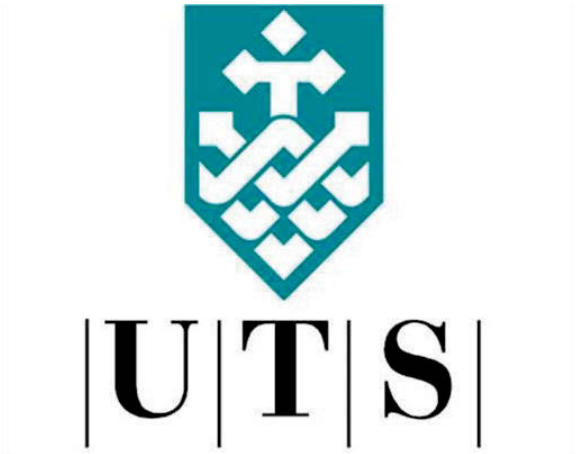
Microparticles Mediate Trait Dominance in Cancer

JAMIE LU

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy



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Jamie Lu

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List of Abbreviations

^0C	Degrees Celsius
µg	Microgram
µM	Micromole
ABC	ATP-binding cassette
ACN	Acetonitrile
ATP	Adenosine triphosphate
cDNA	Complementary deoxyribonucleic acid
BSA	Bovine Serum Albumin
C-terminus	Carboxyl-terminus
CD44	Cluster of differentiation 44
CO_2	Carbon Dioxide
CsA	Cyclosporin A
DNA	Deoxyribonucleic acid
DNR	Daunorubicin
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
FCM	Flow cytometry
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
g	acceleration due to gravity
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Glutathione
GST	Glutathione *S*-Transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
HSP	Heat shock protein

IgG	Immunoglobulin-G
kDa	Kilo Dalton
LC	Liquid chromatography
mAb	Monoclonal antibody
MDR	Multidrug resistance
MFI	Mean fluorescence intensity
miRNA	micro ribonucleic acid
lncRNA	long non-coding ribonucleic acid
MPs	Microparticles
MRP	Multidrug resistance associated protein
mRNA	Messenger Ribonucleic Acid
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
N-terminus	Amino-terminus
ng	Nanogram
PAGE	Polyacrylamide gel electrophoresis
PBC	Probenecid
PBS	Phosphate buffered saline
PE	Phosphatidylethanolamine
P-gp	P-glycoprotein
pmole	picomole
ppm	Parts per million
PS	Phosphatidylserine
PVDF	Polyvinylidene fluoride
qRT-PCR	quantitative Real-Time Polymerase Chain Reaction
RRTA	Rabbit Reticulocyte Translation Assay
RNA	Ribonucleic acid
rpm	Revolutions per minute

S.E.M	Standard Error of the Mean
SEM	Scanning Electron Microscopy
SDS	Sodium Dodecyl Sulphate
TCA	Trichloroacetic acid
TBS	Tris-buffered saline
TBST	Tris-buffered saline-Tween 20
TMD	Transmembrane Domain
Tween-20	Polyoxyethylenesorbitan monolaurate
UTR	Untranslated Region

Publications arising from this work

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Jamie F. Lu; Frederick Luk; Joyce Gong; Ritu Jaiswal; Georges E.R. Grau, Mary Bebawy, **"Microparticles mediate MRP1 intercellular transfer and the re-templating of intrinsic resistance pathways"**, *Pharmacological Research* 2013, DOI: 10.1016/j.phrs.2013.07.009

Jamie F. Lu; Deep Pokharel; Matthew P. Padula; Mary Bebawy. **"A novel method to detect translation of membrane proteins following microvesicle intercellular transfer of nucleic acids**", *Journal of Biochemistry* 2016; doi: 10.1093/jb/mvw033

Jamie F. Lu; Deep Pokharel; Frederick Luk; Mary Bebawy. "**Functional translation of total RNA packaged in microparticles shed from multidrug resistant cancer cells". [abstract]**. *Proceedings of the AACR Precision Medicine Series: Drug Sensitivity and Resistance: Improving Cancer Therapy*; Jun 18-21, 2014; Orlando, FL. Philadelphia (PA): AACR; Clin Cancer Res 2015;21(4 Suppl): Abstract nr B19.

