The Biogenic Pathways of Malignant and Non-Malignant Microvesicles

Jack Taylor

Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

The University of Technology Sydney

GRADUATE SCHOOL OF HEALTH

2019

CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Jack Taylor declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Graduate School of Health at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise reference or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

This research is supported by the Australian Government Research Training Program.

Production Note:
Signature removed
prior to publication.

Jack Taylor

8/11/19

Date

Acknowledgements

First and foremost I would like to thank Professor Mary Bebawy. Thank you for being so generous with your time, knowledge, and advice – I have learnt more than I thought possible thanks to you. For me, the most satisfying part of this journey and the research we have produced is that we have always worked as a team.

To my other supervisors, Dr Ritu Jaiswal, Professor Gregory Monteith, and Dr Michael Johnson, thank you for your support, expertise, and ideas. You have been a source of inspiration throughout my PhD.

I have also been lucky to have worked with The Two Christians: Christian Loebbe who is an absolute master on the AFM, and Christian Evenhuis the image analysis wizard.

To my fellow doctoral candidates in the Graduate School of Hacks. Thanks for the bi-weekly long lunches, coffee runs, beers, and for just being all round legends. Special mentions go to The American, Carmenelocita, and The White Horse – great effort from you guys. Keep it up!

To my UTS-unaffiliated friends, I am so lucky to have you all. The weekends surf trips, early morning surfing session, guitar groups, lunches, catch ups, etc. etc. etc. balanced my life perfectly. You all helped me immensely and I will be forever grateful.

I'd like to thank my most favourite family Emma, Mum, and Dad for the infinite love and support they give me. Mum, I know you would have brought me dinner every night if you could. I love you guys.

Finally, I'd like to thank my sunshine, Aims. Coming home to that smile every day is the best. Thanks for organising our adventures (as I am incapable), infinite laughs, and love, and support. You're unreal, I love you.

Table of Contents

Acknowledgements ..... **iii**

Table of Contents ..... **iv**

List of Figures and Tables ..... **vi**

List of Abbreviations ..... **vii**

Publications arising from this work ..... **x**

Manuscripts under review ..... **x**

Scholarships ..... **xi**

Conference Presentations ..... **xi**

Abstract ..... **xii**

Introduction **1**

Proteins Regulating Microvesicle Biogenesis and Multidrug Resistance in Cancer **1**

Author Contribution Statement – Chapter 1 17

Aims and Objectives **18**

2. Calcium-calpain Dependent Pathways Regulate Vesiculation in Malignant Breast Cells **19**

Author contribution statement – Chapter 2 29

3. Ca^{2+} mediates Extracellular Vesicle Biogenesis Through Alternate Pathways in Malignancy **30**

Author contribution statement – Chapter 3 ..... **45**

4. Membrane to Cytosol Redistribution of αII-Spectrin Drives Extracellular Vesicle Biogenesis in Malignant Cells **46**

4.1. Abstract **48**

4.2. Introduction **50**
4.3. Materials and methods **54**
4.3.1. Chemicals 54
4.3.2. Cell culture 54
4.3.3. Confocal microscopy 54
4.3.4. Cell segmentation and spectrin localisation analysis 55
4.3.5. Atomic force microscopy 57
4.3.6. Statistics 58
4.4. Results **59**
4.4.1. The subcellular localisation of spectrin differs in malignant and non-malignant cells at rest. 59
4.4.2. Increasing intracellular calcium by A23187 results in loss of peripheral spectrin localisation in non-malignant MBE-F cells 63
4.4.3. Inhibition of plasma membrane MV biogenic machinery restores peripheral αII-spectrin borders in MCF-7 cells. 65
4.4.4. Peripheral αII-spectrin borders correlate with increased membrane stiffness. 69
4.5. Discussion **71**
4.6. Conclusions **76**
4.7. References **78**
Author contribution statement – Chapter 4 82
Conclusions and Future directions **83**
Future Directions **92**
References **96**

List of Figures and Tables

Chapter 4

Figure 4. 1. Schematic of the mathematical processing used to segment individual cell nuclei (a) individual cell walls (b). 56

Figure 4. 2. Spectrin is differentially localised non-malignant cells and malignant cells. 60

Figure 4. 3. Quantitative analysis of spectrin distribution in malignant and non-malignant cells. 62

Figure 4. 4. Spectrin membrane localisation diminishes following treatment with calcium ionophore, A23187 in MBE-F cells. 64

Figure 4. 5. Inhibition of the calcium-calpain pathway results in time-dependent restoration of spectrin boarders in MCF-7 cells. 67

Figure 4. 6. Mechanical stiffness of cells correlate with peripheral spectrin distribution. 69

List of Abbreviations

Ab	Antibody
AFM	Atomic force microscope
μM	Micro molar
ABC	ATP-binding cassette
ADP	Adenosine triphosphate
ALLM	N-Acetyl-L-leucyl-L-leucyl-L-methioninal
BAPTA-AM	1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester)
BCRP	Breast cancer resistance protein
BSA	Bovine serum albumin
Ca2+	Calcium ion
DAPI	4′,6-diamidino-2-phenylindole
EBM-2	Endothelial Cell Basal Medium-2
ER	Endoplasm reticulum
ESCRT	Endosomal sorting complexes required for transport
EV	Extracellular vesicle
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
GPG	Gycophorin C
ILV	Intraluminal vesicle
kPa	kilo Pascal
MDR	Multidrug resistance
MEG-1	Mammary Epithelial Cell Growth Medium-1
MP	Microparticle

MRP1	Multidrug Resistance-Associated Protein 1
MV	Microvesicle
MVB	Multivesicular body
NA	Numerical aperture
NBD	Nonbinding domains
nM	nanomolar
nMase	Neutral Sphingomyelinase
PBS	Phosphate buffered saline
PC	phosphatidylcholine
PCH	Pombe Cdc15 homology
PE	phosphatidylethanolamine
PFA	Paraformaldehyde
P-pg	P-glycoprotein
PS	phosphatidylserine
PSS	physiological salt solution
Ptdlns(4,5)P2	Phosphatidylinositol 4,5-bisphosphate
RBC	Red blood cell
RMS	Root mean square
ROCK1	Rho-associated, coiled-coil containing protein kinase 1
ROS	Reactive oxygen species
RPMI	Roswell park media
SD	Standard deviation
SERCA	Sarco/endoplasmic reticulum calcium ATPase
SM	sphingomyelin
SOCE	Store-operated calcium entry

TG	Thapsigargin
TMD	Transmembrane domains
TRPC5	Transient receptor potential channel
YM58483	*N*-[4-[3,5-*Bis*(trifluoromethyl)-1*H*-pyrazol-1-yl]phenyl]-4-methyl-1,2,3-thiadiazole-5-carboxamide

Publications arising from this work

Taylor, J., Jaiswal, R. & Bebawy, M. 2017. Calcium-calpain Dependent Pathways Regulate Vesiculation in Malignant Breast Cells. Current Cancer Drug Targets, 17, 486-494.

Taylor, J. & Bebawy, M. 2019. Proteins Regulating Microvesicle Biogenesis and Multidrug Resistance in Cancer. Proteomics, 19, 1615-9861.

Taylor J., Azimi, I., Monteith, G., & Bebawy, M. 2020. Ca^{2+} mediates extracellular vesicle biogenesis through alternate pathways in malignancy, Journal of Extracellular Vesicles, 9:1, DOI: 10.1080/20013078.2020.1734326

Manuscripts under review

Taylor, J., Patio, K., Morris, M., Evenhuis, C., Johnson, M., Bebawy, M. 2019. Membrane to Cytosol Redistribution of αII-Spectrin Drives Extracellular Vesicle Biogenesis in Malignant cells. (Pre-submission inquiry)

Scholarships

Research

Australian Government Research Training Program Stipend

Travel

Vice-Chancellor's Postgraduate Research Student Conference Fund

Conference Presentations

Sarco/endoplasmic reticulum ATPase inhibition activates calcium signalling pathways for microvesicle biogenesis. International Society of Extracellular Vesicles. May 2nd – May 6th, 2018, Barcelona, Spain.

Sarco/endoplasmic reticulum ATPase inhibition activates calcium signalling pathways for microvesicle biogenesis. Australasian Extracellular Vesicles Conference. 14th – 16th November, 2018, Sydney, Australia.

Abstract

Plasma membrane-derived extracellular vesicles (EV) are released for most cells types and are important mediators of intercellular communication. In the context of cancer, EVs mediate the intercellular transfer of deleterious traits including multidrug resistance (MDR), enhanced metastatic capacity, evasion of immune surveillance, and altered tissue biomechanics. The biogenesis of plasma membrane EVs is characterised by an increase in intracellular calcium followed by successive membrane and cytoskeletal changes. However, the precise biogenic mediators and differences between malignant and non-malignant plasma membrane-derived EV biogenesis remain undefined. Uncovering discrete pathways of biogenesis in malignant and non-malignant cells holds potential in the search for new strategies to circumvent the acquisition and transfer of plasma membrane EV-meditated deleterious traits in cancer.

In this thesis, the molecular pathways that regulate plasma membrane EV biogenesis in malignant and non-malignant cells are investigated.

This study evidences that discrete EV biogenic pathways exist in malignant and non-malignant cells. At rest, biogenesis of cancer EVs is regulated by a calcium-calpain dependent pathway whereas non-malignant biogenesis is driven by alternative pathways. Comparing the surface topography of malignant and non-malignant cells using atomic force microscopy demonstrates malignant cells have intrinsically higher vesiculation at rest and this is shown to be driven by high basal calcium mobilisation. Interrogating the calcium signalling pathways that regulate biogenesis with pharmacological modulators of calcium revealed increases in free cytosolic Ca^{2+} via endoplasmic reticulum (ER) Ca^{2+} store depletion with thapsigargin increases EV biogenesis in both malignant and non-malignant cells. Evidencing a role for the ER in plasma membrane EV biogenesis for the first time. Furthermore, the store-operated calcium entry

(SOCE) plays an essential role in the maintenance of plasma membrane EV biogenesis after store depletion.

The calcium-calpain pathway is also a known regulator of αII-spectrin structural dynamics and this work evidences that it plays a role in increased vesiculation in malignant cells. High resolution confocal microscopy in combination with a custom designed, automated image analysis plugin, provided an unbiased platform to show that the subcellular localisation of spectrin is distinctly different in malignant and non-malignant cells at rest. Non-malignant cells display prominent αII-spectrin borders and are low vesiculators. It is the redistribution of αII-spectrin from the membrane to the cytoplasm that drives plasma membrane-derived EV biogenesis in malignant cells.

These findings provide new insight into the biogenic pathways regulating EV biogenesis in both malignant and non-malignant cells and identifies strategies for the selective modulation of plasma membrane EV biogenesis in malignancy.

Chapter 1

Introduction

Proteins Regulating Microvesicle Biogenesis and Multidrug Resistance in Cancer

Proteomics, **19**, 1615-9861 (2019)

REVIEW
Biogenesis

Proteomics
www.proteomics-journal.com

Proteins Regulating Microvesicle Biogenesis and Multidrug Resistance in Cancer

*Jack Taylor and Mary Bebawy**

Microvesicles (MV) are emerging as important mediators of intercellular communication. While MVs are important signaling vectors for many physiological processes, they are also implicated in cancer pathology and progression. Cellular activation is perhaps the most widely reported initiator of MV biogenesis, however, the precise mechanism remains undefined. Uncovering the proteins involved in regulating MV biogenesis is of interest given their role in the dissemination of deleterious cancer traits. MVs shed from drug-resistant cancer cells transfer multidrug resistance (MDR) proteins to drug-sensitive cells and confer the MDR phenotype in a matter of hours. MDR is attributed to the overexpression of ABC transporters, primarily P-glycoprotein and MRP1. Their expression and functionality is dependent on a number of proteins. In particular, FERM domain proteins have been implicated in supporting the functionality of efflux transporters in drug-resistant cells and in recipient cells during intercellular transfer by vesicles. Herein, the most recent research on the proteins involved in MV biogenesis and in the dissemination of MV-mediated MDR are discussed. Attention is drawn to unanswered questions in the literature that may prove to be of benefit in ongoing efforts to improve clinical response to chemotherapy and circumventing MDR.

J. Taylor, Prof. M. Bebawy
Discipline of Pharmacy
Graduate School of Health
The University of Technology Sydney
Sydney, Australia
E-mail: mary.bebawy@uts.edu.au

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/pmic.201800165

DOI: 10.1002/pmic.201800165

Author Contribution Statement – Chapter 1

J. Taylor wrote the manuscript and made the figures. M. Bebawy was responsible for the overall project, conceptual advice, and revision of the manuscript.

Aims and Objectives

Plasma membrane extracellular vesicles (EVs) play a vital role in the progression of cancer via non-genetic dissemination and acquisition of MDR and other deleterious cancer traits. Despite this, the signalling pathways that regulate their biogenesis remain to be fully elucidated. Furthermore, differences between non-malignant and malignant vesiculation are unknown. This thesis aims to elucidate the biogenic pathways of EVs secreted by malignant and non-malignant cells. The three specific objectives of this thesis include:

1. To establish the role of calpain in vesiculation in resting and calcium activated malignant and non-malignant cells.
2. To determine the role of endoplasmic reticulum (ER) calcium stores and the involvement of store-operated calcium entry in plasma membrane EV biogenesis
3. To establish the subcellular distribution of spectrin in low-vesiculating non-malignant cells and high-vesiculating malignant cells.

Chapter 2

2. Calcium-calpain Dependent Pathways Regulate Vesiculation in Malignant Breast Cells

Current Cancer Drug Targets, 17, 1568-0096 (2017)

Send Orders for Reprints to reprints@benthamscience.ae

Current Cancer Drug Targets, 2017, *17*, 1-9

RESEARCH ARTICLE

Calcium-calpain Dependent Pathways Regulate Vesiculation in Malignant Breast Cells

Jack Taylor, Ritu Jaiswal and Mary Bebawy*

Discipline of Pharmacy, Graduate School of Health, The University of Technology Sydney, NSW, Australia

ARTICLE HISTORY

Received: April 27, 2016
Revised: August 08, 2016
Accepted: September 02, 2016

DOI: 10.2174/1568009616666161026165736

Abstract: ***Background*:** Multidrug resistance in cancer (MDR) occurs when tumours become cross-resistant to a range of different anticancer agents. One mechanism by which MDR can be acquired is through cell to cell communication pathways. Membrane-derived microparticles (MPs) are emerging as important signaling molecules in this process. MPs are released from most eukaryotic cells and transfer functional proteins and nucleic acids to recipient cells conferring deleterious traits within the cancer cell population including MDR, metastasis, and angiogenesis. MP formation is known to be dependent on calpain, an intracellular cysteine protease which acts to cleave the cytoskeleton underlying the plasma membrane, resulting in cellular surface blebbing

***Objective*:** To establish the role of calpain in vesiculation in malignant and non-malignant cells by 1) comparing membrane vesiculation at rest and following the release of intracellular calcium, and 2) comparing vesiculation in the presence and absence of calpain inhibitor II (ALLM).

***Method*:** This study examines the differences in vesiculation between malignant and non-malignant cells using high-resolution Atomic Force Microscopy (AFM). HBEC, MBE-F, MCF-7, and MCF-7/Dx cells were analysed at rest and following treatment with calcium ionophore A23187 for 18 hours. Vesiculation of calcium activated and resting malignant and non-malignant cells was also assessed after 18 hour treatment of calpain inhibitor II (ALLM).

***Results*:** We demonstrate that malignant MCF-7 and MCF-7/Dx cells have an intrinsically higher degree of vesiculation at rest when compared to non-malignant human brain endothelial cells (HBEC) and human mammary epithelial cells (MBE-F). Cellular activation with the calcium ionophore A23187 resulted in an increase in vesiculation in all cell types. We show that calpain-mediated MP biogenesis is the dominant pathway at rest in malignant cells as vesiculation was shown to be inhibited with calpain inhibitor II (ALLM).

***Conclusion*:** These results suggest that differences in the biogenic pathways exist in malignant and non-malignant cells and have important implications in defining novel strategies to selectively ~~target-ing~~ malignant cells for the circumvention of deleterious traits acquired through intercellular exchange of extracellular vesicles.

Keywords: Atomic force microscopy, breast cancer, calcium, calpain, extracellular vesicles, microparticles, multidrug resistance, vesiculation.

*Address correspondence to this author at the Discipline of Pharmacy, Graduate School of Health, The University of Technology Sydney, P.O. Box: 123 Broadway, NSW 2007, Australia; Tel: +61295148305; Fax: +61295148300; E-mail: mary.bebawy@uts.edu.au

1568-0096/17 $58.00+.00

Author contribution statement – Chapter 2

J. Taylor performed all of the experiments, analysed the data and wrote the manuscript. R. Jaiswal was responsible for experimental design, project supervision, and manuscript revision. M. Bebawy was responsible for the overall project, experimental design, project supervision and revision of the manuscript.

Chapter 3

3. Ca^{2+} mediates Extracellular Vesicle Biogenesis Through Alternate Pathways in Malignancy

The Journal of Extracellular Vesicles, 9, 1734326 (2020)

JOURNAL OF EXTRACELLULAR VESICLES
2020, VOL. 9, 1734326
https://doi.org/10.1080/20013078.2020.1734326

Ca^{2+} mediates extracellular vesicle biogenesis through alternate pathways in malignancy

Jack Taylor[a], Iman Azimi[b], Gregory Monteith [c,d,e] and Mary Bebawy[a]

[a]Discipline of Pharmacy, Graduate School of Health, The University of Technology Sydney, Australia; [b]Division of Pharmacy, College of Health and Medicine, University of Tasmania, Australia; [c]School of Pharmacy, The University of Queensland, Brisbane, Australia; [d]Mater Research, Translational Research Institute, the University of Queensland, Brisbane, Australia; [e]Translational Research Institute, The University of Queensland, Brisbane, Australia

ABSTRACT

Extracellular vesicles (EVs) are small extracellular membrane vesicles that serve as important intercellular signalling intermediaries in both malignant and non-malignant cells. For EVs formed by the plasma membrane, their biogenesis is characterized by an increase in intracellular calcium followed by successive membrane and cytoskeletal changes. EV production is significantly higher in malignant cells relative to non-malignant cells and previous work suggests this is dependent on increased calcium mobilization and activity of calpain. However, differences in calcium-signalling pathways in the context of malignant and non-malignant EV biogenesis remain unexplored. Here, we demonstrate vesiculation is greater in malignant MCF-7 cells relative to non-malignant hCMEC-D3 cells, increases in free cytosolic Ca^{2+} via endoplasmic reticulum (ER) Ca^{2+} store depletion with thapsigargin increases EV biogenesis in both cell types, and vesicular induction is abolished by the intracellular Ca^{2+} chelator BAPTA-AM. Store-operated calcium entry (SOCE) plays an essential role in the maintenance of EV biogenesis after store depletion. These findings contribute to furthering our understanding of extracellular vesicle biogenesis. Furthermore, since EVs are key mediators in the intercellular transfer of deleterious cancer traits such as cancer multidrug resistance (MDR), understanding the molecular mechanisms governing their biogenesis in cancer is the crucial first step in finding novel therapeutic targets that circumvent EV-mediated MDR.

ARTICLE HISTORY
Received 8 July 2019
Revised 30 January 2020
Accepted 11 February 2020

KEYWORDS
Biogenesis; extracellular vesicles; microvesicles; calcium; endoplasmic reticulum; sarco/endoplasmic reticulum calcium ATPase; SERCA; store operated calcium entry; cancer; multidrug resistance

Introduction

Extracellular vesicles (EVs) are sub-micron sized membrane-enclosed vesicles released from all cell types [1,2]. Released by all cell types, EVs as signalling vectors, regulate numerous physiological pathways ranging from ageing to inflammation, coagulation to immunity. EVs are also implicated in mediating many disease pathologies including but not limited to; autoimmune disease, infectious disease, cardiovascular disease and cancer. Of particular interest to our laboratory is their contribution to cancer survival via promotion of metastasis, and chemotherapeutic resistance. Tumour EVs mediate the transfer of functional resistance proteins and nucleic acids from drug-resistant to drug-sensitive cells resulting in intercellular conferral of multidrug resistance (MDR) [3–5]. We also showed through the study of EVs that MDR was complex and extending beyond drug – cell interactions. Specifically, EVs from MDR cells could confer increased metastatic capacity [6], active and passive drug sequestration [7], immune evasion [8] and altered tissue biomechanics [9].

The significance of EVs is not restricted to cellular signalling and they have emerged as novel drug-delivery vehicles, and important biomarkers [10,11].

Delineation of extracellular vesicle subtypes is typically based on size and biogenic origin [12,13]. The cellular machinery and signalling pathways involved in EV release also differ between EV subtypes. The process for exosome biogenesis has been extensively described by, Raposo and Stoorvogel [1], Colombo et al. [14] and Kalra et al. [15]. In contrast, the biogenesis of plasma membrane-derived EVs is less well defined, owing in part to early reports defining MVs as simply inert by-products of cellular stress and/or cell death [2,16–18].

Plasma membrane EV release can be initiated through cellular activation in response to stimuli such as exposure to serine proteases, thrombin, calcium ionophores, ADP, inflammatory cytokines, growth factors and shear inducers [19–23]. In breast cancer cells, the calcium channel TRPC5 is important in MDR via extracellular vesicles [24]. Cellular activation and

CONTACT Mary Bebawy mary.bebawy@uts.edu.au Discipline of Pharmacy, Graduate School of Health, the University of Technology Sydney, Sydney, Australia

a subsequent increase in intracellular Ca^{2+} affects both plasma membrane and cytosolic machinery leading to plasma membrane EV biogenesis. At steady state, phospholipids present in the plasma membrane are asymmetrically distributed. The anionic phospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) localize to the inner leaflet whilst phosphatidylcholine (PC) and sphingomyelin (SM) localize to the outer [25]. A network of lipid translocases, modulated by calcium, maintains the asymmetry [26–28]. Increase in intracellular calcium results in the collapse of plasma membrane phospholipid asymmetry resulting in a destabilization of plasma membrane-cytoskeletal anchorage [29,30]. Calcium mobilization concurrently activates calpain, a cysteine protease, which in turn cleaves several cytoskeletal components including actin, ankyrin, protein 4.1 and spectrin [31,32]. Calpain-mediated cleavage of the cytoskeleton further disrupts attachment to the membrane and these localized regions bud outwards to form plasma membrane EVs [20,33,34].

An increase in intracellular calcium appears to be the initiating step in plasma membrane EV biogenesis [20,35–37].

The calcium ion (Ca^{2+}) is a promiscuous signalling molecule, involved in diverse cellular processes ranging from neurological transmission to muscular contraction [38]. In non-excitable cells, cellular calcium activates signalling cascades through effects on Ca^{2+}-regulated proteins and also via Ca^{2+} dependent transcription factors [39].

Cells maintain tight control of compartmental Ca^{2+} homoeostasis through a network of calcium channels, pumps and exchangers. This network facilitates simultaneous operation of calcium signalling pathways in cells. Dysregulation of Ca^{2+} signalling is a key feature of many diseases with calcium modulation forming a significant part of pharmacotherapy [40–43].

In the context of malignancy, dysregulation of Ca^{2+} homoeostasis significantly contributes to disease progression [44] as well as facilitating other cancer hallmarks [43,45]. For this reason, the therapeutic targeting of calcium signalling presents clinical opportunities [40,46]. Despite the known relationship between elevated intracellular calcium and plasma membrane vesiculation, the exact pathway(s) and channels involved in biogenesis remain to be fully defined.

We recently reported the presence of distinct vesiculation pathways in malignant and non-malignant cells at rest [47]. We demonstrated that a calcium–calpain dependent pathway regulated malignant cell biogenesis and an alternative pathway driving biogenesis was proposed for non-malignant cells at rest [47].

In this paper, we investigate the Ca^{2+} signalling pathways involved in regulating plasma membrane EV biogenesis in malignant and non-malignant cells. Specifically, we propose a role for endoplasmic reticulum (ER) calcium stores and store-operated Ca^{2+} entry (SOCE) in EV biogenesis. The discovery of discrete pathways of biogenesis in malignant and non-malignant cells is promising in the search for new strategies to circumvent the acquisition and transfer of plasma membrane EV-mediated deleterious traits in cancer and for advancing knowledge in EV biology.

Methods

Cell culture

The drug-sensitive human breast adenocarcinoma cell line MCF-7 and its drug-resistant sub-line MCF-7/Dx (Dx for simplicity) were routinely cultured in RPMI-1640 (Sigma-Aldrich, NSW, Australia) supplemented with 10% FBS as previously described [48]. Incremental exposure of MCF-7 cells to doxorubicin was used to develop the Dx resistant sub-line as previously described [48–50]. The non-malignant human brain endothelial cell line (hCMEC-D3) was cultured in Endothelial Cell Basal Medium-2 (EBM-2; Lonza, MD, USA) as previously described [51]. All cell lines were maintained at 37°C in a humidified 5% CO_2 incubator. All cell lines were routinely tested for mycoplasma infection.

Atomic force microscopy

The NanoWizard 4 BioScience Atomic Force Microscope (AFM; JPK Instruments, Germany) was used for cell surface topography studies. Cells were fixed in 4% paraformaldehyde (PFA) for 30 min, washed once with phosphate-buffered saline (PBS) and twice with distilled water. Cells were air-dried for ~1 h to allow the membrane-bound vesicles to transform to vesicle-derived pits as previously described [47]. Topographical AFM imaging was carried out in contact mode with sharpened silicon nitride cantilevers (L: 225 µm, W: 46 µm, T: 1.0 µm, SPM Probe Model: SHOCON; AppNano) with a tip radius of < 10 nm and spring constant of 0.6 N/m. Scans were performed in air at a rate of 1 Hz. Three areas were selected at random for scanning for each treatment condition and all experiments were performed in triplicate.

The raw data from AFM height measurements were digitally filtered as previously described by Antonio et al. [52] and Root Mean Square Roughness (R_{RMS}) was computed with the open source software

Gwyddion [53]. The filtration procedure isolated features of interest (plasma membrane EV-derived pits) from features such as the overall cell shape. Given the size range of plasma membrane EVs, the specific filtration procedure applied in this case is separating features ranging from 100 to 1000 nm.

Each AFM image (30 μm^2) was scanned with 512 points (scan step Δx = 58.6 nm) and a filtration procedure was applied with a cut-off normalized frequency (f_c) of 0.07 to remove all features with steps λ longer than 1674.3 nm ($\lambda = 2 \cdot \Delta x/f_c$). Previous work has demonstrated that plasma membrane vesiculation in occurs in perinuclear regions of the membrane [47]. Therefore, a "zoomed" 10 μm^2 area was scanned within each 30 μm^2 was also taken to determine roughness in these areas of surface. These scans consist of 512 points (scan step Δx = 19.5 nm) and are filtered to remove features larger than 975 nm (f_c = 0.04; $\lambda = 2 \cdot \Delta x/f_c$). The Root Mean Square Roughness (R_{RMS}) was then computed as a quantitative measure of surface topography across different treatments.

Intracellular calcium imaging and quantification in MCF-7 cells using imageXpress

Cellular calcium was analysed using the ratiometric fluorescent indicator, Fura-2 AM as previously described [54,55]. Cells were loaded with Fura-2 AM for 30 min at 37°C in the dark with a dye-loading solution comprising 4 µM Fura-2 AM (Invitrogen) in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum with and without 50 µM 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM). Cells were allowed to incubate for a further 15 min in the dark at ambient temperature in physiological salt solution (PSS [nominal]; 10 mM HEPES, 5.9 mM KCl, 1.4 mM MgCl2, 1.2 mM NaH2PO4, 5 mM NaHCO3, 140 mM NaCl, 11.5 mM glucose, 1.8 mM $CaCl_2$, pH 7.3). Loading-dye solution was removed and cells were washed twice with PSS Ca^{2+} (PSS supplemented with 1.8 mM $CaCl_2$) and then twice with PSS no added extracellular (nominal) to ensure less than 100 µM Ca^{2+} was remaining in the test wells [56]. Fresh PSS containing 1.8 mM $CaCl_2$ was added for intracellular calcium measurements.

MCF-7 cells loaded with Fura-2 AM were stimulated with the sarco-endoplasmic reticulum calcium ATPase (SERCA) inhibitor, thapsigargin (TG) (300 nM). Experiments were performed using the ImageXpress^{MICRO} high content imaging system (Molecular Devices, Sunnyvale, CA, USA). Measurements were taken at an excitation wavelength of 340 and 387 nm with fluorescence emission detected using a 510–520 nm band pass filter. Two baseline measurements were taken at 0 and 10 s. TG was loaded into the reagent plate and added to test wells at 20 s. Fura-2 AM ratio data points were acquired every 5 s for a 300 s window. Relative changes in intracellular calcium $[Ca^{2+}]_I$ were determined following background subtraction using the ratio of the two wavelengths, 340 and 387 nm for each time interval.

