12	RPL12	P30050	18	2				101		19	
23	60S ribosomal protein L27	RPL27	P61353	16	2				46		14	
24	60S acidic ribosomal protein P0	RPLP0	P05388	34	6				74		21	
	Protein synthesis											
25	Elongation factor 2	EEF2	P13639	95	6				110		7	
26	Eukaryotic initiation factor 4A-I	EIF4A1	P60842	46	7				213		19	
	Protein transport and secretory cargo											
27	ADP-ribosylation factor 3	ARF3	P61204	21	6				198		44	
28	ADP-ribosylation factor 4	ARF4	P18085	21	2				76		18	
29	Clathrin heavy chain 1	CLTC	Q00610	192	7				143		7	
30	Exportin-2	CSE1L	P55060	110	9				140		10	
31	Ras-related protein Rab-1B	RAB1B	Q9H0U4	22	2				117		15	

32	Transmembrane emp24 domain-containing protein 2	TMED2	Q15363	23	3			113		25		
33	Transmembrane emp24 domain-containing protein 5	TMED5	Q9Y3A6	26	2			93		13		
34	Transmembrane emp24 domain-containing protein 10	TMED10	P49755	25	4			127		22		
	Protein folding and ubiquitination											
35	Polyubiquitin-B	UBB	P0CG47	26	2			50		9		
	Polyubiquitin-C	UBC	P0CG48	77								
	Ubiquitin-60S ribosomal protein L40	UBA52	P62987	15								
	Ubiquitin-40S ribosomal protein S27a	RPS27A	P62979	18								
36	Ubiquitin-like modifier-activating enzyme 1	UBA1	P22314	118	2			61		4		
	Heat shock proteins and chaperones											
37	60 kDa heat shock protein, mitochondrial	HSPD1	P10809	61	8	2		578	110	20	8	
38	78 kDa glucose-regulated protein	HSPA5	P11021	72	1	3	1	100	80	3	7	
39	Endoplasmin[a]	HSP90B1	P14625	92	1			56		2		
40	hCG1639851, partial		gi|119603197	35	2			80		6		
41	Heat shock protein HSP 90-alpha	HSP90AA1	P07900	85	6	1		333	34	12	2	
42	Heat shock protein HSP 90-beta	HSP90AB1	P08238	83	14	2		468	73	22	4	
43	Heat shock 70 kDa protein 1A	HSPA1A	P0DMV8	70	5	2		199	49	5	2	
	Heat shock 70 kDa protein 6	HSPA6	P17066	71								
44	Heat shock cognate 71 kDa protein	HSPA8	P11142	71	6	3	1	305	65	20	20	
45	Stress-70 protein, mitochondrial	HSPA9	P38646	74	8	3		228	99	16	7	
	Immunity											
46	Annexin A1	ANXA1	P04083	39	3	1		171	94	13	5	
47	Complement component 1 Q subcomponent-binding protein, mitochondrial	C1QBP	Q07021	31		2			39		20	
48	High mobility group protein B1	HMGB1	P09429	25	2	8		91	517	26	40	
49	Proteasome activator complex subunit 2	PSME2	Q9UL46	27	2			80		14		
50	Proteasome activator complex subunit 1	PSME1	Q06323	29	3			58		15		
51	Protein S100-A11	S100A11	P31949	12	2			136		24		
	T-complex proteins											
52	T-complex protein 1 subunit alpha	TCP1	P17987	60	6			161		15		
53	T-complex protein 1 subunit beta	CCT2	P78371	57	8			279		20		
54	T-complex protein 1 subunit delta	CCT4	P50991	58	5			142		11		
55	T-complex protein 1 subunit epsilon	CCT5	P48643	60	4			106		10		
56	T-complex protein 1 subunit eta	CCT7	Q99832	59	3			136		7		
57	T-complex protein 1 subunit gamma	CCT3	P49368	61	5			97		10		
58	T-complex protein 1 subunit theta	CCT8	P50990	60	2			56		6		
59	T-complex protein 1 subunit zeta	CCT6A	P40227	58	6			128		11		
	Enzymes											
60	ATP synthase subunit alpha, mitochondrial	ATP5A1	P25705	60	8			245		20		
61	ATP synthase subunit beta, mitochondrial	ATP5B	P06576	57	10			337		29		
62	C-1-tetrahydrofolate synthase, cytoplasmic	MTHFD1	P11586	102	3			76		5		
63	Cytochrome c oxidase subunit 2	MT-CO2	P00403	26	2			76		11		
64	Peroxiredoxin-1	PRDX1	Q06830	22	2			45		12		
65	Peroxiredoxin-6	PRDX6	P30041	25	9		1	570		50		
66	Protein-glutamine gamma-glutamyltransferase 2	TGM2	P21980	77	3			75		7		
67	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	PPP2R1A	P30153	65	5			114		12		
68	cDNA FLJ57235, highly similar to Aldehyde dehydrogenase 1A3 (EC 1.2.1.5)		B4DYU3	49	2			52		6		
	Carbohydrate and lipid metabolism											
69	ATP-citrate synthase	ACLY	P53396	121	3			105		6		

70	Alpha-enolase	ENO1	P06733	47	6				422		20	
71	L-lactate dehydrogenase A chain	LDHA	P00338	37	2				91		8	
72	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	P04406	36	25	8			3512	265	75	39
73	Fatty acid synthase	FASN	P49327	273	10				92		6	
74	Apolipoprotein E	APOE	P02649	36	3				78		11	
75	Acetyl-CoA acetyltransferase, cytosolic (Isoform 2)	ACAT2	Q9BWD1-2	45	2				66		5	
	Cytoskeletal and actin-binding proteins											
76	Cofilin-1	CFL1	P23528	19	2				237		20	
77	F-actin-capping protein subunit alpha-1	CAPZA1	P52907	33	2				111		11	
78	Keratin, type I cuticular Ha5	KRT35	Q92764	50	3				82		3	
79	Keratin, type I cuticular Ha8	KRT38	O76015	50	2				49		2	
80	Myosin-9	MYH9	P35579	227	24	7			507	201	16	6
	Myosin-14	MYH14	Q7Z406	228								
81	Myosin light polypeptide 6	MYL6	P60660	17	2				54		19	
82	Myotilin	MYOT	Q9UBF9	55	2		1		74		5	
83	Tubulin alpha-1B chain	TUBA1B	P68363	50	11	2		1	941	71	36	8
84	Tubulin alpha-4A chain	TUBA4A	P68366	50	3				459		14	
85	Tubulin beta-2A chain	TUBB2A	Q13885	50	21	8			635	68	44	21
	Tubulin beta-8 chain	TUBB8	Q3ZCM7	50								
	cDNA FLJ11352 fis, clone HEMBA1000020, highly similar to Tubulin beta-2C chain		B3KML9	45								
	cDNA FLJ56903, highly similar to Tubulin beta-7 chain		B4DY90	52								
	Cell shape and adhesion											
86	Neuroblast differentiation-associated protein AHNAK	AHNAK	Q09666	629		3				88		1
87	Annexin A2	ANXA2	P07355	39		3				60		12
88	Ezrin	EZR	P15311	69	3				99		7	
	Tyrosine-protein kinase receptor	EZR-ROS1	J7M2B1	99								
89	Lectin galactosidase-binding soluble 3 binding protein[a]	LGALS3BP	Q08380	65	1				90		3	
90	Protein Shroom3	SHROOM3	Q8TF72	217	2				61		1	
91	Talin-1	TLN1	Q9Y490	270	4				65		3	
	Extracellular matrix and membrane proteins											
92	Serpin H1	SERPINH1	P50454	44	3				103		6	
93	Renin receptor	ATP6AP2	O75787	39	6	1			327	55	27	6
94	Fibronectin	FN1	P02751	263	14				386		9	
95	Protein CYR61	CYR61	O00622	42		2				45		6
96	Tyrosine-protein kinase receptor	SDC4-ROS1_S4;R32	M1VKI3	83		2				52		3
	Immunoglobulin											
97	Immunoglobulin heavy chain, partial[b]		gi|578468798	35	18	1				55	19	4
98	immunoglobulin M heavy chain variable region		gi|281186004	11	2				52		18	
99	Immunoglobulin kappa chain variable region, partial		gi|77379536	11	2				115		15	
100	Immunoglobulin kappa light chain variable region		gi|98956304	12	3				174		15	
101	Immunoglobulin kappa light chain variable region		gi|27369027	12	2				93		12	
102	Immunoglobulin kappa light chain variable region[b]		gi|5833869	11	2						23	
103	Immunoglobulin kappa light chain variable region[b]		gi|4323876	11	2						16	
104	Immunoglobulin kappa light chain variable region, partial[b]		gi|77378148	13	2						17	
105	Immunoglobulin kappa light chain variable region, partial		gi|77378142	13	2				98		14	
106	Immunoglobulin light chain		gi|1890132	12	1	1			35	41	15	15
107	Immunoglobulin light chain variable region[b]		gi|13549148	12	2						13	
108	Immunoglobulin variable kappa chain		gi|95007517	11	2				168		15	

	Miscellaneous							
109	ADP/ATP translocase 2	SLC25A5	P05141	33	2		130	10
110	ADP/ATP translocase 3	SLC25A6	P12236	33	3		141	13
111	Sterile alpha motif domain-containing protein 9[b]	SAMD9	Q5K651	184	2			1

For protein entries where the unique peptide(s) identified belong to more than 1 specific protein, all proteins identified are listed.

[a]Proteins identified with 1 unique peptide using IP but were also detected in either hrCNE or pull-down assay with at least 2 unique peptides

[b]Proteins with at least 1 unique peptide identified exclusively using X! Tandem, hence no protein score is available

Exp 1, Experiment 1; Exp 2, Experiment 2

Appendix III. HMGB1-binding proteins identified using pull-down assay coupled to LC-MS/MS.

Appendix III: HMGB1-binding proteins identified using pull-down assay coupled to LC-MS/MS.

#	Identified Proteins	Gene	Accession Number	MW (kDa)	Total unique peptide				Protein score		Sequence coverage (%)	
					HMGB1		Control					
					Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exo 2
	Nuclear structural proteins											
1	Histone H1.2	HIST1H1C	P16403	21	12	2			533	130	23	7
	Histone H1.3	HIST1H1D	P16402	22								
2	Histone H1.5	HIST1H1B	P16401	23	7				314		22	
3	Putative histone H2B type 2-C	HIST2H2BC	Q6DN03	21	2				314		6	
	Putative histone H2B type 2-D	HIST2H2BD	Q6DRA6	18								
4	Histone H3.3	H3F3A/B	P84243	15	2				73		12	
	Histone H3.1t	HIST3H3	Q16695	16								
5	Nucleolin	NCL	P19338	77	32	11			2034	996	26	18
6	Nucleophosmin	NPM1	P06748	33	5	4			612	184	24	21
7	Nucleosome assembly protein 1-like 1	NAP1L1	P55209	45	4	3			139	131	12	16
	Gene transcription											
8	Acidic leucine-rich nuclear phosphoprotein 32 family member A	ANP32A	P39687	29	8	3			320	192	27	10
9	FACT complex subunit SPT16	SUPT16H	Q9Y5B9	120	7				149		9	
10	FACT complex subunit SSRP1	SSRP1	Q08945	81	2				58		3	
11	High mobility group protein HMG-I/HMG-Y	HMGA1	P17096	12	2				103		33	
12	Histone-binding protein RBBP4	RBBP4	Q09028	48	2				46		7	
13	Nuclease-sensitive element-binding protein 1	YBX1	P67809	36	2	2			134	113	11	11
14	Protein SET	SET	Q01105	33	13				1813		47	
	Cell cycle and DNA repair											
15	Acidic leucine-rich nuclear phosphoprotein 32 family member B	ANP32B	Q92688	29	9	4			416	302	41	18
16	Acidic leucine-rich nuclear phosphoprotein 32 family member E	ANP32E	Q9BTT0	31	3	2			121	92	12	12
17	X-ray repair cross-complementing protein 5	XRCC5	P13010	83	17	5			447	122	23	8
18	X-ray repair cross-complementing protein 6	XRCC6	P12956	70	25	9			636	280	46	17
	precursor mRNA processing spliceosome											
19	116 kDa U5 small nuclear ribonucleoprotein component	EFTUD2 SNRP116	Q15029	109	2				76		4	
20	Small nuclear ribonucleoprotein E	SNRPE	P62304	11	2				74		25	
21	Small nuclear ribonucleoprotein Sm D1	SNRPD1	P62314	13	2	1			56	43	20	11
	Heterogeneous nuclear ribonucleoproteins C1/C2	HNRNPC	P07910	34								
22	Heterogeneous nuclear ribonucleoprotein C-like 1	HNRNPCL1	O60812	32	9	2			285	124	31	6
	Heterogeneous nuclear ribonucleoprotein C-like 4	HNRNPCL4	P0DMR1	32								
	Ribosomal proteins											
23	40S ribosomal protein S3a	RPS3A	P61247	30	3				41		14	
24	40S ribosomal protein S4, X isoform	RPS4X	P62701	30	3				103		20	
25	40S ribosomal protein S7	RPS7	P62081	22	4				73		27	
26	40S ribosomal protein S19	RPS19	P39019	16	3				89		22	
27	40S ribosomal protein S25	RPS25	P62851	14	2				79		15	
28	60S ribosomal protein L5	RPL5	P46777	34	3				56		10	
29	60S ribosomal protein L10a	RPL10A	P62906	25	2				50		10	
30	60S ribosomal protein L12	RPL12	P30050	18	5	3			224	72	43	24
31	60S ribosomal protein L22	RPL22	P35268	15	2	1			161	56	19	28
32	60S ribosomal protein L23a	RPL23A	P62750	18	4				188		15	
33	60S ribosomal protein L27[a]	RPL27	P61353	16	1				42		7	
34	60S ribosomal protein L30	RPL30	P62888	13	2				71		24	
35	60S ribosomal protein L31	RPL31	P62899	14	2				47		11	
36	60S ribosomal protein L35	RPL35	P42766	15	2				66		23	
37	60S acidic ribosomal protein P0	RPLP0	P05388	34	11	2			295	122	39	7