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Jamie F. Lu, Ritu Jaiswal, Frederick Luk, Georges E.R. Grau, Mary Bebawy, "**Intercellular Transfer Of Functional MRP1 And The Re-templating Of Intrinsic Resistance Pathways Via Microparticles**", *International society of extracellular vesicles*, April 17-20, 2013; Boston, MA, USA

Jamie F. Lu, Deep Pokharel, Frederick Luk, Mary Bebawy, "**Functional translation of total RNA packaged in microparticles shed from multidrug resistant cancer cells**," *AACR Precision medicine series: Drug sensitivity and resistance: Improving cancer therapy*; 18-21st June, 2014; Orlando, FL, USA.

Other Publications

Deep Pokharel; Matthew P. Padula; **Jamie F. Lu**; Jessica Tacchi; Frederick Luk; Steven P. Djordjevic; Mary Bebawy. "**Proteome analysis of multidrug-resistant, breast cancer-derived microparticles**," *Journal of extracellular vesicles* 2014, DOI: 10.3402/jev.v3.24384.

Deep Pokharel; Matthew P. Padula; **Jamie F. Lu**; Ritu Jaiswal; Steven P. Djordjevic; Mary Bebawy. "**The functional role of CD44 and ERM in conferring multidrug resistance in breast cancer**," *Molecules* 2016, doi:10.3390/molecules21030290

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Abstract

Multidrug resistance (MDR) persists to be a major hindrance to the successful treatment in clinical oncology and is the cause of over 90% of treatment failure in cancer. The two main membrane spanning proteins, P-glycoprotein (*ABCB1*/P-gp) and Multidrug Resistance-associated protein 1 (*ABCC1*/MRP1) are responsible for the efflux of a plethora of unrelated anti-cancer drugs out of cells, resulting in MDR. Cancer cells overexpressing these efflux proteins are insensitive to chemotherapeutic treatments by maintaining sub-lethal intracellular cytotoxic drug concentrations. Given their enormous substrate profile, of which there is significant overlap, the expression of either efflux protein would result in a poor prognosis. In some cancers, the overexpression of these proteins is correlated with clinical stage, with early stage tumours expressing one efflux transporter and substituted by another transporter at an advanced stage. Our group has established the transfer and dissemination of ABC-transporter mediated MDR via a subset of extracellular vesicles known as microparticles (MPs). This study investigates the molecular mechanisms governing the alteration and acquisition of MDR traits in cancer cell populations via MPs.

Spontaneously shed MPs from cancer cells represent a prominent modality for intercellular communication by virtue of their capacity to transport and disseminate bioactive cargo through the vasculature. Their ability to carry large membrane spanning proteins and nucleic acids, imparts their capacity to confer MDR among otherwise drug sensitive tumour cells. Herein, the study validates the MP-transfer and functionality of MRP1 in drug sensitive acute leukaemia cells. The study also introduces

MP-mediated trait dominance and demonstrate the re-templating of a pre-existing MDR phenotype in recipient cells. To validate the transfer and translation of MP packaged nucleic acids, a novel methodology was developed, abolishing the requirement for labelled probes and interspecies models. Using, surface peptide shaving, detection of MP packaged P-gp was removed and showed transcript translation of transferred *ABCB1* in drug sensitive recipient cells after more than 24 h. Finally, the study identifies transcript suppression mechanisms involved in MP-mediated trait dominance and identify a novel relationship between the function of miRNA with a non-target mRNA transcript. Specifically, the presence of a rival transcript *ABCB1* facilitates the *ABCC1* suppression by miR-326. These findings substantially advance our understanding on the molecular mechanisms leading to the alteration of MDR traits and can be translated into clinical oncology by providing prognostic information and additional therapeutic targets.