Cell viability following TG treatment

The effects of TG concentrations (1–300 nM) over a 10 min treatment window on cell viability were assessed by trypan blue dye exclusion at 24-h post treatment as described [20].

MV isolation and quantitative flow cytometric analysis of plasma membrane EVs

Plasma membrane EVs were isolated by a process of differential centrifugation as previously described [3,48]. Briefly, cell supernatants were collected from approximately 1.2 × 10^7 cells and centrifuged for 5 min at 500 × g to remove cells and large cellular debris. Smaller debris was removed by two further spins at 2000 × g for 1 min. The supernatant was collected and centrifuged at 18,000 × g for 30 min at 4°C to pellet EVs. Isolated EVs were resuspended in 200 µL PBS for analysis.

Flow cytometric analysis was conducted on the BD LSRFortessa™ X-20 flow cytometer (BD Biosciences) custom built with a 100 mW blue laser for small particle detection. Plasma membrane EV gates were established as previously described using latex sizing beads 0.3–1.1 µm in diameter (BD Biosciences). The threshold in side scatter was adjusted to 200 arbitrary units to avoid background noise [57]. The predefined gate was applied to all samples during analysis. The performance of lasers was validated before each experiment using BD FACSDiva™ CS&T Research Beads (BD, Australia/New Zealand).

EV samples resuspended in PBS were combined with TruCount™ beads and analysed according to the manufacturer's instructions. The tube was gently vortexed and loaded onto the flow cytometer, after which the fluid stream was allowed to stabilize to a flow rate of 10 events/second for 30 s. To prevent cross-contamination of samples, the sample line was flushed with MilliQ water for at least 30 s between each run. The stop gate was set at a fixed number of 2000 of Trucount™ beads during data acquisition. A PBS blank buffer control was run to ensure

baseline events were low (data not shown). The number of EVs per µL was calculated using the formula: N = (gated MV events/gated Trucount™ events) × total number of Trucount™ beads [11]. EV events were normalized to individual well cell counts to account for variation in cell numbers between samples as previously described [20]. FACSDiva version 8.0.1 (BD Biosciences) was used for analysis of flow cytometry data. Full description of methodologies was also submitted to EV-TRACK (ID: EV190073)

Statistics and data analysis

Data were analysed with a one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparisons test using the GraphPad Prism version 7.02 for Windows (GraphPad Software, La Jolla, CA, USA). Data are presented as the mean or mean ± SD of 3 individual experiments with predictive results value of (****) $P < 0.0001$, (***) $P < 0.0005$, (**) $P < 0.01$ and (*) $P < 0.05$ were considered statistically significant.

Results

Mobilization of intracellular Ca^{2+} increases vesiculation

Using pharmacological modulators of Ca^{2+} release together with topographical AFM analysis we show that intracellular calcium mobilization is required for plasma membrane EV biogenesis. The Ca^{2+} ionophore, A23187 (1 µM) was used to study the effect of increasing intracellular Ca^{2+} on malignant (MCF-7 and Dx) cell and non-malignant (hCMEC-D3) cell vesiculation (Figure 1). Ca^{2+} ionophores mobilize Ca^{2+} through the translocation of Ca^{2+}-ionophore complexes across the plasma membrane, activation of native plasma membrane Ca^{2+} channels, and via mobilization of Ca^{2+} from the ER [58].

As membrane-bound EVs are characteristically soft and deformable when undergoing AFM imaging, we used an indirect method for their examination whereby fixed cells are air-dried and membrane vesicles are transformed into vesicle-derived pits [47,59]. Prior to treatment with the ionophore, hCMEC-D3 cells displayed a smooth surface topography with little to no vesiculation at rest (Figure 1(a), left panel). Contrary to this, both malignant cell types, displayed an abundance of vesicle-derived pits on the cell surface at rest (Figure 1(b,c), left panels).

Increasing intracellular Ca^{2+} following A23187 treatment resulted in a pronounced and modest increase in vesiculation in non-malignant and malignant cells, respectively. Non-malignant cells underwent a pronounced change in topography with numerous vesicle-derived pits observed following treatment with A23187 (Figure 1(a), centre panel). In contrast, the increase in vesicle-derived pits observed in malignant cells treated with A23187 was less pronounced (Figure 1(b,c), centre panels). This is consistent with our previous findings [47]. Both cell types when treated with the calcium ionophore A23187 in combination with the calcium chelator, BAPTA-AM (µM), exhibited a smooth surface morphology devoid of vesicle-derived pits (Figure 1(a, b and c) right panels). Together these results evidence the involvement of intracellular Ca^{2+} mobilization in plasma membrane EV vesiculation in both malignant and non-malignant cells. Notably, the effects on vesiculation following Ca^{2+} activation were less in malignant cells compared with non-malignant cells and this is likely due to the already elevated baseline Ca^{2+} activation in MCF-7 cells at rest [60].

Increasing intracellular Ca^{2+} via store-operated Ca^{2+} entry (SOCE) activates EV vesiculation

The ER is the major intracellular store of calcium and is maintained at higher Ca^{2+} concentrations (100–800 µM) than that of the cytoplasm through the activity of Sarco/endoplasmic reticulum calcium ATPase (SERCA) [61,62]. We used Thapsigargin (TG) to selectively inhibit SERCA, deplete the ER Ca^{2+} stores and in turn activate SOCE [63]. Measurement of intracellular Ca^{2+} transients were carried out using the ImageXpress^{MICRO} high content imaging system as described by [64]. As shown in Figure 2(a) treatment with TG resulted in a gradual increase in relative $[Ca^{2+}]_i$ over a 5 min measurement interval. This corresponded to a significant increase in the F340/F387 ratio – indicative of an increase in intracellular $[Ca^{2+}]_i$ (Figure 2(b)). The pre-treatment of cells with the intracellular Ca^{2+} chelator, BAPTA-AM, (50 µM) abolished the TG-induced increase in $[Ca^{2+}]_i$ (Figure 2(a,b)).

AFM was used to visualize the effects of TG induced $[Ca^{2+}]_i$ increase on EV vesiculation in malignant and non-malignant cells. MCF-7 and hCMEC-D3 cells were treated with TG and the degree of vesiculation assessed for 30 min post treatment. As shown in Figure 2(c) (upper panels), non-malignant hCMEC-D3 cells display a smooth cellular topography supporting the presence of little to no vesiculation at rest. hCMEC-D3 cells treated for 5–30 min with TG displayed an uneven surface topography with a significant increase in Root Mean Squared (RMS) roughness (Figure 2(d)). This change in surface roughness was accompanied by the presence of vesicular pits, predominantly at perinuclear regions. hCMEC-D3 cells treated with TG for 10–30 min induced numerous vesicle-derived pits

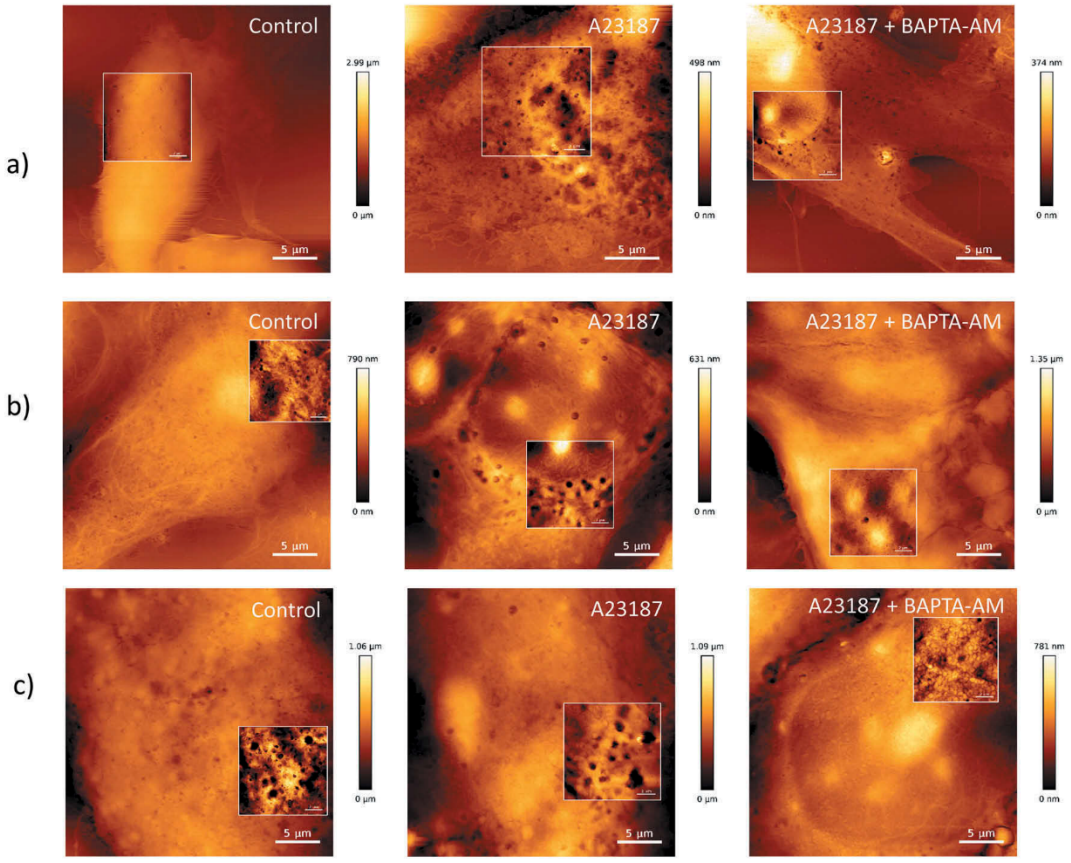

Figure 1. AFM topographical images of vesiculation following increased intracellular calcium in malignant and non-malignant cells.

(a) hCMEC-D3, (b) MCF-7, and (c) Dx cells were imaged using AFM in contact mode (scan rate 1 Hz) in the presence and absence of treatment with the calcium ionophore A23187 (1 μM) ± BAPTA-AM (50 μM) for 18 h. Ca^{2+} activation with A23187 resulted in increased vesiculation in all cells; however, the effect is most prominent in non-malignant cells. The calcium chelator, BAPTA-AM inhibited the formation of EVs in all cells types. Zoomed images shown in focussed panels as shown in frame. Scale bar shown. Image representative of a typical field of view from three independent experiments.

localized in the perinuclear region, consistent with the presence of active vesiculation (Figure 2).

Conversely, the malignant MCF-7 cells are observed to have numerous vesicle derived pits compared to their non-malignant counterparts (Figure 2(c) (lower panels)). This supports these cells to be actively vesiculating at rest, consistent with earlier studies by us [20,47]. Again, there was a modest change in the surface topography following treatment with TG over 30 min in MCF-7 cells following TG treatment (Figure 2(c,d)). These studies demonstrate an increase in vesiculation following TG induced intracellular Ca^{2+} mobilization, particularly pronounced in the non-malignant cells.

Pre-treatment with the intracellular calcium chelator, BAPTA-AM effectively inhibited TG-induced vesiculation in both malignant and non-malignant cells (Figure 3(a)). Together, these results establish a role for $[Ca^{2+}]_i$ in vesiculation in malignant and non-malignant cells.

The high potency and irreversible binding of TG to SERCA means that it can impact cell viability [65,66]. The effects of TG on the viability of hCMEC-D3 and MCF-7 cells were assessed over 24 h using a Trypan Blue Exclusion Assay to ensure viability under experimental conditions. As shown in Figure 3(a,b), 10 nM TG treatment for 5 min resulted in an observable increase in vesiculation, whilst not affecting cell viability. This concentration was chosen for further investigations.

Flow cytometry was used to interrogate the Ca^{2+} signalling pathways regulating plasma membrane EV biogenesis. The gating parameters for the EV region were defined using 0.3 μm latex beads (R1: the lowest possible limit on forward scatter for BD LSRII) and 1.1 μm beads (R2: represents the upper limit for quantitation of the EV population; Figure 4(a)), as previously described [11]. We observed a significant, 1.5 fold increase in EV biogenesis in hCMEC-D3 cells following 300 nM TG treatment (Figure 4(b)). Resting hCMEC-D3 cells treated with the

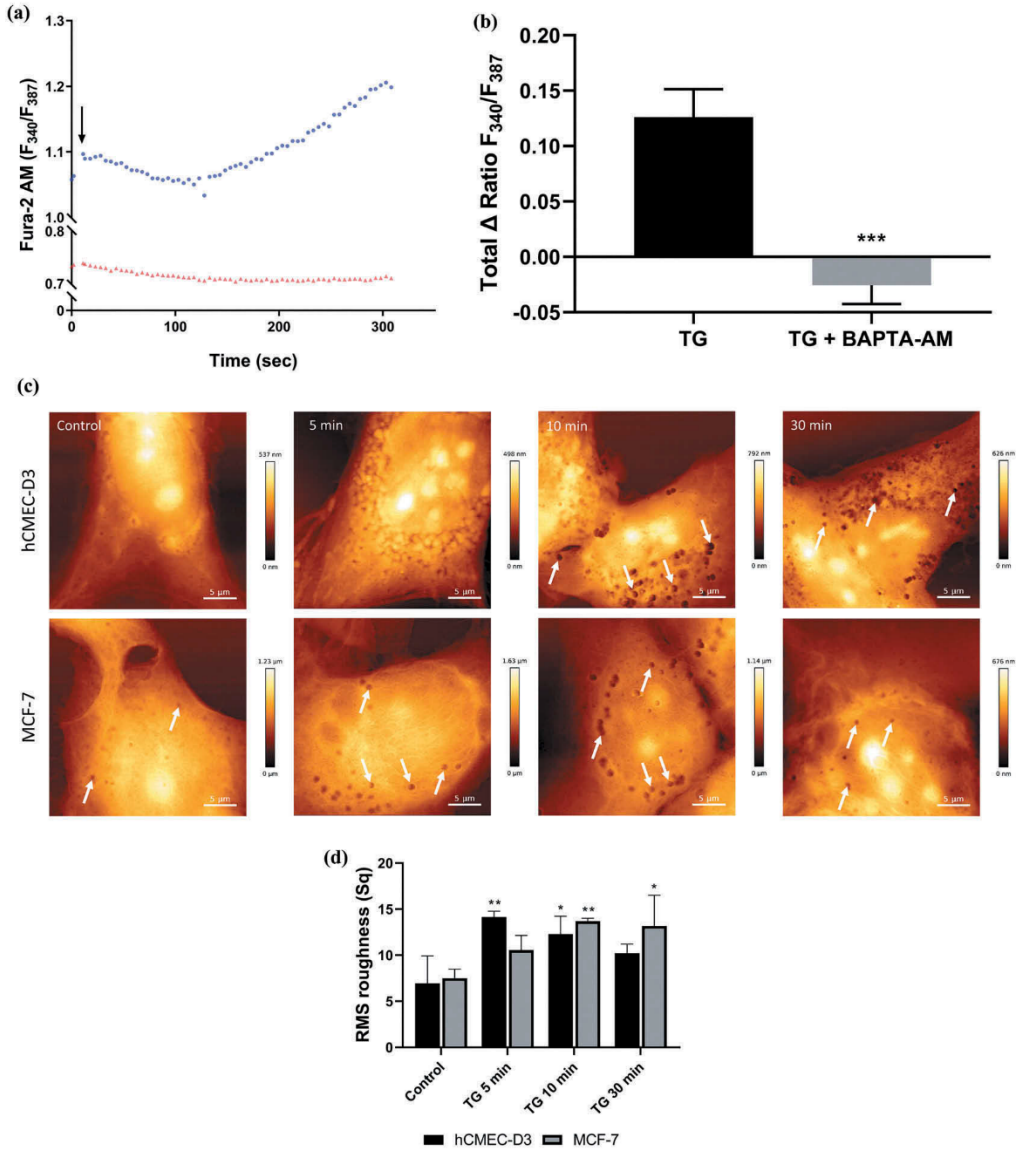

Figure 2. Increasing intracellular calcium with thapsigargin (TG) induces plasma membrane EV biogenesis in malignant MCF-7 cells.

MCF-7 cells were treated with TG (300 nM) for 5 min in the presence and absence of BAPTA-AM (50 uM). Changes to intracellular calcium was analysed using the ImageXpress high content imaging system and ratiometric calcium indicator Fura-2 AM. (a) Representative trace of change in relative fluorescence of Fura-2 AM following treatment with TG alone (●) and in the presence of BAPTA-AM (▲) in MCF-7 cells. The rise in fluorescence indicates increasing intracellular Ca^{2+} over 5 min. (b) Total TG-mediated increase in intracellular Ca^{2+} ± BAPTA-AM indicated by increase in relative fluorescence of Fura-2 AM (340/387) in MCF-7 cells. Data represents the mean ± SD of three experiments. ***$p < 0.001$ (paired t test). (c) Time course of TG-mediated increase in plasma membrane EV biogenesis. hCMEC-D3 (upper panel) and MCF-7 (lower panel) cells treated for 5, 10, and 30 min with TG and EV-derived pits visualized with AFM (arrows). (d) Analysis of hCMEC-D3 and MCF-7 cell topography illustrates there is an increase in surface roughness following thapsigargin treatment. Representative images shown from at least 3 experiments. Data represent the mean ± SD of at least 3 experiments. *$p < 0.05$, **$p < 0.005$ (one way ANOVA).

calpain inhibitor, ALLM (10 μM), displayed a 1.14 fold increase in EV production (Figure 4(b)) consistent with our previous findings [47]. This suggests to us that at rest vesiculation is driven by a calpain-independent pathway in non-malignant HCEM-D3 cells. Pre-treatment of hCMEC-D3 cells with ALLM inhibited the TG-induced plasma membrane EV production consistent with the involvement of a calpain-dependent pathway driving vesiculation following TG treatment. Conversely, the addition of the calpain inhibitor, ALLM to resting MCF-7 cells significantly reduced EV production by 1.4 fold, consistent with the involvement of calpain in EV vesiculation of malignant MCF-7 cells at rest. ALLM also significantly inhibited vesiculation by 2 fold in TG-treated MCF-7

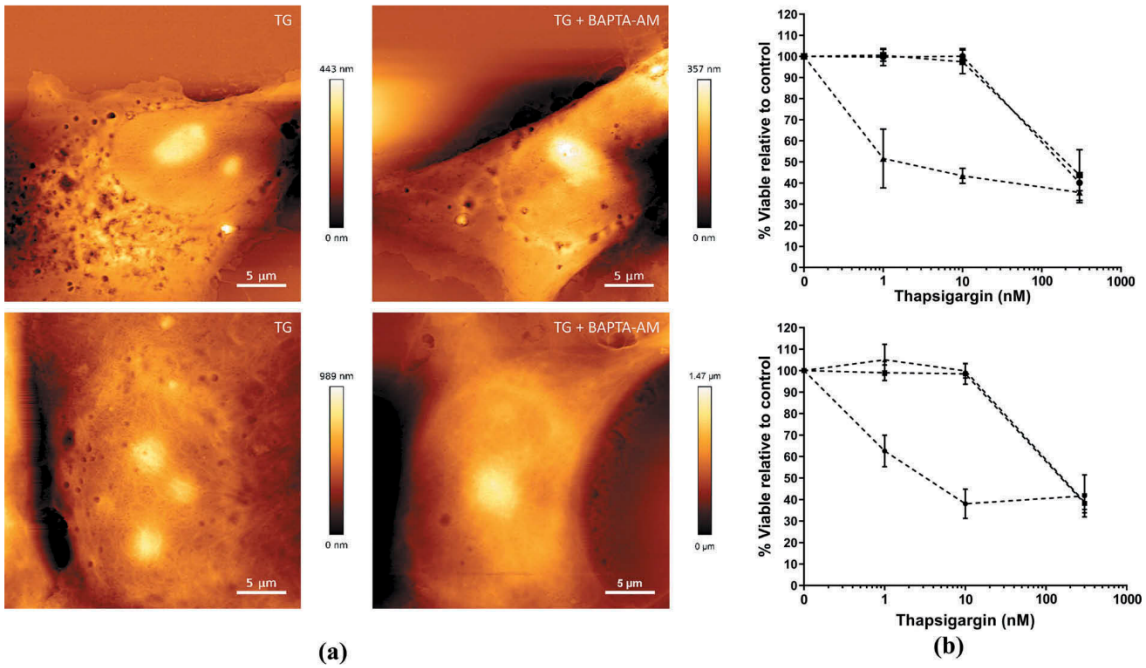

Figure 3. Effects of Thapsigargin (TG)-induced microvesicle (MV) biogenesis on cell viability over 24 h.

hCMEC-D3 and MCF-7 cells were treated with TG (1–300 nM) for up to 10 min. Cells were washed and then incubated for a further 24 h and analysed for viability and plasma membrane EV biogenesis. (a) AFM topographical images following TG (10 nM) treatment for 5 min ± BAPTA-AM (50 µM). hCMEC-D3 (upper panels) and MCF-7 (lower panels). Images representative of three independent experiments. (b) hCMEC-D3 (upper panel) and MCF-7 (lower panel) cell viability was assessed using a Trypan Blue dye Exclusion assay. Cells were treated with 1, 10 and 300 nM TG for 1 min (▲), 5 min (■), and 10 min (●), washed and analysed 24 h post treatment. Data represent the mean ± SD of three experiments.

cells, with the effect on EV vesiculation being greater than ALLM alone (Figure 4(b)).

YM58483 (*N*-[4-[3,5-*Bis*(trifluoromethyl)-1 *H*-pyrazol-1-yl]phenyl]-4-methyl-1,2,3-thiadiazole-5-carboxamide) is a selective inhibitor of SOCE and acts by blocking plasma membrane Ca^{2+} channels which are activated upon ER store depletion [67]. We observed no significant difference in vesiculation in hCMEC-D3 cells treated with 1 µM YM58483 alone compared to untreated control (Figure 4). Inhibition of TG activated SOCE with YM58483 resulted in a 1.5 fold decrease in vesiculation in hCMEC-D3 cells compared to cells treated with TG. This effect was also seen in malignant MCF-7 cells. Collectively, this data suggests that depletion of internal calcium stores by TG induced MVs in both hCMEC-D3 and MCF-7 cells and demonstrates that ER-mediated calcium regulation primarily drives vesiculation in malignant and non-malignant cells. Consistent with this, the lack of reduction in vesiculation of MCF-7 cells by the SOCE blocker YM58483 alone, suggests that elevated basal EVs in MCF-7 cells occurs through a pathway independent of SOCE.

Taken together, these results establish a role for ER Ca^{2+} as well as SOCE in the biogenesis of plasma membrane EVs in malignant and non-malignant cells. Non-malignant hCMEC-D3 cells are low vesiculators at rest and display a large increase in vesiculation following ER Ca^{2+} store depletion and activation of SOCE. Contrary to this, malignant MCF-7 cells are actively vesiculating at rest, and although SOCE can contribute to MVs, SOCE is not the driver of increased EVs in resting MCF-7 cells.

Discussion

An elevated intracellular Ca^{2+} is required in the biogenesis of microvesicles [35–37,47]. Despite the central role that Ca^{2+} plays in plasma membrane EV biogenesis, the source of Ca^{2+} mobilization – intra- or extracellular – driving plasma membrane vesiculation remains undefined. We have previously shown that distinct EV biogenic pathways exist between malignant and non-malignant cells, the differences of which appear related to intracellular calcium levels at rest [47]. Uncovering the mechanistic differences between malignant and non-malignant plasma membrane EV biogenesis is important as it could point to novel and selective treatment strategies for the circumvention of MV-mediated acquisition and dissemination of MDR

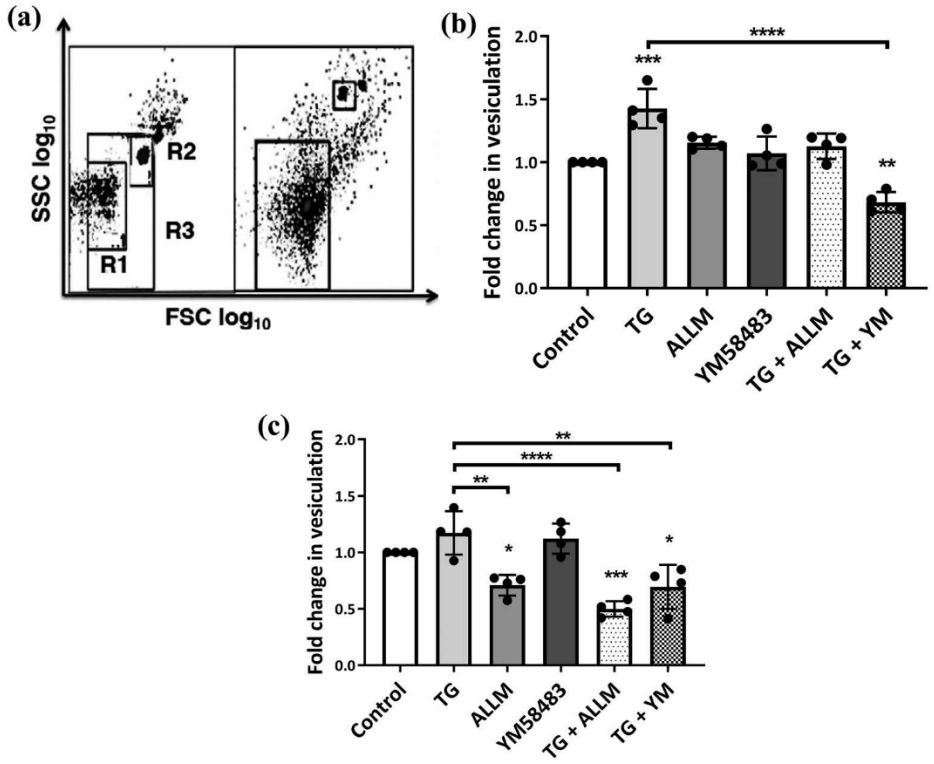

Figure 4. Vesiculation in malignant and non-malignant cells following manipulation of the calcium-calpain plasma membrane EV biogenic pathway.

hCMEC-D3 and MCF-7 cells were treated with modulators of vesiculation and Ca^{2+} for 24 h EV release was quantified by flow cytometry. (a) EV size gating strategy. Latex sizing beads of (0.3–1.1 um diameter) were used to define the gate for EVs. R1 represents the lower (0.3 μm) and R2 represents the upper (1.1 μm) limits set by the beads. R3 defines the region that was used to quantitate EVs and was applied to all samples. The total number of acquired events when the stop gate was set at 5000 TruCount bead counts is shown on the right. Data are expressed as fold change of EV release relative to vehicle control. (b) Non-malignant hCMEC-D3 cells displayed a significant increase in vesiculation following thapsigargin (TG) treatment and a significant reduction in vesiculation when treated with TG and YM58483. While treatments produced a consistent, small increase in vesiculation, it did not reach statistical significance. (c) Treatment with TG only resulted in a modest increase in vesiculation in malignant MCF-7 cells compared to vehicle control. There were significant reductions in malignant cell vesiculation following treatment with calpain inhibitor II (ALLM), TG + ALLM and TG + YM58483. Data represents the mean ± SD of at least 3 experiments. $*p < 0.05$, $**p < 0.005$ $***p < 0.001$ (one way ANOVA).

and other deleterious traits in cancer cell populations. This study sought to identify the calcium signalling pathways involved in the vesiculation of malignant MCF-7 and non-malignant hCMEC-D3 cells. We evidence the involvement of the ER and SOCE pathway in malignant and non-malignant plasma membrane EV biogenesis. Malignant cells are high vesiculators at rest compared to non-malignant cells, however, observed differences do not appear to be related to mobilization of Ca^{2+} following SOCE activation. SOCE does play a role in plasma membrane EV biogenesis; however, it serves as a failsafe mechanism – driving vesiculation in the event ER stores are depleted of Ca^{2+}.

At rest, non-malignant hCMEC-D3 cells are low vesiculators and increases in intracellular Ca^{2+} result in the induction of plasma membrane EV production. Consistent with our previous work, high-resolution AFM images show hCMEC-D3 cells have a smooth topography with very few vesicle-derived pits observable in their resting state (Figure 1(a)). Following cellular activation with the Ca^{2+} ionophore, A23187, plasma membrane vesicle biogenesis is efficiently "turned on" in non-malignant cells. A23187 has been shown previously to elevate $[Ca^{2+}]_i$ and induce plasma membrane EV formation in a number of cell types [58] and these results are consistent with this. Non-malignant cells stimulated with A23187 displayed a dramatically altered surface morphology consistent with cells undergoing active membrane vesiculation.

During plasma membrane EV biogenesis, the increase in intracellular Ca^{2+} triggers a number of cytoplasmic and membrane alterations including: externalization of PS through the activation of lipid translocases, loss of membrane asymmetry, activation of calpain and the remodelling of the cytoskeleton. Ultimately, anchorage of the plasma membrane to the

cytoskeleton is destabilized, localized regions of the membrane form membrane blebs and are released as EVs. These are observed in morphological AFM studies as uneven cellular surfaces with the presence of vesicle derived pits (Figures 1–3).