38	60S acidic ribosomal protein P1	RPLP1	P05386	12	1	1	57	83	14	15
39	60S acidic ribosomal protein P2	RPLP2	P05387	12	6	5	323	259	70	84
	Protein synthesis									
40	Elongation factor 1-beta	EEF1B2	P24534	25	2	1	120	82	12	7
41	Elongation factor 1-delta	EEF1D	P29692	31	5		133		33	
42	Elongation factor 1-gamma	EEF1G	P26641	50	3	3	148	118	8	12
43	Eukaryotic translation initiation factor 3 subunit C	EIF3C	Q99613	105	3		155		4	
44	Eukaryotic translation initiation factor 3 subunit F	EIF3F	O00303	38	2		121		9	
	Protein transport and secretory cargo									
45	General vesicular transport factor p115	USO1	O60763	108	5		176		7	
	Immunity									
46	CD44 antigen	CD44	P16070	82	2	1	147	87	3	5
47	High mobility group protein B1	HMGB1	P09429	25	34	12	3795	1460	58	38
48	Beta-2-microglobulin[a]	B2M	P61769	14	1		52		14	
	Cytoskeletal and actin-binding proteins									
49	Alpha-actinin-1[a]	ACTN1	P12814	103		1		44		2
50	Alpha-actinin-4[a]	ACTN4	O43707	105		1		44		2
51	Myosin-9	MYH9	P35579	227	45		1381		25	
	Myosin-14	MYH14	Q7Z406	228						
52	Myosin light polypeptide 6	MYL6	P60660	17	6		203		48	
53	Myosin regulatory light chain 12A	MYL12A	P19105	20	4		123		28	
	Myosin regulatory light chain 12B	MYL12B	O14950	20						
54	Tubulin alpha-1B chain	TUBA1B	P68363	50	4	5	119	159	14	18
	Tubulin beta-2A chain	Q13885	TUBB2A	50						
55	Tubulin beta-8 chain	Q3ZCM7	TUBB8	50	3	6	78	194	8	11
	cDNA FLJ11352 fis, clone HEMBA1000020, highly similar to Tubulin beta-2C chain		B3KML9	45						
	cDNA FLJ56903, highly similar to Tubulin beta-7 chain		B4DY90	52						
	Heat shock proteins and chaperones									
56	60 kDa heat shock protein, mitochondrial[a]	HSPD1	P10809	61	1		88		4	
57	78 kDa glucose-regulated protein	HSPA5	P11021	72	8		271		15	
58	Endoplasmin	HSP90B1	P14625	92	33	23	1346	1123	41	35
59	Heat shock cognate 71 kDa protein	HSPA8	P11142	71	2		73		4	
60	Heat shock protein HSP 90-alpha	HSP90AA1	P07900	85	1	1	44	92	2	2
61	Heat shock protein HSP 90-beta	HSP90AB1	P08238	83	2	2	202	196	4	4
	ECM and membrane proteins									
62	Agrin - isoform 6	AGRN	O00468-6	215	30	23	1185	1334	17	29
63	Fibronectin	FN1	P02751	263		21		1599		12
64	Laminin subunit alpha-3	LAMA3	Q16787	367	5		148		3	
65	Laminin subunit alpha-5	LAMA5	O15230	400	16	2	529	139	7	2
66	Laminin subunit beta-1	LAMB1	P07942	198	14		412		10	
67	Laminin subunit gamma-1	LAMC1	P11047	178	15		432		12	
68	Laminin subunit gamma-2	LAMC2	Q13753	131	7	2	172	63	8	2
69	Nidogen-1	NID1	P14543	136	5		174		8	
70	Protein CYR61	CYR61	O00622	42	8	4	160	151	21	12
71	Tyrosine-protein kinase receptor	SDC4-ROS1_S4;R32	M1VKI3	83	14	4	2195	752	9	6
	Cell shape and adhesion									
72	Annexin A2	ANXA2	P07355	39	12	2	312	147	39	9
73	Fibroblast growth factor-binding protein 1	FGFBP1	Q14512	26	1	1	131	136	6	6
74	Lectin galactosidase-binding soluble 3 binding protein	LGALS3BP	Q08380	65	10	9	361	484	17	20
	Carbohydrate and lipid metabolism									

75	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	P04406	36	2			86		9		
	Protein folding and ubiquitination											
76	Neutral alpha-glucosidase AB	GANAB	Q14697	107	1	1		30	48	1	2	
77	Polyubiquitin-B[a]	UBB	P0CG47	26	1			44		6		
	Polyubiquitin-C	UBC	P0CG48	77								
	Ubiquitin-60S ribosomal protein L40	UBA52	P62987	15								
	Ubiquitin-40S ribosomal protein S27a	RPS27A	P62979	18								
78	Protein disulfide-isomerase A4	PDIA4	P13667	73		7			163		14	
79	Protein disulfide-isomerase A6	PDIA6	Q15084	48	2	4		80	174	7	13	
80	Transitional endoplasmic reticulum ATPase	VCP	P55072	89	24	7		650	193	36	11	
	Enzymes											
81	Casein kinase II subunit alpha	CSNK2A1	P68400	45	7			222		23		
	Casein kinase II subunit alpha 3	CSNK2A3	Q8NEV1	45								
82	Casein kinase II subunit alpha'	CSNK2A2	P19784	41	2			81		7		
83	Urokinase-type plasminogen activator	PLAU	P00749	49		5			197		20	
84	Protein-glutamine gamma-glutamyltransferase 2[a]	TGM2	P21980	77		1			92		3	
	Miscellaneous											
85	Amyloid beta A4 protein	APP	P05067	87	25	14		1476	790	26	22	
86	Amyloid-like protein 2	APLP2	Q06481	87	9			283		15		
87	Calmodulin	CALM1	P62158	17	4	2		476	258	46	26	
88	Reticulon-4	RTN4	Q9NQC3	130	2			87		3		
89	Single-stranded DNA-binding protein, mitochondrial	SSBP1	Q04837	17	4			92		38		

For protein entries where the unique peptide(s) identified belong to more than 1 specific protein, all proteins identified are listed.

[a]Proteins identified with 1 unique peptide using pull-down assay but were also detected in either hrCNE or IP with at least 2 unique peptides

Exp 1, Experiment 1; Exp 2, Experiment 2

Appendix IV. Evidence of interaction and confidence score for identified primary interactors of HMGB1.

Appendix IV: Evidence of interaction and confidence score for identified primary interactors of HMGB1.

Node 1	Node 2	Node 1 (External ID)	Node 2 (External ID)	Experimentally determined interaction	Coexpression	Automated textmining	Database annotated	Homology	Gene fusion	Neighborhood on chromosome	Phylogenetic cooccurrence	Combined score
UBC	HMGB1	9606.ENSP00000344818	9606.ENSP00000343040	0.958	0.075	0.071	0	0	0	0	0	0.961
HMGB1	SUPT16H	9606.ENSP00000343040	9606.ENSP00000216297	0.629	0	0.87	0	0	0	0	0	0.949
HMGB1	CAPZA1	9606.ENSP00000343040	9606.ENSP00000263168	0	0	0.128	0.9	0	0	0	0	0.909
HMGB1	APP	9606.ENSP00000343040	9606.ENSP00000284981	0	0	0.113	0.9	0	0	0	0	0.907
HMGB1	SSRP1	9606.ENSP00000343040	9606.ENSP00000278412	0.792	0.073	0.725	0	0.731	0	0	0	0.837
HMGB1	HSPA8	9606.ENSP00000343040	9606.ENSP00000227378	0.576	0	0.267	0	0	0	0	0	0.675
HMGB1	HMGA1	9606.ENSP00000343040	9606.ENSP00000308227	0.376	0	0.439	0	0	0	0	0	0.635
HNRNPK	HMGB1	9606.ENSP00000365439	9606.ENSP00000343040	0.457	0	0.191	0	0	0	0	0	0.541
HMGB1	GAPDH	9606.ENSP00000343040	9606.ENSP00000229239	0	0	0.503	0	0	0	0	0	0.503
XRCC6	HMGB1	9606.ENSP00000352257	9606.ENSP00000343040	0.297	0	0.31	0	0	0	0	0	0.494
HMGB1	H3F3B	9606.ENSP00000343040	9606.ENSP00000254810	0.363	0	0.154	0	0	0	0	0	0.438
HMGB1	HSP90B1	9606.ENSP00000343040	9606.ENSP00000299767	0	0.077	0.417	0	0	0	0	0	0.438
XRCC5	HMGB1	9606.ENSP00000375977	9606.ENSP00000343040	0.213	0	0.291	0	0	0	0	0	0.418
HMGB1	HSP90AA1	9606.ENSP00000343040	9606.ENSP00000335153	0.075	0	0.391	0	0	0	0	0	0.412

Appendix V. Gene ontology term analysis (cellular component) of the 37 HMGB1-binding proteins identified using 2 or more techniques.

Appendix V: Gene ontology term analysis (Cellular component) of the 37 HMGB1-binding proteins identified using 2 or more techniques.