Chapter 1

Introduction

1. MRP1 and its role in anticancer drug resistance

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REVIEW ARTICLE

MRP1 and its role in anticancer drug resistance

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Abstract

The phenomenon of multidrug resistance (MDR) in cancer is associated with the overexpression of the ATP-binding cassette (ABC) transporter proteins, including multidrug resistance-associated protein 1 (MRP1) and P-glycoprotein. MRP1 plays an active role in protecting cells by its ability to efflux a vast array of drugs to sub-lethal levels. There has been much effort in elucidating the mechanisms of action, structure and substrates and substrate binding sites of MRP1 in the last decade. In this review, we detail our current understanding of MRP1, its clinical relevance and highlight the current environment in the search for MRP1 inhibitors. We also look at the capacity for the rapid intercellular transfer of MRP1 phenotype from spontaneously shed membrane vesicles known as microparticles and discuss the clinical and therapeutic significance of this in the context of cancer MDR.

Keywords

Microparticles, multidrug resistance, multidrug resistance-associated protein 1, intercellular transfer, P-glycoprotein, trait dominance, cancer

History

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Introduction

The use of combinations of cytotoxic drugs in chemotherapy has led to vast improvements in patient survival. Previously fatal malignancies such as testicular cancer, Hodgkin's disease and many leukemia's are now manageable diseases (Dryver et al., 2003; Farber et al., 1980; Frei, 1985). Despite this, the development of drug resistance in cancer generally is a frequent occurrence and is a major impediment to successful treatment (Gong et al., 2012).

After an initial positive response to drug treatment, a tumor may fail to respond to subsequent chemotherapy despite the use of structurally and functionally unrelated drugs. This type of unique resistance is known as multidrug resistance (MDR). A tumor exhibiting MDR is unresponsive to a plethora of modern chemotherapeutics. MDR is responsible for more than 90% of treatment failure of metastatic cancer using adjuvant chemotherapy (Saraswathy & Gong, 2013).

One of the most intensely studied mechanisms of MDR is the drug efflux capacity of certain proteins found overexpressed on the surface of resistant cancer cells. These efflux proteins are typically members of the ATP-binding cassette (ABC) superfamily of membrane transporters (Leslie et al., 2001b, 2005; Tran et al., 2011; Winter et al., 2013). Structurally, these transmembrane proteins consist of transmembrane domain(s) (TMD), which comprise the drug-binding region, and the nucleotide-binding domains (NBDs) which hydrolyze ATP required to drive drug efflux (Cornwell et al., 1987; Mimmack et al., 1989). To date, there are 49 ABC-transporter proteins expressed in humans, however, only a handful are implicated in MDR (Tarling et al., 2013). The most intensely studied of these transporters which contribute to MDR are P-glycoprotein (P-gp/*ABCB1*), multidrug resistance-associated protein 1 (MRP1/*ABCC1*) and to a lesser extent, the breast cancer resistance protein (BCRP/*ABCG1*) (Falasca & Linton, 2012; Rees et al., 2009).

Recently much attention has been focused on the role of extracellular vesicles and how they contribute to cancer cell biology (Bebawy et al., 2009, 2013; Gong et al., 2012, 2013; Jaiswal et al., 2012a, b, 2013; Lu et al., 2013; Ma et al., 2013; Pokharel et al., 2014). Their capacity to carry cell surface antigens, biologically functional nuclear cargo and proteins has provoked much interest in their molecular roles as mediators of cell–cell communication and the transfer of ABC-transporter-mediated MDR (Bebawy et al., 2009, 2013; Diehl et al., 2012; Distler et al., 2006; Gong et al., 2013; Jaiswal et al., 2012a, b, 2013; Lu et al., 2013; Morel et al., 2008; Pokharel et al., 2014). Herein, we provide a recent review on the research on MRP1/*ABCC1* and discuss its clinical relevance. Overexpression of MRP1 in a diverse range of tumors has prompted investigations into its influence on patient survivability. We further examine the controversy on the clinical implications of MRP1 on a range of tumors and discuss the variability in the literature. In addition, we provide an overview of the current situation for the search of MRP1 inhibitors. Finally, we discuss the dynamic and active role of MPs in MDR and the potential for these vesicles to provide a novel therapeutic drug target in clinical oncology.