Co-treatment of hCMEC-D3 cells with A23187 and the intracellular Ca^{2+} chelator, BAPTA-AM, inhibited vesiculation, again evidencing the essential role of Ca^{2+} mobilization in MV biogenesis (Figure 1(a)). Interestingly, inhibiting calpain with ALLM in resting hCMEC-D3 cells resulted in a modest increase in vesiculation compared to untreated control (Figures 1(a), 4(b)). Calpains are highly conserved cysteine proteases that, when activated by Ca^{2+} cleave a number of substrates including kinases and phosphatases, membrane receptors, and cytoskeletal proteins and associated proteins [68]. Some calpain substrates are responsible for alternative MV biogenesis pathways. For example, calpain directly inhibits the small GTPase, RhoA that is an upstream activator of ROCK1-mediated MV biogenesis [21]. Thus, inhibition of this can activate the RhoA mediated vesiculation pathway. Given chelation of intracellular Ca^{2+} with BAPTA-AM prevents calpain activation, one would expect to see a similar rise in MV production compared to treatment with ALLM. However, treatment with BAPTA-AM completely blocks vesiculation in resting non-malignant cells. This result suggests the alternative plasma membrane EV biogenic pathway may also be Ca^{2+} dependent.

Conversely, resting malignant cells produce significantly more plasma membrane vesicles than their non-malignant counterparts [20] (Figure 1). Our results show that the higher resting level of vesiculation in malignant cells is dependent on both intracellular calcium and the activity of calpain as production can be inhibited by both calcium modulators and the calpain inhibitor ALLM (Figures 1 and 4). Resting malignant cells display a surface topography consistent with actively vesiculating cells: with an abundance of vesicle-derived pits (Figure 1(b,c)). MCF-7 cells treated with the calcium chelator, BAPTA-AM were devoid of vesicle-derived pits and displayed a smooth surface morphology (Figure 1(b,c)). Quantitative flow cytometry verified these observations and demonstrated a significant 1.4-fold reduction in vesiculation in MCF-7 cells treated with ALLM (Figure 4(c)). Quantitative analysis of surface topography with AFM on fixed cells is somewhat cumbersome as image acquisition is slow. This disadvantage makes this approach unsuited to analysis of broader changes in vesiculation. AFM is however, capable of resolving fine details in the cellular surface such as vesicle-derived pits down to 100 nm in diameter. It is also important to note that while analysis of surface roughness provides a measure of topographical surface changes in different cells types and treatment conditions, only the formation of plasma membrane EV-derived pits has been correlated to production of plasma membrane EVs previously.

These results evidence our proposal that resting malignant cell plasma membrane EV biogenesis is dependent on both Ca^{2+} and calpain and supports the presence of discrete biogenic pathways in malignant and non-malignant cells at rest [47].

A network of pumps, channels and exchangers maintain tight regulatory control of intracellular Ca^{2+} homoeostasis [69]. This enables a multitude of signalling modalities to be simultaneously operational in cells [70]. Ca^{2+} is a highly promiscuous intracellular signalling molecule and cellular effects are dependent on the size, kinetics and subcellular localization of incoming Ca^{2+} signals [71]. An increase in intracellular Ca^{2+} is the initiating stimulus for both the collapse of membrane phospholipid asymmetry, and for the activation of calpain and cytoskeletal remodelling required for plasma membrane EV biogenesis. What is intriguing is the propensity of malignant cells to shed spontaneous EVs at rest. This is contrary to non-malignant cells, which require cellular activation. The process in malignant cells is Ca^{2+}-dependent as chelation of intracellular Ca^{2+} attenuates biogenesis (Figure 1). In adriamycin-resistant breast cancer cells, TRPC5 calcium channels promote the formation of MVs [24]. While this study demonstrated the importance of calcium signalling in vesiculation, the differences in the biogenic pathways between malignant and non-malignant cells were not examined.

The ER is the primary intracellular store of Ca^{2+}, which can be mobilized in response to numerous stimuli [61,72]. It provides the ideal starting point for the interrogation of the specific Ca^{2+} signalling pathways involved in plasma membrane EV biogenesis as it is well documented to be involved in many cellular processes. TG is commonly employed as an experimental tool to interrogate Ca^{2+} signalling pathways and is has high selectivity and potency as an irreversible inhibitor of SERCA [73]. SERCA is responsible for the maintenance of high Ca^{2+} concentration in the ER (≈100–-800 μM) and its inhibition ultimately results in an influx of Ca^{2+} into the cytosol. (Figure 5).

SERCA inhibitors such as TG are favoured in the Ca^{2+} signalling experimental toolkit, as direct depletion of Ca^{2+} from the ER bypasses other, interfering biochemical signals. As TG has also been employed in the study of numerous cellular processes ranging from cytokine release regulation in lymphocytes [74] to the dysregulation of Ca^{2+} homoeostasis in cancer [40,54,71], it was

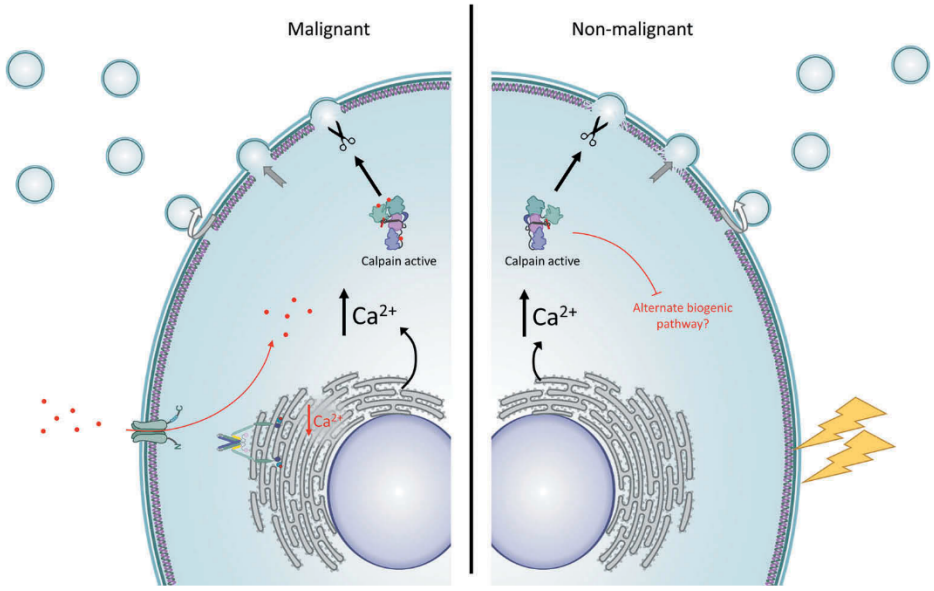

Figure 5. Proposed model for the role of the endoplasmic reticulum and SOCE in MV biogenesis.

Malignant cells have high basal production of plasma membrane EVs compared to non-malignant cells which is driven by a calcium–calpain dependent pathway. Resting vesiculation in MCF-7 cells is dependent on mobilization of calcium from stores within the endoplasmic reticulum rather than from activity of plasma membrane channels. In the event of ER store-depletion – as observed when SERCA is inhibited with thapsigargin – the store-operated calcium entry (SOCE) pathway is activated to restore ER calcium stores. Depleting ER calcium stores and blocking the activity of Orai1 channels with a selective inhibitor of SOCE, YM58483, prevents intracellular calcium increases and inhibits the production of EVs. Calcium signalling pathways are not activated in resting non-malignant hCMEC-D3 cells. Consequently, these cells are relatively low vesiculators in the resting state. Following cellular activation (lightning bolt) or activation of SOCE with thapsigargin, there is an increase in cytosolic calcium and activation of calpain. Calpain mediates remodelling of cytoskeletal proteins, disrupts anchorage of the plasma membrane to the cytoskeleton and results in an increase in plasma membrane EV biogenesis.

chosen to interrogate Ca^{2+} signalling and plasma membrane EV biogenesis in our cell lines. We demonstrate that TG induces an increase in $[Ca^{2+}]_i$ within 5 min in MCF-7 cells (Figure 2(a,b)). As expected, pre-treatment with BAPTA-AM inhibited TG-mediated $[Ca^{2+}]_i$ increases (Figure 2(a,b)). Importantly, corresponding to Ca^{2+} increases, we observed a time-dependent change in surface morphology in both MCF-7 and hCMEC-D3 cells indicative of an increase in vesiculation (Figure 2(c,d)). Demonstrating for the first time that TG-mediated activation of SOCE can generate production of plasma membrane EVs.

Having established TG as a suitable molecule for activation of plasma membrane EV biogenesis and a concentration that does not compromise cell viability (Figure 3(b)), we interrogated the role of ER and SOCE pathway in MV biogenesis.

We demonstrate that SOCE is involved in plasma membrane EV biogenesis in both malignant MCF-7 and non-malignant hCMEC-D3 cells. Upon depletion of intracellular stores of Ca^{2+} with TG, a significant increase in vesicle biogenesis is observed in hCMEC-D3 cells. While statistical significance was not reached by flow cytometry, there is a consistent increase in MCF-7 vesiculation observable in these and in our AFM studies (Figures 1–2). Some malignant cells lines have been reported to maintain higher basal Ca^{2+} concentrations than normal cell lines [60], which could explain why there is such a stark difference in vesiculation before stimulation between hCMEC-D3 and MCF-7 cells. Roseblade et al. [20] proposed that higher basal Ca^{2+} and activity of calpain contribute to higher levels of basal vesiculation in malignant cells. Indeed, ALLM pre-treatment of non-malignant cells reduced TG-mediated vesicle production to levels comparable to control (Figure 4(b)). In malignant cells, ALLM significantly reduces TG-mediated vesiculation by 0.5 fold to levels well below control (Figure 4(c)). This further supports a calcium–calpain pathway driving malignant cell plasma membrane vesiculation at rest and demonstrates a role for SOCE in the pathway.

Dysregulation of Ca^{2+} homoeostasis and signalling is complex and highly varied between cancer types [45]. Malignant cells often have remodelled Ca^{2+} signalling pathways that provide them with a survival advantage [43]. In the present study, differences between ER Ca^{2+} signalling pathways and, in particular, the SOCE pathway in breast cancer cells was assessed in terms of plasma membrane EV production with the selective SOCE inhibitor, YM58483. The pyrazole analogue YM58483

potently inhibits TG-mediated intracellular Ca^{2+} increases in non-excitable cells [67], effectively inhibits SOCE in breast cancer cells [75], as well as inhibiting T-cell activation mediated hypersensitivity reactions in mice [76]. At rest, both malignant and non-malignant vesiculation was unaffected when treated with YM58483 alone (Figure 4(a,b)). This demonstrates that basal vesiculation in both cell types is not dependent on SOCE– hCMEC-D3 cells remain low vesiculators and MCF-7 cells remain high vesiculators.

We show that treatment of non-malignant hCMEC-D3 cells with YM58483 induced a significant reduction in TG-promoted vesiculation compare to control (Figure 4(b)). In this case, TG inhibits SERCA and results in Ca^{2+} depletion from the ER, however SOCE is blocked and so less extracellular Ca^{2+} enters cells. This renders the plasma membrane EV biogenic machinery inactive and less vesicles are released. These results demonstrate that SOCE is involved in the biogenesis of plasma membrane EVs from both MCF-7 and hCMEC-D3 cells. However, it appears that SOCE acts as a secondary pathway of Ca^{2+} entry that is activated down stream of ER store depletion. While this does not completely explain differences in vesiculation at rest between MCF-7 and hCMEC-D3 cells, it does demonstrate differences in EV production between these malignant and non-malignant cells could be related to altered ER Ca^{2+} mobilization pathways (Figure 5). Importantly, results reported here mainly focus on larger (≈1000 nm) EV population only as particle analysis with flow cytometry is limited in the detection of particles below a few hundred nanometres. This particle size detection limitation prevents analysis on small EVs and therefore changes in their production are not considered.

These results also provide interesting avenues for future work. Malignant cells have higher basal Ca^{2+} activity and some reports suggest this could be related to impaired intracellular Ca^{2+} storage [77]. On the other hand, malignant cells have also been reported to have increased expression and activity of ER Ca^{2+} channels – resulting in more frequent mobilization from intracellular stores. In each case, the ability of malignant cells to maintain high Ca^{2+} concentrations within intracellular Ca^{2+} store is impaired and is therefore more susceptible to SERCA inhibition and sensitive to store depletion. Future work aims to determine if malignant cell vesiculation is vulnerable to store depletion as this could prove to be useful in endeavours to mitigate EV-mediated cancer progression with novel therapeutics.

Conclusion

Cancer EVs are capable of transferring function proteins and genetic material to recipient cells, bestowing upon them a complex MDR phenotype [3,7,9,78,79]. In the effort to develop novel strategies for the circumvention of EV-mediated cancer progression, resistance and survival it is imperative to understand the biogenic mechanisms that govern vesiculation. Here, we have demonstrated that malignant MCF-7 cells are high vesiculators at rest compared to non-malignant hCMEC-D3 cells and this is dependent on Ca^{2+} mobilization and activation of calpain. We found that the ER is the primary source of Ca^{2+} for plasma membrane EV biogenesis and that SOCE acts as a backup in the event of store depletion. Further, we report that differences in malignant and non-malignant vesiculation are related to altered mobilization of Ca^{2+} from ER stores. Pharmacological manipulation of ER Ca^{2+} stores has been a focus for many researchers attempting target Ca^{2+} signalling in the treatment of cancer. It now appears Ca^{2+} signalling may also be a novel avenue to pursue in the effort to reduce EV-mediated MDR and other deleterious cancer traits.

Declaration

GRM has had projects associated with UniQuest the commercialisation company of the University of Queensland.

Funding

GRM was supported by the Mater Foundation. The Translational Research Institute is supported by a grant from the Australian Government.

ORCID

Gregory Monteith http://orcid.org/0000-0002-4345-530X

References

[1] Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol. 2013;200:373–383.
[2] Wolf P. Nature and significance of platelet products in human plasma. Br J Haematol. 1967;13:269-&.
[3] Bebawy M, Combes V, Lee E, et al. Membrane microparticles mediate transfer of P-glycoprotein to drug sensitive cancer cells. Leukemia. 2009;23:1643–1649.
[4] Lu JF, Luk F, Gong J, et al. Microparticles mediate MRP1 intercellular transfer and the re-templating of intrinsic resistance pathways. Pharmacol Res. 2013;76:77–83.
[5] Lu JF, Pokharel D, Padula MP, et al. A novel method to detect translation of membrane proteins following microvesicle intercellular transfer of nucleic acids. J Biochem. 2016;160:281–289.
[6] Gong J, Luk F, Jaiswal R, et al. Microparticles mediate the intercellular regulation of microRNA-503 and proline-rich tyrosine Kinase 2 to alter the migration

and invasion capacity of breast cancer cells. Front Oncol. 2014;4:220.
[7] Gong J, Luk F, Jaiswal R, et al. Microparticle drug sequestration provides a parallel pathway in the acquisition of cancer drug resistance. Eur J Pharmacol. 2013;721:116–125.
[8] Jaiswal R, Johnson MS, Pokharel D, et al. Microparticles shed from multidrug resistant breast cancer cells provide a parallel survival pathway through immune evasion. Bmc Cancer. 2017;17:12.
[9] Pokharel D, Wijesinghe P, Oenarto V, et al. Deciphering cell-to-cell communication in acquisition of cancer traits: extracellular membrane vesicles are regulators of tissue biomechanics. OMICS. 2016;20:462–469.
[10] De Rubis G, Krishnan SR, Bebawy M. Liquid biopsies in cancer diagnosis, monitoring, and prognosis. Trends Pharmacol Sci. 2019;40:172–186.
[11] Krishnan SR, Luk F, Brown RD, et al. Isolation of human CD138(+) microparticles from the plasma of patients with multiple myeloma. Neoplasia. 2016;18:25–32.
[12] Lötvall J, Hill AF, Hochberg F, et al. Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the international society for extracellular vesicles. J Extracell Vesicles. 2014;3:26913.
[13] Witwer KW, Buzás EI, Bemis LT, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. J Extracell Vesicles. 2013;2:20360.
[14] Colombo M, Raposo G, Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. In: Schekman R, Lehmann R, editors. Annual review of cell and developmental biology. Vol. 30. Palo Alto: Annual Reviews; 2014:255–289.
[15] Kalra H, Drummen GPC, Mathivanan S. Focus on extracellular vesicles: introducing the next small big thing. Int J Mol Sci. 2016;17:30.
[16] Chargaff E, West R. The biological significance of the thromboplastic protein of blood. J Biol Chem. 1946;166:189–197.
[17] Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. Trends Cell Biol. 2009;19:43–51.
[18] Warren BA, Vales O. Release of vesicles from platelets following adhesion to vessel walls in-vitro. Br J Exp Pathol. 1972;53:206-&.
[19] Freyssinet JM, Toti F. Formation of procoagulant microparticles and properties. Thromb Res. 2010;125:S46–S48.
[20] Roseblade A, LUK F, UNG A, et al. Targeting microparticle biogenesis: a novel approach to the circumvention of cancer multidrug resistance. Curr Cancer Drug Targets. 2015;15:205–214.
[21] Sapet C, Simoncini S, Loriod B, et al. Thrombin-induced endothelial microparticle generation: identification of a novel pathway involving ROCK-II activation by caspase-2. Blood. 2006;108:1868–1876.
[22] Vanwijk MJ, Vanbavel E, Sturk A, et al. Microparticles in cardiovascular diseases. Cardiovasc Res. 2003;59:277–287.
[23] Zwaal RFA, Comfurius P, Bevers EM. Surface exposure of phosphatidylserine in pathological cells. Cell Mol Life Sci. 2005;62:971–988.
[24] Ma X, Chen Z, Hua D, et al. Essential role for TrpC5-containing extracellular vesicles in breast cancer with chemotherapeutic resistance. Proc Natl Acad Sci U S A. 2014;111:6389–6394.
[25] Bretscher MS. Asymmetrical lipid bilayer structure for biological-membranes. Nat New Biol. 1972;236:11-+.
[26] Daleke DL. Regulation of transbilayer plasma membrane phospholipid asymmetry. J Lipid Res. 2003;44:233–242.
[27] Daleke DL, Lyles JV. Identification and purification of aminophospholipid flippases. Biochim Biophys Acta Mol Cell Biol Lipids. 2000;1486:108–127.
[28] Daleke DL, Lyles JV, Nemergut E, et al. Purification and substrate specificity of the human erythrocyte aminophospholipid transporter. In: Trafficking of intracellular membranes: from molecular sorting to membrane fusion. Vol. 91. 1995. p. 49–59. Heidelberger: Springer-Verlag Berlin.
[29] Kunzelmann-marche C, Freyssinet JM, Martinez MC. Regulation of phosphatidylserine transbilayer redistribution by store-operated Ca2+ entry - role of actin cytoskeleton. J Biol Chem. 2001;276:5134–5139.
[30] Martin SJ, Reutelingsperger CPM, Mcgahon AJ, et al. Early redistribution of plasma-membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus - inhibition by overexpression of BCL-2 and ABL. J Exp Med. 1995;182:1545–1556.
[31] Perrin BJ, Amann KJ, Huttenlocher A. Proteolysis of cortactin by calpain regulates membrane protrusion during cell migration. Mol Biol Cell. 2006;17:239–250.
[32] Storr SJ, Carragher NO, Frame MC, et al. The calpain system and cancer. Nat Rev Cancer. 2011;11:364–374.
[33] Basse F, Gaffet P, Bienvenue A. Correlation between inhibition of cytoskeleton proteolysis and anti-vesiculation effect of calpeptin during A23187-induced activation of human platelets - are vesicles shed by filopod fragmentation. Biochim Biophys Acta, Biomembr. 1994;1190:217–224.
[34] Fox JEB, Austin CD, Reynolds CC, et al. Evidence that agonist-induced activation of calpain causes the shedding of procoagulant-containing microvesicles from the membrane of aggregating platelets. J Biol Chem. 1991;266:13289–13295.
[35] Distler JHW, Huber LC, Hueber AJ, et al. The release of microparticles by apoptotic cells and their effects on macrophages. Apoptosis. 2005;10:731–741.
[36] Morrell AE, Brown GN, Robinson ST, et al. Mechanically induced Ca2+ oscillations in osteocytes release extracellular vesicles and enhance bone formation. Bone Res. 2018;6:11.
[37] Pasquet JM, Dachary-prigent J, Nurden AT. Calcium influx is a determining factor of calpain activation and microparticle formation in platelets. Eur J Biochem. 1996;239:647–654.
[38] Berridge MJ, Bootman MD, Roderick HL. Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol. 2003;4:517–529.
[39] Berridge MJ. Calcium signalling remodelling and disease. Biochem Soc Trans. 2012;40:297–309.

[40] Azimi I, Roberts-Thomson SJ, Monteith GR. Calcium influx pathways in breast cancer: opportunities for pharmacological intervention. Br J Pharmacol. 2014;171:945–960.
[41] Brini M, Carafoli E. Calcium pumps in health and disease. Physiol Rev. 2009;89:1341–1378.
[42] Missiaen L, Robberecht W, Van Den Bosch L, et al. Abnormal intracellular Ca2+ homeostasis and disease. Cell Calcium. 2000;28:1–21.
[43] Monteith GR, Davis FM, Roberts-Thomson SJ. Calcium channels and pumps in cancer: changes and consequences. J Biol Chem. 2012;287:31666–31673.
[44] Monteith GR, Mcandrew D, Faddy HM, et al. Calcium and cancer: targeting Ca2+ transport. Nat Rev Cancer. 2007;7:519–530.
[45] Stewart TA, Yapa K, Monteith GR. Altered calcium signaling in cancer cells. Biochim Biophys Acta, Biomembr. 2015;1848:2502–2511.
[46] Pera E, Kaemmerer E, Milevskiy MJG, et al. The voltage gated Ca2+-channel Ca(v)3.2 and therapeutic responses in breast cancer. Cancer Cell Int. 2016;16:15.
[47] Taylor J, Jaiswal R, Bebawy M. Calcium-calpain dependent pathways regulate vesiculation in malignant breast cells. Curr Cancer Drug Targets. 2017;17:486–494.
[48] Jaiswal R, Gong J, Sambasivam S, et al. Microparticle-associated nucleic acids mediate trait dominance in cancer. Faseb J. 2012a;26:420–429.
[49] Donmez Y, Akhmetova L, Iseri OD, et al. Effect of MDR modulators verapamil and promethazine on gene expression levels of MDR1 and MRP1 in doxorubicin-resistant MCF-7 cells. Cancer Chemother Pharmacol. 2011;67:823–828.
[50] Kars MD, Iseri OD, Gunduz U, et al. Development of rational in vitro models for drug resistance in breast cancer and modulation of MDR by selected compounds. Anticancer Res. 2006;26:4559–4568.
[51] Jaiswal R, Luk F, Dalla PV, et al. Breast cancer-derived microparticles display tissue selectivity in the transfer of resistance proteins to cells. Plos One. 2013;8:10.
[52] Antonio PD, Lasalvia M, Perna G, et al. Scale-independent roughness value of cell membranes studied by means of AFM technique. Biochim Biophys Acta, Biomembr. 2012;1818:3141–3148.
[53] Necas D, Klapetek P. Gwyddion: an open-source software for SPM data analysis. Cent Eur J Phys. 2012;10:181–188.
[54] Feng MY, Grice DM, Faddy HM, et al. Store-independent activation of orai1 by SPCA2 in Mammary Tumors. Cell. 2010;143:84–98.
[55] Robinson JA, Jenkins NS, Holman NA, et al. Ratiometric and nonratiometric Ca2+ indicators for the assessment of intracellular free Ca2+ in a breast cancer cell line using a fluorescence microplate reader. J Biochem Biophys Methods. 2004;58:227–237.
[56] Azimi I, Flanagan JU, Stevenson RJ, et al. Evaluation of known and novel inhibitors of orai1-mediated store operated Ca2+ entry in MDA-MB-231 breast cancer cells using a fluorescence imaging plate reader assay. Bioorg Med Chem. 2017;25:440–449.
[57] Lacroix R, Robert S, Poncelet P, et al. Overcoming limitations of microparticle measurement by flow cytometry. Semin Thromb Hemost. 2010;36:807–818.
[58] Dedkova EN, Sigova AA, Zinchenko VP. Mechanism of action of calcium ionophores on intact cells: ionophore-resistant cells. Membr cell biol. 2000;13:357–368.
[59] Zhang X, Chen Y, Chen Y. An AFM-based pit-measuring method for indirect measurements of cell-surface membrane vesicles. Biochem Biophys Res Commun. 2014;446:375–379.
[60] Pottle J, Sun C, Gray L, et al. Exploiting MCF-7 Cells¡¯ calcium dependence with interlaced therapy. J Cancer Ther. 2013;04(7):9.
[61] Marchi S, Patergnani S, Missiroli S, et al. Mitochondrial and endoplasmic reticulum calcium homeostasis and cell death. Cell Calcium. 2018;69:62–72.
[62] Pinton P, Giorgi C, Siviero R, et al. Calcium and apoptosis: ER-mitochondria Ca2+ transfer in the control of apoptosis. Oncogene. 2008;27:6407–6418.
[63] Treiman M, Caspersen C, Christensen SB. A tool coming of age: thapsigargin as an inhibitor of sarcoendoplasmic reticulum Ca2+-ATPases. Trends Pharmacol Sci. 1998;19:131–135.
[64] Peters AA, Simpson PT, Bassett JJ, et al. Calcium channel TRPV6 as a potential therapeutic target in estrogen receptor-negative breast cancer. Mol Cancer Ther. 2012;11:2158–2168.
[65] Sagara Y, Wade JB, Inesi G. A conformational mechanism for formation of a dead-end complex by the sarcoplasmic-reticulum atpase with thapsigargin. J Biol Chem. 1992;267:1286–1292.
[66] Sehgal P, Szalai P, Olesen C, et al. Inhibition of the sarco/endoplasmic reticulum (ER) Ca2+-ATPase by thapsigargin analogs induces cell death via ER Ca2+ depletion and the unfolded protein response. J Biol Chem. 2017;292:19656–19673.
[67] Ishikawa J, Ohga K, Yoshino T, et al. A pyrazole derivative, YM-58483, potently inhibits store-operated sustained Ca2+ influx and IL-2 production in T lymphocytes. J Iimmunol. 2003;170:4441–4449.
[68] Chan SL, Mattson MP. Caspase and calpain substrates: roles in synaptic plasticity and cell death. J Neurosci Res. 1999;58:167–190.
[69] Carafoli E. Calcium signaling: a tale for all seasons. Proc Natl Acad Sci U S A. 2002;99:1115–1122.
[70] Lipskaia L, Lompre AM. Alteration in temporal kinetics of Ca2+ signaling and control of growth and proliferation. Biol Cell. 2004;96:55–68.
[71] Monteith GR, Prevarskaya N, Roberts-Thomson SJ. The calcium-cancer signalling nexus. Nat Rev Cancer. 2017;17:367–380.
[72] Rizzuto R, Pinton P, Ferrari D, et al. Calcium and apoptosis: facts and hypotheses. Oncogene. 2003;22:8619–8627.
[73] Putney JW. Pharmacology of store-operated calcium channels. Mol Interv. 2010;10:209–218.
[74] Takezawa R, Cheng H, Beck A, et al. A pyrazole derivative potently inhibits lymphocyte Ca2+ influx and cytokine production by facilitating transient receptor potential melastatin 4 channel activity. Mol Pharmacol. 2006;69:1413–1420.
[75] Azimi I, Bong AH, Poo GXH, et al. Pharmacological inhibition of store-operated calcium entry in MDA-MB-468 basal A breast cancer cells: consequences on

calcium signalling, cell migration and proliferation. Cell Mol Life Sci. 2018;75:4525–4537.
[76] Ohga K, Takezawa R, Arakida Y, et al. Characterization of YM-58483/BTP2, a novel store-operated Ca(2+) entry blocker, on T cell-mediated immune responses in vivo. Int Immunopharmacol. 2008;8:1787–1792.
[77] Pinton P, Ferrari D, Magalhaes P, et al. Reduced loading of intracellular Ca2+ stores and downregulation of capacitative Ca2+ influx in Bcl-2-overexpressing cells. J Cell Biol. 2000;148:857–862.
[78] Jaiswal R, Luk F, Gong J, et al. Microparticle conferred microRNA profiles - implications in the transfer and dominance of cancer traits. Mol Cancer. 2012b;11:13.
[79] Lu JF, Pokharel D, Bebawy M. A novel mechanism governing the transcriptional regulation of ABC transporters in MDR cancer cells. Drug Deliv Transl Res. 2017;7:276–285.