Pathway ID	Pathway description	Observed gene count	False discovery rate	Matching proteins in your network (labels)
GO.0043227	membrane-bounded organelle	39	7.16E-05	ACAT2,ACTN1,ACTN4,APP,CFL1,ENO1,FN1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HNRNPC,HNRNPCL1, HSP90AA1,HSP90AB1,HSPA5,HSPA6,HSPA8,HSPD1,LGALS3BP,MYH14,MYH9,MYL6,NPM1,RPL12,RPL27,RPLP0,RPS27A,TGM2,TUBA1B, TUBB2A,TUBB8,UBB,UBC,XRCC6
GO.0044421	extracellular region part	37	1.79E-18	ACAT2,ACTN1,ACTN4,ANXA2,APP,CFL1,CYR61,ENO1,FN1,GAPDH,H3F3B,HIST1H1B,HIST3H3,HMGB1,HNRNPC,HSP90AA1,HSP90AB1, HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1,LDHA,LGALS3BP,MYH14,MYH9,MYL6,RPL12,RPL27,RPLP0,RPS27A,TGM2,TUBA1B,TUBB2A,TUBB8, UBB,UBC
GO.0005576	extracellular region	37	6.04E-16	ACAT2,ACTN1,ACTN4,ANXA2,APP,CFL1,CYR61,ENO1,FN1,GAPDH,H3F3B,HIST1H1B,HIST3H3,HMGB1,HNRNPC,HSP90AA1,HSP90AB1, HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1,LDHA,LGALS3BP,MYH14,MYH9,MYL6,RPL12,RPL27,RPLP0,RPS27A,TGM2,TUBA1B,TUBB2A,TUBB8, UBB,UBC
GO.0070062	extracellular exosome	35	8.27E-20	ACAT2,ACTN1,ACTN4,ANXA2,APP,CFL1,ENO1,FN1,GAPDH,H3F3B,HIST1H1B,HIST3H3,HNRNPC,HSP90AA1,HSP90AB1,HSP90B1,HSPA5, HSPA6,HSPA8,HSPD1,LDHA,LGALS3BP,MYH14,MYH9,MYL6,RPL12,RPL27,RPLP0,RPS27A,TGM2,TUBA1B,TUBB2A,TUBB8,UBB,UBC
GO.0031988	membrane-bounded vesicle	34	6.93E-16	ACAT2,ACTN1,ACTN4,ANXA2,CFL1,ENO1,FN1,GAPDH,H3F3B,HIST1H1B,HIST3H3,HNRNPC,HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA6, HSPA8,HSPD1,LDHA,LGALS3BP,MYH14,MYH9,MYL6,RPL12,RPL27,RPLP0,RPS27A,TGM2,TUBA1B,TUBB2A,TUBB8,UBB,UBC
GO.0005634	nucleus	32	3.78E-06	ACAT2,ACTN4,ANXA2,APP,CFL1,ENO1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HNRNPC,HNRNPCL1, HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA8,LDHA,MYH9,NPM1,RPL27,RPLP0,RPS27A,TUBB2A,UBA52,UBB,UBC,XRCC6
GO.0044422	organelle part	32	7.04E-05	ACAT2,ACTN1,ANXA2,APP,B2M,CFL1,ENO1,FN1,GAPDH,H3F3B,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AA1,HSP90AB1,HSPA5,HSPA6, HSPA8,HSPD1,LDHA,MYL6,NPM1,RPL12,RPL27,RPLP0,RPS27A,TUBB2A,TUBB8,UBB,UBC,XRCC6
GO.0043231	intracellular membrane-bounded organelle	32	0.0216	ACAT2,ACTN1,ACTN4,APP,CFL1,ENO1,FN1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HNRNPC,HNRNPCL1, HSP90AA1,HSP90AB1,HSPA5,HSPA8,HSPD1,MYH9,NPM1,RPL27,RPLP0,RPS27A,TGM2,TUBB2A,UBB,UBC,XRCC6
GO.0044446	intracellular organelle part	30	0.000458	ACAT2,ACTN1,ANXA2,APP,B2M,CFL1,FN1,GAPDH,H3F3B,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AA1,HSP90AB1,HSPA5,HSPA6,HSPA8, HSPD1,MYL6,NPM1,RPL12,RPL27,RPLP0,RPS27A,TUBB2A,TUBB8,UBB,UBC,XRCC6
GO.0043233	organelle lumen	27	2.16E-07	ACAT2,ACTN1,ACTN4,APP,B2M,CFL1,FN1,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AA1,HSP90AB1, HSP90B1,HSPA5,HSPA8,HSPD1,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0032991	macromolecular complex	27	1.20E-06	ACTN4,ANXA2,APP,B2M,ENO1,FN1,GAPDH,H3F3B,HIST3H3,HMGA1,HNRNPC,HNRNPCL1,HSP90B1,HSPA5,HSPA8,HSPD1,MYH14,MYH9, MYL6,NPM1,RPL12,RPL27,RPLP0,RPS27A,TUBB2A,TUBB8,XRCC6
GO.0044444	cytoplasmic part	26	0.0156	ACTN1,CFL1,FN1,GAPDH,HMGA1,HNRNPC,HSP90AA1,HSP90AB1,HSPA5,HSPA6,HSPA8,HSPD1,MYH14,MYH9,MYL6,NPM1,ROS1,RPL12, RPL27,RPLP0,RPS27A,TGM2,TUBA1B,UBB,UBC,XRCC6
GO.0005829	cytosol	24	2.47E-07	ACTN1,APP,GAPDH,HMGA1,HNRNPC,HSP90AA1,HSP90AB1,HSP90B1,HSPA6,HSPA8,HSPD1,LDHA,MYH14,MYH9,MYL6,NPM1,RPL12,RPL27, RPLP0,RPS27A,TGM2,UBB,UBC,XRCC6
GO.0043232	intracellular non-membrane-bounded organelle	24	1.20E-06	ACAT2,ACTN1,ACTN4,ANXA2,APP,CFL1,ENO1,GAPDH,H3F3B,HIST3H3,HMGA1,HMGB1,HNRNPC,HSPA6,HSPA8,MYL6,NPM1,RPL12,RPL27, RPLP0,RPS27A,TUBB2A,TUBB8,XRCC6
GO.0043234	protein complex	24	6.68E-06	ACTN4,ANXA2,APP,B2M,ENO1,FN1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HNRNPC,HSP90B1,HSPA5,HSPA8, HSPD1,MYH14,MYH9,MYL6,TUBB2A,TUBB8,XRCC6

GO.0070013	intracellular organelle lumen	24	1.42E-05	ACAT2,APP,B2M,CFL1,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AA1,HSP90AB1,HSP90B1,HSPA5, HSPA8,HSPD1,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0044428	nuclear part	21	0.00017	ACAT2,APP,CFL1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AA1,HSP90AB1,HSPA8,NPM1, RPS27A,UBA52,UBB,UBC,XRCC6
GO.0071944	cell periphery	20	0.0102	ACTN1,ACTN4,ANXA2,APP,CFL1,ENO1,FN1,GAPDH,HSP90AB1,HSP90B1,HSPA5,HSPA8,HSPD1,MYH9,ROS1,RPS27A,TGM2,UBA52,UBB, UBC
GO.0031981	nuclear lumen	19	0.000647	ACAT2,CFL1,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AA1,HSP90AB1,HSPA8,NPM1,RPS27A, UBA52,UBB,UBC,XRCC6
GO.0005886	plasma membrane	19	0.0207	ACTN1,ANXA2,APP,CFL1,ENO1,FN1,GAPDH,HSP90AB1,HSP90B1,HSPA5,HSPA8,HSPD1,MYH9,ROS1,RPS27A,TGM2,UBA52,UBB,UBC
GO.0016023	cytoplasmic membrane-bounded vesicle	15	1.36E-07	ACTN1,ACTN4,ANXA2,B2M,FN1,HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA8,HSPD1,RPS27A,UBA52,UBB,UBC
GO.0005654	nucleoplasm	15	0.0113	ACAT2,H3F3B,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AA1,HSP90AB1,HSPA8,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0005925	focal adhesion	13	1.15E-10	ACTN1,ACTN4,CFL1,HMGA1,HSP90B1,HSPA5,HSPA8,MYH9,NPM1,RPL12,RPL27,RPLP0,TGM2
GO.0005912	adherens junction	12	1.06E-08	ACTN4,CFL1,HMGA1,HSP90B1,HSPA5,HSPA8,MYH9,NPM1,RPL12,RPL27,RPLP0,TGM2
GO.0044433	cytoplasmic vesicle part	12	8.91E-08	ACTN1,ACTN4,APP,B2M,FN1,HSP90AA1,HSP90B1,HSPA8,RPS27A,UBA52,UBB,UBC
GO.0005615	extracellular space	12	0.000308	ACTN1,ACTN4,ANXA2,APP,CFL1,ENO1,FN1,HMGB1,HSPA6,HSPA8,HSPD1,LGALS3BP
GO.0098805	whole membrane	12	0.00826	ANXA2,APP,B2M,CFL1,FN1,HSP90AA1,HSP90AB1,HSPA8,HSPD1,RPS27A,UBB,UBC
GO.0030054	cell junction	11	0.000425	APP,HMGA1,HSP90B1,HSPA5,HSPA8,MYH9,NPM1,RPL12,RPL27,RPLP0,TGM2
GO.0030529	ribonucleoprotein complex	10	4.08E-05	ACTN4,GAPDH,HNRNPC,HNRNPCL1,HSPA8,NPM1,RPL12,RPL27,RPLP0,RPS27A
GO.0009986	cell surface	10	7.16E-05	ANXA2,APP,B2M,HMGB1,HSP90AB1,HSPA5,HSPD1,MYH9,NPM1,ROS1
GO.0044430	cytoskeletal part	9	0.029	ACTN1,ACTN4,APP,CFL1,HSPA6,MYL6,NPM1,TUBB2A,TUBB8
GO.0044454	nuclear chromosome part	8	4.15E-05	H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HNRNPC,NPM1,XRCC6

GO.0000228	nuclear chromosome	8	8.76E-05	H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HNRNPC,NPM1,XRCC6
GO.0043209	myelin sheath	7	3.58E-06	ANXA2,HSP90AA1,HSPA5,HSPA8,HSPD1,MYH14,TUBA1B
GO.0030139	endocytic vesicle	7	2.33E-05	B2M,HSP90AA1,HSP90B1,RPS27A,UBA52,UBB,UBC
GO.0000790	nuclear chromatin	7	4.49E-05	H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HNRNPC,NPM1
GO.0044440	endosomal part	7	0.000647	ANXA2,APP,B2M,RPS27A,UBA52,UBB,UBC
GO.0005694	chromosome	7	0.0164	H3F3B,HIST3H3,HMGA1,HMGB1,HNRNPC,NPM1,XRCC6
GO.0005768	endosome	7	0.0184	APP,B2M,HSPD1,RPS27A,UBA52,UBB,UBC
GO.0060205	cytoplasmic membrane-bounded vesicle lumen	6	2.17E-06	ACTN1,ACTN4,APP,FN1,HSP90AA1,HSP90B1
GO.0042470	melanosome	6	5.16E-06	ANXA2,HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA8
GO.0032993	protein-DNA complex	6	2.97E-05	H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,XRCC6
GO.0010008	endosome membrane	6	0.00402	ANXA2,B2M,RPS27A,UBA52,UBB,UBC
GO.0030659	cytoplasmic vesicle membrane	6	0.00506	B2M,HSPA8,RPS27A,UBA52,UBB,UBC
GO.0015629	actin cytoskeleton	6	0.00526	ACTN1,ACTN4,CFL1,MYH9,MYL6,NPM1
GO.0048471	perinuclear region of cytoplasm	6	0.0366	ACTN4,ANXA2,APP,GAPDH,HSP90B1,ROS1
GO.0044427	chromosomal part	6	0.043	H3F3B,HIST3H3,HMGA1,HNRNPC,NPM1,XRCC6
GO.0000786	nucleosome	5	5.65E-05	H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3
GO.0005903	brush border	5	7.38E-05	ACTN1,HSP90AB1,MYH14,MYH9,MYL6

GO.0030666	endocytic vesicle membrane	5	0.000273	B2M,RPS27A,UBA52,UBB,UBC
GO.0001726	ruffle	5	0.000586	ACTN1,ANXA2,CFL1,HSP90AA1,MYH9
GO.0044445	cytosolic part	5	0.00102	ENO1,RPL12,RPL27,RPLP0,RPS27A
GO.0030141	secretory granule	5	0.0105	ACTN1,ACTN4,APP,FN1,HSPD1
GO.0031252	cell leading edge	5	0.0161	ACTN1,ANXA2,CFL1,HSP90AA1,MYH9
GO.0000785	chromatin	5	0.0281	H3F3B,HIST3H3,HMGA1,HNRNPC,NPM1
GO.0001725	stress fiber	4	0.000108	ACTN1,ACTN4,MYH14,MYH9
GO.0031093	platelet alpha granule lumen	4	0.000151	ACTN1,ACTN4,APP,FN1
GO.0031091	platelet alpha granule	4	0.000332	ACTN1,ACTN4,APP,FN1
GO.0022626	cytosolic ribosome	4	0.00128	RPL12,RPL27,RPLP0,RPS27A
GO.0072562	blood microparticle	4	0.00261	FN1,HSPA6,HSPA8,LGALS3BP
GO.0044391	ribosomal subunit	4	0.00506	RPL12,RPL27,RPLP0,RPS27A
GO.0030135	coated vesicle	4	0.012	APP,B2M,HSPA8,HSPD1
GO.0005938	cell cortex	4	0.0207	ACTN4,ANXA2,CFL1,MYH9
GO.0022625	cytosolic large ribosomal subunit	3	0.00432	RPL12,RPL27,RPLP0
GO.0042641	actomyosin	3	0.00525	ACTN1,ACTN4,MYH9
GO.0016459	myosin complex	3	0.00955	MYH14,MYH9,MYL6

GO.0030863	cortical cytoskeleton	3	0.00985	ACTN4,CFL1,MYH9
GO.0030496	midbody	3	0.0445	ANXA2,HSP90B1,HSPA5
GO.0097513	myosin II filament	2	0.000458	MYH14,MYH9
GO.0034663	endoplasmic reticulum chaperone complex	2	0.00656	HSP90B1,HSPA5
GO.0031143	pseudopodium	2	0.0113	ACTN1,ACTN4
GO.0031904	endosome lumen	2	0.0138	APP,B2M
GO.0071682	endocytic vesicle lumen	2	0.0138	HSP90AA1,HSP90B1
GO.0000788	nuclear nucleosome	2	0.0243	H3F3B,HIST3H3
GO.0005719	nuclear euchromatin	2	0.0243	HIST1H1C,HIST1H1D
GO.0016460	myosin II complex	2	0.0258	MYH14,MYH9
GO.0005790	smooth endoplasmic reticulum	2	0.0426	APP,HSPA5

Appendix VI. Gene ontology term analysis (molecular function) of the 37 HMGB1-binding proteins identified using 2 or more techniques.

Appendix VI: Gene ontology term analysis (Molecular function) of the 37 HMGB1-binding proteins identified using 2 or more techniques.