General overview of MRP1

The clinical relevance of P-gp overexpression is unequivocal, given the correlation with its overexpression and treatment

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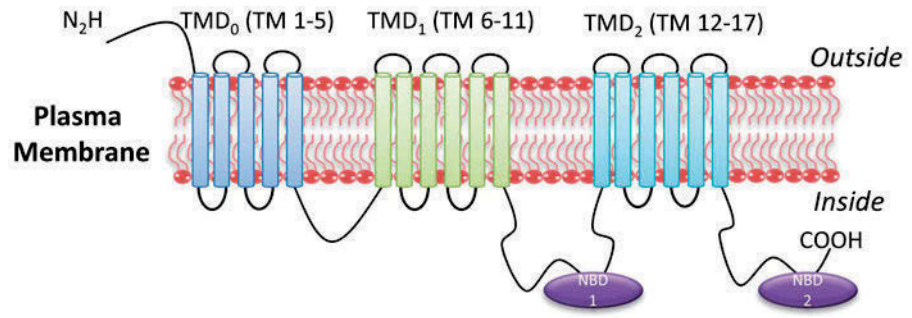

Figure 1. The orientation and topology of MRP1. MRP1 includes three transmembrane domains (TMD0, TMD1 and TMD2) composed of 17 transmembrane segments (TM). There are also two nucleotide-binding domains (NBD1 and NBD2) within the cytosol.

failure in cancer treatment (Atalay et al., 2006; Campos et al., 1992; Kuwazuru et al., 1990; Leith et al., 1999; Sun et al., 2004; Tamaki et al., 2011). It was first discovered in 1976 and is the most well-characterized ABC-transporter contributing to MDR (Juliano & Ling, 1976). For a period after its discovery, P-gp was widely believed to be the exclusive cause of MDR (Ambudkar et al., 1999). During the late 1980s, increasing evidence of an MDR phenotype in the absence of P-gp emerged. MDR cells were identified that lacked any detectable P-gp, despite having undergone drug selection protocols, which would typically result in P-gp overexpression (Cole, 1986; Mirski et al., 1987; Slovak et al., 1988). Rather, the resistance was found to be strongly correlated with another protein from the same superfamily, known as MRP1 (Cole et al., 1992).

The gene encoding MRP1/*ABCC1* has been mapped to 16p13.1 and represents a 1531-amino acid protein (Slovak et al., 1993). MRP1 is widely expressed in normal tissues and cellular organelles, particularly in the testes, kidneys, placenta and at pharmacological barriers (Leslie et al., 2005). MRP1's capacity for drug efflux prevents effective treatment of a range of diseases, including clinical depression, cancer and epilepsy (Bao et al., 2011; Chen et al., 2013; Kanigur et al., 2010; Lee et al., 2010; Mueller et al., 2010). The overexpression of MRP1 across a range of cancers has led to relapse and drastically reduced overall survival (OS) in cancer patients (Alisi et al., 2013; Diestra et al., 2003; Haber et al., 2006; Semsei et al., 2012; Vulsteke et al., 2013; Winter et al., 2013; Zhang et al., 2012).

Currently, the most widely accepted topology of MRP1 includes three TMDs (TMD_0, TMD_1, TMD_2) and two NBDs (NBD_1, NDB_2) (Cole, 2014; Hollenstein et al., 2007) (Figure 1). This configuration is consistent throughout the MRP family of proteins, except in MRP4, MRP5 and CTFR, which do not possess the additional TMD_0 domain (Borst et al., 2000; Patrick & Thomas, 2012). There are 17 transmembrane (TM) segments distributed among these three TMDs: TM 1–5 (TMD_0), TM 6–11 (TMD_1) and TM 12–17 (TMD_2) (DeGorter et al., 2008).

