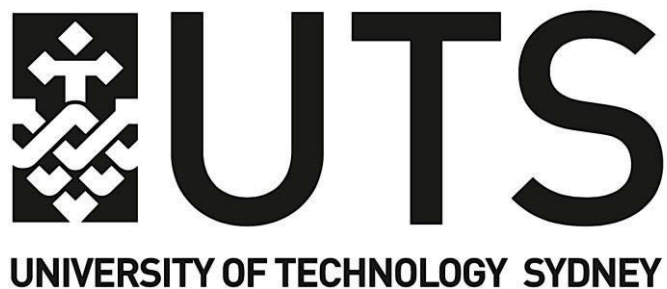


# **Investigation of the proteins SPARC and HMGB1 in chronic airways disease**

A thesis submitted for the degree of  
Doctor of Philosophy

Sharon Wong  
BPharm (Hons)

Graduate School of Health



March 2017

# CERTIFICATE OF ORIGINAL AUTHORSHIP

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as part of the collaborative doctoral degree and/or fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Signature of Student:

Production Note:  
Signature removed prior to publication.

Date: 3<sup>rd</sup> March 2017

# ACKNOWLEDGEMENTS

I would like to express my heartfelt thanks to my primary supervisor Dr Maria Sukkar who has been supportive from day 1 of my studies and has taught me many things, in research and in life. I am also thankful to my co-supervisor Dr Matthew Padula for his invaluable guidance and for introducing me to the fascinating world of proteomics.

A special thanks also to Dr Jerran Santos, A/Prof Sheila Donnelly and Professor Steven Djordjevic for their intellectual input and help with proteomic analysis in my studies; and to Dr Lyn Moir for her help in my thesis writing.

I would like to express my sincere gratitude to Aini and Michele who have shared the ups and downs of this journey with me; I appreciate all your encouragement and advice. Thanks also to Raj who has always been so thoughtful and helpful in the lab. I would like to thank Mona, Riana and Lucia for their support, motivation and company on the weekends and late nights.

I would like to especially thank Joyce To, Jess Tacchi, Ben Raymond and Michael Widjaja who have helped me around in the lab on countless occasions. A special thanks also to Dia Xenaki for her assistance with the primary cell cultures.

I would like to express my gratitude to the University of Technology Sydney (UTS) for supporting me throughout my studies with the UTS President Scholarship and UTS International Research Scholarship.

Lastly, I would like to thank my parents and brothers who have always been there for me, and cannot be more reassuring when I lose sight of the important things in life. Special thanks also to Auntie Angela and Uncle Kai Lok for having me for the last 4 years of my life in Sydney and for treating me as your own.

## **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

**Wong SL** and Sukkar MB

British Journal of Pharmacology (2017) 174(1):3–14

### **Presentations**

Potential role of SPARC, a downstream mediator of TGF- $\beta$  in chronic airways disease

**Wong SL**, Ibrahim ZA, Wark PA, Sukkar MB

European Respiratory Journal 2015, Vol 46, Issue S59, PA904

DOI: 10.1183/13993003.congress-2015.PA904

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

**Wong SL**, Ibrahim ZA, Wark PA, Sukkar MB

Respirology 2015, Vol 20, Issue S2, p. 144

Potential role of SPARC in chronic airways disease

2nd Annual Respiratory Epithelium Workshop 2014

Potential role of SPARC in inflammatory airways disease

Woolcock Institute of Medical Research Symposium 2014

University of Technology Sydney 3 Minute Thesis (3MT) 2014

### **Awards linked to abstracts**

The Thoracic Society of Australia and New Zealand (TSANZ) Travel Award 2014

# TABLE OF CONTENTS

<b>Certificate of Original Authorship .....</b>	<b>ii</b>
<b>Acknowledgements .....</b>	<b>iii</b>
<b>Publications and Presentations During Candidature .....</b>	<b>v</b>
<b>Table of Contents.....</b>	<b>vi</b>
<b>List of Figures .....</b>	<b>xi</b>
<b>List of Tables.....</b>	<b>xiii</b>
<b>List of Abbreviations .....</b>	<b>xiv</b>
<b>Abstract .....</b>	<b>xvi</b>