Author contribution statement – Chapter 3

J. Taylor performed all of the experiments, analysed the data and composed the manuscript. I. Azimi provided conceptual guidance for the intracellular calcium imaging and quantification, and revision of the manuscript. G. Monteith was responsible for experimental design for the intracellular calcium imaging and quantification, project supervision, and revision of the manuscript. M. Bebawy was responsible for the overall project, experimental design, project supervision, and revision of the manuscript.

Chapter 4

4. Membrane to Cytosol Redistribution of αII-Spectrin Drives Extracellular Vesicle Biogenesis in Malignant Cells

Membrane to Cytosol Redistribution of αII-Spectrin Drives Extracellular vesicle Biogenesis in Malignant Cells

Taylor, J[1]., Patio, K[1]., Morris, M[2]., Evenhuis, C[3]., Johnson, M[3]., Bebawy, M[*1]

[1]Discipline of Pharmacy, Graduate School of Health, The University of Technology Sydney,

[2]Discipline of Physiology and Bosch Institute, School of Medical Sciences, Faculty of Medicine and Health, University of Sydney, New South Wales, Australia,

[3]School of Life Sciences, The University of Technology Sydney,

4.1. Abstract

Spectrin is a ubiquitous cytoskeletal protein that provides structural stability and supports membrane integrity. In erythrocytes, spectrin proteolysis leads to the biogenesis of plasma membrane extracellular vesicles (EVs) however, it's role in non-erythroid or cancer-derived plasma membrane EVs biogenesis is unknown. Malignant cells generate significantly more EVs compared to non-malignant cells which is driven by a calcium – calpain mediated pathway at rest. Given calcium and calpain are known regulators of spectrin structural dynamics, we propose that spectrin remodelling would serve a role in malignant cell vesiculation. This study aims to examine the role of αII-spectrin in malignant and non-malignant plasma membrane vesiculation. We have designed and written a custom automated cell segmentation plugin for the open source image processing software, Fiji to be combined with high resolution confocal microscopy that allows for an unbiased assessment of subcellular distribution of αII-spectrin. We show that in low vesiculating non-malignant MBE-F breast cells, prominent spectrin borders localise to the cell periphery at rest. In comparison, these peripheral spectrin borders are diminished in high vesiculating malignant MCF-7 breast cells at rest. The subcellular distribution of spectrin towards the membrane periphery correlated with increased biomechanical stiffness as measured by Atomic Force Microscopy. We also show that peripheral spectrin borders can be restored in malignant MCF-7 cells when treated with known vesiculation modulators including the calcium chelator, BAPTA-AM or with the calpain inhibitor II (ALLM). These results demonstrate the subcellular localisation of spectrin is distinctly different in malignant and non-malignant cells at rest and shows that the redistribution of αII-spectrin from the membrane to the cytoplasm drives plasma membrane-derived EV biogenesis in malignant cells. These results again support delineated vesiculation pathways in malignant and non-malignant cells at rest and support strategies for selective modulation of biogenesis.

Keywords: Extracellular vesicles, cytoskeleton, biogenesis, multidrug resistance, spectrin, microvesicles, cancer, cancer cell biology.

4.2. Introduction

Microvesicles (MV) are extracellular vesicles measuring approximately 0.1 – 1 μm in diameter that are shed from the plasma membrane of cells following the loss phospholipid asymmetry and cytoskeletal reorganisation (Bebawy et al., 2009a). MVs are characteristic of their cell of origin and contain markers such as functional proteins, bioactive molecules and genetic material. They are released by both erythroid and non-erythroid cell types and have emerged as important intercellular signalling mediators (Abels and Breakefield, 2016, Al-Nedawi et al., 2009, de Souza et al., 2016, Lener et al., 2015, Malik, 2015). MVs have been implicated across diverse physiological processes such as vascular haemostasis, coagulation, and inflammation (Gong et al., 2012) as well as being recognised as key mediators in the progression of different cancers. Bebawy et al. were the first to show that EVs, specifically MVs were capable of intercellular conferral of multidrug resistance (MDR) in cancer cells through the transfer of functional proteins and nucleic acids (Bebawy et al., 2009b, Lu et al., 2013, Lu et al., 2016). MDR is a unique type of resistance which presents a major obstacle to successful cancer treatment and occurs when tumours become cross-resistant to a wide range of structurally and mechanistically different chemotherapeutic agents (Biedler and Riehm, 1970). It is attributed to the over expression of energy-dependent drug efflux pumps, the ATP-binding cassette (ABC) transporters P-glycoprotein (P-gp) and Multidrug Resistance-Associated Protein 1 (MRP1) (Juliano and Ling, 1976, Cole et al., 1992) amongst others. MVs have been implicated in the dissemination of other deleterious cancer traits including; drug sequestration (Gong et al., 2013), angiogenesis, increased metastatic capacity (Gong et al., 2014a, Pokharel et al., 2016b), evasion of immune surveillance (Jaiswal et al., 2017), and altered tissue biomechanics (Pokharel et al., 2016a).

While knowledge on exosome biogenesis is comprehensive and well defined (Raposo and Stoorvogel, 2013, Colombo et al., 2014), the precise biogenic pathway and mediators involved

in MV production are yet to be elucidated. MVs are released simultaneously, in response to various external stimuli, following cellular activation, as well as during programmed cells death. In the case of cellular activation, mobilisation of intracellular calcium drives plasma membrane and cytosolic alterations. Calcium modulates the activity of several lipid translocases in the plasma membrane resulting in the externalisation of anionic phospholipids and collapse of membrane asymmetry (Martin et al., 1995). Calcium also activates proteolytic enzymes – such as calpain – responsible for cytoskeletal remodelling (Perrin et al., 2006, Storr et al., 2011). These concerted events compromise membrane anchorage to the cytoskeleton and allow regions of membrane to bud away from the cell, ultimately to be released as MVs.

The lipid membrane is tethered to the underlying cytoskeletal meshwork via various transmembrane proteins and negatively charged components of the lipid membrane (Bogusławska et al., 2014). The membrane-cytoskeleton interface is a dynamic environment that provides both structural stability as well as the ability to undergo changes in response to internal and external signals. One of the major contributors to the glove-like adhesion of the plasma membrane to the cytoskeleton is spectrin. Spectrin is a ubiquitous protein that is part of the F-actin cross-linking superfamily and is made up of dimerised α- and β-spectrin subunits (Zhang et al., 2013a, Liem, 2016a). The elongated spectrin dimers form repeating hexamer units with junctional protein complexes consisting of actin, protein 4.1, and ankyrin – giving the spectrin meshwork beneath the membrane a distinct honeycomb appearance (Lux, 2016). The major sites of plasma membrane attachment to the network of spectrin filaments is to the junctional complexes, however, the phospholipid phosphatidylserine (PS) also binds at site on α- and β-spectrin (An et al., 2004b). PS binding sites on β-spectrin are localised close to junctional complexes and this has been reported to have a two-fold consequence for membrane-cytoskeletal adhesion: 1) There is a sizable ‘adhesion patch’ formed by lipid-cytoskeletal interactions around spectrin junctions that stabilise membrane adhesion (An et al., 2004a), and

2) PS binding to spectrin may modulate the protein-protein interactions in erythroid and non-erythroid membrane (Boguslawska et al., 2014). It is the combined phospholipid-membrane-protein-spectrin interactions that provides cells with mechanical strength, stability, and elasticity. In the context of MV biogenesis, localised remodelling of spectrin results in loss of membrane attachment to the underlying cytoskeleton and an increase in localised pressure causes the membrane to bleb and form plasma membrane EVs.

Spectrin has long been associated with membrane integrity and the production of plasma membrane vesicles in erythrocytes. First reports demonstrated spectrin oxidation to be associated with the release of membrane vesicles in stored red blood cells (Wagner et al., 1987). However, the role it plays in non-erythroid biogenesis remains unexplored. Spectrin's ability to adhere to both membrane-proteins and the lipid membrane itself provides the basis for hypothesising spectrin structure is central to MV biogenesis.

MVs are released from all cell types, however, there are distinct differences in malignant and non-malignant vesiculation (Roseblade et al., 2013). For example, malignant cells shed significantly more MVs at rest compared to non-malignant cells and this is attributed to different biogenic pathways (Pap, 2011). At rest, malignant cell MV biogenesis occurs via a calcium-calpain-dependent pathway, while non-malignant MVs are produced via alternative pathways (Taylor et al., 2017). The inhibition of calpain significantly reduced vesiculation in malignant cells *in vitro*, returning it to levels similar to non-malignant cells (Taylor et al., 2017). Calpain is a cysteine protease, which upon activation by intracellular calcium, cleaves numerous substrates including: cytoskeletal proteins (e.g. actin, ankyrin, protein 4.1, and spectrin) (Weber et al., 2009, Boivin et al., 1990), membrane-associated proteins (e.g. ion channels, pumps and receptors), enzymes involved in signal transduction, transcription factors and others (Wang and Yuen, 1999). Spectrin as a substrate of calpain is of particular interest in the study of malignant MV biogenesis as remodelling of this cytoskeletal component could

result in loss of plasma membrane anchorage and promotion of MV release. Specifically, calpain cleaves apart the α- and β-spectrin subunits (Harris et al., 1988, Harris et al., 1989, Harris and Morrow, 1990). The individual subunits then interact with calmodulin, which promote further cleavage of α- and β-spectrin by calpain (Harris et al., 1988, Harris et al., 1989, Harris and Morrow, 1990).

This study, aims to determine the role of spectrin in plasma membrane MV biogenesis in malignant cells, and to examine the presence of any differences in spectrin subcellular distribution in malignant and non-malignant cells. We propose that spectrin remodelling and alterations in its subcellular localisation play a pivotal role in MV biogenesis in malignant cells. Our results demonstrate that the redistribution of αII-spectrin from the membrane to the cytoplasm drives plasma membrane-derived EV biogenesis in malignant cells. These results again support delineated vesiculation pathways in malignant and non-malignant cells at rest and support strategies for selective modulation of biogenesis.

4.3. Materials and methods

4.3.1. Chemicals

Foetal bovine serum (FBS) (Sigma-Aldrich, NSW, Australia), Roswell Park Memorial Institute medium (RPMI-1640) (Sigma-Aldrich, USA), , Mammary Epithelial Cell Growth Medium-1 (MEG-1) (Zen-Bio, USA), 4′,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, USA), CF™ 594 hydrazide (Sigma-Aldrich), Anti-Alpha Fodrin antibody [3D7] (ab131575) (Abcam, Australia), Bovine serum albumin (BSA) (Sigma-Aldrich, NSW, Australia), Triton X-100 (Thermo Fisher Scientific, USA), Glycerol (Thermo Fisher Scientific, USA), Phosphate buffer saline (PBS) (Sigma-Aldrich, USA), Glycine (Thermo Fisher Scientific), Paraformaldehyde (Sigma-Aldrich, NSW, Australia), 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM; Thermo Fisher Scientific, USA).

4.3.2. Cell culture

Cell lines used in this study were the human breast adenocarcinoma cell line MCF-7, and human mammary basal epithelial cell line, MBE-F. MCF-7 cells were grown in RPMI-1640 media (Sigma-Aldrich, NSW, Australia), and MBE-F cells were grown in MEG-1 media (Zen-Bio, North Carolina, USA). Culture media was supplemented with 10% heat inactivated FBS (Invitrogen, Life Technologies, VIC, Australia) and maintained at 37°C and 5% CO_2. Cells were routinely tested for mycoplasma infection.

4.3.3. Confocal microscopy

MBE-F and MCF-7 cells were seeded at a density of 5 x 10^6 cells/mL on glass cover slips in sterile 6-well plates (Corning) and allowed to adhere overnight. Following culture for a further 24 hours in the presence and absence of MV biogenesis modulators, cells were fixed in 4% PFA for 30 minutes and washed 3 times with PBS. Cells were permeabilised with 0.1% Triton

X-100 for 1 minute then washed 3 times with PBS. Excess aldehyde was quenched with 100 mM glycine and washed 3 times with PBS. 2% BSA in 0.1% Triton X-100 was added to cells and incubated for 1 hour to prevent non-specific binding. Primary antibody (Anti-Alpha Fodrin antibody [3D7] (ab131575)) was added to cells for spectrin staining and incubated for 1 hour. Cells were washed 3 times with PBS. Secondary antibody (CF™ 594 hydrazide) was added to cells and incubated for 1 hour under foil to preserve fluorophores. Cell nuclei were stained with DAPI (1 µg/mL) for 15 minutes then washed 3 times with PBS. Coverslips were mounted in 90% glycerol and sealed with nail polish. Images of 3 different fields of view were acquired using the 60x oil lens and 1.4 NA with using the Nikon A1 laser scanning confocal microscope (Nikon). Images were analysed using the NIS Elements software package and a custom designed Fiji (Image J) plugin. Experiments were performed in triplicate.

4.3.4. Cell segmentation and spectrin localisation analysis

High resolution confocal images were imported into Fiji (Image J) image processing suite for analysis of αII-spectrin subcellular distribution. Confocal images were passed through an automated cell segmentation processor which was a custom designed Fiji plugin. Cells were segmented using marker controlled watershed (Legland et al., 2016) which uses a gradient image, a set of marker points, and an optional mask. This process allowed for analysis of individual cells.

Briefly, for the nuclear segmentation (Fig. 1 a), marker points were created by first blurring the DAPI channel at small and large scales by applying a morphological opening and a Gaussian blur filter. The gradient image was created by taking the internal gradient of the large scale blurred image. The marker points were created by thresholding the large scale burred image, filling holes, performing a Euclidian distance transform, then locating maxima. The mask image was created from the small scale blurred image by Otsu thresholding and filling holes.

The gradient, marker and mask images were used as input to the marker based watershed algorithm to generate labelled segmentation of the nuclei.

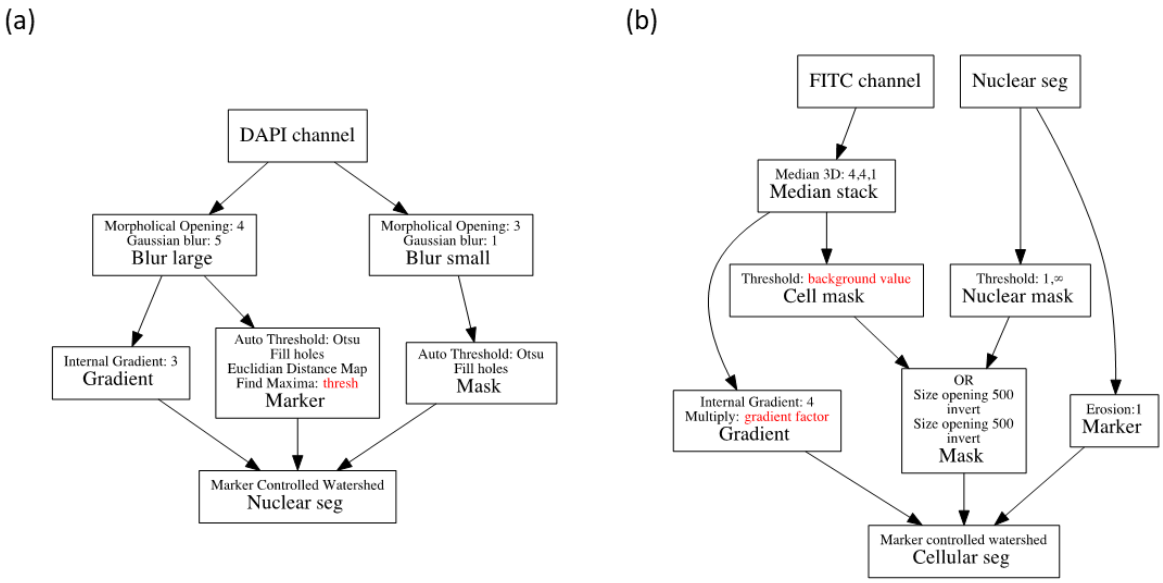

Figure 4. 1. Schematic of the mathematical processing used to segment individual cell nuclei (a) individual cell walls (b).

To define the membrane boarders of individual cells, a dilation function was applied to each seed point (Fig. 1 b). Expansion rate of the dilation front was restricted by the background mask limits, high intensity signal in the αII-spectrin channel, and contact with dilation front from other seed points.

Finally, the nuclear and cellular segmentations were used to create cell membrane and cytoplasm regions of interest (RoIs). For each label in the segmented images the label image was dilated by 3 pixels and eroded by 3 pixels. The cell membrane RoI was created by subtracting the eroded label from the dilated label. The cytoplasm RoI was determined by subtracting the nuclear label image from the eroded label image. Segmented edges were banded (1 µm = 4.76 px) to define the area of the cell membrane. The average fluorescence of the cell

membrane area was divided by the average fluorescence of the cytoplasmic area to determine if the distribution of αII-spectrin is in the periphery or in the cytoplasm. An αII-spectrin membrane:cytoplasm (M:C) distribution ratio of close to 1 indicates there is no distinct propensity of spectrin to localise in the periphery.

4.3.5. Atomic force microscopy

Atomic force microscopy (AFM) was used for cell surface topography studies and the biomechanical measurements of malignant and non-malignant cells. Sharpened silicon cantilevers (L: 225 μm, W: 28 μm, T: 3 μm, BudgetSensors) with a tip radius of 10 nm and a nominal spring constant of 3 N/m were mounted on the JPK Nanowizard SENSE AFM (JPK instruments, Germany). The exact spring constant was determined immediately prior to measurements via the thermal noise method (Hutter and Bechhoefer, 1993) included in the SFM software. The AFM was set up on an inverted optical microscope (Nikon Eclipse Ti-U, Japan for precise positioning of the cantilever over the centre of cells that ensured consistent force measurements. For surface topography studies, Cells were fixed in 4% paraformaldehyde (PFA) for 30 minutes, washed once with phosphate-buffered saline (PBS) and twice with distilled water. Cells were air-dried for ~1 hour to allow the membrane-bound vesicles to transform to vesicle-derived pits as previously described (Zhang et al., 2014, Zhang et al., 2015, Taylor et al., 2017). Scans were performed in air at a rate of 1 Hz. Three areas were selected at random for scanning for each treatment condition and all experiments were performed in triplicate. For biomechanical measurements of cells, Force-displacement curves were recorded with a tip approach speed of 1.0 μm/s. All measurements were performed in cell culture media at 37˚C using force spectrometry mode. Force-displacement curves for each measurement were analysed using JPK SPM software (Version 6.6.142, JPK Instruments) and the Young's Modulus computed.

4.3.6. Statistics

Data were analysed using one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparisons test or an unpaired t test using GraphPad Prism version 8.2.1 for windows (GraphPad Software, La Jolla, CA, USA). Data is presented as the mean or mean ± SD of at least 3 individual experiments with predictive results value of (****) $P < 0.0001$, (***) $P < 0.0005$, (**) $P < 0.01$, and (*) $P < 0.05$ being considered statistically significant.

4.4. Results

4.4.1. The subcellular localisation of spectrin differs in malignant and non-malignant cells at rest.

In determining the role of spectrin in MV biogenesis, we first examined its subcellular localisation in cells of differing vesiculation at rest. We have previously shown that non-malignant cells and malignant cells have differing basal rates of vesiculation with the former, being low vesiculators whilst malignant cells display high levels of vesiculation at rest (Roseblade et al., 2015, Taylor et al., 2017). We used high-resolution confocal microscopy to visualise both MCF-7 and MBE-F cells at rest following labelling with monoclonal mouse anti αII-Fodrin (spectrin; Abcam). We observed a clear localisation of αII-spectrin predominantly in the periphery of non-malignant MBE-F cells at rest (Fig. 2 a, top panels). While there was also diffuse cytoplasmic distribution of spectrin, the formation of defined spectrin borders at the edge of cells was evident in these non-malignant cells at rest. The αII-spectrin borders were very prominent in z-planes approaching the apical side of cells. In comparison, high vesiculating MCF-7 cells display primarily diffuse cytoplasmic distribution of αII-spectrin with minimal peripheral fluorescence (Fig. 2 a, lower panels). Basal to apical polarity of spectrin distribution was not observed in the malignant cells.

Non-malignant MBE-F and malignant MCF-7 cells were imaged with AFM was to assess degree of vesiculation in the resting state. Resting MBE-F cells display a smooth cellular surface with minimal vesicle-derived pits observed (Fig. 2 b, upper panel). Conversely, AFM shows that resting MCF-7 cells are high vesiculators at rest with many vesicle-derived pits present on the cellular surface (Fig. 2 b, lower panel). These results are consistent with previous work (Taylor et al., 2017) and evidence a role for peripheral spectrin in inhibiting the formation of plasma membrane vesicles.

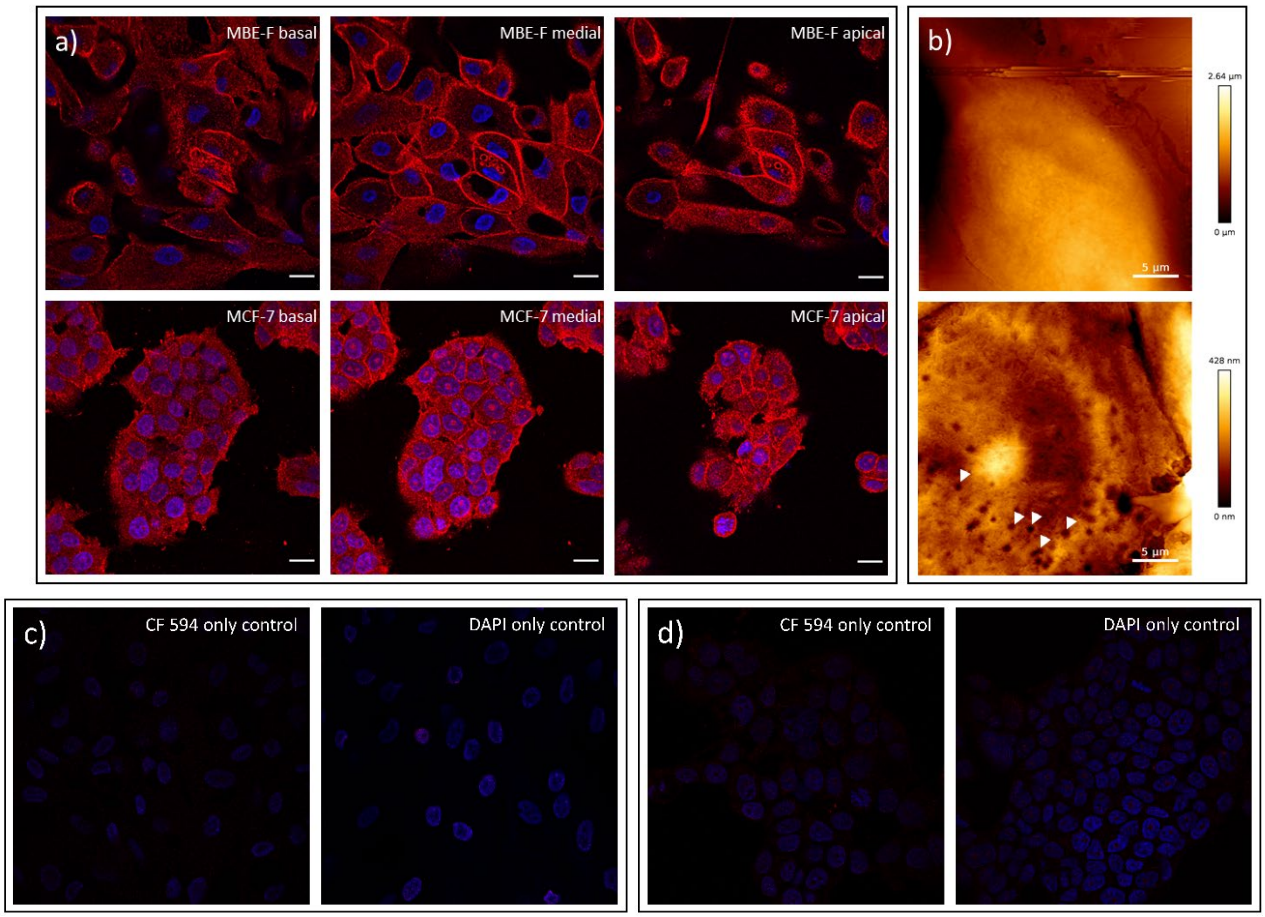

Figure 4. 2. Spectrin is differentially localised non-malignant cells and malignant cells.

a) High resolution confocal microscopy of basal, middle, and apical z-planes shows αII-spectrin (red) is predominantly localised at the edge and towards the apical surface of non-malignant MBE-F cells. In contrast, spectrin is distributed throughout the cytoplasm in basal, middle, and apical z-planes in MCF-7 cells. Scale bar is representative of 20 µm. 60x magnification. b) Atomic force microscopy topographical images of low vesiculating MBE-F (upper panel) and high vesiculating MCF-7 (lower panel) cells at rest. Arrow heads indicate vesicle-derived pits. Scan areas 30 µm^2. Secondary antibody (CF 954) and DAPI only unstained negative control of MBE-F (c) and MCF-7 (d) cells. 60x magnification. Images representative of typical cells from three individual experiments.

We designed and wrote the script for an automated cell segmentation plugin to be run in the Fiji image processing software to allow for an unbiased measure of αII-spectrin distribution in individual cells (Fig. 3 a). Fig. 3 a. shows a schematic of automated image processing and segmentation workflow and include: 1) analysis of individual z-planes of up to 150 cells per field of view, 2) marker controlled segmentation of individual nuclei, 3) background thresholding to isolate cell clusters, and 4) automated marker controlled segmentation of individual cells. Following segmentation of individual cells, the ratio of membrane and cytoplasmic αII-spectrin was calculated as the αII-spectrin Membrane:Cytoplasm (M:C) distribution ratio (Fig. 3. b). We compared the M:C distribution ratio in MBE-F and MCF-7 cells at rest. At rest, non-malignant MBE-F cells display spectrin M:C ratio of 1.74 (± 0.065) indicative of a localised distribution of spectrin in the membrane with respect to the cytoplasm. In comparison, MCF-7 cells display significantly lower M:C ratio of spectrin with a distribution ration of 1.16 (± 0.036, $p < 0.0001$) evidencing a greater cytoplasmic localisation compared to non-malignant cells.

Spectrin plays a role in numerous cell functions including maintenance of cellular morphology and migration as well as the regulation of transmembrane proteins, cell adhesion, cell spreading and cell cycle (Machnicka et al., 2012, Zhang et al., 2013b, Gascard and Mohandas, 2000). These functions are dependent subcellular localisation as it allows spectrin to associate with the other proteins involved in each process. Given the function of spectrin can be regulated by its subcellular localisation, we also examined the distribution of αII-spectrin across different z-planes of from the basal to apical surface (Fig. 3. c). Non-malignant cells have increasing spectrin borders as the z-planes move towards the apical surface of the cell. This polarity is not observed in malignant MCF-7 cells (Fig. 3. c). These results show that spectrin in distributed to the apical surface in resting non-malignant cells whilst in malignant cells, spectrin is primarily distributed within the cytoplasmic region.

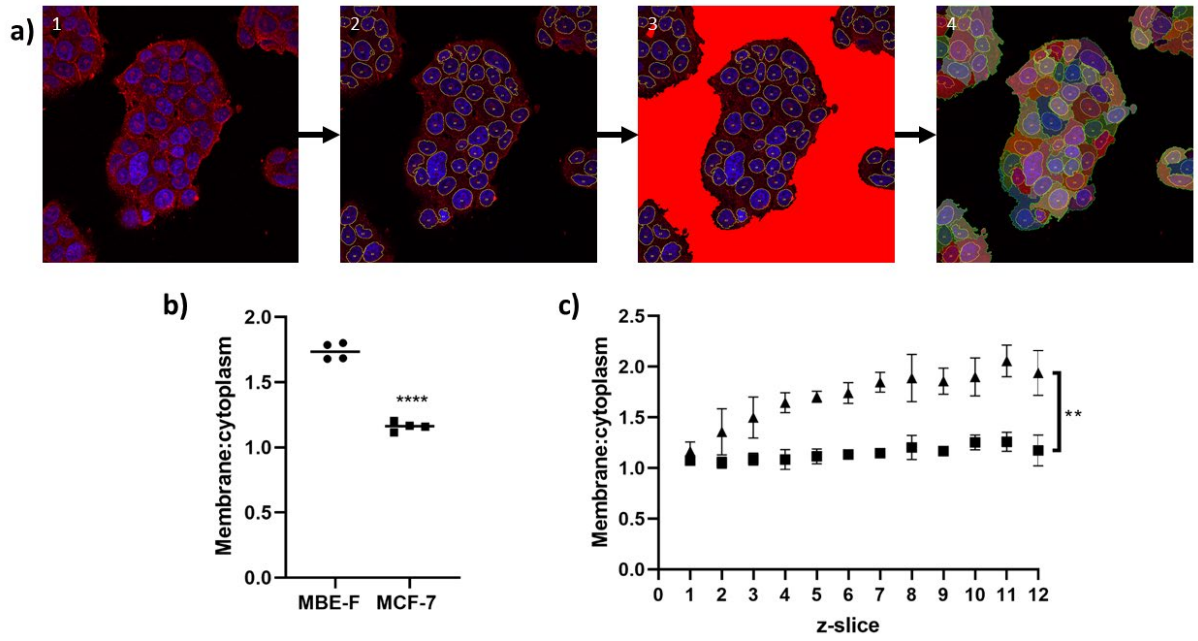

Figure 4. 3. Quantitative analysis of spectrin distribution in malignant and non-malignant cells.