Pathway ID	Pathway description	Observed gene count	False discovery rate	Matching proteins in your network (labels)
GO.0005488	binding	37	0.00037	ANXA2,APP,B2M,FN1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AA1,HSP90B1,HSPA5,HSPA6, HSPA8,HSPD1,LDHA,MYH14,MYH9,MYL6,NPM1,ROS1,RPL12,RPL27,RPLP0,RPS27A,TGM2,TUBA1B,TUBB2A,TUBB8,UBA52,UBB,UBC,XRCC6
GO.1901363	heterocyclic compound binding	35	7.12E-10	ACTN1,ACTN4,ANXA2,APP,ENO1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AA1,HSP90B1, HSPA5,HSPA6,HSPA8,HSPD1,LDHA,MYH14,MYH9,ROS1,RPL12,RPL27,RPLP0,RPS27A,TUBA1B,TUBB2A,TUBB8,UBA52,UBB,UBC,XRCC6
GO.0097159	organic cyclic compound binding	35	8.04E-10	ACTN1,ACTN4,ANXA2,APP,ENO1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AA1,HSP90B1, HSPA5,HSPA6,HSPA8,HSPD1,LDHA,MYH14,MYH9,ROS1,RPL12,RPL27,RPLP0,RPS27A,TUBA1B,TUBB2A,TUBB8,UBA52,UBB,UBC,XRCC6
GO.0005515	protein binding	31	8.14E-09	ACTN1,ACTN4,ANXA2,APP,B2M,CYR61,ENO1,FN1,GAPDH,H3F3B,HIST1H1B,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AA1,HSP90AB1, HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1,MYH9,NPM1,ROS1,RPS27A,TUBA1B,UBA52,UBB,UBC,XRCC6
GO.0003676	nucleic acid binding	29	4.06E-09	ACTN1,ACTN4,ANXA2,APP,ENO1,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HNRNPC,HNRNPCL1,HSP90AA1, HSP90AB1,HSP90B1,HSPA8,HSPD1,MYH9,RPL12,RPL27,RPLP0,RPS27A,TUBA1B,UBA52,UBB,UBC,XRCC6
GO.0003723	RNA binding	26	8.51E-15	ACTN1,ACTN4,ANXA2,ENO1,HIST1H1B,HIST1H1C,HIST1H1D,HMGB1,HNRNPC,HNRNPCL1,HSP90AA1,HSP90AB1,HSP90B1,HSPA8, HSPD1,MYH9,NPM1,RPL12,RPL27,RPLP0,RPS27A,TUBA1B,UBA52,UBB,UBC,XRCC6
GO.0043167	ion binding	24	0.0316	ACTN1,ACTN4,APP,CYR61,ENO1,FN1,HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1,MYH14,MYH9,MYL6,NPM1,ROS1, RPS27A,TGM2,TUBA1B,TUBB2A,TUBB8,XRCC6
GO.0044822	poly(A) RNA binding	23	3.75E-14	ACTN4,ANXA2,ENO1,HIST1H1B,HIST1H1C,HIST1H1D,HMGB1,HNRNPC,HNRNPCL1,HSP90AA1,HSP90AB1,HSPA8,HSPD1,MYH9,NPM1, RPL12,RPL27,RPLP0,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0036094	small molecule binding	20	2.37E-05	ACTN4,GAPDH,HNRNPC,HNRNPCL1,HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1,LDHA,MYH14,MYH9,NPM1,ROS1, TUBA1B,TUBB2A,TUBB8,XRCC6
GO.0019899	enzyme binding	19	4.72E-09	APP,ENO1,FN1,HIST1H1B,HMGA1,HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1,NPM1,ROS1,RPS27A,TUBA1B,UBA52, UBB,UBC
GO.0097367	carbohydrate derivative binding	19	1.08E-05	ACTN4,APP,B2M,CYR61,FN1,HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1,MYH14,MYH9,ROS1,TUBA1B,TUBB2A,TUBB8, XRCC6
GO.0043168	anion binding	19	0.000133	ANXA2,APP,CYR61,FN1,HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1,MYH14,MYH9,NPM1,ROS1,TUBA1B,TUBB2A, TUBB8,XRCC6
GO.0000166	nucleotide binding	18	0.000116	GAPDH,HNRNPC,HNRNPCL1,HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1,LDHA,MYH14,MYH9,ROS1,TUBA1B,TUBB2A, TUBB8,XRCC6
GO.0044877	macromolecular complex binding	17	1.46E-08	ACTN1,ACTN4,CYR61,FN1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGB1,HNRNPC,HSP90AA1,HSP90AB1,HSPA5,HSPA8, MYH9,NPM1
GO.0001882	nucleoside binding	15	0.000577	ACTN4,HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1,MYH14,MYH9,ROS1,TUBA1B,TUBB2A,TUBB8,XRCC6
GO.0032550	purine ribonucleoside binding	14	0.00209	HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1,MYH14,MYH9,ROS1,TUBA1B,TUBB2A,TUBB8,XRCC6

GO.0035639	purine ribonucleoside triphosphate binding	14	0.00209	HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1,MYH14,MYH9,ROS1,TUBA1B,TUBB2A,TUBB8,XRCC6
GO.0032555	purine ribonucleotide binding	14	0.00241	HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1,MYH14,MYH9,ROS1,TUBA1B,TUBB2A,TUBB8,XRCC6
GO.0016787	hydrolase activity	13	0.0423	HMGA1,HSP90AA1,HSPA5,HSPA6,HSPA8,HSPD1,MYH14,MYH9,MYL6,TUBA1B,TUBB2A,TUBB8,XRCC6
GO.0017111	nucleoside-triphosphatase activity	12	7.69E-06	HSP90AA1,HSPA5,HSPA6,HSPA8,HSPD1,MYH14,MYH9,MYL6,TUBA1B,TUBB2A,TUBB8,XRCC6
GO.0032403	protein complex binding	11	5.21E-06	ACTN1,ACTN4,CYR61,FN1,GAPDH,H3F3B,HNRNPC,HSP90AA1,HSP90AB1,HSPA8,MYH9
GO.0005524	ATP binding	11	0.0188	HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1,MYH14,MYH9,ROS1,XRCC6
GO.0043566	structure-specific DNA binding	10	1.71E-08	ACTN4,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGB1,HNRNPC,HSPD1,NPM1,XRCC6
GO.0005102	receptor binding	10	0.0076	ACTN1,ACTN4,APP,CYR61,FN1,HMGA1,HMGB1,HSP90B1,HSPA8,NPM1
GO.0016887	ATPase activity	9	1.61E-05	HSP90AA1,HSPA5,HSPA6,HSPA8,HSPD1,MYH14,MYH9,MYL6,XRCC6
GO.0042802	identical protein binding	9	0.0139	ACTN1,ACTN4,APP,B2M,GAPDH,HNRNPC,HSP90AA1,MYH9,NPM1
GO.0003682	chromatin binding	8	0.00109	ACTN4,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGB1,HNRNPC,NPM1
GO.0031490	chromatin DNA binding	7	1.80E-08	ACTN4,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HNRNPC,NPM1
GO.0019904	protein domain specific binding	7	0.00159	APP,HSP90AA1,HSP90AB1,HSPA5,HSPA8,MYH9,NPM1
GO.0005198	structural molecule activity	7	0.0175	MYL6,RPL12,RPLP0,RPS27A,TUBA1B,TUBB2A,TUBB8
GO.0042623	ATPase activity, coupled	6	0.00209	HSPA6,HSPA8,MYH14,MYH9,MYL6,XRCC6
GO.0051082	unfolded protein binding	5	0.0001	HSPA5,HSPA6,HSPA8,HSPD1,NPM1
GO.0002020	protease binding	5	0.000222	FN1,RPS27A,UBA52,UBB,UBC
GO.0003725	double-stranded RNA binding	4	0.00119	ACTN1,HSP90AB1,HSPD1,TUBA1B

GO.0005178	integrin binding	4	0.00241	ACTN1,ACTN4,CYR61,FN1
GO.0023026	MHC class II protein complex binding	3	0.000297	HSP90AA1,HSP90AB1,HSPA8
GO.0030898	actin-dependent ATPase activity	3	0.00037	MYH14,MYH9,MYL6
GO.0000146	microfilament motor activity	3	0.00209	MYH14,MYH9,MYL6
GO.0030374	ligand-dependent nuclear receptor transcription coactivator activity	3	0.0179	ACTN1,ACTN4,HMGA1
GO.0016835	carbon-oxygen lyase activity	3	0.0249	ENO1,HMGA1,XRCC6
GO.0051015	actin filament binding	3	0.0362	ACTN1,ACTN4,MYH9
GO.0030911	TPR domain binding	2	0.00283	HSP90AA1,HSP90AB1
GO.0051575	5 -deoxyribose-5-phosphate lyase activity	2	0.00283	HMGA1,XRCC6
GO.0030235	nitric-oxide synthase regulator activity	2	0.0069	HSP90AA1,HSP90AB1
GO.0031492	nucleosomal DNA binding	2	0.0154	H3F3B,HNRNPC
GO.0042974	retinoic acid receptor binding	2	0.0362	ACTN4,HMGA1

Appendix VII. Gene ontology term analysis (biological process) of the 37 HMGB1-binding proteins identified using 2 or more techniques.

Appendix VII: Gene ontology term analysis (Biological process) of the 37 HMGB1-binding proteins identified using 2 or more techniques.

Pathway ID	Pathway description	Observed gene count	False discovery rate	Matching proteins in your network (labels)
GO.0051716	cellular response to stimulus	29	2.57E-05	ACTN4,APP,B2M,CFL1,CYR61,GAPDH,H3F3B,HMGA1,HMGB1,HSP90AA1,HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1, LDHA,LGALS3BP,MYH14,MYH9,MYL6,NPM1,ROS1,RPS27A,TGM2,TUBA1B,UBA52,UBB,UBC,XRCC6
GO.0016043	cellular component organization	28	3.04E-06	ACTN4,CYR61,GAPDH,H3F3B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AA1,HSP90AB1,HSP90B1, HSPD1,MYH14,MYL6,NPM1,RPL12,RPL27,RPLP0,RPS27A,TUBA1B,TUBB2A,TUBB8,UBA52,UBB,UBC,XRCC6
GO.0044237	cellular metabolic process	28	0.0151	APP,B2M,CFL1,CYR61,ENO1,FN1,GAPDH,H3F3B,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AB1,HSP90B1,HSPA5, HSPA6,HSPA8,HSPD1,NPM1,RPL12,RPLP0,RPS27A,TGM2,TUBA1B,TUBB2A,UBB,UBC,XRCC6
GO.0044238	primary metabolic process	28	0.0187	ACAT2,B2M,CFL1,ENO1,FN1,GAPDH,H3F3B,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AB1,HSP90B1,HSPA5,HSPA6, HSPA8,HSPD1,MYH9,NPM1,RPL12,RPLP0,RPS27A,TGM2,TUBA1B,TUBB2A,UBB,UBC,XRCC6
GO.0071704	organic substance metabolic process	28	0.0272	ACAT2,B2M,CFL1,ENO1,FN1,GAPDH,H3F3B,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AB1,HSP90B1,HSPA5,HSPA6, HSPA8,HSPD1,MYH9,NPM1,RPL12,RPLP0,RPS27A,TGM2,TUBA1B,TUBB2A,UBB,UBC,XRCC6
GO.0050794	regulation of cellular process	28	0.0478	ACTN1,ANXA2,B2M,CYR61,ENO1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HSP90AA1, HSP90B1,HSPA5,HSPA8,HSPD1,LDHA,LGALS3BP,MYH14,MYL6,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0071840	cellular component organization or biogenesis	27	1.37E-05	ACTN4,CYR61,GAPDH,H3F3B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AA1,HSP90AB1,HSP90B1, HSPD1,MYH14,MYL6,NPM1,RPL12,RPL27,RPS27A,TUBA1B,TUBB2A,TUBB8,UBA52,UBB,UBC,XRCC6
GO.0043170	macromolecule metabolic process	27	0.00504	APP,B2M,CFL1,ENO1,FN1,GAPDH,H3F3B,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AB1,HSP90B1,HSPA5,HSPA6, HSPD1,MYH9,NPM1,RPL12,RPLP0,RPS27A,TGM2,TUBA1B,TUBB2A,UBB,UBC,XRCC6
GO.0044260	cellular macromolecule metabolic process	26	0.00375	APP,B2M,CFL1,ENO1,FN1,GAPDH,H3F3B,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AB1,HSP90B1,HSPA5,HSPA6, HSPD1,NPM1,RPL12,RPLP0,RPS27A,TGM2,TUBA1B,TUBB2A,UBB,UBC,XRCC6
GO.0050896	response to stimulus	26	0.00526	ACTN1,CYR61,ENO1,FN1,GAPDH,HMGA1,HMGB1,HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1,LDHA,LGALS3BP,MYH14, MYH9,MYL6,NPM1,ROS1,RPS27A,TGM2,TUBA1B,UBA52,UBB,UBC,XRCC6
GO.0043933	macromolecular complex subunit organization	24	1.30E-09	ACTN4,CFL1,FN1,H3F3B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AA1,HSP90B1,HSPA8,HSPD1, MYH9,NPM1,RPL12,RPL27,RPLP0,RPS27A,TUBA1B,TUBB2A,TUBB8,UBA52
GO.0007154	cell communication	24	0.000533	ACTN4,APP,B2M,CFL1,CYR61,H3F3B,HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA8,HSPD1,LDHA,LGALS3BP,MYH14, MYH9,MYL6,NPM1,ROS1,RPS27A,TGM2,UBA52,UBB,UBC
GO.0031323	regulation of cellular metabolic process	24	0.00184	ACTN1,ACTN4,ANXA2,CYR61,ENO1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGA1,HMGB1,HSP90AA1, HSP90B1,HSPA5,HSPD1,MYH9,NPM1,ROS1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0019222	regulation of metabolic process	24	0.0101	ACTN1,ACTN4,ANXA2,CYR61,ENO1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGA1,HMGB1,HNRNPC, HSP90AA1,HSP90B1,HSPA5,HSPD1,MYH9,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0071822	protein complex subunit organization	23	7.36E-12	ACTN4,CFL1,FN1,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HSP90AA1,HSP90B1,HSPA8,HSPD1, MYH9,NPM1,RPL12,RPL27,RPLP0,RPS27A,TUBA1B,TUBB2A,TUBB8,UBA52
GO.0006950	response to stress	23	1.24E-05	ACTN1,B2M,CFL1,CYR61,FN1,GAPDH,H3F3B,HMGA1,HMGB1,HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1,LDHA,LGALS3BP, MYH9,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0044700	single organism signaling	23	0.00105	ACTN4,APP,B2M,CFL1,CYR61,H3F3B,HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA8,HSPD1,LGALS3BP,MYH14, MYH9,MYL6,NPM1,ROS1,RPS27A,TGM2,UBA52,UBB,UBC
GO.0060255	regulation of macromolecule metabolic process	23	0.00274	ACTN1,ACTN4,ANXA2,CYR61,ENO1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGA1,HMGB1,HNRNPC, HSP90B1,HSPA5,HSPD1,MYH9,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0080090	regulation of primary metabolic process	23	0.00311	ACTN1,ACTN4,ANXA2,CYR61,ENO1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGA1,HMGB1,HSP90B1, HSPA5,HSPD1,MYH9,NPM1,ROS1,RPS27A,UBA52,UBB,UBC,XRCC6