The mechanisms of MRP1 trafficking, maturation and degradation still remain poorly understood. Newly synthesized MRP1 is a 170 kDa polypeptide immature protein, which is rapidly processed into its 190 kDa form following *N*-terminal glycosylation (Almquist et al., 1995; Krishnamachary et al., 1993). *In vitro* transcription and translational analysis have revealed the critical role of the second transmembrane segment (TM 2) in the cellular translocation and topologic folding of TMD_0 during synthesis (Zhang, 2000). It was found that TM2 ensures the correct right-side out orientation of MRP1 on the plasma membrane (N-terminus outside, C-terminus inside) (Zhang, 2000). In addition, it can translocate post-translationally into the endoplasmic reticulum for additional stringency for the correct folding of the remaining domains in MRP1 (Zhang, 2000).

MRP1 has also been found to be localized within the cell (Roundhill & Burchill, 2012). MRP1 expression in the mitochondria appears to be more efficient than plasma MRP1 (Roundhill & Burchill, 2012). The presence of transporters in this organelle may protect mitochondrial DNA from damage and mitochondrial-induced cell death. MRP1 has also been detected in other subcellular organelles such as endoplasmic reticulum and endocytic vesicles, possibly serving as a sequestering mechanism to prevent drugs from reaching their intracellular target (Rajagopal & Simon, 2003). When cultured astrocytes are exposed to bilirubin (an MRP1 substrate), MRP1 was found to rapidly translocate onto the plasma membrane from subcellular organelles (Gennuso et al., 2004). Using confocal microscopy and immunofluorescence, MRP1 was found to be co-localized to the Golgi apparatus (Gennuso et al., 2004). After exposure to low amounts of bilirubin (40 nm), a 5-fold increase in MRP1 immunofluorescence was detected within a 30–120 min time frame. The inhibition of MRP1 in astrocytes resulted in increased bilirubin-induced damage (Gennuso et al., 2004). This demonstrates that intracellular MRP1 may serve as a reservoir, where cell surface expression may be conferred rapidly when required.

ABC-transporters in membrane vesicles not only can actively sequester drugs within cells, but also outside cells, before they even reach their target. Our group recently reported the inside-out orientation of P-gp within circulating extracellular vesicles, enabling an influx of doxorubicin opposed to typical efflux mechanisms (Gong et al., 2013). The result is an overall reduction in intracellular accumulation of drugs, representative of an additional parallel pathway in MDR. It is likely that similar sequestration mechanisms occur with MRP1; however, this remains to be elucidated.

Mechanisms of action

The structure of typical ABC-transporters is comprised of two conserved NBDs (NBD_1, NBD_2) and two TMDs (TMD_1, TMD_2). The two TMDs work collaboratively to form a permeable pore in which substrates may travel from the cytoplasmic region to the extracellular space in a selective and energy-dependent manner (Rees et al., 2009). These