*a) Schematic of automated image processing in Fiji. 1) Each z-plane is analysed individually. 2) Nuclei (blue) are passed through an automated segmentation process and labelled as seed points for each cell. 3) A background threshold is applied to isolate groups of cells. 4) An automated seed point dilation function is used to find the edges of individual cells. b) Ratio of membrane associated and cytoplasmic spectrin fluorescence in resting MBE-F and MCF-7 cells. Data represents mean fluorescence of four individual experiments. ****p < 0.0001 (unpaired t test). c) Membrane:cytoplasm ratio of spectrin fluorescence in each confocal z-plane from basal to apical side MBE-F (▲) and MCF-7 (■) cells in.** p < 0.01 (unpaired t test).*

4.4.2. Increasing intracellular calcium by A23187 results in loss of peripheral spectrin localisation in non-malignant MBE-F cells

MV biogenesis can be activated in non-malignant cells by increasing intracellular calcium through the use of the calcium ionophore A23187 (Taylor et al., 2017, Pasquet et al., 1996). Increasing intracellular Ca^{2+} results in the activation of calpain which cleaves cytoskeletal proteins including spectrin (Pasquet et al., 1996, Morel et al., 2011). In erythrocytes, remodelling of spectrin compromises membrane-cytoskeleton anchorage and allows for the formation of MVs (Leal et al., 2018). However, the distribution of non-erythroid spectrin has not been examined in the context of MV biogenesis. To determine the effect of increasing intracellular calcium on spectrin distribution, MBE-F cells were treated with the calcium ionophore, A23187 (1 μM) for 24 h. High resolution confocal microscopy was used to visualise αII-spectrin in MBE-F cells in response to activation with A23187. The images reveal a primarily diffuse fluorescence staining in the cytoplasm (Fig. 4. A, lower panels) with peripheral spectrin localisation being substantially reduced relative to untreated cells. AFM images show that calcium activation of MBE-F cells with A23187 increases the biogenesis of plasma membrane vesicles (Fig. 4 b, lower panel). This demonstrates that an increase in intracellular calcium results in the disruption of membrane-associated spectrin borders and the production of MVs.

Quantitative analysis of spectrin distribution following automated segmentation also support these observations. As shown in Fig. 4. c, MBE-F cells treated with A23187 displayed a significant reduction from 1.75 to 1.385 ($p < 0.014$) in the M:C ratio indicating that spectrin borders were diminished following an increase in intracellular calcium. Furthermore, this was observed in each z-plane from the basal to apical regions, suggesting that the remodelling of spectrin occurs throughout the cell with loss of spectrin polarity (Fig. 4 d).

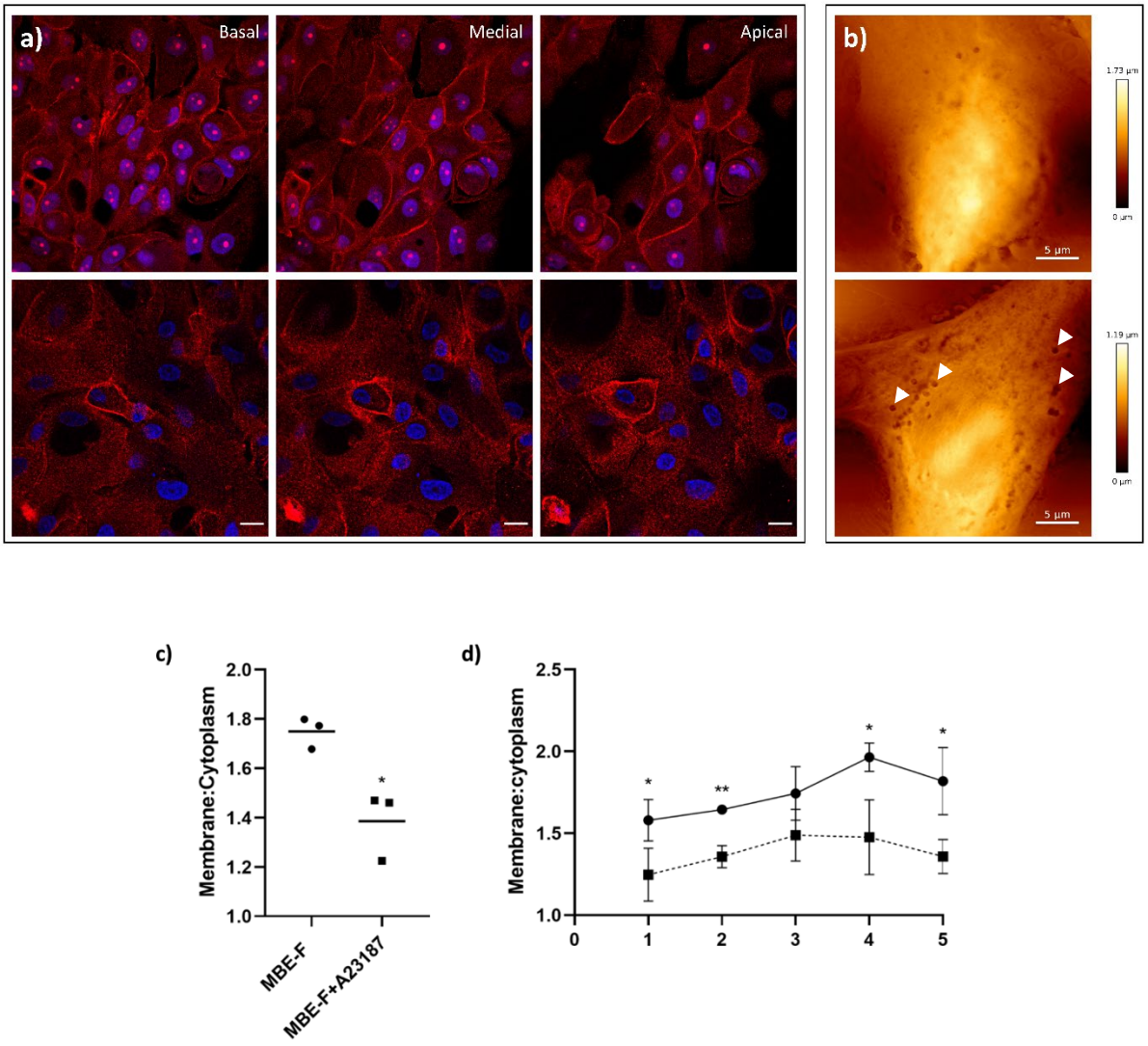

Figure 4. 4. Spectrin membrane localisation diminishes following treatment with calcium ionophore, A23187 in MBE-F cells.

MBE-F cells were treated with A23187 (1 µM) for 24 hours and αII-spectrin was visualised using high resolution confocal microscopy. a) Confocal images in the basal, middle, and apical z-planes show peripheral αII-spectrin (red) borders present in untreated cells (upper panels). Contrary to this, αII-spectrin is not peripherally localised on the cell surface following treatment with 1µM A23187 (lower panels), rather are localised diffusely throughout the cell cytoplasm. Images are representative of a typical cluster of cells from a single experiment. Scale bar is representative of 20 µm. 60x magnification. b) AFM topographical image of MBE-

*F cell at rest (upper panel) and following treatment with A23187 (lower panel) showing MV-derived pits on the surface (arrow heads). Scan area 30 μm^2. Image representative of typical cell. c) Ratio of membranous and cytoplasmic spectrin fluorescence in resting MBE-F cells and cells activated with A21387. Data represents mean fluorescence ratio of three individual experiments. *p < 0.0144. d) Quantitative analysis of confocal z-planes from basal to apical (1-5) following automated segmentation of MBE-F cells ± A23187. αII-spectrin localises in the membrane periphery in apical z-planes in resting MBE-F cells (●) consistent with a high membrane:cytoplasm ratio. MBE-F cells treated with A23187 (■) display diminished peripheral spectrin boarders consistent with a smaller ratio.*

4.4.3. Inhibition of plasma membrane MV biogenic machinery restores peripheral αII-spectrin borders in MCF-7 cells.

MCF-7 cells are high vesiculators at rest due to high basal calcium levels and a high basal level of calcium-calpain dependent biogenesis (Goll et al., 2003, Pottle et al., 2013, Roseblade et al., 2015, Taylor et al., 2017). Inhibition of this pathway through the use of calpain inhibitor II (ALLM) and intracellular chelation of calcium with BAPTA-AM has been shown to significantly reduce the production of plasma membrane EVs (Roseblade et al., 2015, Taylor et al., 2017). To examine the effect of inhibiting the calcium-calpain pathway on the subcellular distribution αII-spectrin, MCF-7 cells were treated with the calpain inhibitor II, ALLM (10 μM) and the intracellular calcium chelator BAPTA-AM (50 μM) up to 48 hours.

Figure 5 (a) shows resting MCF-7 cells display diffuse cytoplasmic spectrin and diminished peripheral borders. These cells also display a high degree of vesiculation evidenced by numerous vesicle-derived pits in AFM images (Fig. 5 a). We also show that inhibition of calpain resulted in a time-dependent restoration of peripheral spectrin borders in MCF-7 cells.

Cells treated with ALLM for 48 hours displayed prominent peripheral borders (Fig. 5. b, middle panel) and significantly higher M:C distribution ratio in each z-plane compared to control (Fig. 5. c). This suggests that calpain activation is responsible for the lack of peripheral spectrin near the apical membrane of MCF-7 cells. We also show that treatment with ALLM resulted in the inhibition of MV biogenesis (Fig. 5 b, left panel) suggesting MV biogenesis and spectrin remodelling is regulated by a calpain-dependent pathway. This effect was time dependent as only modest increase in peripheral spectrin borders was observed compared to untreated controls in MCF-7 cells treated with ALLM for 24 hours (Fig. 5 b and c) suggesting the restoration of spectrin borders is time-dependent.

We also examined the effect of chelating intracellular calcium with BAPTA-AM on the distribution of αII-spectrin in MCF-7 cells. Similarly to treatment with ALLM, cells treated with BAPTA-AM displayed a time-dependent restoration of peripheral spectrin borders (Fig. 5. b, left and middle panels). AFM images show that chelation of intracellular calcium with BAPTA-AM resulted in a concomitant inhibition of MV biogenesis (Fig. 5 b, right panel). As shown in Fig 5, treatment of high vesiculating MCF-7 cells with BAPTA-AM for 48 hours, resulted in a significant increase in M:C spectrin distribution ratio in all z-planes from basolateral to apical (Fig. 5 d). However, the increase was more pronounced in z-planes closer to the apical surface (Fig. 5 d). These results suggest the restoration of spectrin borders and inhibition of MV biogenesis can be induced by a reduction in free intracellular calcium.

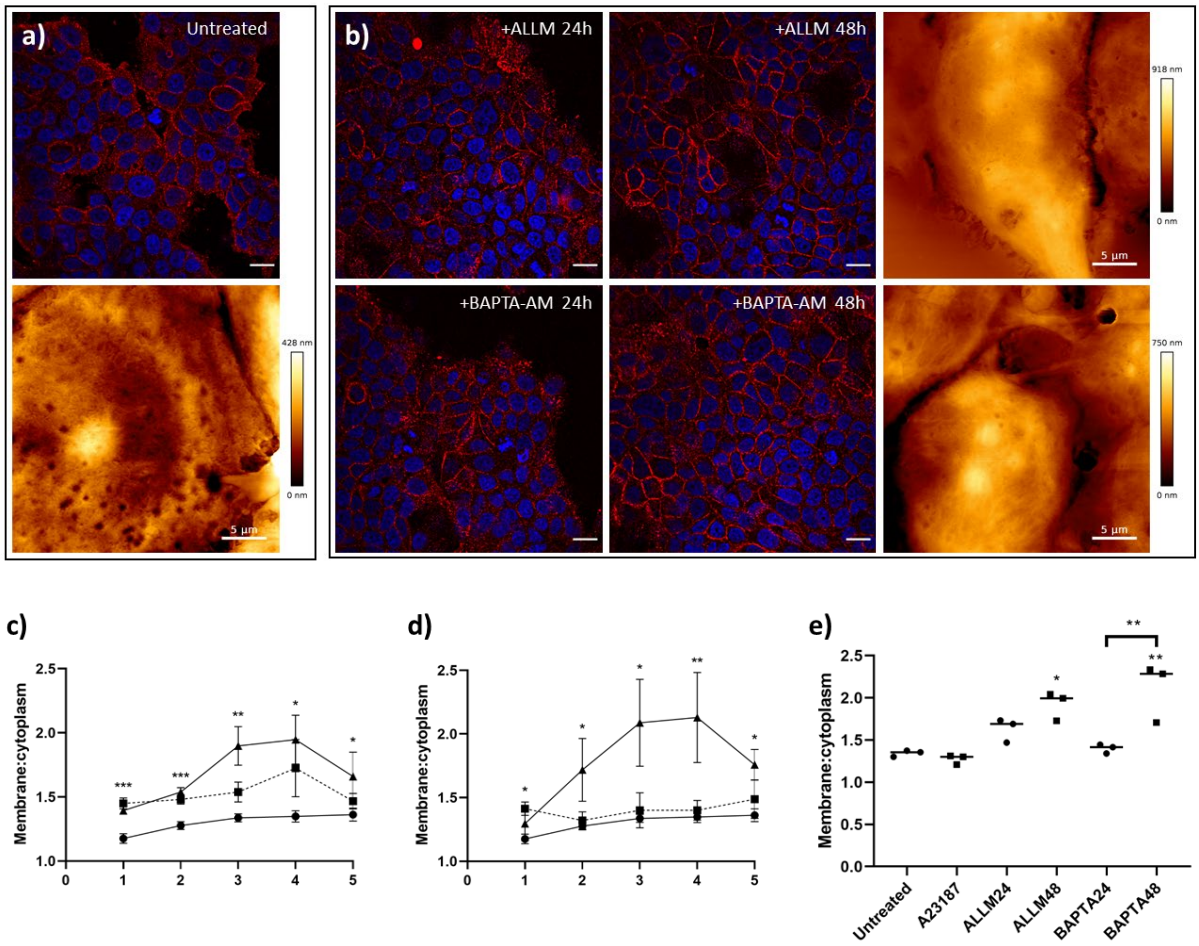

Figure 4. 5. Inhibition of the calcium-calpain pathway results in time-dependent restoration of spectrin boarders in MCF-7 cells.

MCF-7 cells were treated with the calpain inhibitor II (ALLM) or intracellular calcium chelator BAPTA-AM for up to 48 hours. Cells were then fixed and αII-spectrin (red) localisation was visualised with confocal microscopy. a) Confocal (upper panel) and AFM (lower panel) images of MCF-7 cells at rest. The images show diminished peripheral spectrin borders correlated with an increase in MV-derived pits. b) MCF-7 cells treated with the calpain inhibitor ALLM (upper panels) and BAPTA-AM (lower panels) for 24 (left panel) and 48 hours (middle panel). Spectrin borders in the periphery of cells are being restored in a time-dependent manner. Time dependent spectrin border restoration correlated with a decrease in MV-derived pits as illustrated in AFM images (right panel). Confocal images are

*representative of a typical cluster of cells from a single experiment. Scale bar is representative of 20 µm. 60x magnification. AFM images representative of a typical cells. Scan area 30 µm^2. c) Quantification of peripheral vs cytosolic spectrin fluorescence from basal to apical (1-5) side of MCF-7 cells at rest (●), and treated with ALLM (10 µM) for 24 (■) and 48 (▲) hours. d) z-plane analysis of MCF-7 cells at rest (●), and treated with BAPTA-AM (50 µM) for 24 (■) and 48 (▲) hours. Peripheral spectrin borders are more prominent towards the apical side of the cells treated with both ALLM (c) and BAPTA-AM (d). e) Comparison of membrane:cytoplasm distribution ratio of spectrin in apical z-planes of MCF-7 cells following treatment with A23187 (1 µM), ALLM (10 µM), and BAPTA-AM (50 µM). Treatment with ALLM and BAPTA-AM resulted in the restoration of spectrin borders in a time-dependent manner. Data represents mean fluorescence ratio of three individual experiments. *p < 0.05, **p < 0.01, ***p < 0.001 (one way ANOVA).*

MCF-7 cells were also treated with calcium ionophore, A23187 (1 µM) for 24 hours. However, mobilisation of intracellular calcium with A23187 had no effect on the distribution of αII-spectrin (Fig 5 d). This is likely due to MCF-7 cells having high basal calcium activation and therefore higher activation of calpain at rest.

Fig. 5 e shows that inhibition of the calcium-calpain MV biogenic pathway results in the time-dependent restoration of αII-spectrin borders in the apical surface of MCF-7 cells. MCF-7 cells displayed a significant increase of 0.39 ($p = 0.0036$) and 0.766 ($p = 0.0089$) in M:C distribution ratio of spectrin fluorescence following treatment with ALLM and BAPTA-AM respectively compared to untreated controls (Fig 5. e). These results illustrate that spectrin is distributed to the periphery to form borders when biogenic machinery is inhibited. Whereas treatment with A23187 did not change the distribution of spectrin. Collectively, these results suggest that the

subcellular localisation of spectrin is regulated by a calpain-calcium dependent pathway – the same pathway that regulates malignant MV biogenesis.

4.4.4. Peripheral αII-spectrin borders correlate with increased membrane stiffness.

As spectrin plays an integral role in the maintenance of cell shape and stability, we next examined the differences in mechanical properties of non-malignant and malignant cells. To determine if biomechanical properties are affected by subcellular distribution of αII-spectrin, Atomic force spectroscopy was performed on MBE-F and MCF-7 cells and the Young's Modulus was calculated for resting cells, as well as cells treated with A23187 (1 μM), ALLM (10 μM), and BAPTA-AM (50 μM). MBE-F cells with prominent spectrin borders exhibit an elastic modulus of 40 kPa ± SD (Fig. 6. a). In high vesiculating MCF-7 cells lacking spectrin borders, the Young's Modulus is significantly lower, 28 kPa ± SD. This demonstrates that peripheral spectrin borders correlate with increased biomechanical stiffness in non-malignant cells.

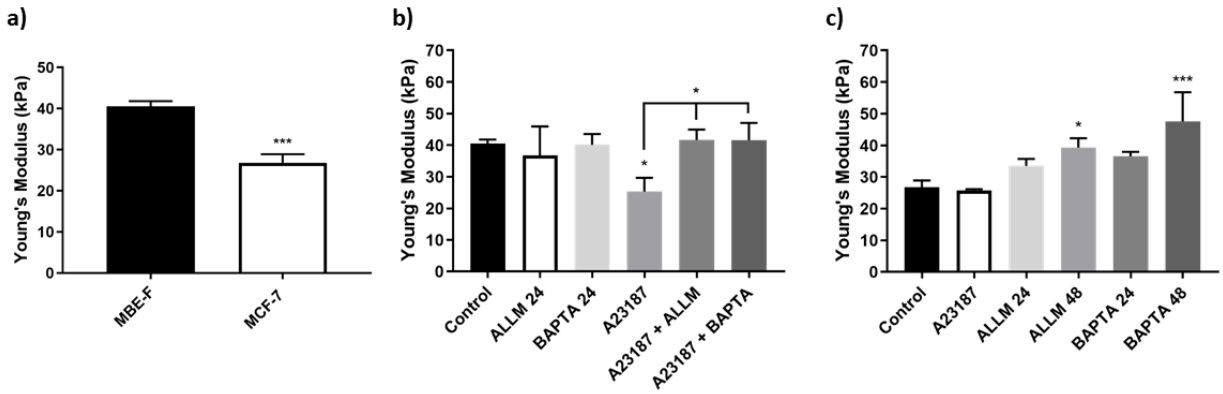

Figure 4. 6. Mechanical stiffness of cells correlate with peripheral spectrin distribution.

Non-malignant MBE-F cells and malignant MCF-7 cells were treated with calcium ionophore A23187 (1 μM), ALLM (10 μM), and BAPTA-AM (50 μM) for 24 or 48 hours. Atomic Force spectroscopy was performed and the elastic modulus was determine. a) Non-malignant MBE-F cells that have prominent spectrin borders display higher elastic modulus at rest compared

*to malignant MCF-7 cells. b) Treatment with A23187 results in loss of spectrin borders and a significant decrease in membrane stiffness in MBE-F cells. Pre-treatment with either ALLM or BAPTA-AM prevented A23187-induced reduction in membrane stiffness. c) At rest, high vesiculating MCF-7 cells have lower stiffness. Treatment with MV biogenesis inhibitors ALLM or BAPTA-AM increases stiffness in a time-dependent manner. Data represents mean ± SD from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 (one way ANOVA).*

MBE-F and MCF-7 cellular elasticity was also examined in the presence of molecules that activate and inhibit plasma membrane EV biogenesis. We observed no change in the Young's Modulus in MBE-F cells treated with inhibitors of the calcium-calpain dependent EV biogenic pathway, ALLM or BAPTA-AM. Conversely, treatment with calcium ionophore resulted in a significant reduction in young's modulus to approximately half of untreated cells (Fig. 6. b). Pre-treatment of MBE-F cells with either ALLM or BAPTA-AM for 15 mins prevented the A23187-mediated reduction in Young's modulus. These results suggest that increases in intracellular calcium reduces membrane stiffness via activation of a calcium-calpain dependent pathway.

As shown in Fig 6. c, MCF-7 cells treated with A23187 displayed no change in Young's modulus compared to untreated control. In cells treated with the calpain inhibitor, we observed a modest and significant increase in Young's Modulus after 24 and 48 hours respectively. Similarly, BAPTA-AM treated MCF-7 cells displayed a time-dependent increase in Young's modulus (Fig. 6. c). These results suggest malignant cell biomechanical stiffness can be increased by inhibition of the established pathway of calcium-calpain-dependent plasma membrane biogenesis.

4.5. Discussion

Localised loss of membrane-cytoskeletal anchorage is a key step in the biogenesis of plasma membrane EVs. In erythroid cells, peripheral spectrin is vital for membrane integrity and calpain-mediated remodelling of spectrin promotes the generation of plasma membrane microvesicles (An et al., 2004a, Gascard and Mohandas, 2000, Machnicka et al., 2012, Wagner et al., 1987). However, the subcellular distribution of spectrin and its role in plasma membrane EV biogenesis in non-erythroid cells remains to be defined. Plasma membrane EVs are formed by discrete biogenic pathways in malignant and non-malignant cells. We have previously shown that malignant cells are high vesiculators rest and biogenesis is driven by a calcium-calpain dependent pathway (Taylor et al., 2017). In this study, we sought to define the role of spectrin in the biogenesis of EVs in malignant MCF-7 and non-malignant MBE-F cells. We demonstrate αII-spectrin is differentially distributed in these cell types – prominent peripheral spectrin borders are only observed in low vesiculating non-malignant cells whereas they are absent in high vesiculating MCF-7 cells. Furthermore, we show for the first time that inhibition of the plasma membrane MV biogenic pathway restores spectrin borders in a time-dependent manner in MCF-7 cells.

At rest, prominent peripheral spectrin borders are evident is non-malignant cells and not in malignant cells. High resolution confocal images show low vesiculating MBE-F cells with strong αII-spectrin fluorescence in borders localised in the periphery with some diffuse cytoplasmic staining (Fig. 2). It was also observed that peripheral spectrin borders are more prominent towards the apical surface of MBE-F cells (Fig 2). These observations are perhaps not surprising considering spectrin is a multifunctional protein that is important not only for providing strength and stability to the plasma membrane (Marchesi and Steers, 1968, Machnicka et al., 2014) and organisation of cytoskeleton (Machnicka et al., 2012), but they are also vital for cell polarity (Black et al., 1988), regulation of ion channels (Lubbers et al., 2019),

and assembly of excitable axon domains (Liu and Rasband, 2019). It is well established that the spectrin meshwork under the plasma membrane of erythrocytes hinders the release of plasma membrane EVs (An et al., 2004b). As red blood cells age, they begin to release more plasma membrane EVs as a result of calcium-mediated proteolysis of membrane-associated spectrin (Leal et al., 2018). However, it is important to note that erythroid spectrin isotypes are considerably different to the non-erythroid isotypes present in these cells (Leto et al., 1988, Moon and McMahon, 1990).

In contrast, high vesiculating MCF-7, do not exhibit spectrin borders in the periphery at rest (Fig. 2). These cells only exhibit diffuse spectrin labelling throughout the cytoplasm and there no is observable change between the basal and apical regions (Fig. 2). This is consistent with early reports that high grade tumours show cytoplasmic accumulation of spectrin (Sormunen et al., 1999). Further studies have implicated loss of membrane-associated spectrin and cellular polarity with increase proliferative and invasive capacity in malignancy (Tuominen et al., 1996). However, localisation and function of αII-spectrin in malignancy varies among different cancers. Another explanation for this, is the high basal level of intracellular calcium in malignant cells, which is significantly higher than in non-malignant cells (Goll et al., 2003, Pottle et al., 2013). We have previously demonstrated high basal calcium in malignant MCF-7 cells facilitates the biogenesis of plasma membrane EVs via activation of calpain. Calpain is a cysteine protease that is activated by an increase in intracellular calcium (Roseblade et al., 2013). Upon activation, calpain cleaves cytoskeletal spectrin proteins resulting in the local detachment of the cell membrane from the underlying cytoskeleton (Weber et al., 2009, Boivin et al., 1990, Roseblade et al., 2013).

To further interrogate the role of spectrin in plasma membrane MVs biogenesis, non-malignant MBE-F cells were treated with a well-established plasma membrane vesicle biogenesis activator, calcium ionophore, A23187 (Basse et al., 1994, Pasquet et al., 1996). High resolution

confocal microscopy shows MBE-F cells lose peripheral spectrin borders following cellular activation with A23187. Quantitative analysis of the spectrin M:C distribution ratio confirms visual observations with a significant decrease in membrane fluorescence compared to untreated controls. The loss of spectrin borders in the apical surface of the cells also shows a loss of polarity in A23187 treated cells. These results suggest degradation of αII-spectrin at the plasma membrane is mediated by a calcium-calpain dependent pathway. It is known that A23187-mediated increases in intracellular calcium results in the activation of calpain and the proteolysis of αII-spectrin into two proteolytic fragments calcium in many cell types (Gov et al., 2009, Wang et al., 1996, Saido et al., 1992). The pathway for spectrin remodelling holds remarkable similarity to the plasma membrane MB biogenic pathway. These results demonstrate that activation of MV biogenesis via increasing intracellular calcium results in the loss of peripheral spectrin borders in low-vesiculating MBE-F cells.

Our lab was the first to show that EVs, specifically MVs released from malignant cells are involved in the non-genetic transfer of multidrug resistance in cancer (Bebawy et al., 2009b). MVs are now implicated in conferral of many other deleterious cancer traits such as increased metastatic capacity, active and passive drug sequestration, evasion of immune surveillance, and altered tissue biomechanics (Gong et al., 2014b, Gong et al., 2013, Jaiswal et al., 2017, Pokharel et al., 2016a). Understanding the specific biogenic pathways involved in malignant vesiculation holds potential for development of novel therapeutic strategies to circumvent EV-mediated cancer progression, however, precise pathways remain to be fully elucidated.

At rest malignant cells shed large quantities of plasma membrane vesicles and we have previously shown inhibition of calcium signalling or calpain activity effectively blocks production of membrane EVs (Roseblade et al., 2015, Taylor et al., 2017). Considering that spectrin also is cleaved by calpain, it raises the question of whether inhibiting calpain in MCF-7 cells will prevent spectrin proteolysis and restore distribution at the plasma membrane. In

this study, we demonstrate for the first time that inhibition of these biogenic pathways restores peripheral spectrin borders in MCF-7 cells in a time dependent manner. Treatment of malignant MCF-7 cells with calpain inhibitor II (ALLM) for 24 hours resulted in an increase in M:C distribution of spectrin – indicating localisation of spectrin had shifted towards the membrane. This peripheral distribution was also polarised with spectrin borders more prominent towards the apical surface of MCF-7 cells. Increasing the treatment time of MCF-7 cells with ALLM to 48 hours resulted in further restoration of peripheral spectrin borders. This was quantified as αII-spectrin M:C distribution ratio of 2.109 (Fig. 5 d) – similar to levels observed in non-malignant cells. Clearly demonstrating for the first time that peripheral spectrin can be recovered in malignant cells by inhibition of calpain. This suggests the loss of peripheral spectrin borders correlates with increased MV biogenesis in malignant cells.