GO.0044765	single-organism transport	22	4.31E-06	ACTN1,ACTN4,ANXA2,APP,CFL1,FN1,HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA8,MYH9,NPM1,RPL12,RPL27, RPLP0,RPS27A,TGM2,TUBA1B,UBA52,UBB,UBC
GO.0048519	negative regulation of biological process	22	0.000444	ACTN1,ANXA2,CFL1,ENO1,FN1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HSP90B1, HSPA8,NPM1,ROS1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0007165	signal transduction	22	0.00129	ACTN4,APP,B2M,CFL1,CYR61,H3F3B,HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPD1,LGALS3BP,MYH14,MYH9,MYL6, NPM1,ROS1,RPS27A,TGM2,UBA52,UBB,UBC
GO.0044085	cellular component biogenesis	21	1.69E-07	ACTN4,ANXA2,APP,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HSP90AA1,HSP90B1,HSPD1,NPM1, RPLP0,RPS27A,TUBA1B,TUBB2A,TUBB8,UBA52,UBB,UBC
GO.0051641	cellular localization	21	2.56E-07	ACTN1,ACTN4,ANXA2,APP,CFL1,FN1,HIST1H1B,HSP90AA1,HSP90B1,HSPA5,HSPA8,MYH9,NPM1,RPL12,RPL27, RPLP0,RPS27A,TUBA1B,UBA52,UBB,UBC
GO.1902578	single-organism localization	21	2.57E-05	ACTN1,ACTN4,ANXA2,APP,CFL1,FN1,HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA8,NPM1,RPL12,RPL27,RPLP0, RPS27A,TGM2,TUBA1B,UBA52,UBB,UBC
GO.0042221	response to chemical	21	0.000287	APP,CYR61,GAPDH,H3F3B,HMGB1,HSP90AA1,HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1,LDHA,MYH14,MYH9,MYL6, NPM1,RPS27A,TUBA1B,UBA52,UBB,UBC
GO.0031326	regulation of cellular biosynthetic process	21	0.000518	ACTN1,ACTN4,CYR61,ENO1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGA1,HMGB1,HSP90AA1,HSP90AB1, HSPA5,HSPA8,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0051649	establishment of localization in cell	20	1.69E-07	ACTN1,ACTN4,ANXA2,APP,CFL1,FN1,HSP90AA1,HSP90B1,HSPA5,HSPA8,MYH9,NPM1,RPL12,RPL27,RPLP0, RPS27A,TUBA1B,UBA52,UBB,UBC
GO.0022607	cellular component assembly	20	2.06E-07	ACTN4,ANXA2,APP,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HSP90AA1,HSP90B1,HSPD1,NPM1, RPS27A,TUBA1B,TUBB2A,TUBB8,UBA52,UBB,UBC
GO.0006810	transport	20	0.000302	ACTN1,ACTN4,APP,CFL1,FN1,HSP90AA1,HSP90AB1,HSP90B1,LGALS3BP,MYH9,NPM1,RPL12,RPL27,RPLP0, RPS27A,TGM2,TUBA1B,UBA52,UBB,UBC
GO.0051234	establishment of localization	20	0.00043	ACTN1,ACTN4,APP,CFL1,FN1,HIST1H1B,HSP90AA1,HSP90AB1,HSP90B1,LGALS3BP,NPM1,RPL12,RPL27,RPLP0, RPS27A,TGM2,TUBA1B,UBA52,UBB,UBC
GO.0048523	negative regulation of cellular process	20	0.00142	ACTN1,ANXA2,CFL1,ENO1,FN1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HSP90B1, HSPA8,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0010468	regulation of gene expression	20	0.00152	CYR61,ENO1,FN1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGA1,HMGB1,HNRNPC,HSPA5,MYH9,NPM1, ROS1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0019538	protein metabolic process	20	0.00161	APP,B2M,CFL1,FN1,GAPDH,HSP90AB1,HSP90B1,HSPA5,HSPA6,HSPD1,MYH9,NPM1,RPL12,RPLP0,RPS27A,TGM2, TUBA1B,TUBB2A,UBB,UBC
GO.0051179	localization	20	0.00413	ACTN1,APP,CYR61,FN1,HIST1H1B,HMGB1,HSP90AA1,HSP90AB1,HSP90B1,LGALS3BP,NPM1,RPL12,RPL27,RPLP0, RPS27A,TGM2,TUBA1B,UBA52,UBB,UBC
GO.0048583	regulation of response to stimulus	19	0.000684	ACTN4,ANXA2,APP,CFL1,CYR61,FN1,HMGA1,HSP90AA1,HSP90AB1,HSP90B1,HSPA8,HSPD1,MYH9,NPM1,ROS1, RPS27A,UBA52,UBB,UBC
GO.1903506	regulation of nucleic acid-templated transcription	19	0.000719	ACTN1,ACTN4,APP,CYR61,ENO1,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGA1,HMGB1,HSPA5,HSPA8,NPM1, RPS27A,UBA52,UBB,UBC,XRCC6
GO.0044267	cellular protein metabolic process	19	0.000825	APP,B2M,CFL1,FN1,GAPDH,HSP90AB1,HSP90B1,HSPA5,HSPA6,HSPD1,NPM1,RPL12,RPLP0,RPS27A,TGM2,TUBA1B, TUBB2A,UBB,UBC
GO.0010556	regulation of macromolecule biosynthetic process	19	0.00233	ACTN1,ACTN4,CYR61,ENO1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGA1,HMGB1,HSPA5,HSPA8,NPM1, RPS27A,UBA52,UBB,UBC,XRCC6
GO.0051171	regulation of nitrogen compound metabolic process	19	0.00542	ACTN1,ACTN4,CYR61,ENO1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGA1,HMGB1,HSP90AA1,HSP90AB1, NPM1,RPS27A,UBA52,UBB,UBC,XRCC6

GO.0044710	single-organism metabolic process	19	0.00822	ACAT2,APP,ENO1,GAPDH,HIST3H3,HMGA1,HMGB1,HSP90AA1,HSP90B1,HSPA5,HSPA8,HSPD1,NPM1,ROS1,RPS27A, UBA52,UBB,UBC,XRCC6
GO.0006139	nucleobase-containing compound metabolic process	19	0.0181	APP,ENO1,GAPDH,H3F3B,HIST3H3,HMGA1,HMGB1,HNRNPC,HSPA8,HSPD1,NPM1,RPL12,RPL27,RPLP0,RPS27A, UBA52,UBB,UBC,XRCC6
GO.0010605	negative regulation of macromolecule metabolic process	18	3.11E-05	ANXA2,APP,ENO1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGA1,HMGB1,HSP90AB1,HSPA8,ROS1,RPS27A, UBA52,UBB,UBC,XRCC6
GO.0065008	regulation of biological quality	18	0.000676	ACTN1,ACTN4,ANXA2,GAPDH,H3F3B,HIST1H1B,HIST3H3,HNRNPC,HSP90AB1,HSP90B1,HSPA5,HSPA8,HSPD1, MYH14,MYH9,TGM2,UBB,XRCC6
GO.0051252	regulation of RNA metabolic process	18	0.00282	ACTN1,ACTN4,APP,CYR61,ENO1,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGA1,HMGB1,HSPA5,NPM1,RPS27A, UBA52,UBB,UBC,XRCC6
GO.0090304	nucleic acid metabolic process	18	0.0148	APP,ENO1,H3F3B,HIST3H3,HMGA1,HMGB1,HNRNPC,HSPA8,HSPD1,NPM1,RPL12,RPL27,RPLP0,RPS27A,UBA52, UBB,UBC,XRCC6
GO.0048522	positive regulation of cellular process	18	0.0308	CFL1,CYR61,H3F3B,HIST1H1B,HMGA1,HMGB1,HSP90AA1,HSPA8,HSPD1,LDHA,MYH9,NPM1,RPS27A,TGM2,UBA52, UBB,UBC,XRCC6
GO.0009605	response to external stimulus	17	2.01E-05	APP,B2M,CFL1,CYR61,ENO1,HMGA1,HMGB1,HSP90AA1,HSP90AB1,HSPA5,HSPA8,HSPD1,LDHA,MYH14,MYH9, MYL6,NPM1
GO.0031324	negative regulation of cellular metabolic process	17	0.000103	ANXA2,APP,ENO1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGA1,HMGB1,HSP90AB1,HSPA8,RPS27A,UBA52, UBB,UBC,XRCC6
GO.0031325	positive regulation of cellular metabolic process	17	0.00105	ANXA2,APP,CYR61,FN1,HIST1H1B,HMGA1,HMGB1,HSP90AA1,HSPA8,HSPD1,MYH9,NPM1,RPS27A,UBA52,UBB,UBC, XRCC6
GO.0006355	regulation of transcription, DNA-templated	17	0.00597	APP,CYR61,ENO1,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGA1,HMGB1,HSPA5,HSPA8,NPM1,RPS27A,UBA52,UBB, UBC,XRCC6
GO.2000112	regulation of cellular macromolecule biosynthetic process	17	0.0148	CYR61,ENO1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGA1,HMGB1,HSPA5,HSPA8,NPM1,RPS27A,UBA52, UBB,UBC,XRCC6
GO.0019219	regulation of nucleobase-containing compound metabolic process	17	0.0177	ACTN1,ACTN4,APP,CYR61,ENO1,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGA1,HMGB1,NPM1,RPS27A,UBA52,UBB, UBC,XRCC6
GO.0002376	immune system process	16	0.000111	ACTN1,ANXA2,APP,CFL1,FN1,GAPDH,HMGB1,HSP90AA1,HSP90AB1,HSP90B1,HSPD1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0010033	response to organic substance	16	0.000892	CFL1,GAPDH,H3F3B,HSP90AA1,HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1,LDHA,NPM1,RPS27A,TUBA1B,UBA52,UBB,UBC
GO.0010604	positive regulation of macromolecule metabolic process	16	0.0017	ANXA2,APP,CYR61,HIST1H1B,HMGA1,HMGB1,HSP90AB1,HSPA8,HSPD1,MYH9,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0006996	organelle organization	16	0.00381	ACTN4,ANXA2,GAPDH,H3F3B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HNRNPC,HSP90AA1,HSP90B1,MYH14, NPM1,TUBA1B,XRCC6
GO.0009893	positive regulation of metabolic process	16	0.0239	ANXA2,APP,CYR61,HIST1H1B,HMGA1,HMGB1,HSP90AA1,HSPA8,HSPD1,MYH9,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0048731	system development	16	0.0288	ACTN1,ANXA2,B2M,CYR61,FN1,H3F3B,HMGB1,HSP90AA1,HSPA8,HSPD1,MYH14,MYH9,MYL6,NPM1,TGM2,UBB
GO.0032270	positive regulation of cellular protein metabolic process	15	1.18E-05	ANXA2,APP,CYR61,FN1,HIST1H1B,HMGB1,HSP90AB1,HSPA5,HSPD1,MYH9,NPM1,RPS27A,UBA52,UBB,UBC
GO.0042981	regulation of apoptotic process	15	1.80E-05	ACTN1,ACTN4,CFL1,CYR61,HMGB1,HSP90AB1,HSP90B1,HSPA5,HSPD1,LDHA,RPS27A,TGM2,UBA52,UBB,UBC