<b>General Introduction.....</b>	<b>1</b>
Introduction.....	2
Pathophysiology of asthma and COPD .....	3
Airway inflammation .....	4
Airway remodeling.....	8
TGF- $\beta$ is a master regulator of airway remodeling .....	10
Matricellular proteins regulate ECM deposition and assembly .....	13
SPARC as a potential player in chronic airways disease .....	13
Endoplasmic reticulum stress is implicated in chronic airways disease.....	15
HMGB1 is emerging as an important mediator of chronic airways disease ...	20
Hypotheses and Aims.....	23
References .....	25

<b>The SPARC protein: an overview of its role in lung cancer and pulmonary fibrosis and its potential role in chronic airways disease.....</b>	<b>39</b>
Abstract .....	40

Introduction .....	41
SPARC and lung cancer .....	45
SPARC expression in NSCLC tissues is associated with disease prognosis .....	45
SPARC promotes metastasis in NSCLC by promoting cell invasion and development of the tumoral vasculature network .....	47
SPARC and pulmonary fibrosis .....	48
SPARC confers resistance to apoptosis in lung fibroblasts and is a downstream effector of TGF- $\beta$ -induced fibrosis in IPF .....	48
Mouse models of bleomycin-induced IPF reveal a distinct role for SPARC in inflammatory versus fibrotic components of the disease .....	50
SPARC and chronic airways disease .....	52
SPARC activity overlaps with TGF- $\beta$ , a key mediator in asthma and COPD .....	52
Potential role of SPARC in airway wall remodeling .....	53
Evidence for SPARC in the immune and inflammatory response .....	57
Therapeutic implications .....	60
Conclusion and future directions .....	62
References .....	65
<b>Expression and function of SPARC in human airway epithelial cells .....</b>	<b>79</b>
Introduction .....	80
Materials and Methods .....	82
Human airway epithelial cells .....	82
Airway epithelial cell stimulation .....	83
ELISA .....	83
Immunoblotting .....	83
Statistical analysis .....	85

Results.....	85
Effect of TGF- $\beta$ on SPARC protein expression and secretion in human AECs .....	85
Effect of type 1 and type 2 cytokines on SPARC protein expression and secretion in asthmatic and non-asthmatic AECs .....	88
Comparative effect of TGF- $\beta$ and SPARC on cytokine and chemokine secretion in human AECs .....	94
Comparative effect of TGF- $\beta$ and SPARC on epithelial and mesenchymal markers in human AECs.....	98
Discussion .....	100
References .....	107

**SPARC expression in airway smooth muscle is regulated by the unfolded protein response and is diminished in chronic obstructive pulmonary disease..... 116**

Introduction.....	117
Materials and Methods .....	119
Human ASM cell culture and stimulation .....	119
ELISA .....	123
Immunoblotting.....	123
Statistical analysis .....	124
Results.....	125
TGF- $\beta$ augments cell-associated SPARC expression and induces SPARC secretion in human ASM cells .....	125
TGF- $\beta$ -induced expression of cell-associated and secreted SPARC in human ASM cells is reversed in the presence of chemical chaperones ...	127
Chemical inducers of ER stress inhibit basal SPARC secretion, but do not modulate cell-associated SPARC expression in human ASM cells.....	129



TGF- $\beta$ and thapsigargin induce different levels of ER stress in ASM cells, but this does not explain their differential effect on SPARC secretion .....	131
Thapsigargin mediated inhibition of SPARC secretion is not due to IRE1 $\alpha$ /RIDD dependent signalling.....	135
SPARC secretion is reduced in ASM cells from subjects with COPD .....	141
Discussion .....	142
References .....	149