Furthermore, we show that chelation of intracellular calcium with BAPTA-AM restored peripheral spectrin in a time-dependent manner. Calcium is a highly diversified signalling molecule involved in numerous physiological and pathophysiological cellular processes (Berridge, 2012). In the context of plasma membrane EV biogenesis, mobilisation of calcium from intracellular stores is essential. Considering calpain is a calcium-dependent protease and its inhibition restored spectrin borders, it was expected that chelation of intracellular calcium would be similarly efficient in spectrin border restoration compared to ALLM. Interestingly, treatment of MCF-7 cells with BAPTA-AM for 24 hours showed minimal change is spectrin distribution compared to resting MCF-7 cells (Fig. 5) despite increases in intracellular calcium being upstream of calpain in the proposed pathway. Although, spectrin peripheral borders being more prominent in MCF-7 cells treated with BAPTA-AM for 48 hours compared to ALLM for 48 hour (fig. 5), the delayed effect is unexpected. We cannot rule out the possibility of alternative pathways involved in preventing the formation of peripheral spectrin borders in MCF-7 cells. However, given calcium chelation ultimately results in restoration of the borders,

these alternative pathways appear to be secondary in nature. While the aim of this work was to determine the subcellular localisation of spectrin in malignant and non-malignant cells, gene expression of spectrin is another important variable that needs to be addressed in future work. It has been reported that altered spectrin may be involved in the development and progression of some cancers (Ackermann and Brieger, 2019).The mechanical elasticity of cells is primarily dependent on spectrin complexes that provide stability to otherwise mechanically fragile cells (Baines, 2010). In fig. 7, we illustrate the stark differences observed in spectrin localisation between low vesiculating MBE-F and high vesiculating MCF-7 cells are reflected in their mechanical properties. Non-malignant cells have significantly higher Young's modulus compared to malignant cells. Similar to the loss of peripheral spectrin fluorescence, treatment of non-malignant cells with vesicle production activator, A23187 resulted in the reduction of cellular stiffness. Treatment of MCF-7 cells with inhibitors of calcium-calpain plasma membrane biogenic pathway displayed a time-dependent increase in young's modulus (fig. 7). These result highlights the importance of subcellular distribution on the function of αII-spectrin. Spectrin makes up approximately 2-3% of the total protein in non-erythroid cells (Goodman et al., 2015) and is implicated in a diverse range of functions (Ackermann and Brieger, 2019, Wu et al., 2017, Liem, 2016a, Goodman et al., 2015). For example, membranous spectrin is pivotal for maintaining the overall elasticity of osteocytes as well as cell cortex stiffness. Disruption of the spectrin network resulted in obvious softening of osteocytes and resulted in significant influx of calcium (Wu et al., 2017). Increases in intracellular Ca^{2+} also result in 'contractions' within the actin network and production of EVs in osteocytes (Morrell et al., 2018). Clearly, the integrity of the plasma membrane and biogenesis of EVs depends on a structural network formed with spectrin and a number of other structural proteins including actin, protein 4.1, and ankyrin. The regulation of these other cytoskeletal components remains

critical in understanding spectrin's role in plasma membrane EV biogenesis in malignant and non-malignant cells.

In the context of malignancy, increases in cytoplasmic spectrin is implicated in the proliferative and invasive capacity of cells and suggested as a marker of neoplasia (Sormunen et al., 1999, Ho, 1992). The lack of a physical αII-spectrin barrier at the plasma membrane and concurrent lack of mechanical stiffness appears to provide malignant cells with the ideal platform to shed plasma membrane EVs.

4.6. Conclusions

The role of spectrin in RBC vesiculation is well characterised where its remodelling allow the shedding of plasma membrane EVs. However, its role in non-erythroid plasma membrane EV biogenesis remains to be fully elucidated. In this work, we have demonstrated for the first time that the subcellular distribution of αII-spectrin is different in malignant and non-malignant cells. We show the presence of spectrin borders is feature of low vesiculating MBE-F cells whereas there are minimal spectrin borders observed in high vesiculating MCF-7 cells. Furthermore, we demonstrate inhibition of the calpain-calcium dependent pathway for EV biogenesis restores spectrin borders in a time-dependent manner. These results establish a role for subcellular distribution spectrin in malignant MV biogenesis and suggest that a lack spectrin borders may be an indicator of vesicle biogenesis. Lastly, we have shown that restoration of peripheral spectrin increases cellular stiffness by the inhibition of calcium-calpain dependent pathway. However, it remains an important step to investigate whether these observations are conserved in other cells types. Collectively, this work characterises αII-spectrin and sheds light on how it is involved in the biogenesis of plasma membrane EVs. These findings provide valuable insight to the potential roles of spectrin in microvesicle biogenesis and malignancy and provide a first-step to uncovering the role that spectrin plays in malignancy.

List of abbreviations

ALLM: N-Acetyl-L-leucyl-L-leucyl-L-methioninal

BAPTA-AM:

1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester)

BSA: Bovine serum albumin

DAPI: 4′,6-diamidino-2-phenylindole

FBS: Foetal bovine serum

MEG-1: Mammary Epithelial Cell Growth Medium-1(Blikstad et al., 1983)

MV: Microvesicle

NA: Numerical Aperture

PBS: Phosphate-buffered saline

RPMI: Roswell Park Memorial Institute medium

Conflict of interest

The authors confirm that this article content has no conflict of interest.

4.7. References

1. Bebawy, M., et al., *Membrane microparticles mediate transfer of P-glycoprotein to drug sensitive cancer cells.* Leukemia, 2009. **23**: p. 1643.
2. Abels, E.R. and X.O. Breakefield, *Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake.* Cellular and Molecular Neurobiology, 2016. **36**(3): p. 301-312.
3. Al-Nedawi, K., B. Meehan, and J. Rak, *Microvesicles Messengers and mediators of tumor progression.* Cell Cycle, 2009. **8**(13): p. 2014-2018.
4. De Souza, P.S., et al., *Membrane microparticles: shedding new light into cancer cell communication.* Journal of Cancer Research and Clinical Oncology, 2016. **142**(7): p. 1395-1406.
5. Lener, T., et al., *Applying extracellular vesicles based therapeutics in clinical trials - an ISEV position paper.* Journal of Extracellular Vesicles, 2015. **4**: p. 31.
6. Malik, M.F.A., *Influence of Microvesicles in Breast Cancer Metastasis and their Therapeutic Implications.* Archives of Iranian Medicine, 2015. **18**(3): p. 189-192.
7. Gong, J., et al., *Microparticles and their emerging role in cancer multidrug resistance.* Cancer Treatment Reviews, 2012. **38**(3): p. 226-234.
8. Bebawy, M., et al., *Membrane microparticles mediate transfer of P-glycoprotein to drug sensitive cancer cells.* Leukemia, 2009. **23**(9): p. 1643-1649.
9. Lu, J.F., et al., *Microparticles mediate MRP1 intercellular transfer and the re-templating of intrinsic resistance pathways.* Pharmacological Research, 2013. **76**: p. 77-83.
10. Lu, J.F., et al., *A novel method to detect translation of membrane proteins following microvesicle intercellular transfer of nucleic acids.* Journal of Biochemistry, 2016. **160**(5): p. 281-289.
11. Biedler, J.L. and H. Riehm, *Cellular resistance to actinomycin-d in chinese hamster cells in-vitro - cross-resistance, radioautographic, and cytogenetic studies.* Cancer Research, 1970. **30**(4): p. 1174-&.
12. Juliano, R.L. and V. Ling, *Surface glycoprotein modulating drug permeability in chinese-hamster ovary cell mutants.* Biochimica Et Biophysica Acta, 1976. **455**(1): p. 152-162.
13. Cole, S.P.C., et al., *Overexpression of a transporter gene in a multidrug-resistant human lung-cancer cell-line.* Science, 1992. **258**(5088): p. 1650-1654.
14. Gong, J., et al., *Microparticle drug sequestration provides a parallel pathway in the acquisition of cancer drug resistance.* European Journal of Pharmacology, 2013. **721**(1-3): p. 116-125.
15. Gong, J., et al., *Microparticles Mediate the Intercellular Regulation of microrna-503 and Proline-Rich Tyrosine Kinase 2 to Alter the Migration and Invasion Capacity of Breast Cancer Cells.* Frontiers in Oncology, 2014. **4**(220).
16. Pokharel, D., et al., *Deciphering Cell-to-Cell Communication in Acquisition of Cancer Traits: Extracellular Membrane Vesicles Are Regulators of Tissue Biomechanics.* OMICS: A Journal of Integrative Biology, 2016. **20**(8): p. 462-469.
17. Jaiswal, R., et al., *Microparticles shed from multidrug resistant breast cancer cells provide a parallel survival pathway through immune evasion.* Bmc Cancer, 2017. **17**: p. 12.
18. Pokharel, D., et al., *Deciphering Cell-to-Cell Communication in Acquisition of Cancer Traits: Extracellular Membrane Vesicles Are Regulators of Tissue Biomechanics.* Omics-a Journal of Integrative Biology, 2016. **20**(8): p. 462-469.

19. Raposo, G. And W. Stoorvogel, *Extracellular vesicles: Exosomes, microvesicles, and friends.* Journal of Cell Biology, 2013. **200**(4): p. 373-383.
20. Colombo, M., G. Raposo, and C. Thery, *Biogenesis, Secretion, and Intercellular Interactions of Exosomes and Other Extracellular Vesicles*, in *Annual Review of Cell and Developmental Biology, Vol 30*, R. Schekman and R. Lehmann, Editors. 2014, Annual Reviews: Palo Alto. P. 255-289.
21. Martin, S.J., et al., *Early redistribution of plasma-membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus - inhibition by overexpression of BCL-2 AND ABL.* Journal of Experimental Medicine, 1995. **182**(5): p. 1545-1556.
22. Perrin, B.J., K.J. Amann, and A. Huttenlocher, *Proteolysis of cortactin by calpain regulates membrane protrusion during cell migration.* Molecular Biology of the Cell, 2006. **17**(1): p. 239-250.
23. Storr, S.J., et al., *The calpain system and cancer.* Nature Reviews Cancer, 2011. **11**(5): p. 364-374.
24. Bogusławska, D.M., et al., *Spectrin and phospholipids — the current picture of their fascinating interplay.* Cellular & Molecular Biology Letters, 2014. **19**(1): p. 158-179.
25. Zhang, R., et al., *Spectrin: Structure, function and disease.* Science China Life Sciences, 2013. **56**(12): p. 1076-1085.
26. Liem, R.K., *Cytoskeletal integrators: the spectrin superfamily.* Cold Spring Harbor perspectives in biology, 2016. **8**(10): p. A018259.
27. Lux, S.E., *Anatomy of the red cell membrane skeleton: unanswered questions.* Blood, 2016. **127**(2): p. 187-199.
28. An, X.L., et al., *Phosphatidylserine binding sites in red cell spectrin.* Blood Cells Molecules and Diseases, 2004. **32**(3): p. 430-432.
29. An, X.L., et al., *Phosphatidylserine binding sites in erythroid spectrin: Location and implications for membrane stability.* Biochemistry, 2004. **43**(2): p. 310-315.
30. Boguslawska, D.M., et al., *Spectrin and phospholipids - the current picture of their fascinating interplay.* Cellular & Molecular Biology Letters, 2014. **19**(1): p. 158-179.
31. Wagner, G.M., et al., *Spectrin oxidation correlates with membrane vesiculation in stored RBCS.* Blood, 1987. **69**(6): p. 1777-1781.
32. Roseblade, A., et al., *Cell-derived microparticles: new targets in the therapeutic management of disease.* Journal of Pharmacy & Pharmaceutical Sciences, 2013. **16**(2): p. 238-253.
33. Pap, E., *The role of microvesicles in malignancies*, in *Cell Fusion in Health and Disease*. 2011, Springer. P. 183-199.
34. Taylor, J., R. Jaiswal, and M. Bebawy, *Calcium-calpain Dependent Pathways Regulate Vesiculation in Malignant Breast Cells.* Current Cancer Drug Targets, 2017. **17**(5): p. 486-494.
35. Weber, H., et al., *Calpain-mediated breakdown of cytoskeletal proteins contributes to cholecystokinin-induced damage of rat pancreatic acini.* International Journal of Experimental Pathology, 2009. **90**(4): p. 387-399.
36. Boivin, P., C. Galand, and D. Dhermy, *In vitro digestion of spectrin, protein 4.1 and ankyrin by erythrocyte calcium dependent neutral protease (Calpain I).* International Journal of Biochemistry, 1990. **22**(12): p. 1479-1489.
37. Wang, K. And P.-w. Yuen, *Calpain: The Pharmacology and Toxicology of Calcium-Dependent Protease*. 1999.
38. Harris, A.S., D.E. Croall, and J.S. Morrow, *The calmodulin-binding site in alpha-fodrin is near the calcium-dependent protease-I cleavage site.* Journal of Biological Chemistry, 1988. **263**(30): p. 15754-61.

39. Harris, A.S., D.E. Croall, and J.S. Morrow, *Calmodulin regulates fodrin susceptibility to cleavage by calcium-dependent protease I.* Journal of Biological Chemistry, 1989. **264**(29): p. 17401-17408.
40. Harris, A.S. and J.S. Morrow, *Calmodulin and calcium-dependent protease I coordinately regulate the interaction of fodrin with actin.* Proceedings of the National Academy of Sciences, 1990. **87**(8): p. 3009.
41. Legland, D., I. Arganda-Carreras, and P. Andrey, *morpholibj: integrated library and plugins for mathematical morphology with imagej.* Bioinformatics, 2016. **32**(22): p. 3532-3534.
42. Hutter, J.L. and J. Bechhoefer, *Calibration of atomic-force microscope tips.* Review of Scientific Instruments, 1993. **64**(7): p. 1868-1873.
43. Zhang, X., Y. Chen, and Y. Chen, *An AFM-based pit-measuring method for indirect measurements of cell-surface membrane vesicles.* Biochemical and Biophysical Research Communications, 2014. **446**(1): p. 375-379.
44. Zhang, X.J., et al., *AFM visualization of cortical filaments/network under cell-bound membrane vesicles.* Biochimica Et Biophysica Acta-Biomembranes, 2015. **1848**(10): p. 2225-2232.
45. Roseblade, A., et al., *Targeting Microparticle Biogenesis: A Novel Approach to the Circumvention of Cancer Multidrug Resistance.* Current Cancer Drug Targets, 2015. **15**(3): p. 205-214.
46. Machnicka, B., et al., *Spectrin-based skeleton as an actor in cell signaling.* Cellular and Molecular Life Sciences, 2012. **69**(2): p. 191-201.
47. Zhang, R., et al., *Spectrin: Structure, function and disease.* Science China-Life Sciences, 2013. **56**(12): p. 1076-1085.
48. Gascard, P. And N. Mohandas, *New insights into functions of erythroid proteins in nonerythroid cells.* Current Opinion in Hematology, 2000. **7**(2): p. 123-129.
49. Pasquet, J.M., J. Dachary-prigent, and A.T. Nurden, *Calcium influx is a determining factor of calpain activation and microparticle formation in platelets.* European Journal of Biochemistry, 1996. **239**(3): p. 647-654.
50. Morel, O., et al., *Cellular Mechanisms Underlying the Formation of Circulating Microparticles.* Arteriosclerosis Thrombosis and Vascular Biology, 2011. **31**(1): p. 15-26.
51. Leal, J.K.F., M.J.W. Adjobo-Hermans, and G. Bosman, *Red Blood Cell Homeostasis: Mechanisms and Effects of Microvesicle Generation in Health and Disease.* Frontiers in Physiology, 2018. **9**: p. 7.
52. Goll, D.E., et al., *The calpain system.* Physiological Reviews, 2003. **83**(3): p. 731-801.
53. Pottle, J., et al., *Exploiting MCF-7 Cells¡¯Calcium Dependence with Interlaced Therapy.* Journal of Cancer Therapy, 2013. **Vol.04No.07**: p. 9.
54. Marchesi, V.T. and E. Steers, *Selective Solubilization of a Protein Component of the Red Cell Membrane.* Science, 1968. **159**(3811): p. 203.
55. Machnicka, B., et al., *Spectrins: A structural platform for stabilization and activation of membrane channels, receptors and transporters.* Biochimica Et Biophysica Acta-Biomembranes, 2014. **1838**(2): p. 620-634.
56. Black, J.D., et al., *Heterogeneity in lymphocyte spectrin distribution - ultrastructural identification of a new spectrin-rich cytoplasmic structure.* Journal of Cell Biology, 1988. **106**(1): p. 97-109.
57. Leto, T.L., et al., *COMPARISON OF NONERYTHROID ALPHA-SPECTRIN GENES REVEALS STRICT HOMOLOGY AMONG DIVERSE SPECIES.* Molecular and Cellular Biology, 1988. **8**(1): p. 1-9.

58. Moon, R.T. and A.P. mcmahon, *Generation of diversity in nonerythroid spectrins - multiple polypeptides are predicted by sequence-analysis of cdnas encompassing the coding region of human nonerythroid alpha-spectrin.* Journal of Biological Chemistry, 1990. **265**(8): p. 4427-4433.
59. Sormunen, R.T., et al., *Immunolocalization of the fodrin, E-cadherin, and beta-catenin adhesion complex in infiltrating ductal carcinoma of the breast - Comparison with an in vitro model.* Journal of Pathology, 1999. **187**(4): p. 416-423.
60. Tuominen, H., R. Sormunen, and M. Kallioinen, *Non-erythroid spectrin (fodrin) in cutaneous tumours: Diminished in cell membranes, increased in the cytoplasm.* British Journal of Dermatology, 1996. **135**(4): p. 576-580.
61. Basse, F., P. Gaffet, and A. Bienvenue, *Correlation between inhibition of cytoskeleton proteolysis and anti-vesiculation effect of calpeptin during A23187-induced activation of human platelets - are vesicles shed by filopod fragmentation.* Biochimica Et Biophysica Acta-Biomembranes, 1994. **1190**(2): p. 217-224.
62. Gov, N., et al., *Cytoskeletal control of red blood cell shape: theory and practice of vesicle formation.* Advances in planar lipid bilayers and liposomes, 2009. **10**: p. 95-119.
63. Wang, K.K.W., et al., *An alpha-mercaptoacrylic acid derivative is a selective nonpeptide cell-permeable calpain inhibitor and is neuroprotective.* Proceedings of the National Academy of Sciences of the United States of America, 1996. **93**(13): p. 6687-6692.
64. Saido, T.C., et al., *Positive regulation of Mu-calpain action by polyphosphoinositides.* Journal of Biological Chemistry, 1992. **267**(34): p. 24585-24590.
65. Gong, J., et al., *Microparticles Mediate the Intercellular Regulation of microrna-503 and Proline-Rich Tyrosine Kinase 2 to Alter the Migration and Invasion Capacity of Breast Cancer Cells.* Frontiers in Oncology, 2014. **4**: p. 220.
66. Berridge, M.J., *Calcium signalling remodelling and disease.* Biochemical Society Transactions, 2012. **40**: p. 297-309.
67. Baines, A.J., *The spectrin-ankyrin-4.1-adducin membrane skeleton: adapting eukaryotic cells to the demands of animal life.* Protoplasma, 2010. **244**(1-4): p. 99-131.
68. Goodman, S.R., R.P. Chapa, and W.E. Zimmer, *Spectrin's chimeric E2/E3 enzymatic activity.* Experimental Biology and Medicine, 2015. **240**(8): p. 1039-1049.
69. Ackermann, A. And A. Brieger, *The Role of Nonerythroid Spectrin alpha II in Cancer.* Journal of Oncology, 2019: p. 14.
70. Wu, X.T., et al., *The potential role of spectrin network in the mechanotransduction of MLO-Y4 osteocytes.* Scientific Reports, 2017. **7**: p. 12.
71. Ho, S.B., *Cytoskeleton and other differentiation markers in the colon.* Journal of Cellular Biochemistry, 1992: p. 119-128.
72. Blikstad, I., et al., *Synthesis and assembly of spectrin during avian erythropoiesis: stoichiometric assembly but unequal synthesis of α and β spectrin.* Cell, 1983. **32**(4): p. 1081-1091.

Author contribution statement – Chapter 4

J. Taylor designed and performed experiments, analysed the data and composed the manuscript. K. Patio performed confocal microscopy experiments. M. Johnson provided conceptual guidance, assisted in the design of confocal experiments, and project supervision. C. Evenhuis wrote the script and developed the macro plugin for Fiji image analysis. M. Bebawy was responsible for the overall project, experimental design, project supervision, and revision of the manuscript.

Chapter 5

Conclusions and Future directions

Extracellular vesicles (EVs) are small membrane enclosed vesicles that are important for intercellular communication. Currently, EVs are classified into three broad subtypes – exosomes, microvesicles (MVs), and apoptotic bodies – based on size, molecular composition, and biogenic origin (Thery et al., 2018). Exosomes are typically 70-150 nm in diameter and are formed in the endosomal compartment and released following the fusion of multi-vesicular bodies with the plasma membrane (Lener et al., 2015). Direct budding of the plasma membrane gives rise to MVs which are 150-1000 nm in diameter (Harding et al., 1983). Apoptotic bodies are generally larger (<1000-50000 nm in diameter), and also formed directly from the plasma membrane, however they are released from cells during apoptosis (Ihara et al., 1998, Poon et al., 2019). EVs contain a variety of proteins, nucleic acids, and bioactive molecules characteristic of their parent cells. They also bear surface molecules that directs tropism to specific recipient cell populations where delivery of EV cargo results in the modification of the recipient cells biochemical and biological state (Mesri and Altieri, 1999, Mallat et al., 2000, Ratajczak et al., 2006).

EVs have broad utility as signalling vectors and can induce effects in a wide range of physiological systems including: vascular homeostasis, reticulocyte maturation, innate and acquired immunity, embryonic development, bone calcification, and tissue repair (Burnier et

al., 2009, Vidal, 2010, Wang et al., 2011, Nolte-'t Hoen and Wauben, 2012, Machtinger et al., 2016, Golub, 2009, Quesenberry and Aliotta, 2008). EVs are also implicated across many pathophysiological conditions including neurodegenerative disease (Guo et al., 2015), inflammatory conditions (Cloutier et al., 2013, Huber et al., 2002, Qiu et al., 2013), and cancer. Cancer cells and cells of the tumour micro-environment release EVs in significant numbers and these play important roles in cancer biology. Of particular interest to our laboratory, is the role of plasma membrane-derived EV's in the dissemination and acquisition of cancer multidrug resistance.

MDR is the phenomenon whereby cancer cells become cross-resistant to wide range of structurally and mechanistically different anticancer agents following exposure to a single agent (Kartner et al., 1983, Cole et al., 1992, Gottesman et al., 2002). This phenotype is predominantly associated with the over expression of drug efflux transporters P-glycoprotein (P-gp/*ABCB1*) and Multidrug Resistance-Associated Protein 1 (MRP1/*ABCC1*) which belong to the ATP-binding cassette (ABC) superfamily of membrane transporters (Juliano and Ling, 1976, Cole et al., 1992). EVs shed from MDR cells harbour functional resistance proteins and are capable of conferring drug resistance to previously drug-sensitive cells *in vitro* and *in vivo* (Bebawy et al., 2009b, Lu et al., 2013, Jaiswal et al., 2013). Subsequently, EVs have been implicated in the dissemination of other deleterious cancer traits associated with MDR including increased metastatic capacity (Gong et al., 2014b), evasion of immune surveillance (Jaiswal et al., 2017), active and passive drug sequestration (Gong et al., 2013), and altered tissue biomechanics (Pokharel et al., 2016a).

The ability of EVs to bestow such a wide array of cancer traits has garnered substantial interest in the development of novel therapeutics that specifically target cancer EV biogenesis. Circulating tumour-derived EVs are also being explored as potential diagnostic and prognostic biomarkers to monitor cancer progression (De Rubis et al., 2019, Krishnan et al., 2016b, Sun

et al., 2016, Burger et al., 2013). Although the clinical potential of EVs is evident, there remain challenges before clinical application can be realised.

Understanding the precise biogenic pathways involved in cancer EV biogenesis is imperative if safe and effective therapeutics are to be developed which target this pathway. Exosomes are perhaps the most studied EVs within the field and their biogenesis has been extensively characterised (Raposo and Stoorvogel, 2013, Tkach and Thery, 2016, Colombo et al., 2013, Colombo et al., 2014, Andreu and Yanez-Mo, 2015, Verweij et al., 2018, Verweij et al., 2011). Plasma membrane EV biogenesis in comparison, remains to be fully elucidated. Plasma membrane EVs are released spontaneously in cancer or following cellular activation, or in response to pre-apoptotic signals (Gyorgy et al., 2011, Hugel et al., 2005, Gong et al., 2012). An increase in intracellular calcium initiates EV biogenesis and this is followed by successive membrane and cytoplasmic alterations, which ultimately result in the release of EVs from the plasma membrane (Pasquet et al., 1996, Freyssinet and Toti, 2010). However, this basic biology does not illuminate specific drug targets that would enable novel drug development, particularly those which are selective to certain cell types and mitigate potential off-target effects. Specifically, it is unclear as to how vesiculation pathways differ between malignant and non-malignant cells and whether there are opportunities for selective targeting of MV biogenesis in cancer management.

In this thesis, we evidence discrete plasma membrane EV biogenic pathways for malignant and non-malignant cells. By examining the molecular machinery of EV production, we demonstrate malignant cell EV biogenesis is dependent on a calcium-calpain pathway at rest and that non-malignant cells vesiculate via alternative pathways (chapter 2; (Taylor et al., 2017)). Furthermore, we show the endoplasmic reticulum plays a role mobilisation of calcium and EV biogenesis in both malignant and non-malignant cells, while the store-operated calcium entry (SOCE) pathway an important role in the event of store depletion (Chapter 3; (Taylor et al.,

2020)). Finally, we have established the redistribution of spectrin from the membrane to the cytoplasm drives plasma membrane-derived EV biogenesis in malignant cells (Chapter 4).

Malignant cells produce significantly more EVs than non-malignant cells at rest and given their role in the acquisition of MDR and other cancer traits, they present as attractive novel therapeutic targets. Previous work in our laboratory has identified calpain inhibitors as effective agents in stopping the production of EVs from resting and activated cancer cells (Roseblade et al., 2015). Calpain is a cysteine protease that is activated by intracellular calcium and which cleaves a variety of substrates including: cytoskeletal proteins, membrane bound channels, and others (Goll et al., 2003). However, as calpain operates as a multifunctional protease and is required for normal physiological functions, further delineation of its role in EV biogenesis in malignant and non-malignant cells is required. In Chapter 2, we examine vesiculation in malignant and non-malignant cells using high-resolution atomic force microscopy (AFM) to determine if differences in biogenic pathways exist. We compared malignant and non-malignant MV biogenesis at rest, following mobilisation of intracellular calcium, and in the presence and absence of the calpain inhibitor, ALLM. We evidence that a calcium-calpain dependent pathway drives vesiculation in malignant cells. The study of plasma membrane EVs presents a number of challenges and is mostly restricted to analysing EVs isolated from cell culture supernatant or biological fluids. In particular, few reports exist that examine EV biogenesis at a cellular level or their in situ release from the plasma membrane (Arasu et al., 2017, Lai et al., 2015). In order to study the biogenesis of EVs in malignant and non-malignant cells we required an imaging technique with high spatial resolution, which retained the cellular native structures, and one that did not require markers for vesicle identification. AFM was chosen as a suitable platform for this study as it has been shown to successfully characterise numerous cellular constituents and provides topographical images in sub-nanometre resolution

(Alessandrini and Facci, 2011, Binnig et al., 1986, Lal and Proksch, 1997, Park et al., 2013, Xu et al., 2013).

In our study, topographical AFM analysis showed that malignant cells have an intrinsically higher degree of vesiculation at rest compared to non-malignant cells. Unstimulated non-malignant cells display a smooth cellular surface with minimal vesicles evident (Fig 1 a-b). Contrary to this, unstimulated malignant cells exhibit a surface topography that is rough, corrugated, and with copious membrane-bound EVs visible (Fig 1 c-d).

The stark differences observed in vesiculation between malignant and non-malignant cells at rest, suggests that different pathways regulate biogenesis in these cell types. Given calpain inhibition effectively attenuates EV production in drug sensitive and drug resistant cells (Roseblade et al., 2015), it was first examined as the likely candidate contributing to these differences. To define the role of calpain in the biogenesis of EVs in malignant and non-malignant cells, resting cells were treated with the calpain inhibitor II (ALLM; Fig. 2-3). There was a significant reduction in vesicle production in malignant cancer cells treated with ALLM (Fig. 2). Vesicle-derived pits were absent and cell morphology was smooth. Non-malignant cells treated with ALLM displayed an increase in the number of vesicle-derived pits compared to untreated control (Fig. 3 a-b). This distinctly different effect by ALLM suggests the presence of alternative biogenic pathways in operation in resting non-malignant cells that are not dependent on calpain activity.