GO.0006952	defense response	15	2.01E-05	APP,B2M,CFL1,FN1,GAPDH,HMGB1,HSP90AA1,HSP90AB1,HSP90B1,LGALS3BP,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0010629	negative regulation of gene expression	15	2.30E-05	ENO1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGA1,HMGB1,HSPA8,ROS1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0048468	cell development	15	6.65E-05	ACTN1,ANXA2,APP,CYR61,FN1,HMGB1,HSP90AA1,HSP90AB1,HSPA8,MYH14,MYH9,MYL6,NPM1,ROS1,UBB
GO.0051704	multi-organism process	15	0.00105	CFL1,ENO1,HMGA1,HSP90AB1,HSPA8,HSPD1,ROS1,RPL12,RPL27,RPLP0,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0016032	viral process	14	2.56E-07	B2M,HMGA1,HSP90AB1,HSPA8,HSPD1,NPM1,RPL12,RPL27,RPLP0,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0006461	protein complex assembly	14	3.99E-06	ANXA2,FN1,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HSP90AA1,HSPD1,NPM1,TUBA1B,TUBB2A,TUBB8
GO.0070271	protein complex biogenesis	14	3.99E-06	ANXA2,FN1,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HSP90AA1,HSPD1,NPM1,TUBA1B,TUBB2A,TUBB8
GO.0016192	vesicle-mediated transport	14	1.47E-05	ACTN1,ACTN4,ANXA2,APP,CFL1,FN1,HSP90AA1,HSP90AB1,HSP90B1,HSPA5,LGALS3BP,MYH9,NPM1,TGM2
GO.2000113	negative regulation of cellular macromolecule biosynthetic process	14	3.60E-05	ENO1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGA1,HMGB1,HSPA8,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0046907	intracellular transport	14	4.25E-05	ANXA2,APP,HSP90AA1,HSP90B1,HSPA8,NPM1,RPL12,RPL27,RPLP0,RPS27A,TUBA1B,UBA52,UBB,UBC
GO.0006955	immune response	14	4.45E-05	APP,B2M,CFL1,GAPDH,HMGB1,HSP90AA1,HSP90AB1,HSP90B1,HSPD1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0051172	negative regulation of nitrogen compound metabolic process	14	8.00E-05	ENO1,GAPDH,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGA1,HMGB1,HSPA8,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0080134	regulation of response to stress	14	8.00E-05	ANXA2,B2M,HMGA1,HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA8,HSPD1,NPM1,RPS27A,UBA52,UBB,UBC
GO.0010941	regulation of cell death	14	0.000103	ACTN1,ACTN4,CFL1,HMGB1,HSP90AB1,HSP90B1,HSPA5,HSPD1,LDHA,RPS27A,TGM2,UBA52,UBB,UBC
GO.0031399	regulation of protein modification process	14	0.000203	ANXA2,APP,CYR61,FN1,HIST1H1B,HSP90AB1,HSP90B1,HSPA5,NPM1,ROS1,RPS27A,UBA52,UBB,UBC
GO.0033554	cellular response to stress	14	0.000264	HMGA1,HMGB1,HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA6,HSPA8,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0006357	regulation of transcription from RNA polymerase II promoter	14	0.000324	APP,CYR61,ENO1,HIST1H1B,HIST1H1C,HIST1H1D,HMGB1,HSPA5,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0010628	positive regulation of gene expression	14	0.000332	APP,CYR61,FN1,HMGA1,HMGB1,HSPA5,HSPA8,MYH9,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0044093	positive regulation of molecular function	14	0.000358	ACTN4,ANXA2,APP,CYR61,FN1,HMGB1,HSP90AB1,HSPA5,HSPD1,NPM1,RPS27A,UBA52,UBB,UBC
GO.0048584	positive regulation of response to stimulus	14	0.000945	ACTN4,B2M,CFL1,CYR61,HMGA1,HSP90AA1,HSP90AB1,HSP90B1,HSPD1,NPM1,RPS27A,UBA52,UBB,UBC
GO.0009653	anatomical structure morphogenesis	14	0.00208	ACTN1,ANXA2,APP,CYR61,FN1,HSP90AA1,HSP90AB1,HSPA5,HSPA8,MYH14,MYL6,NPM1,TGM2,UBB

GO.0032268	regulation of cellular protein metabolic process	14	0.00433	ANXA2,CYR61,GAPDH,HIST1H1B,HMGB1,HSP90B1,HSPA5,HSPD1,MYH9,ROS1,RPS27A,UBA52,UBB,UBC
GO.0065009	regulation of molecular function	14	0.0179	ACTN4,ANXA2,B2M,CYR61,FN1,HMGB1,HSP90AA1,HSP90B1,HSPD1,NPM1,RPS27A,UBA52,UBB,UBC
GO.0045087	innate immune response	13	1.37E-05	APP,B2M,CFL1,GAPDH,HMGB1,HSP90AA1,HSP90AB1,HSP90B1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0045892	negative regulation of transcription, DNA-templated	13	5.24E-05	ENO1,H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HMGA1,HMGB1,HSPA8,RPS27A,UBA52,UBB,UBC,XRCC6
GO.1902582	single-organism intracellular transport	13	5.70E-05	ANXA2,APP,HSP90AA1,HSP90B1,HSPA8,RPL12,RPL27,RPLP0,RPS27A,TUBA1B,UBA52,UBB,UBC
GO.0019220	regulation of phosphate metabolic process	13	0.000511	ANXA2,APP,CYR61,FN1,HSP90AB1,HSP90B1,HSPA5,NPM1,ROS1,RPS27A,UBA52,UBB,UBC
GO.1901575	organic substance catabolic process	13	0.00053	ENO1,GAPDH,HMGB1,HSP90B1,HSPA5,LDHA,RPL12,RPL27,RPLP0,RPS27A,UBA52,UBB,UBC
GO.0008104	protein localization	13	0.0012	ACTN4,ANXA2,HIST1H1B,HSP90AA1,HSP90B1,HSPA5,MYH9,NPM1,RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0031328	positive regulation of cellular biosynthetic process	13	0.00141	APP,CYR61,HMGA1,HMGB1,HSP90AA1,HSP90AB1,HSPA5,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0051173	positive regulation of nitrogen compound metabolic process	13	0.00153	APP,CYR61,HMGA1,HMGB1,HSP90AA1,HSP90AB1,HSPA8,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0007166	cell surface receptor signaling pathway	13	0.00611	APP,B2M,CFL1,HSP90AA1,HSP90AB1,MYH14,MYH9,MYL6,ROS1,RPS27A,UBA52,UBB,UBC
GO.0050790	regulation of catalytic activity	13	0.0104	ANXA2,CYR61,FN1,HMGB1,HSP90AA1,HSP90B1,HSPA5,HSPD1,NPM1,RPS27A,UBA52,UBB,UBC
GO.0048513	organ development	13	0.0474	ACTN1,ANXA2,APP,B2M,CFL1,CYR61,H3F3B,HSP90AB1,MYH14,MYH9,MYL6,NPM1,TGM2
GO.0009628	response to abiotic stimulus	12	0.00019	ACTN4,APP,HSP90AA1,HSP90B1,HSPA6,HSPA8,HSPD1,LDHA,RPS27A,UBA52,UBB,UBC
GO.0001932	regulation of protein phosphorylation	12	0.000294	ANXA2,APP,CYR61,FN1,HSP90AB1,HSPA5,NPM1,ROS1,RPS27A,UBA52,UBB,UBC
GO.0045184	establishment of protein localization	12	0.000655	ACTN4,ANXA2,HIST1H1B,HSP90AA1,HSP90B1,MYH9,NPM1,RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0051254	positive regulation of RNA metabolic process	12	0.00152	APP,CYR61,HMGA1,HMGB1,HSPA5,HSPA8,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0007399	nervous system development	12	0.0145	FN1,H3F3B,HMGB1,HSP90AA1,HSP90AB1,HSPA8,LDHA,MYH14,MYH9,MYL6,NPM1,UBB
GO.0051128	regulation of cellular component organization	12	0.0258	ANXA2,CYR61,ENO1,FN1,H3F3B,HIST1H1B,HIST3H3,HMGA1,HSPA8,MYH14,MYH9,NPM1
GO.0050778	positive regulation of immune response	11	8.15E-06	B2M,CFL1,HSP90AA1,HSP90AB1,HSP90B1,HSPD1,NPM1,RPS27A,UBA52,UBB,UBC
GO.0034622	cellular macromolecular complex assembly	11	1.24E-05	H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HSP90AA1,HSPD1,NPM1,TUBA1B,TUBB2A,TUBB8

GO.0006259	DNA metabolic process	11	3.25E-05	H3F3B,HIST3H3,HMGA1,HMGB1,HSPD1,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0061024	membrane organization	11	0.000136	APP,HSP90AA1,HSPA8,MYH9,RPL12,RPL27,RPLP0,RPS27A,UBA52,UBB,UBC
GO.0000902	cell morphogenesis	11	0.000244	ACTN1,APP,CFL1,FN1,HSP90AA1,HSP90AB1,HSPA8,MYH14,MYH9,MYL6,UBB
GO.0034613	cellular protein localization	11	0.000877	ANXA2,HIST1H1B,HSP90AA1,HSP90B1,HSPA5,NPM1,RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0015031	protein transport	11	0.00148	ACTN4,ANXA2,HSP90AA1,HSP90B1,MYH9,NPM1,RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0006928	movement of cell or subcellular component	11	0.0025	ACTN4,APP,CYR61,FN1,HMGB1,HSP90AA1,HSP90AB1,HSPA8,MYH9,MYL6,UBB
GO.0045893	positive regulation of transcription, DNA-templated	11	0.00416	APP,CYR61,HMGA1,HMGB1,HSPA5,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0043085	positive regulation of catalytic activity	11	0.00433	APP,CYR61,FN1,HMGB1,HSP90AB1,HSPA5,HSPD1,RPS27A,UBA52,UBB,UBC
GO.0045935	positive regulation of nucleobase-containing compound metabolic process	11	0.0131	APP,CYR61,HMGA1,HMGB1,HSPA8,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0045862	positive regulation of proteolysis	10	3.04E-06	APP,CYR61,FN1,HMGB1,HSPD1,MYH9,RPS27A,UBA52,UBB,UBC
GO.0022411	cellular component disassembly	10	2.45E-05	CFL1,FN1,HMGA1,HMGB1,HSPA8,RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0006935	chemotaxis	10	8.00E-05	APP,CFL1,CYR61,HMGB1,HSP90AA1,HSP90AB1,HSPA8,MYH14,MYH9,MYL6
GO.0000904	cell morphogenesis involved in differentiation	10	0.000103	ACTN1,APP,CFL1,FN1,HSP90AA1,HSP90AB1,HSPA8,MYH14,MYH9,MYL6
GO.0031175	neuron projection development	10	0.000104	APP,CFL1,HMGB1,HSP90AA1,HSP90AB1,HSPA8,MYH14,MYH9,MYL6,UBB
GO.0007169	transmembrane receptor protein tyrosine kinase signaling pathway	10	0.00015	CFL1,HSP90AA1,MYH14,MYH9,MYL6,ROS1,RPS27A,UBA52,UBB,UBC
GO.0030162	regulation of proteolysis	10	0.00027	CYR61,FN1,HMGB1,HSP90AB1,HSPD1,MYH9,RPS27A,UBA52,UBB,UBC
GO.0000122	negative regulation of transcription from RNA polymerase II promoter	10	0.000275	ENO1,HIST1H1B,HIST1H1C,HIST1H1D,HMGB1,NPM1,RPS27A,UBA52,UBB,UBC
GO.0044265	cellular macromolecule catabolic process	10	0.000313	HMGB1,HSP90B1,HSPA5,RPL12,RPL27,RPLP0,RPS27A,UBA52,UBB,UBC
GO.0048666	neuron development	10	0.000382	APP,CFL1,HMGB1,HSP90AA1,HSP90AB1,HSPA8,MYH14,MYH9,MYL6,UBB
GO.0050776	regulation of immune response	10	0.000382	CFL1,HSP90AA1,HSP90AB1,HSP90B1,HSPD1,NPM1,RPS27A,UBA52,UBB,UBC
GO.1902580	single-organism cellular localization	10	0.000512	ACTN4,ANXA2,HSP90AA1,MYH9,RPL12,RPL27,RPLP0,RPS27A,UBA52,UBB