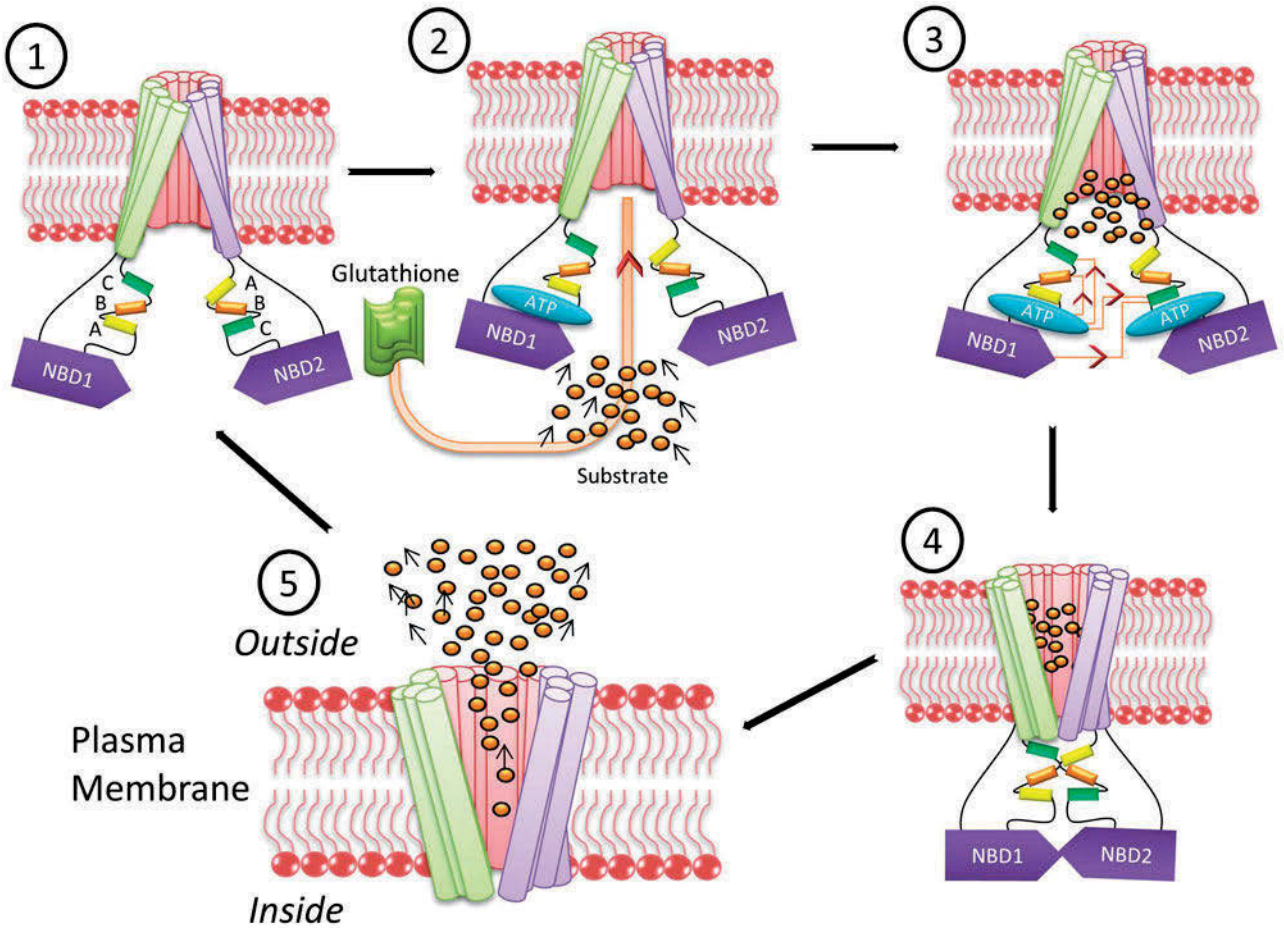

Figure 2. Multidrug resistance-associated protein 1 (MRP1) protein transport cycle. (1) MRP1 transport cycle begins in its resting state. (2) A substrate conjugated to GSH or GSH alone binds to a high-affinity site causing conformational changes, resulting in the recruitment of ATP to NBDs. (3) The two NBDs dimerize in a head to the tail manner, causing further conformational changes in the TMDs. (4) The closing of the NBDs triggers a flipping of the TMDs from ''right-side out'' to ''inside-out.'' (5) This allows the bound substrate to be moved into the extracellular environment and released. ATP is hydrolyzed into ADP, the NBD dimer is destabilized and the TMDs are returned to resting state (1).

ABC-transporters derive their energy through the hydrolysis of ATP after binding to their designated NBDs. The NBDs on all ABC-transporters are composed of three highly conserved amino acid sequences; Walker A, B and C (Chaptal et al., 2014). Walker A and Walker B motifs are required for ATP-binding and hydrolysis reactions, respectively, and are present on all ATP-binding proteins (Chaptal et al., 2014). The Walker A motif binds to the α- and γ- phosphates of di- or tri-nucleotides and the Walker B motif helps manage Mg^{+} ions. Walker C (LSGGQ) is a signature to ABC-transporters and has been proposed to be important for NDB_1 and NDB_2 dimerization (Smith et al., 2002).