The first step in the production of plasma membrane EVs is cellular activation and subsequent mobilisation of intracellular calcium and an increase in the available concentration (Basse et al., 1994). We used the calcium ionophore, A23187 – a well characterised calcium ionophore – as an agonist of EV biogenesis. Treatment with the ionophore resulted in a significant increase in biogenesis in non-malignant cells. Malignant cells displayed only a modest increase

in vesiculation following calcium activation (Fig. 4 c-d). Inhibition of calpain in cells activated with A23187 resulted in complete attenuation of vesiculation in malignant cells, while also significantly reducing EV production in non-malignant cells (Fig 5).

These results demonstrate that biogenic pathways for vesiculation in malignant cells in the resting state are calcium-calpain dependent and generate more EVs due to higher basal calcium levels. They also support our hypothesis that distinct biogenic pathways regulate vesiculation in malignant and non-malignant cells.

Having established EV biogenesis in cancer cells is dependent on a calcium-calpain dependent pathway, in Chapter 3, we explore the calcium-signalling pathways driving EV biogenesis in both malignant and non-malignant cells. A vast network of pumps, channels, and exchangers operate in harmony to control compartmental calcium concentrations and maintain intracellular calcium homeostasis (Berridge et al., 2003, Brini and Carafoli, 2009, Clapham, 2007). The endoplasmic reticulum (ER) is the primary intracellular store of calcium and is involved in numerous signalling pathways (Carreras-Sureda et al., 2018, Prakriya and Lewis, 2015, Putney, 2010); however, its role in EV biogenesis is unknown. We investigated the role of endoplasmic reticulum (ER) calcium stores and store-operated calcium entry (SOCE) in EV biogenesis.

Calcium is a highly promiscuous ion that is involved in many cellular signalling modalities. In the context of cancer, dysregulation of cancer signalling is a common feature in each of the hallmarks of cancer and contributes to disease progression (Azimi et al., 2014, Monteith et al., 2012, Monteith et al., 2017, Stewart et al., 2015). Despite the well-established relationship between high intracellular calcium and increased EV biogenesis, the regulation calcium signalling driving plasme membrane EV biogenesis remains poorly characterised. We show that malignant cells generate significantly more vesicles at rest than non-malignant cells and

show the intracellular calcium chelator, BAPTA-AM results in attenuation of vesiculation (Fig. 1).

The involvement ER calcium stores was studied using the sarco/endoplasmic reticulum ATPase (SERCA) inhibitor, thapsigargin (TG). SERCA operates to pump calcium into the ER and is responsible for maintaining higher concentrations of calcium with intracellular stores (Brini et al., 2000). TG is a well-characterised molecule in the calcium signalling toolkit, which selectively inhibits SERCA and results in the activation of the SOCE pathway (Lytton et al., 1991, Sehgal et al., 2017, Thastrup et al., 1990). We show treatment of malignant cells with TG results in an increase in intracellular calcium and a synchronised increase in EV biogenesis. Non-malignant cells display a more pronounced increase in vesiculation in comparison to malignant cells following TG treatment (Fig. 2). The difference in response likely due to malignant cells having high basal calcium levels and therefore higher baseline production of EVs compared to non-malignant cells (Pottle et al., 2013). Thus, further calcium activation of malignant cells results in only a modest increase in vesiculation. BAPTA-AM effectively abolishes TG-mediated increase in calcium as well as EV production in both cell types. Importantly, BAPTA-AM inhibits vesiculation in malignant cells, supporting the hypothesis that resting malignant vesiculation is driven by high basal calcium levels.

Having demonstrated TG can activate EV biogenesis, we sought to determine the role of store-operated calcium entry (SOCE) and whether this calcium signalling pathway is responsible for the observed differences in resting vesiculation between malignant and non-malignant cells. We show that inhibition of SOCE with the selective inhibitor YM58483 blocks TG-mediated vesiculation in both cell types (Fig. 4). However, the high basal vesiculation of malignant cells is not affected by YM584783 treatment alone. As TG induces mobilisation of calcium via activation of the SOCE, these results demonstrate it is involved in EV biogenesis. However, SOCE pathway appears to operate as a secondary pathway which is activated in the event of

ER store depletion. Collectively, our results suggest differences in resting vesiculation in malignant and non-malignant cells are related to altered ER calcium mobilisation pathways and differences in calpain activity.

In Chapter 3, we have established higher basal vesiculation in malignant cells is dependent on mobilisation of intracellular calcium stores and the activity of calpain. In chapter 4, we turn our attention to the down-stream effects of calcium-calpain activation in high vesiculating malignant and low vesiculating non-malignant cells. Specifically, we examine the role of αII-spectrin in EV biogenesis. Spectrin is a ubiquitous cytoskeletal protein and provides cells with mechanical strength, structural stability and elasticity (An et al., 2004a, Liem, 2016b, Zhang et al., 2013b). In erythrocytes, spectrin proteolysis by calpain, mediates the production of plasma membrane EVs (Wagner et al., 1987), however, its role in non-erythroid or cancer cell EV biogenesis is unknown.

Using high resolution confocal microscopy, we show the subcellular localisation of αII-spectrin differs in malignant and non-malignant cells at rest (Fig. 2). In non-malignant cells, αII-spectrin is forms a prominent border in the cell periphery, whereas, spectrin borders are diminished in malignant cells. Qualitative observations were confirmed by quantitatively assessing the ratio of peripheral and cytoplasmic αII-spectrin fluorescence – termed the membrane:cytoplasm (M:C) αII-spectrin distribution ratio. To achieve this, we developed an automated cell segmentation Fiji plugin that allowed for an unbiased measure of spectrin distribution in individual cells. Using this method, we show non-malignant cells display a significantly higher ratio of spectrin distribution to the membrane compared to malignant cells. Given these observations, the prominent spectrin borders in the apical membrane of non-malignant cells likely stabilise membrane-cytoskeletal anchorage and prevent basal vesiculation. On the other hand, the absence of spectrin peripheral borders in resting malignant cells correlates strongly

with a high degree of vesiculation at rest (Fig. 2-3). This evidences a role for spectrin in the biogenesis of plasma membrane EVs in resting malignant cells.

Having shown an absence of peripheral spectrin borders results in increased vesiculation in malignant cells, we also demonstrated that restoration of spectrin borders can be achieved with inhibitors of the calcium-calpain mediated pathway (Fig. 5). Chelation of intracellular calcium with BAPTA-AM and inhibition of calpain with ALLM resulted in a significant increase in the M:C αII-spectrin distribution ratio in malignant cells. Calpain inhibition prevented the remodelling of peripheral spectrin in malignant cells and this was accompanied by inhibition of EV biogenesis. This shows that loss of peripheral spectrin drives EV biogenesis in malignant cells at rest (Fig 2 and 5). These findings provide new insight into the biogenic pathway regulating EV biogenesis in both malignant and non-malignant cells and identifies strategies for the selective modulation of plasma membrane EV biogenesis.

Future Directions

EVs are regarded as important signalling vectors involved in the non-genetic conferral of MDR in cancer (Bebawy et al., 2009b, Jaiswal et al., 2012, Lu et al., 2013, Lu et al., 2017). Their capacity to transfer bioactive cargo from drug-resistant cells to drug-sensitive cells makes them attractive entities for targeted therapies. Indeed, our laboratory has explored the use of selective inhibitors of calpain as potential EV biogenesis modulators with encouraging results (Roseblade et al., 2015). However, EVs also play an important, multifaceted role in physiological functions and future anticancer agents will therefore be required to specifically block cancer MVs while leaving non-malignant MV production unaffected. Clear delineation of the biogenic pathways in malignant and non-malignant cells is required before novel therapeutic targets can be identified.

Unlike exosomes, the biogenesis of vesicles from the plasma membrane is poorly characterised. Although there is scientific consensus on the basic steps for plasma membrane vesicles formation, these mechanisms are often described using 'broad-strokes' – lacking intricacy, specificity, or precision. The marked heterogeneity of plasma membrane EVs has proffered substantial challenges in the development of comprehensive characterisation criteria. One of the other challenges facing the EV research field is drawing together the different aspects of plasma membrane EV biogenesis. In this thesis and for the first time, we have examined in greater detail the biogenic machinery involved at different stages of the vesiculation pathway. Furthermore, we compare biogenesis in malignant and non-malignant cells. We demonstrate the involvement the ER in delivering calcium to the cytoplasm required for biogenesis, the secondary role that SOCE plays, as well as the importance of spectrin in the stabilisation of the plasma membrane. However, many facets of EV biogenesis remain elusive. Ultimately, the goal is to understand the precise regulators of plasma membrane EV biogenesis in malignant and non-malignant cells.

Malignant cells display a propensity to shed high numbers of EVs even in an unstimulated state (Pottle et al., 2013). A characteristic that allows cancer cells to exchange information, enhance growth, and acquire survival traits at accelerated rates. As we have shown, unstimulated malignant vesiculation is dependent on a calcium-calpain pathway, pharmacological manipulation of this pathway holds potential in mitigating EV-mediated cancer progression. However, both calcium signalling and the calpain system are complex and challenges remain in elucidating suitable therapeutic targets. In the context of EV biogenesis, our results suggest differences in resting vesiculation rates in malignant and non-malignant cells which appear to be related to altered ER calcium mobilisation pathways. While this is a promising finding, dysregulation of ER calcium homeostasis in malignancy is common and remodelling of a number of regulators cannot be ruled out. Reports suggest malignant cells have higher basal calcium activity and this could be related to overexpression or hyper-activity of calcium specific channels. Given results presented in thesis, interrogation of specific ER channels, pumps, and exchangers with selective inhibitors is one avenue worthy of exploration in endeavours to clearly define why malignant cells shed large quantities of EVs at rest.

Another line of investigation that has become important for researchers interested in the dysregulation of calcium signalling in malignancy is the type of calcium signal being transmitted (Monteith et al., 2017). Calcium signals vary greatly in their magnitude, as well as their temporal and spatial characteristics and it has been proposed these characteristics could be exploited for specific therapeutic benefit. For example, large persistent increases in calcium concentration is often associated with cell death and in cancers that are characterised by over expression of calcium channels, calcium overload is has been suggested as a viable way to specifically target death of those cancer cells (Azimi et al., 2018, Azimi et al., 2017, Monteith et al., 2007, Peters et al., 2012, Stewart et al., 2015). On the other hand, microdomains of highly localised calcium increases – known as ‘flickers’ or ‘sparks’ – have been shown to govern cell

migration (Wei et al., 2009) and may be selectively targeted to alter cancer cell mobility and inhibit metastasis. Calcium microdomains tend to form when multiple calcium channels of the same subtype group together in clusters (Berridge, 2006) and selective inhibition of that channel prevents influx of calcium into the cytoplasm. In the context of EV biogenesis, we have shown there are regions in the membrane of dense vesiculation – termed vesiculation "hotspots" – in activated cells that resemble calcium microdomains (Rizzuto and Pozzan, 2006, Visser et al., 2013, Wei et al., 2009). By using carefully controlled buffering of intracellular calcium indicators and simultaneous confocal/AFM imaging, it would be possible to visualise the localised bursts of calcium and determine if these correlate with dense regions of high vesiculation. The advent of techniques that allow for higher resolution live-cell imagining such as quantum dot and protein vehicle fluorophores (Beliu and Sauer, 2020), and improved live-cell super resolution microscopy will further enrich this approach and allow for real-time characterisation or plasma membrane EVs. Identifying the channel subtypes involved would further characterise the biogenic pathways involved in production plasma membrane EVs. Comparing malignant and non-malignant cells would provide valuable information regarding the EV release characteristics in activated cells and illuminate if calcium microdomains are altered following malignant transformation.

The work detailed in this thesis follows on from previous work aimed at curtailing EV-mediated dissemination of cancer traits by inhibiting EV production (Roseblade et al., 2015). However, a deeper understanding of the effects of calpain inhibition and the role it plays in vesiculation in malignant and non-malignant cells is required if safe and effective compounds are to be designed. Examination of calpain substrate structure provides valuable insight into the activity of the protease in high vesiculating cells and low vesiculating cells. Spectrin is one such substrate of calpain and is an integral part of the network of proteins that maintains plasma membrane anchorage to the cytoskeleton. Our results demonstrate for the first time the

subcellular distribution of spectrin has been linked to the propensity of cells to generate plasma membrane EVs as rest. It appears that dynamic remodelling of spectrin is greatly amplified in malignant cells and this is driven by active calpain. While it is tempting to suggest future therapies will likely be aimed at stabilising spectrin borders or inhibiting calpain, there could well be other factors that need to be considered. For example, defective spectrin isoforms have been reported in some diseases that prevent the end-to-end fusion of spectrin chains (Costa et al., 2005, Gallagher, 2004, Gallagher et al., 1992). Malformation of the spectrin subunits results in less stable chains that are more prone to protease cleavage. Furthermore, cells that encounter problems in consolidating structural protein networks often have reduced protein expression while retaining normal or even increased transcription of mRNA (Lefferts and Lambert, 2003). Gene sequencing techniques could very well uncover increased mRNA for mutant spectrin or even mutant variants of associated proteins supporting spectrin formation.

In conclusion, EV-mediated conferral of deleterious cancer traits is a potential problem in cancer management (Krishnan et al., 2016a, Krishnan et al., 2016b). Clear delineation of biogenic pathways involved in EV production is imperative not only for the development of novel therapeutic strategies, but also to fully appreciate the potential of EVs as non-invasive biomarkers of disease. We have demonstrated there are differences in the biogenic pathways in malignant and non-malignant cells, however, it is imperative to fully elucidate the drivers of malignant plasma membrane EV biogenesis before safe and efficacious selectively targeted therapies can be developed.

References

ABELS, E. R. & BREAKEFIELD, X. O. 2016. Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake. *Cellular and Molecular Neurobiology,* 36**,** 301-312.

ACKERMANN, A. & BRIEGER, A. 2019. The Role of Nonerythroid Spectrin alpha II in Cancer. *Journal of Oncology***,** 14.

AL-NEDAWI, K., MEEHAN, B. & RAK, J. 2009. Microvesicles Messengers and mediators of tumor progression. *Cell Cycle,* 8**,** 2014-2018.

ALESSANDRINI, A. & FACCI, P. 2011. Unraveling lipid/protein interaction in model lipid bilayers by Atomic Force Microscopy. *Journal of Molecular Recognition,* 24**,** 387-396.

AN, X. L., GUO, X. H., SUM, H., MORROW, J., GRATZER, W. & MOHANDAS, N. 2004a. Phosphatidylserine binding sites in erythroid spectrin: Location and implications for membrane stability. *Biochemistry,* 43**,** 310-315.

AN, X. L., GUO, X. H., WU, Y. & MOHANDAS, N. 2004b. Phosphatidylserine binding sites in red cell spectrin. *Blood Cells Molecules and Diseases,* 32**,** 430-432.

ANDREU, Z. & YANEZ-MO, M. 2015. Tetraspanins in extracellular vesicle formation and function. *Frontiers in Immunology,* 5**,** 12.

ARASU, U. T., KARNA, R., HARKONEN, K., OIKARI, S., KOISTINEN, A., KROGER, H., QU, C. J., LAMMI, M. J. & RILLA, K. 2017. Human mesenchymal stem cells secrete hyaluronan-coated extracellular vesicles. *Matrix Biology,* 64**,** 54-68.

AZIMI, I., BONG, A. H., POO, G. X. H., ARMITAGE, K., LOK, D., ROBERTS-THOMSON, S. J. & MONTEITH, G. R. 2018. Pharmacological inhibition of store-operated calcium entry in MDA-MB-468 basal A breast cancer cells: consequences on calcium signalling, cell migration and proliferation. *Cellular and Molecular Life Sciences,* 75**,** 4525-4537.

AZIMI, I., FLANAGAN, J. U., STEVENSON, R. J., INSERRA, M., VETTER, I., MONTEITH, G. R. & DENNY, W. A. 2017. Evaluation of known and novel inhibitors of Orai1-mediated store operated Ca2+ entry in MDA-MB-231 breast cancer cells using a Fluorescence Imaging Plate Reader assay. *Bioorganic & Medicinal Chemistry,* 25**,** 440-449.

AZIMI, I., ROBERTS-THOMSON, S. J. & MONTEITH, G. R. 2014. Calcium influx pathways in breast cancer: opportunities for pharmacological intervention. *British Journal of Pharmacology,* 171**,** 945-960.

BAINES, A. J. 2010. The spectrin-ankyrin-4.1-adducin membrane skeleton: adapting eukaryotic cells to the demands of animal life. *Protoplasma,* 244**,** 99-131.

BASSE, F., GAFFET, P. & BIENVENUE, A. 1994. CORRELATION BETWEEN INHIBITION OF CYTOSKELETON PROTEOLYSIS AND ANTI-VESICULATION EFFECT OF CALPEPTIN DURING A23187-INDUCED ACTIVATION OF HUMAN PLATELETS - ARE VESICLES SHED BY FILOPOD FRAGMENTATION. *Biochimica Et Biophysica Acta-Biomembranes,* 1190**,** 217-224.

BEBAWY, M., COMBES, V., LEE, E., JAISWAL, R., GONG, J., BONHOURE, A. & GRAU, G. E. R. 2009a. Membrane microparticles mediate transfer of P-glycoprotein to drug sensitive cancer cells. *Leukemia,* 23**,** 1643.

BEBAWY, M., COMBES, V., LEE, E., JAISWAL, R., GONG, J., BONHOURE, A. & GRAU, G. E. R. 2009b. Membrane microparticles mediate transfer of P-glycoprotein to drug sensitive cancer cells. *Leukemia,* 23**,** 1643-1649.

BELIU, G. & SAUER, M. 2020. A Trojan Horse for live-cell super-resolution microscopy. *Light-Science & Applications,* 9**,** 2.

BERRIDGE, M. J. 2006. Calcium microdomains: Organization and function. *Cell Calcium,* 40**,** 405-412.

BERRIDGE, M. J. 2012. Calcium signalling remodelling and disease. *Biochemical Society Transactions,* 40**,** 297-309.

BERRIDGE, M. J., BOOTMAN, M. D. & RODERICK, H. L. 2003. Calcium signalling: Dynamics, homeostasis and remodelling. *Nature Reviews Molecular Cell Biology,* 4**,** 517-529.

BIEDLER, J. L. & RIEHM, H. 1970. CELLULAR RESISTANCE TO ACTINOMYCIN-D IN CHINESE HAMSTER CELLS IN-VITRO - CROSS-RESISTANCE, RADIOAUTOGRAPHIC, AND CYTOGENETIC STUDIES. *Cancer Research,* 30**,** 1174-&.

BINNIG, G., QUATE, C. F. & GERBER, C. 1986. ATOMIC FORCE MICROSCOPE. *Physical Review Letters,* 56**,** 930-933.

BLACK, J. D., KOURY, S. T., BANKERT, R. B. & REPASKY, E. A. 1988. HETEROGENEITY IN LYMPHOCYTE SPECTRIN DISTRIBUTION - ULTRASTRUCTURAL IDENTIFICATION OF A NEW SPECTRIN-RICH CYTOPLASMIC STRUCTURE. *Journal of Cell Biology,* 106**,** 97-109.

BLIKSTAD, I., NELSON, W. J., MOON, R. T. & LAZARIDES, E. 1983. Synthesis and assembly of spectrin during avian erythropoiesis: stoichiometric assembly but unequal synthesis of α and β spectrin. *Cell,* 32**,** 1081-1091.

BOGUSLAWSKA, D. M., MACHNICKA, B., HRYNIEWICZ-JANKOWSKA, A. & CZOGALLA, A. 2014. SPECTRIN AND PHOSPHOLIPIDS - THE CURRENT PICTURE OF THEIR FASCINATING INTERPLAY. *Cellular & Molecular Biology Letters,* 19**,** 158-179.

BOGUSŁAWSKA, D. M., MACHNICKA, B., HRYNIEWICZ-JANKOWSKA, A. & CZOGALLA, A. 2014. Spectrin and phospholipids — the current picture of their fascinating interplay. *Cellular & Molecular Biology Letters,* 19**,** 158-179.

BOIVIN, P., GALAND, C. & DHERMY, D. 1990. In vitro digestion of spectrin, protein 4.1 and ankyrin by erythrocyte calcium dependent neutral protease (Calpain I). *International Journal of Biochemistry,* 22**,** 1479-1489.

BRINI, M., BANO, D., MANNI, S., RIZZUTO, R. & CARAFOLI, E. 2000. Effects of PMCA and SERCA pump overexpression on the kinetics of cell Ca2+ signalling. *Embo Journal,* 19**,** 4926-4935.

BRINI, M. & CARAFOLI, E. 2009. Calcium Pumps in Health and Disease. *Physiological Reviews,* 89**,** 1341-1378.

BURGER, D., SCHOCK, S., THOMPSON, C. S., MONTEZANO, A. C., HAKIM, A. M. & TOUYZ, R. M. 2013. Microparticles: biomarkers and beyond. *Clinical Science,* 124**,** 423-441.

BURNIER, L., FONTANA, P., KWAK, B. R. & ANGELILLO-SCHERRER, A. 2009. Cell-derived microparticles in haemostasis and vascular medicine. *Thrombosis and Haemostasis,* 101**,** 439-451.

CARRERAS-SUREDA, A., PIHAN, P. & HETZ, C. 2018. Calcium signaling at the endoplasmic reticulum: fine-tuning stress responses. *Cell Calcium,* 70**,** 24-31.

CLAPHAM, D. E. 2007. Calcium signaling. *Cell,* 131**,** 1047-1058.

CLOUTIER, N., TAN, S., BOUDREAU, L. H., CRAMB, C., SUBBAIAH, R., LAHEY, L., ALBERT, A., SHNAYDER, R., GOBEZIE, R., NIGROVIC, P. A., FARNDALE, R. W., ROBINSON, W. H., BRISSON, A., LEE, D. M. & BOILARD, E. 2013. The exposure of autoantigens by microparticles underlies the formation of potent inflammatory components: the microparticle-associated immune complexes. *Embo Molecular Medicine,* 5**,** 235-249.

COLE, S. P. C., BHARDWAJ, G., GERLACH, J. H., MACKIE, J. E., GRANT, C. E., ALMQUIST, K. C., STEWART, A. J., KURZ, E. U., DUNCAN, A. M. V. & DEELEY, R. G. 1992. OVEREXPRESSION OF A TRANSPORTER GENE IN A MULTIDRUG-RESISTANT HUMAN LUNG-CANCER CELL-LINE. *Science,* 258**,** 1650-1654.

COLOMBO, M., MOITA, C., VAN NIEL, G., KOWAL, J., VIGNERON, J., BENAROCH, P., MANEL, N., MOITA, L. F., THERY, C. & RAPOSO, G. 2013. Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *Journal of Cell Science,* 126**,** 5553-5565.

COLOMBO, M., RAPOSO, G. & THERY, C. 2014. Biogenesis, Secretion, and Intercellular Interactions of Exosomes and Other Extracellular Vesicles. *In:* SCHEKMAN, R. & LEHMANN, R. (eds.) *Annual Review of Cell and Developmental Biology, Vol 30.* Palo Alto: Annual Reviews.

COSTA, D. B., LOZOVATSKY, L., GALLAGHER, P. G. & FORGET, B. G. 2005. A novel splicing mutation of the alpha-spectrin gene in the original hereditary pyropoikilocytosis kindred. *Blood,* 106**,** 4367-4369.

DE RUBIS, G., KRISHNAN, S. R. & BEBAWY, M. 2019. Liquid Biopsies in Cancer Diagnosis, Monitoring, and Prognosis. *Trends in Pharmacological Sciences,* 40**,** 172-186.

DE SOUZA, P. S., FACCION, R. S., BERNARDO, P. S. & MAIA, R. C. 2016. Membrane microparticles: shedding new light into cancer cell communication. *Journal of Cancer Research and Clinical Oncology,* 142**,** 1395-1406.

FREYSSINET, J. M. & TOTI, F. 2010. Formation of procoagulant microparticles and properties. *Thrombosis Research,* 125**,** S46-S48.

GALLAGHER, P. G. 2004. Hereditary elliptocytosis: Spectrin and protein 4.1R. *Seminars in Hematology,* 41**,** 142-164.

GALLAGHER, P. G., TSE, W. T., COETZER, T., LECOMTE, M. C., GARBARZ, M., ZARKOWSKY, H. S., BARUCHEL, A., BALLAS, S. K., DHERMY, D., PALEK, J. & FORGET, B. G. 1992. A COMMON TYPE OF THE SPECTRIN ALPHA-I 46-50A-KD PEPTIDE ABNORMALITY IN HEREDITARY ELLIPTOCYTOSIS AND PYROPOIKILOCYTOSIS IS ASSOCIATED WITH A MUTATION DISTANT FROM THE PROTEOLYTIC CLEAVAGE SITE - EVIDENCE FOR THE FUNCTIONAL IMPORTANCE OF THE TRIPLE HELICAL MODEL OF SPECTRIN. *Journal of Clinical Investigation,* 89**,** 892-898.

GASCARD, P. & MOHANDAS, N. 2000. New insights into functions of erythroid proteins in nonerythroid cells. *Current Opinion in Hematology,* 7**,** 123-129.

GOLL, D. E., THOMPSON, V. F., LI, H. Q., WEI, W. & CONG, J. Y. 2003. The calpain system. *Physiological Reviews,* 83**,** 731-801.

GOLUB, E. E. 2009. Role of matrix vesicles in biomineralization. *Biochimica Et Biophysica Acta-General Subjects,* 1790**,** 1592-1598.

GONG, J., JAISWAL, R., MATHYS, J. M., COMBES, V., GRAU, G. E. R. & BEBAWY, M. 2012. Microparticles and their emerging role in cancer multidrug resistance. *Cancer Treatment Reviews,* 38**,** 226-234.

GONG, J., LUK, F., JAISWAL, R. & BEBAWY, M. 2014a. Microparticles Mediate the Intercellular Regulation of microRNA-503 and Proline-Rich Tyrosine Kinase 2 to Alter the Migration and Invasion Capacity of Breast Cancer Cells. *Frontiers in Oncology,* 4.

GONG, J., LUK, F., JAISWAL, R. & BEBAWY, M. 2014b. Microparticles Mediate the Intercellular Regulation of microRNA-503 and Proline-Rich Tyrosine Kinase 2 to Alter the Migration and Invasion Capacity of Breast Cancer Cells. *Frontiers in Oncology,* 4**,** 220.

GONG, J., LUK, F., JAISWAL, R., GEORGE, A. M., GRAU, G. E. R. & BEBAWY, M. 2013. Microparticle drug sequestration provides a parallel pathway in the acquisition of cancer drug resistance. *European Journal of Pharmacology,* 721**,** 116-125.

GOODMAN, S. R., CHAPA, R. P. & ZIMMER, W. E. 2015. Spectrin's chimeric E2/E3 enzymatic activity. *Experimental Biology and Medicine,* 240**,** 1039-1049.

GOTTESMAN, M. M., FOJO, T. & BATES, S. E. 2002. Multidrug resistance in cancer: Role of ATP-dependent transporters. *Nature Reviews Cancer,* 2**,** 48-58.

GOV, N., CLUITMANS, J., SENS, P. & BOSMAN, G. 2009. Cytoskeletal control of red blood cell shape: theory and practice of vesicle formation. *Advances in planar lipid bilayers and liposomes,* 10**,** 95-119.

GUO, B. B., BELLINGHAM, S. A. & HILL, A. F. 2015. The Neutral Sphingomyelinase Pathway Regulates Packaging of the Prion Protein into Exosomes. *Journal of Biological Chemistry,* 290**,** 3455-3467.

GYORGY, B., SZABO, T. G., PASZTOI, M., PAL, Z., MISJAK, P., ARADI, B., LASZLO, V., PALLINGER, E., PAP, E., KITTEL, A., NAGY, G., FALUS, A. & BUZAS, E. I. 2011. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cellular and Molecular Life Sciences,* 68**,** 2667-2688.

HARDING, C., HEUSER, J. & STAHL, P. 1983. RECEPTOR-MEDIATED ENDOCYTOSIS OF TRANSFERRIN AND RECYCLING OF THE TRANSFERRIN RECEPTOR IN RAT RETICULOCYTES. *Journal of Cell Biology,* 97**,** 329-339.

HARRIS, A. S., CROALL, D. E. & MORROW, J. S. 1988. The calmodulin-binding site in alpha-fodrin is near the calcium-dependent protease-I cleavage site. *Journal of Biological Chemistry,* 263**,** 15754-61.

HARRIS, A. S., CROALL, D. E. & MORROW, J. S. 1989. Calmodulin regulates fodrin susceptibility to cleavage by calcium-dependent protease I. *Journal of Biological Chemistry,* 264**,** 17401-17408.

HARRIS, A. S. & MORROW, J. S. 1990. Calmodulin and calcium-dependent protease I coordinately regulate the interaction of fodrin with actin. *Proceedings of the National Academy of Sciences,* 87**,** 3009.