GO.0030182	neuron differentiation	10	0.00129	APP,CFL1,HMGB1,HSP90AA1,HSP90AB1,HSPA8,MYH14,MYH9,MYL6,UBB
GO.0045944	positive regulation of transcription from RNA polymerase II promoter	10	0.00153	APP,CYR61,HMGB1,HSPA5,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0016310	phosphorylation	10	0.00241	APP,CFL1,ENO1,GAPDH,LDHA,NPM1,RPS27A,UBA52,UBB,UBC
GO.0040011	locomotion	10	0.00478	APP,CYR61,FN1,HMGB1,HSP90AA1,HSP90AB1,HSPA8,MYH14,MYH9,MYL6
GO.0048699	generation of neurons	10	0.00979	CFL1,FN1,HMGB1,HSP90AA1,HSP90AB1,HSPA8,MYH14,MYH9,MYL6,UBB
GO.0071103	DNA conformation change	9	7.52E-07	H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,NPM1,XRCC6
GO.0002757	immune response-activating signal transduction	9	2.30E-05	CFL1,HSP90AA1,HSP90AB1,HSP90B1,HSPD1,RPS27A,UBA52,UBB,UBC
GO.0006897	endocytosis	9	0.000103	APP,CFL1,HSP90AA1,HSP90AB1,HSP90B1,LGALS3BP,MYH9,NPM1,TGM2
GO.0071345	cellular response to cytokine stimulus	9	0.000139	B2M,GAPDH,HSP90AB1,HSPA5,RPS27A,TUBA1B,UBA52,UBB,UBC
GO.0048812	neuron projection morphogenesis	9	0.00017	APP,CFL1,HSP90AA1,HSP90AB1,HSPA8,MYH14,MYH9,MYL6,UBB
GO.0043065	positive regulation of apoptotic process	9	0.000193	CYR61,HMGB1,HSPD1,LDHA,RPS27A,TGM2,UBA52,UBB,UBC
GO.0001775	cell activation	9	0.000403	ACTN1,ACTN4,APP,B2M,CFL1,FN1,HMGB1,HSPA5,HSPD1
GO.0050878	regulation of body fluid levels	9	0.000655	ACTN1,ACTN4,ANXA2,APP,CFL1,FN1,H3F3B,HSPA5,MYH9
GO.0045859	regulation of protein kinase activity	9	0.000684	APP,CYR61,HSP90AB1,HSPA5,NPM1,RPS27A,UBA52,UBB,UBC
GO.0042060	wound healing	9	0.0007	ACTN1,ACTN4,APP,CFL1,CYR61,FN1,H3F3B,HSPA5,MYH9
GO.0006886	intracellular protein transport	9	0.000935	ANXA2,HSP90AA1,HSP90B1,NPM1,RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0043066	negative regulation of apoptotic process	9	0.00221	CFL1,CYR61,HSP90AB1,HSP90B1,HSPA5,RPS27A,UBA52,UBB,UBC
GO.0051276	chromosome organization	9	0.00284	H3F3B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HNRNPC,NPM1,XRCC6
GO.0051241	negative regulation of multicellular organismal process	9	0.00534	ANXA2,APP,FN1,HMGB1,NPM1,RPS27A,UBA52,UBB,UBC
GO.0030030	cell projection organization	9	0.00591	APP,CFL1,HMGB1,HSP90AA1,HSP90AB1,HSPA8,MYH14,MYL6,UBB
GO.0031401	positive regulation of protein modification process	9	0.00822	ANXA2,CYR61,HIST1H1B,HSP90AB1,HSPA5,RPS27A,UBA52,UBB,UBC

GO.0006915	apoptotic process	9	0.00975	APP,CYR61,GAPDH,HMGB1,HSPD1,RPS27A,UBA52,UBB,UBC
GO.0044092	negative regulation of molecular function	9	0.0104	ANXA2,APP,B2M,HSPA5,NPM1,RPS27A,UBA52,UBB,UBC
GO.0051240	positive regulation of multicellular organismal process	9	0.0367	CYR61,FN1,HSPD1,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0009719	response to endogenous stimulus	9	0.0476	CFL1,H3F3B,HSP90B1,HSPD1,LDHA,RPS27A,UBA52,UBB,UBC
GO.0030522	intracellular receptor signaling pathway	8	4.31E-06	ACTN4,APP,HSP90AB1,NPM1,RPS27A,UBA52,UBB,UBC
GO.0006457	protein folding	8	7.57E-06	B2M,HSP90AB1,HSP90B1,HSPA5,HSPA6,HSPD1,TUBA1B,TUBB2A
GO.0043241	protein complex disassembly	8	1.24E-05	CFL1,HMGA1,HSPA8,RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0019058	viral life cycle	8	1.79E-05	HSP90AB1,RPL12,RPL27,RPLP0,RPS27A,UBA52,UBB,UBC
GO.0001666	response to hypoxia	8	2.45E-05	ACTN4,HSP90B1,HSPD1,LDHA,RPS27A,UBA52,UBB,UBC
GO.0007411	axon guidance	8	0.000166	APP,CFL1,HSP90AA1,HSP90AB1,HSPA8,MYH14,MYH9,MYL6
GO.0006281	DNA repair	8	0.000265	HMGA1,HMGB1,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0007017	microtubule-based process	8	0.000525	APP,GAPDH,MYH9,NPM1,TUBA1B,TUBB2A,TUBB8,UBB
GO.0007409	axonogenesis	8	0.000533	APP,CFL1,HSP90AA1,HSP90AB1,HSPA8,MYH14,MYH9,MYL6
GO.0061564	axon development	8	0.000671	APP,CFL1,HSP90AA1,HSP90AB1,HSPA8,MYH14,MYH9,MYL6
GO.0001817	regulation of cytokine production	8	0.000732	FN1,HSPD1,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0048667	cell morphogenesis involved in neuron differentiation	8	0.000842	APP,CFL1,HSP90AA1,HSP90AB1,HSPA8,MYH14,MYH9,MYL6
GO.0033365	protein localization to organelle	8	0.000859	HIST1H1B,HSP90AA1,HSPA5,RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0007596	blood coagulation	8	0.000887	ACTN1,ACTN4,APP,CFL1,FN1,H3F3B,HSPA5,MYH9
GO.0016071	mRNA metabolic process	8	0.00127	APP,HNRNPC,HSPA8,RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0046903	secretion	8	0.00134	ACTN1,ACTN4,ANXA2,APP,CFL1,FN1,HSPA5,HSPA8
GO.0006325	chromatin organization	8	0.00217	H3F3B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,HMGB1,HNRNPC,NPM1

GO.0016482	cytoplasmic transport	8	0.00413	HSP90AA1,HSP90B1,NPM1,RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0031347	regulation of defense response	8	0.00478	B2M,HSP90AB1,HSP90B1,HSPD1,RPS27A,UBA52,UBB,UBC
GO.0001934	positive regulation of protein phosphorylation	8	0.00766	ANXA2,CYR61,HSP90AB1,HSPA5,RPS27A,UBA52,UBB,UBC
GO.0060548	negative regulation of cell death	8	0.0148	CFL1,HSP90AB1,HSP90B1,HSPA5,RPS27A,UBA52,UBB,UBC
GO.0032269	negative regulation of cellular protein metabolic process	8	0.0199	ANXA2,APP,GAPDH,HSP90AB1,RPS27A,UBA52,UBB,UBC
GO.0006508	proteolysis	8	0.0397	HSP90B1,HSPA5,HSPD1,MYH9,RPS27A,UBA52,UBB,UBC
GO.0031497	chromatin assembly	7	5.49E-06	H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,NPM1
GO.0019080	viral gene expression	7	5.89E-06	RPL12,RPL27,RPLP0,RPS27A,UBA52,UBB,UBC
GO.0034728	nucleosome organization	7	8.22E-06	H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,NPM1
GO.0006323	DNA packaging	7	1.45E-05	H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,HMGA1,NPM1
GO.0006986	response to unfolded protein	7	1.45E-05	HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA6,HSPA8,HSPD1
GO.0043624	cellular protein complex disassembly	7	5.24E-05	CFL1,HSPA8,RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0038093	Fc receptor signaling pathway	7	8.00E-05	CFL1,HSP90AA1,HSP90AB1,RPS27A,UBA52,UBB,UBC
GO.0030168	platelet activation	7	9.10E-05	ACTN1,ACTN4,APP,CFL1,FN1,HSPA5,MYH9
GO.0090150	establishment of protein localization to membrane	7	0.000116	ANXA2,HSP90AA1,RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0051098	regulation of binding	7	0.00015	ANXA2,APP,B2M,HMGB1,HSP90AB1,HSPA5,NPM1
GO.0002429	immune response-activating cell surface receptor signaling pathway	7	0.000168	CFL1,HSP90AA1,HSP90AB1,RPS27A,UBA52,UBB,UBC
GO.0001819	positive regulation of cytokine production	7	0.000648	HSPD1,NPM1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0072594	establishment of protein localization to organelle	7	0.000655	HIST1H1B,HSP90AA1,RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0045088	regulation of innate immune response	7	0.000696	HSP90AB1,HSP90B1,HSPD1,RPS27A,UBA52,UBB,UBC
GO.0072657	protein localization to membrane	7	0.000793	ANXA2,HSP90AA1,RPL12,RPL27,RPLP0,RPS27A,UBA52

GO.0006091	generation of precursor metabolites and energy	7	0.00112	ENO1,GAPDH,LDHA,RPS27A,UBA52,UBB,UBC
GO.0006605	protein targeting	7	0.00134	ANXA2,HSP90AA1,RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0045860	positive regulation of protein kinase activity	7	0.00156	CYR61,HSP90AB1,HSPA5,RPS27A,UBA52,UBB,UBC
GO.0032940	secretion by cell	7	0.00253	ACTN1,ACTN4,APP,CFL1,FN1,HSPA5,HSPA8
GO.0044723	single-organism carbohydrate metabolic process	7	0.00768	ENO1,GAPDH,LDHA,RPS27A,UBA52,UBB,UBC
GO.0043408	regulation of MAPK cascade	7	0.00822	CYR61,FN1,ROS1,RPS27A,UBA52,UBB,UBC
GO.0009607	response to biotic stimulus	7	0.0171	B2M,CFL1,ENO1,HMGA1,HSPA5,HSPD1,NPM1
GO.0006468	protein phosphorylation	7	0.0319	APP,CFL1,NPM1,RPS27A,UBA52,UBB,UBC
GO.0043086	negative regulation of catalytic activity	7	0.0379	ANXA2,APP,NPM1,RPS27A,UBA52,UBB,UBC
GO.0035872	nucleotide-binding domain, leucine rich repeat containing receptor signaling pathway	6	8.56E-07	APP,HSP90AB1,RPS27A,UBA52,UBB,UBC
GO.0032481	positive regulation of type I interferon production	6	1.18E-05	HSPD1,RPS27A,UBA52,UBB,UBC,XRCC6
GO.0002576	platelet degranulation	6	1.37E-05	ACTN1,ACTN4,APP,CFL1,FN1,HSPA5
GO.0006334	nucleosome assembly	6	3.10E-05	H3F3B,HIST1H1B,HIST1H1C,HIST1H1D,HIST3H3,NPM1
GO.0002224	toll-like receptor signaling pathway	6	4.64E-05	HSP90B1,HSPD1,RPS27A,UBA52,UBB,UBC
GO.0016051	carbohydrate biosynthetic process	6	4.75E-05	ENO1,GAPDH,RPS27A,UBA52,UBB,UBC
GO.0070972	protein localization to endoplasmic reticulum	6	8.00E-05	HSPA5,RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0006006	glucose metabolic process	6	8.86E-05	ENO1,GAPDH,RPS27A,UBA52,UBB,UBC
GO.0006909	phagocytosis	6	0.000154	CFL1,HSP90AA1,HSP90AB1,MYH9,NPM1,TGM2
GO.0006612	protein targeting to membrane	6	0.000175	ANXA2,RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0001818	negative regulation of cytokine production	6	0.000387	FN1,NPM1,RPS27A,UBA52,UBB,UBC
GO.0090092	regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	6	0.00043	CYR61,HSPA5,RPS27A,UBA52,UBB,UBC

GO.0090287	regulation of cellular response to growth factor stimulus	6	0.000705	CYR61,HSPA5,RPS27A,UBA52,UBB,UBC
GO.1903362	regulation of cellular protein catabolic process	6	0.00205	ANXA2,HSP90AB1,RPS27A,UBA52,UBB,UBC
GO.0043161	proteasome-mediated ubiquitin-dependent protein catabolic process	6	0.00208	HSP90B1,HSPA5,RPS27A,UBA52,UBB,UBC
GO.0034655	nucleobase-containing compound catabolic process	6	0.00339	HMGB1,RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0030198	extracellular matrix organization	6	0.00426	ACTN1,ANXA2,APP,CYR61,FN1,HSP90AB1
GO.0044257	cellular protein catabolic process	6	0.0179	HSP90B1,HSPA5,RPS27A,UBA52,UBB,UBC
GO.0010564	regulation of cell cycle process	6	0.0217	APP,NPM1,RPS27A,UBA52,UBB,UBC
GO.0051129	negative regulation of cellular component organization	6	0.0277	ACTN4,ANXA2,HIST3H3,HMGA1,HSPA8,NPM1
GO.0030155	regulation of cell adhesion	6	0.0308	ACTN4,CYR61,FN1,HSPD1,NPM1,TGM2
GO.0002520	immune system development	6	0.0351	ACTN1,ANXA2,B2M,HMGB1,HSPD1,MYH9
GO.0040008	regulation of growth	6	0.0466	APP,CYR61,ENO1,FN1,H3F3B,HIST1H1B
GO.0042026	protein refolding	5	6.54E-07	B2M,HSP90AA1,HSPA6,HSPA8,HSPD1
GO.0043618	regulation of transcription from RNA polymerase II promoter in response to stress	5	3.86E-05	HSPA5,RPS27A,UBA52,UBB,UBC
GO.0002479	antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent	5	6.65E-05	B2M,RPS27A,UBA52,UBB,UBC
GO.0030512	negative regulation of transforming growth factor beta receptor signaling pathway	5	8.00E-05	HSPA5,RPS27A,UBA52,UBB,UBC
GO.0042058	regulation of epidermal growth factor receptor signaling pathway	5	8.33E-05	APP,RPS27A,UBA52,UBB,UBC
GO.0002755	MyD88-dependent toll-like receptor signaling pathway	5	0.000111	HSPD1,RPS27A,UBA52,UBB,UBC
GO.0006614	SRP-dependent cotranslational protein targeting to membrane	5	0.000346	RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0019083	viral transcription	5	0.000403	RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0000184	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	5	0.000459	RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0051092	positive regulation of NF-kappaB transcription factor activity	5	0.000533	NPM1,RPS27A,UBA52,UBB,UBC