The most accepted transport cycle is as follows (Chaptal et al., 2014; Hollenstein et al., 2007; Manciu et al., 2003; Smith et al., 2002) (Figure 2): First the substrate, which may be conjugated to glutathione (GSH) or GSH alone, binds to a high-affinity site on MRP1. This interaction, in turn, induces conformational transformations, which causes recruitment of ATP to the NBD_1. This causes further conformational changes and interaction of NBD_1 with Walker C signature of NBD2, which recruits a second ATP molecule NBD_2. The two NBDs are arranged in a head facing tail manner with the two ATP molecules positioned in-between. The ATP interacts with the Walker A and B motifs of one NBD and the C signature of the other domain (Smith et al., 2002). The dimerization of the two NBDs causes conformations changes in the TMD. This ''closing'' of the two NBD domains is the key to the unidirectional movement of substrates across membranes. The closing of the NBD helices appears to trigger a flipping of the TMDs from a ''right-side out'' to an ''inside-out'' conformation. In this conformation, the substrate is moved to a low-affinity state and is released into the environment (Amram et al., 2014). The transport cycle concludes with the hydrolysis of ATP and the release of ADP, which destabilizes the NBD dimer and returns the protein into resting state, ready to recruit new substrates for export.

As mentioned, MRP1 is atypical of ABC-transporters where it possesses an additional TMD connected to TMD_1 by an intracellular linker region known as L_0. Deletion of the atypical TMD_0, however, did not adversely affect transporter function (Bakos et al., 1998), although it is believed to play a role in localization (Westlake et al., 2004, 2005), with a significant amount of MRP1 lacking TMD_0 found in subcellular organelles (Bakos et al., 1998; Westlake et al.,

2005). These studies established that TMD_0 plays an important role for the cell surface expression of MRP1, however, the reasons why remain less clear. It is possible that the TMD_0 may be essential for plasma membrane retention and its loss accelerates the endocytosis of MRP1.

Relevance of MRP1 to drug disposition

The ABC efflux transporters play a major part in drug distribution. MRP1 is found in most tissues, with elevated amounts in organs such as testes, lung, skin, skeletal muscles, heart, kidneys and small intestines, which are important pharmacological barriers in drug absorption and elimination. In addition to restricting absorption and substantially reducing the bioavailability of drugs, MRP1 also determines drug elimination into urine and feces (Mueller et al., 2010; Plasschaert et al., 2005; Wijnholds et al., 1997).

Knockout mice ($MRP1^{-/-}$) have greatly contributed to our understanding of the role of MRP1 on drug disposition and the compensatory responses to xenobiotic toxicity. The first generation of MRP1 deficient mice demonstrated its importance as a drug transporter and urged the development of MRP1 modulators (Wijnholds et al., 1997). It was found that $MRP1^{-/-}$ mice were hypersensitive to etoposide treatment; however, the tissue distribution of radiolabeled drug remained unchanged. It was later found that this result was consistent with the distribution of 17-β-estradiol-D-17β-glucuronide, vincristine and doxorubicin across the blood-brain barrier (BBB) in $MRP1^{-/-}$ mice (Jungsuwadee et al., 2012; Lee et al., 2004). This resistance may be a result of intracellularly localized MRP1. In 2003, a group showed that intracellular localized MRP1 can confer MDR (Rajagopal & Simon, 2003). Using a cell impermeable inhibitor, the group showed that doxorubicin was actively sequestered within lysosomes and prevented its cytotoxicity. Therefore, it is unsurprising to observe no changes in tissue distribution of drugs along with MDR when targeting MRP1. It should be pointed out, however, that the kinetic difference between murine and human may be substantial and should be taken into consideration. For instance, some anthracyclines are not substrates for murine MRP1, making their disposition irrelevant in humans (Stride et al., 1997).