HO, S. B. 1992. CYTOSKELETON AND OTHER DIFFERENTIATION MARKERS IN THE COLON. *Journal of Cellular Biochemistry***,** 119-128.

HUBER, J., VALES, A., MITULOVIC, G., BLUMER, M., SCHMID, R., WITZTUM, J. L., BINDER, B. R. & LEITINGER, N. 2002. Oxidized membrane vesicles and blebs from apoptotic cells contain biologically active oxidized phospholipids that induce monocyte-endothelial interactions. *Arteriosclerosis Thrombosis and Vascular Biology,* 22**,** 101-107.

HUGEL, B., CARMEN, M., MARTINEZ, M. C., KUNZELMANN, C. & FREYSSINET, J. M. 2005. Membrane microparticles: Two sides of the coin. *Physiology,* 20**,** 22-27.

HUTTER, J. L. & BECHHOEFER, J. 1993. CALIBRATION OF ATOMIC-FORCE MICROSCOPE TIPS. *Review of Scientific Instruments,* 64**,** 1868-1873.

IHARA, T., YAMAMOTO, T., SUGAMATA, M., OKUMURA, H. & UENO, Y. 1998. The process of ultrastructural changes from nuclei to apoptotic body. *Virchows Archiv-an International Journal of Pathology,* 433**,** 443-447.

JAISWAL, R., GONG, J., SAMBASIVAM, S., COMBES, V., MATHYS, J. M., DAVEY, R., GRAU, G. E. R. & BEBAWY, M. 2012. Microparticle-associated nucleic acids mediate trait dominance in cancer. *Faseb Journal,* 26**,** 420-429.

JAISWAL, R., JOHNSON, M. S., POKHAREL, D., KRISHNAN, S. R. & BEBAWY, M. 2017. Microparticles shed from multidrug resistant breast cancer cells provide a parallel survival pathway through immune evasion. *Bmc Cancer,* 17**,** 12.

JAISWAL, R., LUK, F., DALLA, P. V., GRAU, G. E. R. & BEBAWY, M. 2013. Breast Cancer-Derived Microparticles Display Tissue Selectivity in the Transfer of Resistance Proteins to Cells. *Plos One,* 8**,** 10.

JULIANO, R. L. & LING, V. 1976. SURFACE GLYCOPROTEIN MODULATING DRUG PERMEABILITY IN CHINESE-HAMSTER OVARY CELL MUTANTS. *Biochimica Et Biophysica Acta,* 455**,** 152-162.

KARTNER, N., RIORDAN, J. R. & LING, V. 1983. CELL-SURFACE P-GLYCOPROTEIN ASSOCIATED WITH MULTIDRUG RESISTANCE IN MAMMALIAN-CELL LINES. *Science,* 221**,** 1285-1288.

KRISHNAN, S. R., JAISWAL, R., BROWN, R. D., LUK, F. & BEBAWY, M. 2016a. Multiple myeloma and persistence of drug resistance in the age of novel drugs. *International Journal of Oncology,* 49**,** 33-50.

KRISHNAN, S. R., LUK, F., BROWN, R. D., SUEN, H., KWAN, Y. & BEBAWY, M. 2016b. Isolation of Human CD138(+) Microparticles from the Plasma of Patients with Multiple Myeloma. *Neoplasia,* 18**,** 25-32.

LAI, C. P., KIM, E. Y., BADR, C. E., WEISSLEDER, R., MEMPEL, T. R., TANNOUS, B. A. & BREAKEFIELD, X. O. 2015. Visualization and tracking of tumour extracellular vesicle delivery and RNA translation using multiplexed reporters. *Nature Communications,* 6**,** 12.

LAL, R. & PROKSCH, R. 1997. Multimodal atomic force microscopy: Biological imaging using atomic force microscopy combined with light fluorescence and confocal microscopies and electrophysiologic recording. *International Journal of Imaging Systems and Technology,* 8**,** 293-300.

LEAL, J. K. F., ADJOBO-HERMANS, M. J. W. & BOSMAN, G. 2018. Red Blood Cell Homeostasis: Mechanisms and Effects of Microvesicle Generation in Health and Disease. *Frontiers in Physiology,* 9**,** 7.

LEFFERTS, J. A. & LAMBERT, M. W. 2003. Fanconi anemia cell lines deficient in alpha II spectrin express normal levels of alpha II spectrin mRNA. *Biochemical and Biophysical Research Communications,* 307**,** 510-515.

LEGLAND, D., ARGANDA-CARRERAS, I. & ANDREY, P. 2016. MorphoLibJ: integrated library and plugins for mathematical morphology with ImageJ. *Bioinformatics,* 32**,** 3532-3534.

LENER, T., GIMONA, M., AIGNER, L., BORGER, V., BUZAS, E., CAMUSSI, G., CHAPUT, N., CHATTERJEE, D., COURT, F. A., DEL PORTILLO, H. A., O'DRISCOLL, L., FAIS, S., FALCON-PEREZ, J. M., FELDERHOFF-MUESER, U., FRAILE, L., GHO, Y. S., GORGENS, A., GUPTA, R. C., HENDRIX, A., HERMANN, D. M., HILL, A. F., HOCHBERG, F., HORN, P. A., DE KLEIJN, D., KORDELAS, L., KRAMER, B. W., KRAMER-ALBERS, E. M., LANER-PLAMBERGER, S., LAITINEN, S., LEONARDI, T., LORENOWICZ, M. J., LIM, S. K., LOTVALL, J., MAGUIRE, C. A., MARCILLA, A., NAZARENKO, I., OCHIYA, T., PATEL, T., PEDERSEN, S., POCSFALVI, G., PLUCHINO, S., QUESENBERRY, P., REISCHL, I. G., RIVERA, F. J., SANZENBACHER, R., SCHALLMOSER, K., SLAPER-CORTENBACH, I., STRUNK, D., TONN, T., VADER, P., VAN BALKOM, B. W. M., WAUBEN, M., EL ANDALOUSSI, S., THERY, C., ROHDE, E. & GIEBEL, B. 2015. Applying extracellular vesicles based therapeutics in clinical trials - an ISEV position paper. *Journal of Extracellular Vesicles,* 4**,** 31.

LETO, T. L., FORTUGNOERIKSON, D., BARTON, D., YANGFENG, T. L., FRANCKE, U., HARRIS, A. S., MORROW, J. S., MARCHESI, V. T. & BENZ, E. J. 1988. COMPARISON OF NONERYTHROID ALPHA-SPECTRIN GENES REVEALS STRICT HOMOLOGY AMONG DIVERSE SPECIES. *Molecular and Cellular Biology,* 8**,** 1-9.

LIEM, R. K. 2016a. Cytoskeletal integrators: the spectrin superfamily. *Cold Spring Harbor perspectives in biology,* 8**,** a018259.

LIEM, R. K. H. 2016b. Cytoskeletal Integrators: The Spectrin Superfamily. *Cold Spring Harbor Perspectives in Biology,* 8**,** 10.

LIU, C. H. & RASBAND, M. N. 2019. Axonal Spectrins: Nanoscale Organization, Functional Domains and Spectrinopathies. *Frontiers in Cellular Neuroscience,* 13**,** 10.

LU, J. F., LUK, F., GONG, J., JAISWAL, R., GRAU, G. E. R. & BEBAWY, M. 2013. Microparticles mediate MRP1 intercellular transfer and the re-templating of intrinsic resistance pathways. *Pharmacological Research,* 76**,** 77-83.

LU, J. F., POKHAREL, D. & BEBAWY, M. 2017. A novel mechanism governing the transcriptional regulation of ABC transporters in MDR cancer cells. *Drug Delivery and Translational Research,* 7**,** 276-285.

LU, J. F., POKHAREL, D., PADULA, M. P. & BEBAWY, M. 2016. A novel method to detect translation of membrane proteins following microvesicle intercellular transfer of nucleic acids. *Journal of Biochemistry,* 160**,** 281-289.

LUBBERS, E. R., MURPHY, N. P., MUSA, H., HUANG, C. Y. M., GUPTA, R., PRICE, M. V., HAN, M., DAOUD, G., GRATZ, D., EL REFAEY, M., XU, X. Y., HOEFLINGER, N. K., FRIEL, E. L., LANCIONE, P., WALLACE, M. J., CAVUS, O., SIMMONS, S. L., WILLIAMS, J. L., SKAF, M., KOENIG, S. N., JANSSEN, P. M. L., RASBAND, M. N., HUND, T. J. & MOHLER, P. J. 2019. Defining new mechanistic roles for II spectrin in cardiac function. *Journal of Biological Chemistry,* 294**,** 9576-9591.

LUX, S. E. 2016. Anatomy of the red cell membrane skeleton: unanswered questions. *Blood,* 127**,** 187-199.

LYTTON, J., WESTLIN, M. & HANLEY, M. R. 1991. THAPSIGARGIN INHIBITS THE SARCOPLASMIC OR ENDOPLASMIC-RETICULUM CA-ATPASE FAMILY OF CALCIUM PUMPS. *Journal of Biological Chemistry,* 266**,** 17067-17071.

MACHNICKA, B., CZOGALLA, A., HRYNIEWICZ-JANKOWSKA, A., BOGUSLAWSKA, D. M., GROCHOWALSKA, R., HEGER, E. & SIKORSKI, A. F. 2014. Spectrins: A structural platform for stabilization and activation of membrane channels, receptors and transporters. *Biochimica Et Biophysica Acta-Biomembranes,* 1838**,** 620-634.

MACHNICKA, B., GROCHOWALSKA, R., BOGUSLAWSKA, D. M., SIKORSKI, A. F. & LECOMTE, M. C. 2012. Spectrin-based skeleton as an actor in cell signaling. *Cellular and Molecular Life Sciences,* 69**,** 191-201.

MACHTINGER, R., LAURENT, L. C. & BACCARELLI, A. A. 2016. Extracellular vesicles: roles in gamete maturation, fertilization and embryo implantation. *Human Reproduction Update,* 22**,** 182-193.

MALIK, M. F. A. 2015. Influence of Microvesicles in Breast Cancer Metastasis and their Therapeutic Implications. *Archives of Iranian Medicine,* 18**,** 189-192.

MALLAT, Z., BENAMER, H., HUGEL, B., BENESSIANO, J., STEG, P. G., FREYSSINET, J. M. & TEDGUI, A. 2000. Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. *Circulation,* 101**,** 841-843.

MARCHESI, V. T. & STEERS, E. 1968. Selective Solubilization of a Protein Component of the Red Cell Membrane. *Science,* 159**,** 203.

MARTIN, S. J., REUTELINGSPERGER, C. P. M., MCGAHON, A. J., RADER, J. A., VANSCHIE, R., LAFACE, D. M. & GREEN, D. R. 1995. EARLY REDISTRIBUTION OF PLASMA-MEMBRANE PHOSPHATIDYLSERINE IS A GENERAL FEATURE OF APOPTOSIS REGARDLESS OF THE INITIATING STIMULUS - INHIBITION BY OVEREXPRESSION OF BCL-2 AND ABL. *Journal of Experimental Medicine,* 182**,** 1545-1556.

MESRI, M. & ALTIERI, D. C. 1999. Leukocyte microparticles stimulate endothelial cell cytokine release and tissue factor induction in a JNK1 signaling pathway. *Journal of Biological Chemistry,* 274**,** 23111-23118.

MONTEITH, G. R., DAVIS, F. M. & ROBERTS-THOMSON, S. J. 2012. Calcium Channels and Pumps in Cancer: Changes and Consequences. *Journal of Biological Chemistry,* 287**,** 31666-31673.

MONTEITH, G. R., MCANDREW, D., FADDY, H. M. & ROBERTS-THOMSON, S. J. 2007. Calcium and cancer: targeting Ca2+ transport. *Nature Reviews Cancer,* 7**,** 519-530.

MONTEITH, G. R., PREVARSKAYA, N. & ROBERTS-THOMSON, S. J. 2017. The calcium-cancer signalling nexus. *Nature Reviews Cancer,* 17**,** 367-380.

MOON, R. T. & MCMAHON, A. P. 1990. GENERATION OF DIVERSITY IN NONERYTHROID SPECTRINS - MULTIPLE POLYPEPTIDES ARE PREDICTED BY SEQUENCE-ANALYSIS OF CDNAS ENCOMPASSING THE CODING REGION OF HUMAN NONERYTHROID ALPHA-SPECTRIN. *Journal of Biological Chemistry,* 265**,** 4427-4433.

MOREL, O., JESEL, L., FREYSSINET, J. M. & TOTI, F. 2011. Cellular Mechanisms Underlying the Formation of Circulating Microparticles. *Arteriosclerosis Thrombosis and Vascular Biology,* 31**,** 15-26.

MORRELL, A. E., BROWN, G. N., ROBINSON, S. T., SATTLER, R. L., BAIK, A. D., ZHEN, G. H., CAO, X., BONEWALD, L. F., JIN, W. Y., KAM, L. C. & GUO, X. E. 2018. Mechanically induced Ca2+ oscillations in osteocytes release extracellular vesicles and enhance bone formation. *Bone Research,* 6**,** 11.

NOLTE-'T HOEN, E. N. M. & WAUBEN, M. H. M. 2012. Immune Cell-derived Vesicles: Modulators and Mediators of Inflammation. *Current Pharmaceutical Design,* 18**,** 2357-2368.

PAP, E. 2011. The role of microvesicles in malignancies. *Cell Fusion in Health and Disease.* Springer.

PARK, A. Y., CHAE, Y. S., LEE, S. H., KAANG, B. K. & LEE, S. 2013. Presynaptic Structure of Aplysia Single Live Neuron by Atomic Force and Confocal Laser Scanning Microscope. *Journal of Physical Chemistry B,* 117**,** 4779-4788.

PASQUET, J. M., DACHARYPRIGENT, J. & NURDEN, A. T. 1996. Calcium influx is a determining factor of calpain activation and microparticle formation in platelets. *European Journal of Biochemistry,* 239**,** 647-654.

PERRIN, B. J., AMANN, K. J. & HUTTENLOCHER, A. 2006. Proteolysis of cortactin by calpain regulates membrane protrusion during cell migration. *Molecular Biology of the Cell,* 17**,** 239-250.

PETERS, A. A., SIMPSON, P. T., BASSETT, J. J., LEE, J. M., DA SILVA, L., REID, L. E., SONG, S., PARAT, M. O., LAKHANI, S. R., KENNY, P. A., ROBERTS-THOMSON, S. J. & MONTEITH, G. R. 2012. Calcium Channel TRPV6 as a Potential Therapeutic Target in Estrogen Receptor-Negative Breast Cancer. *Molecular Cancer Therapeutics,* 11**,** 2158-2168.

POKHAREL, D., WIJESINGHE, P., OENARTO, V., LU, J. F., SAMPSON, D. D., KENNEDY, B. F., WALLACE, V. P. & BEBAWY, M. 2016a. Deciphering Cell-to-Cell Communication in Acquisition of Cancer Traits: Extracellular Membrane Vesicles Are Regulators of Tissue Biomechanics. *Omics-a Journal of Integrative Biology,* 20**,** 462-469.

POKHAREL, D., WIJESINGHE, P., OENARTO, V., LU, J. F., SAMPSON, D. D., KENNEDY, B. F., WALLACE, V. P. & BEBAWY, M. 2016b. Deciphering Cell-to-Cell Communication in Acquisition of Cancer Traits: Extracellular Membrane Vesicles Are Regulators of Tissue Biomechanics. *OMICS: A Journal of Integrative Biology,* 20**,** 462-469.

POON, I. K. H., PARKES, M. A. F., JIANG, L. Z., ATKIN-SMITH, G. K., TIXEIRA, R., GREGORY, C. D., OZKOCAK, D. C., RUTTER, S. F., CARUSO, S., SANTAVANOND, J. P., PAONE, S., SHI, B., HODGE, A. L., HULETT, M. D., CHOW, J. D. Y., PHAN, T. K. & BAXTER, A. A. 2019. Moving beyond size and phosphatidylserine exposure: evidence for a diversity of apoptotic cell-derived extracellular vesicles in vitro. *Journal of Extracellular Vesicles,* 8**,** 14.

POTTLE, J., SUN, C., GRAY, L. & LI, M. 2013. Exploiting MCF-7 Cells¡¯ Calcium Dependence with Interlaced Therapy. *Journal of Cancer Therapy,* Vol.04No.07**,** 9.

PRAKRIYA, M. & LEWIS, R. S. 2015. STORE-OPERATED CALCIUM CHANNELS. *Physiological Reviews,* 95**,** 1383-1436.

PUTNEY, J. W. 2010. Pharmacology of Store-operated Calcium Channels. *Molecular Interventions,* 10**,** 209-218.

QIU, Q., XIONG, W., YANG, C., GAGNON, C. & HARDY, P. 2013. Lymphocyte-derived microparticles induce bronchial epithelial cells' pro-inflammatory cytokine production and apoptosis. *Molecular Immunology,* 55**,** 220-230.

QUESENBERRY, P. J. & ALIOTTA, J. M. 2008. The paradoxical dynamism of marrow stem cells: Considerations of stem cells, niches, and microvesicles. *Stem Cell Reviews,* 4**,** 137-147.

RAPOSO, G. & STOORVOGEL, W. 2013. Extracellular vesicles: Exosomes, microvesicles, and friends. *Journal of Cell Biology,* 200**,** 373-383.

RATAJCZAK, J., WYSOCZYNSKI, M., HAYEK, F., JANOWSKA-WIECZOREK, A. & RATAJCZAK, M. Z. 2006. Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. *Leukemia,* 20**,** 1487-1495.

RIZZUTO, R. & POZZAN, T. 2006. Microdomains of intracellular Ca2+: Molecular determinants and functional consequences. *Physiological Reviews,* 86**,** 369-408.

ROSEBLADE, A., LUK, F., RAWLING, T., UNG, A., GRAU, G. E. & BEBAWY, M. 2013. Cell-derived microparticles: new targets in the therapeutic management of disease. *Journal of Pharmacy & Pharmaceutical Sciences,* 16**,** 238-253.

ROSEBLADE, A., LUK, F., UNG, A. & BEBAWY, M. 2015. Targeting Microparticle Biogenesis: A Novel Approach to the Circumvention of Cancer Multidrug Resistance. *Current Cancer Drug Targets,* 15**,** 205-214.

SAIDO, T. C., SHIBATA, M., TAKENAWA, T., MUROFUSHI, H. & SUZUKI, K. 1992. POSITIVE REGULATION OF MU-CALPAIN ACTION BY POLYPHOSPHOINOSITIDES. *Journal of Biological Chemistry,* 267**,** 24585-24590.

SEHGAL, P., SZALAI, P., OLESEN, C., PRAETORIUS, H. A., NISSEN, P., CHRISTENSEN, S. B., ENGEDAL, N. & MOLLER, J. V. 2017. Inhibition of the sarco/endoplasmic reticulum (ER) Ca2+-ATPase by thapsigargin analogs induces cell death via ER Ca2+ depletion and the unfolded protein response. *Journal of Biological Chemistry,* 292**,** 19656-19673.

SORMUNEN, R. T., LEONG, A. S. Y., VAARANIEMI, J. P., FERNANDO, S. S. E. & ESKELINEN, S. M. 1999. Immunolocalization of the fodrin, E-cadherin, and beta-catenin adhesion complex in

infiltrating ductal carcinoma of the breast - Comparison with an in vitro model. *Journal of Pathology,* 187**,** 416-423.

STEWART, T. A., YAPA, K. & MONTEITH, G. R. 2015. Altered calcium signaling in cancer cells. *Biochimica Et Biophysica Acta-Biomembranes,* 1848**,** 2502-2511.

STORR, S. J., CARRAGHER, N. O., FRAME, M. C., PARR, T. & MARTIN, S. G. 2011. The calpain system and cancer. *Nature Reviews Cancer,* 11**,** 364-374.

SUN, Y., XIA, Z. J., SHANG, Z., SUN, K. B., NIU, X. M., QIAN, L. Q., FAN, L. Y., CAO, C. X. & XIAO, H. 2016. Facile preparation of salivary extracellular vesicles for cancer proteomics. *Scientific Reports,* 6**,** 11.

TAYLOR, J., AZIMI, I., MONTEITH, G. & BEBAWY, M. 2020. Ca2+ mediates extracellular vesicle biogenesis through alternate pathways in malignancy. *Journal of Extracellular Vesicles,* 9**,** 14.

TAYLOR, J., JAISWAL, R. & BEBAWY, M. 2017. Calcium-calpain Dependent Pathways Regulate Vesiculation in Malignant Breast Cells. *Current Cancer Drug Targets,* 17**,** 486-494.

THASTRUP, O., CULLEN, P. J., DROBAK, B. K., HANLEY, M. R. & DAWSON, A. P. 1990. THAPSIGARGIN, A TUMOR PROMOTER, DISCHARGES INTRACELLULAR CA-2+ STORES BY SPECIFIC-INHIBITION OF THE ENDOPLASMIC-RETICULUM CA-2+-ATPASE. *Proceedings of the National Academy of Sciences of the United States of America,* 87**,** 2466-2470.

THERY, C., WITWER, K. W., AIKAWA, E., ALCARAZ, M. J., ANDERSON, J. D., ANDRIANTSITOHAINA, R., ANTONIOU, A., ARAB, T., ARCHER, F., ATKIN-SMITH, G. K., AYRE, D. C., BACH, J. M., BACHURSKI, D., BAHARVAND, H., BALAJ, L., BALDACCHINO, S., BAUER, N. N., BAXTER, A. A., BEBAWY, M., BECKHAM, C., ZAVEC, A. B., BENMOUSSA, A., BERARDI, A. C., BERGESE, P., BIELSKA, E., BLENKIRON, C., BOBIS-WOZOWICZ, S., BOILARD, E., BOIREAU, W., BONGIOVANNI, A., BORRAS, F. E., BOSCH, S., BOULANGER, C. M., BREAKEFIELD, X., BREGLIO, A. M., BRENNAN, M. A., BRIGSTOCK, D. R., BRISSON, A., BROEKMAN, M. L. D., BROMBERG, J. F., BRYL-GORECKA, P., BUCH, S., BUCK, A. H., BURGER, D., BUSATTO, S., BUSCHMANN, D., BUSSOLATI, B., BUZAS, E. I., BYRD, J. B., CAMUSSI, G., CARTER, D. R. F., CARUSO, S., CHAMLEY, L. W., CHANG, Y. T., CHEN, C. C., CHEN, S., CHENG, L., CHIN, A. R., CLAYTON, A., CLERICI, S. P., COCKS, A., COCUCCI, E., COFFEY, R. J., CORDEIRO-DA-SILVA, A., COUCH, Y., COUMANS, F. A. W., COYLE, B., CRESCITELLI, R., CRIADO, M. F., D'SOUZA-SCHOREY, C., DAS, S., CHAUDHURI, A. D., DE CANDIA, P., DE SANTANA, E. F., DE WEVER, O., DEL PORTILLO, H. A., DEMARET, T., DEVILLE, S., DEVITT, A., DHONDT, B., DI VIZIO, D., DIETERICH, L. C., DOLO, V., RUBIO, A. P. D., DOMINICI, M., DOURADO, M. R., DRIEDONKS, T. A. P., DUARTE, F. V., DUNCAN, H. M., EICHENBERGER, R. M., EKSTROM, K., ANDALOUSSI, S. E. L., ELIE-CAILLE, C., ERDBRUGGER, U., FALCON-PEREZ, J. M., FATIMA, F., FISH, J. E., FLORES-BELLVER, M., FORSONITS, A., FRELET-BARRAND, A., et al. 2018. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *Journal of Extracellular Vesicles,* 7**,** 43.

TKACH, M. & THERY, C. 2016. Communication by Extracellular Vesicles: Where We Are and Where We Need to Go. *Cell,* 164**,** 1226-1232.

TUOMINEN, H., SORMUNEN, R. & KALLIOINEN, M. 1996. Non-erythroid spectrin (fodrin) in cutaneous tumours: Diminished in cell membranes, increased in the cytoplasm. *British Journal of Dermatology,* 135**,** 576-580.

VERWEIJ, F. J., BEBELMAN, M. P., JIMENEZ, C. R., GARCIA-VALLEJO, J. J., JANSSEN, H., NEEFJES, J., KNOL, J. C., DE GOEIJ-DE HAAS, R., PIERSMA, S. R., BAGLIO, S. R., VERHAGE, M., MIDDELDORP, J. M., ZOMER, A., VAN RHEENEN, J., COPPOLINO, M. G., HURBAIN, I., RAPOSO, G., SMIT, M. J., TOONEN, R. F. G., VAN NIEL, G. & PEGTEL, D. M. 2018. Quantifying exosome secretion from single cells reveals a modulatory role for GPCR signaling. *The Journal of Cell Biology*.

VERWEIJ, F. J., VAN EIJNDHOVEN, M. A. J., HOPMANS, E. S., VENDRIG, T., WURDINGER, T., CAHIR-MCFARLAND, E., KIEFF, E., GEERTS, D., VAN DER KANT, R., NEEFJES, J., MIDDELDORP, J. M. &

PEGTEL, D. M. 2011. LMP1 association with CD63 in endosomes and secretion via exosomes limits constitutive NF-kappa B activation. *Embo Journal,* 30**,** 2115-2129.
VIDAL, M. 2010. Exosomes in erythropoiesis. *Transfusion Clinique Et Biologique,* 17**,** 131-137.
VISSER, D., LANGESLAG, M., KEDZIORA, K. M., KLARENBEEK, J., KAMERMANS, A., HORGEN, F. D., FLEIG, A., VAN LEEUWEN, F. N. & JALINK, K. 2013. TRPM7 triggers Ca2+ sparks and invadosome formation in neuroblastoma cells. *Cell Calcium,* 54**,** 404-415.
WAGNER, G. M., CHIU, D. T. Y., QJU, J. H., HEATH, R. H. & LUBIN, B. H. 1987. SPECTRIN OXIDATION CORRELATES WITH MEMBRANE VESICULATION IN STORED RBCS. *Blood,* 69**,** 1777-1781.
WANG, J. G., WILLIAMS, J. C., DAVIS, B. K., JACOBSON, K., DOERSCHUK, C. M., TING, J. P. Y. & MACKMAN, N. 2011. Monocytic microparticles activate endothelial cells in an IL-1 beta-dependent manner. *Blood,* 118**,** 2366-2374.
WANG, K. & YUEN, P.-W. 1999. *Calpain: The Pharmacology and Toxicology of Calcium-Dependent Protease*.
WANG, K. K. W., NATH, R., POSNER, A., RASER, K. J., BUROKERKILGORE, M., HAJIMOHAMMADREZA, I., PROBERT, A. W., MARCOUX, F. W., YE, Q. H., TAKANO, E., HATANAKA, M., MAKI, M., CANER, H., COLLINS, J. L., FERGUS, A., LEE, K. S., LUNNEY, E. A., HAYS, S. J. & YUEN, P. W. 1996. An alpha-mercaptoacrylic acid derivative is a selective nonpeptide cell-permeable calpain inhibitor and is neuroprotective. *Proceedings of the National Academy of Sciences of the United States of America,* 93**,** 6687-6692.
WEBER, H., HÜHNS, S., LÜTHEN, F. & JONAS, L. 2009. Calpain-mediated breakdown of cytoskeletal proteins contributes to cholecystokinin-induced damage of rat pancreatic acini. *International Journal of Experimental Pathology,* 90**,** 387-399.
WEI, C. L., WANG, X. H., CHEN, M., OUYANG, K., SONG, L. S. & CHENG, H. P. 2009. Calcium flickers steer cell migration. *Nature,* 457**,** 901-905.
WU, X. T., SUN, L. W., YANG, X., DING, D., HAN, D. & FAN, Y. B. 2017. The potential role of spectrin network in the mechanotransduction of MLO-Y4 osteocytes. *Scientific Reports,* 7**,** 12.
XU, H. J., SU, W. H., CAI, M. J., JIANG, J. G., ZENG, X. L. & WANG, H. D. 2013. The Asymmetrical Structure of Golgi Apparatus Membranes Revealed by In situ Atomic Force Microscope. *Plos One,* 8**,** 10.
ZHANG, R., ZHANG, C., ZHAO, Q. & LI, D. 2013a. Spectrin: Structure, function and disease. *Science China Life Sciences,* 56**,** 1076-1085.
ZHANG, R., ZHANG, C. Y., ZHAO, Q. & LI, D. H. 2013b. Spectrin: Structure, function and disease. *Science China-Life Sciences,* 56**,** 1076-1085.
ZHANG, X., CHEN, Y. & CHEN, Y. 2014. An AFM-based pit-measuring method for indirect measurements of cell-surface membrane vesicles. *Biochemical and Biophysical Research Communications,* 446**,** 375-379.
ZHANG, X. J., TANG, Q. S., WU, L., HUANG, J. & CHEN, Y. 2015. AFM visualization of cortical filaments/network under cell-bound membrane vesicles. *Biochimica Et Biophysica Acta-Biomembranes,* 1848**,** 2225-2232.