GO.0007219	Notch signaling pathway	5	0.00072	APP,RPS27A,UBA52,UBB,UBC
GO.0000086	G2/M transition of mitotic cell cycle	5	0.000892	HSP90AA1,RPS27A,UBA52,UBB,UBC
GO.0009408	response to heat	5	0.00107	HSP90AA1,HSP90AB1,HSPA6,HSPA8,HSPD1
GO.0043393	regulation of protein binding	5	0.00113	ANXA2,APP,B2M,HSP90AB1,HSPA5
GO.0010952	positive regulation of peptidase activity	5	0.00129	APP,CYR61,FN1,HMGB1,HSPD1
GO.0031398	positive regulation of protein ubiquitination	5	0.00152	HSPA5,RPS27A,UBA52,UBB,UBC
GO.0006415	translational termination	5	0.00181	RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0006414	translational elongation	5	0.00253	RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0006413	translational initiation	5	0.00504	RPL12,RPL27,RPLP0,RPS27A,UBA52
GO.0010948	negative regulation of cell cycle process	5	0.00553	NPM1,RPS27A,UBA52,UBB,UBC
GO.0016485	protein processing	5	0.00611	HSPD1,RPS27A,UBA52,UBB,UBC
GO.0090068	positive regulation of cell cycle process	5	0.00648	APP,RPS27A,UBA52,UBB,UBC
GO.0023014	signal transduction by protein phosphorylation	5	0.00671	ROS1,RPS27A,UBA52,UBB,UBC
GO.1901990	regulation of mitotic cell cycle phase transition	5	0.00696	APP,RPS27A,UBA52,UBB,UBC
GO.0006367	transcription initiation from RNA polymerase II promoter	5	0.00735	NPM1,RPS27A,UBA52,UBB,UBC
GO.0071902	positive regulation of protein serine/threonine kinase activity	5	0.00952	HSP90AB1,RPS27A,UBA52,UBB,UBC
GO.1903050	regulation of proteolysis involved in cellular protein catabolic process	5	0.0115	HSP90AB1,RPS27A,UBA52,UBB,UBC
GO.0043623	cellular protein complex assembly	5	0.0127	HSP90AA1,HSPD1,TUBA1B,TUBB2A,TUBB8
GO.0045787	positive regulation of cell cycle	5	0.0151	NPM1,RPS27A,UBA52,UBB,UBC
GO.0051348	negative regulation of transferase activity	5	0.0179	NPM1,RPS27A,UBA52,UBB,UBC
GO.0045785	positive regulation of cell adhesion	5	0.0193	CYR61,FN1,HSPD1,NPM1,TGM2

GO.0001558	regulation of cell growth	5	0.0248	CYR61,ENO1,FN1,H3F3B,HIST1H1B
GO.0019221	cytokine-mediated signaling pathway	5	0.0319	B2M,RPS27A,UBA52,UBB,UBC
GO.0019082	viral protein processing	4	5.03E-06	RPS27A,UBA52,UBB,UBC
GO.0070987	error-free translesion synthesis	4	1.96E-05	RPS27A,UBA52,UBB,UBC
GO.0042276	error-prone translesion synthesis	4	2.30E-05	RPS27A,UBA52,UBB,UBC
GO.0007220	Notch receptor processing	4	2.57E-05	RPS27A,UBA52,UBB,UBC
GO.0005978	glycogen biosynthetic process	4	4.01E-05	RPS27A,UBA52,UBB,UBC
GO.0070266	necroptotic process	4	5.70E-05	RPS27A,UBA52,UBB,UBC
GO.0070423	nucleotide-binding oligomerization domain containing signaling pathway	4	6.46E-05	RPS27A,UBA52,UBB,UBC
GO.0010939	regulation of necrotic cell death	4	7.23E-05	RPS27A,UBA52,UBB,UBC
GO.0075733	intracellular transport of virus	4	8.02E-05	RPS27A,UBA52,UBB,UBC
GO.0019068	virion assembly	4	0.000103	RPS27A,UBA52,UBB,UBC
GO.0061418	regulation of transcription from RNA polymerase II promoter in response to hypoxia	4	0.000103	RPS27A,UBA52,UBB,UBC
GO.0042769	DNA damage response, detection of DNA damage	4	0.000111	RPS27A,UBA52,UBB,UBC
GO.0032480	negative regulation of type I interferon production	4	0.000225	RPS27A,UBA52,UBB,UBC
GO.0042059	negative regulation of epidermal growth factor receptor signaling pathway	4	0.000225	RPS27A,UBA52,UBB,UBC
GO.0051084	de novo posttranslational protein folding	4	0.000322	HSPA8,HSPD1,TUBA1B,TUBB2A
GO.0007249	I-kappaB kinase/NF-kappaB signaling	4	0.000533	RPS27A,UBA52,UBB,UBC
GO.0030048	actin filament-based movement	4	0.000811	ACTN4,MYH14,MYH9,MYL6
GO.0034146	toll-like receptor 5 signaling pathway	4	0.000811	RPS27A,UBA52,UBB,UBC
GO.0034166	toll-like receptor 10 signaling pathway	4	0.000811	RPS27A,UBA52,UBB,UBC

GO.0006977	DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest	4	0.000851	RPS27A,UBA52,UBB,UBC
GO.0038123	toll-like receptor TLR1:TLR2 signaling pathway	4	0.00102	RPS27A,UBA52,UBB,UBC
GO.0038124	toll-like receptor TLR6:TLR2 signaling pathway	4	0.00102	RPS27A,UBA52,UBB,UBC
GO.0034162	toll-like receptor 9 signaling pathway	4	0.00105	RPS27A,UBA52,UBB,UBC
GO.0051437	positive regulation of ubiquitin-protein ligase activity involved in regulation of mitotic cell cycle transition	4	0.00105	RPS27A,UBA52,UBB,UBC
GO.0034134	toll-like receptor 2 signaling pathway	4	0.0011	RPS27A,UBA52,UBB,UBC
GO.0031571	mitotic G1 DNA damage checkpoint	4	0.00126	RPS27A,UBA52,UBB,UBC
GO.0035666	TRIF-dependent toll-like receptor signaling pathway	4	0.00129	RPS27A,UBA52,UBB,UBC
GO.0051436	negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	4	0.00152	RPS27A,UBA52,UBB,UBC
GO.0031145	anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process	4	0.00156	RPS27A,UBA52,UBB,UBC
GO.0034138	toll-like receptor 3 signaling pathway	4	0.00156	RPS27A,UBA52,UBB,UBC
GO.0007254	JNK cascade	4	0.00163	RPS27A,UBA52,UBB,UBC
GO.0050852	T cell receptor signaling pathway	4	0.00183	RPS27A,UBA52,UBB,UBC
GO.0048013	ephrin receptor signaling pathway	4	0.0021	CFL1,MYH14,MYH9,MYL6
GO.0034142	toll-like receptor 4 signaling pathway	4	0.00269	RPS27A,UBA52,UBB,UBC
GO.0034605	cellular response to heat	4	0.0031	HSP90AA1,HSP90AB1,HSPA6,HSPA8
GO.0051099	positive regulation of binding	4	0.00413	ANXA2,HMGB1,HSP90AB1,NPM1
GO.0071456	cellular response to hypoxia	4	0.00476	RPS27A,UBA52,UBB,UBC
GO.0002223	stimulatory C-type lectin receptor signaling pathway	4	0.00541	RPS27A,UBA52,UBB,UBC
GO.0090263	positive regulation of canonical Wnt signaling pathway	4	0.00656	RPS27A,UBA52,UBB,UBC
GO.0007179	transforming growth factor beta receptor signaling pathway	4	0.00768	RPS27A,UBA52,UBB,UBC

GO.0000187	activation of MAPK activity	4	0.00841	RPS27A,UBA52,UBB,UBC
GO.0007623	circadian rhythm	4	0.00879	RPS27A,UBA52,UBB,UBC
GO.0002478	antigen processing and presentation of exogenous peptide antigen	4	0.0103	RPS27A,UBA52,UBB,UBC
GO.0090090	negative regulation of canonical Wnt signaling pathway	4	0.011	RPS27A,UBA52,UBB,UBC
GO.0006338	chromatin remodeling	4	0.0117	HMGA1,HMGB1,HNRNPC,NPM1
GO.0046034	ATP metabolic process	4	0.0136	ENO1,GAPDH,HSPA8,LDHA
GO.0008543	fibroblast growth factor receptor signaling pathway	4	0.0139	RPS27A,UBA52,UBB,UBC
GO.0038095	Fc-epsilon receptor signaling pathway	4	0.0152	RPS27A,UBA52,UBB,UBC
GO.0000082	G1/S transition of mitotic cell cycle	4	0.0161	RPS27A,UBA52,UBB,UBC
GO.0043123	positive regulation of I-kappaB kinase/NF-kappaB signaling	4	0.0174	RPS27A,UBA52,UBB,UBC
GO.0071560	cellular response to transforming growth factor beta stimulus	4	0.0179	RPS27A,UBA52,UBB,UBC
GO.0044344	cellular response to fibroblast growth factor stimulus	4	0.0199	RPS27A,UBA52,UBB,UBC
GO.0000209	protein polyubiquitination	4	0.0239	RPS27A,UBA52,UBB,UBC
GO.0007173	epidermal growth factor receptor signaling pathway	4	0.0268	RPS27A,UBA52,UBB,UBC
GO.0016197	endosomal transport	4	0.0323	RPS27A,UBA52,UBB,UBC
GO.0071353	cellular response to interleukin-4	3	0.00148	HSP90AB1,HSPA5,TUBA1B
GO.0006096	glycolytic process	3	0.00398	ENO1,GAPDH,LDHA
GO.0019674	NAD metabolic process	3	0.00883	ENO1,GAPDH,LDHA
GO.0032392	DNA geometric change	3	0.00883	HMGA1,HMGB1,XRCC6
GO.0032091	negative regulation of protein binding	3	0.0138	ANXA2,B2M,HSPA5
GO.1900034	regulation of cellular response to heat	3	0.0148	HSP90AA1,HSP90AB1,HSPA8

GO.0038096	Fc-gamma receptor signaling pathway involved in phagocytosis	3	0.0175	CFL1,HSP90AA1,HSP90AB1
GO.0051258	protein polymerization	3	0.0244	TUBA1B,TUBB2A,TUBB8
GO.0030705	cytoskeleton-dependent intracellular transport	3	0.0306	APP,TUBA1B,UBB
GO.0050821	protein stabilization	3	0.0413	GAPDH,HIST1H1B,HSPD1
GO.0060591	chondroblast differentiation	2	0.00411	CYR61,NPM1
GO.0016584	nucleosome positioning	2	0.0051	HIST1H1C,HIST1H1D
GO.0034975	protein folding in endoplasmic reticulum	2	0.0051	HSP90B1,HSPA5
GO.0075713	establishment of integrated proviral latency	2	0.0051	HMGA1,XRCC6
GO.0036500	ATF6-mediated unfolded protein response	2	0.00623	HSP90B1,HSPA5
GO.0051085	chaperone mediated protein folding requiring cofactor	2	0.00623	HSPA8,HSPD1
GO.0006266	DNA ligation	2	0.0088	HMGB1,XRCC6
GO.0019042	viral latency	2	0.0088	HMGA1,XRCC6
GO.0051131	chaperone-mediated protein complex assembly	2	0.0101	HSP90AA1,HSPD1
GO.1900121	negative regulation of receptor binding	2	0.0101	ANXA2,B2M
GO.0030220	platelet formation	2	0.0182	ACTN1,MYH9
GO.0032069	regulation of nuclease activity	2	0.0199	HSPA5,NPM1
GO.0032469	endoplasmic reticulum calcium ion homeostasis	2	0.0199	APP,TGM2
GO.0036344	platelet morphogenesis	2	0.0199	ACTN1,MYH9
GO.0048384	retinoic acid receptor signaling pathway	2	0.022	ACTN4,NPM1
GO.0051261	protein depolymerization	2	0.0333	CFL1,HSPA8
GO.0051290	protein heterotetramerization	2	0.0358	ANXA2,HIST3H3

GO.0002711	positive regulation of T cell mediated immunity	2	0.0384	B2M,HSPD1
GO.0043277	apoptotic cell clearance	2	0.0408	NPM1,TGM2
GO.0061621	canonical glycolysis	2	0.0408	ENO1,GAPDH
GO.0018149	peptide cross-linking	2	0.0467	FN1,TGM2
GO.0033198	response to ATP	2	0.0496	HSP90B1,HSPD1

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