**Proteomic analysis of extracellular HMGB1 identifies binding partners and exposes its potential role in airway epithelial cell homeostasis ..... 157**

Abstract .....	158
Introduction.....	159
Materials and Methods .....	161
Generation of airway epithelial cell culture supernatants .....	161
Separation of protein complexes using 1D hrCNE and 2D hrCNE/SDS-PAGE .....	162
Detection of HMGB1 using immunoblotting.....	163
Isolation of HMGB1-binding proteins using immunoprecipitation .....	164
Isolation of HMGB1-binding proteins using pull-down assay.....	165
Identification of HMGB1-binding proteins using LC-MS/MS .....	167
Protein-protein interaction network and Gene Ontology term enrichment analysis .....	168
Results.....	168
Extracellular HMGB1 exists in a multimeric state in unstimulated human airway epithelial cell cultures .....	168
Enrichment of extracellular HMGB1 and identification of its binding proteins using immunoprecipitation.....	172
Identification of HMGB1-binding proteins via pull-down assay .....	174

Combined profile of HMGB1-binding proteins identified using clear native electrophoresis, immunoprecipitation and pull-down assays.....	176
Bioinformatic analysis reveals novel HMGB1-binding proteins and predicts homeostatic functions of extracellular HMGB1 .....	180
Discussion .....	185
References .....	194
<b>General Discussion .....</b>	<b>206</b>
References .....	216
<b>Appendices.....</b>	<b>225</b>
Appendix I. HMGB1-binding proteins identified using high resolution clear native electrophoresis (hrCNE) coupled to LC-MS/MS.....	226
Appendix II. HMGB1-binding proteins identified using immunoprecipitation (IP) coupled to LC-MS/MS.....	229
Appendix III. HMGB1-binding proteins identified using pull-down assay coupled to LC-MS/MS.....	234
Appendix IV. Evidence of interaction and confidence score for identified primary interactors of HMGB1. ....	238
Appendix V. Gene ontology term analysis (cellular component) of the 37 HMGB1-binding proteins identified using 2 or more techniques. ....	240
Appendix VI. Gene ontology term analysis (molecular function) of the 37 HMGB1-binding proteins identified using 2 or more techniques. ....	246
Appendix VII. Gene ontology term analysis (biological process) of the 37 HMGB1-binding proteins identified using 2 or more techniques. ....	250
Appendix VIII. Copyright permission to reprint published manuscript.....	268

# LIST OF FIGURES

## Chapter 1

Figure 1.1: Overview of airway inflammation and remodeling in chronic airways disease. ....	7
Figure 1.2: Distinct histopathologies in the asthmatic and chronic obstructive pulmonary disease (COPD) airways. ....	10
Figure 1.3: The signaling arms of the unfolded protein response (UPR). ....	17

## Chapter 2

Figure 2.1: Proposed role of SPARC in airway and vascular remodelling in asthma and COPD. ....	54
--	----

## Chapter 3

Figure 3.1: Effect of TGF- $\beta$ on secreted SPARC in human airway epithelial cells 16-HBE14o-. ....	86
Figure 3.2: Effect of TGF- $\beta$ on cell-associated and secreted SPARC in primary human airway epithelial cells. ....	87
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- $\beta$ . ....	90
Figure 3.4: Effect of type 1 cytokines on cell-associated and secreted SPARC in asthmatic and non-asthmatic airway epithelial cells. ....	92
Figure 3.5: Effect of type 2 cytokines on cell-associated and secreted SPARC in asthmatic and non-asthmatic airway epithelial cells. ....	93
Figure 3.6: Effect of SPARC on cytokine and chemokine secretion in primary human airway epithelial cells. ....	95
Figure 3.7: Effect of TGF- $\beta$ on cytokine and chemokine secretion in primary human airway epithelial cells. ....	97
Figure 3.8: Comparative effect of SPARC and TGF- $\beta$ on epithelial-mesenchymal transition markers in primary human airway epithelial cells. ....	99

## Chapter 4

Figure 4.1: TGF- $\beta$ induces cell-associated and secreted SPARC in human airway smooth muscle cells. ....	126
Figure 4.2: Chemical chaperones reverse TGF- $\beta$ -induced cell-associated and secreted SPARC in human airway smooth muscle cells.....	128
Figure 4.3: Effect of ER stress inducers and calcium chelator on cell-associated and secreted SPARC in human airway smooth muscle cells.....	130
Figure 4.4: Thapsigargin induces greater ER stress than TGF- $\beta$ in human airway smooth muscle cells. ....	133
Figure 4.5: Loss of SPARC under conditions of severe ER stress could not be reversed by chemical chaperones. ....	134
Figure 4.6: Thapsigargin, but not TGF- $\beta$ activates the IRE1 $\alpha$ /XBP-1 pathway in human airway smooth muscle cells. ....	137
Figure 4.7: Loss of SPARC under conditions of severe ER stress is not due to IRE1 $\alpha$ /RIDD dependent signalling. ....	138
Figure 4.8: Effect of IRE1 $\alpha$ kinase inhibitors on IRE1 $\alpha$ /XBP-1 signalling. ....	139
Figure 4.9: Effect of IRE1 $\alpha$ RNase inhibitors on IRE1 $\alpha$ /XBP-1 signalling. ....	140
Figure 4.10: TGF- $\beta$ -induced SPARC expression is attenuated in COPD airway smooth muscle cells.....	141

## Chapter 5

Figure 5.1: Experimental approach used to identify HMGB1-binding proteins in airway epithelial cell culture supernatants.....	162
Figure 5.2: Structure and protein sequence of His-tagged recombinant human HMGB1.....	166
Figure 5.3: Analysis of extracellular HMGB1 using high resolution clear native electrophoresis.....	171
Figure 5.4: Analysis of extracellular HMGB1 using immunoprecipitation. ....	173
Figure 5.5: Analysis of extracellular HMGB1 using pull-down assays.....	176
Figure 5.6: HMGB1-binding proteins detected using different approaches. ....	177
Figure 5.7: Protein network analysis of HMGB1-binding proteins.....	181
Figure 5.8: KEGG pathway analysis of identified HMGB1-binding proteins....	185

# LIST OF TABLES

## Chapter 1

Table 1.1: Roles of airway epithelial cells and airway smooth muscle cells in airway inflammation and remodeling in chronic airways disease.....	12
--	----

## Chapter 2

Table 2.1: Classification of SPARC family proteins.....	41
Table 2.2: Role of SPARC in different cellular compartments.....	44

## Chapter 4

Table 4.1: COPD and non-COPD ASM cell donors in which SPARC secretion was assessed . .....	121
Table 4.2: List of chemical inducers and inhibitors of ER stress used for ASM cell treatment. ....	122

## Chapter 5

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.....	178
Table 5.2: Top 15 Gene Ontology terms enriched in HMGB1-binding proteins identified using 2 or more techniques.....	183

# 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 <sup>2+</sup>	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)- <i>N,N,N',N'</i> -tetraacetic acid
eIF2 $\alpha$	Eukaryotic translation initiator factor 2 $\alpha$
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
GO	Gene Ontology
GRP78	Glucose-regulated protein 78
GSK-3 $\beta$	Glycogen-synthase kinase-3 beta
HMGB1	High mobility group box 1
hrCNE	High resolution clear native electrophoresis
IFN- $\gamma$	Interferon gamma
ILC2s	Type 2 innate lymphoid cells
ILC3s	Type 3 innate lymphoid cells
IPF	Idiopathic pulmonary fibrosis
IRE1 $\alpha$	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 <sup>2+</sup> -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 lymphopietin
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- $\beta$ ), 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- $\beta$  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- $\beta$ -mediated SPARC expression in AECs, and showed that TGF- $\beta$ -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.