MRP1 and the heart

Cardiomyopathy is a serious condition attributed by the dose-dependent toxicity of drugs used in chemotherapy. Anthracyclines are among the most successful antineoplastics used in chemotherapy; however, they are limited by their cardiotoxicity. The cardioprotective role of MRP1 has been demonstrated in a number of studies (Krause et al., 2007; Semsei et al., 2012). MRP1 serves to protect the heart during chemotherapy by the efflux of toxic products causing oxidative stress from mitochondrion and cardiomyocytes (Krause et al., 2007; Semsei et al., 2012). These knockout mice studies have established the inherent role MRP1 plays in protecting the heart from xenobiotics.

MRP1 and the brain

A poor prognosis in brain cancer is partly due to the inaccessibility of chemotherapeutics due to the BBB. MRP1 is constitutively expressed at a relatively high level in the BBB and serves to prevent drug uptake into the brain. Using probenecid and MK571, which are MRP family selective inhibitors, MRP1 was shown to play a fundamental intrinsic role in chemoresistance at the human BBB (Brandmann et al., 2012; Spiegl-Kreinecker et al., 2002). These MRP-specific inhibitors were shown to have a sensitizing effect on all primary brain tumors. Normal astrocytes and gliomas displayed very little to no P-gp expression and high MRP1 levels, indicative of its constitutive role as a chemoprotectant. Not only does MRP1 prevent drug permeability, but the presence of a given substrate will also stimulate export of GSH, subsequently regulating intracellular GSH levels (Brandmann et al., 2012; Tadepalle et al., 2014). This may have consequences on GSH homeostasis and/or GSH-dependent detoxification pathways in the brain and contribute to neurotoxicity.

Clinical correlations of MRP1 in cancer

MRP1 is overexpressed in a diverse range of cancers such as, but not limited to, acute lymphoblastic leukemia (ALL), breast, acute myeloid leukemia (AML), tongue, brain, pancreatic, prostate, non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC) (Bakos & Homolya, 2007; Lu et al., 2013; Munoz et al., 2007; Nies et al., 2004; Schaich et al., 2005; Sullivan et al., 2000; Taheri & Mahjoubi, 2013; Zhang et al., 2012). However, clinical correlations of MRP1 overexpression are reported to be extremely variable amongst tumor types, with some malignancies more controversial than others (Table 1).

Breast cancer

The clinical prognostic value of MRP1 remains debatable in breast cancer, despites its well-established capacity to confer MDR *in vitro* (Tamaki et al., 2011; Winter et al., 2013). Conflicting reports have emerged linking MRP1 to clinical outcome, with some studies finding no association with therapeutic response (Larkin et al., 2004; Legrand et al., 1999; Leith et al., 1999; Nooter et al., 1997; van der Kolk et al., 2001). In locally advanced breast cancer tumors, 80% of patients exhibited MRP1 positivity; however, there was no significant correlation to clinical responsiveness after treatment (Atalay et al., 2006). This result is consistent with a recent study which report little to no ‘‘patient disease-free survival’’ predictive value in MRP1 expression pre- and post-chemotherapy treatment for breast cancer patients (Kim et al., 2013). In the study, a consistently low MRP1 expression was detected pretreatment and modest up-regulation post-treatment was observed (Kim et al., 2013). Interestingly, the same group had earlier reported a high predictive value for MRP1 when pretreatment expression of MRP1 was consistently high (Larkin et al., 2004). This discrepancy may be owing to the difference in patient cohorts and variations in methodology.

In an early-stage breast cancer study examining the efficacy of cyclophosphamide, methotrexate, fluorouracil treatment with increasing expression of MRP1, a strong association with OS (Filipits et al., 2005) was observed. Using immunostaining of tissue sections, MRP1 expression was independently predictive of reduced OS and relapse-free

Table 1. Clinical correlations of MRP1 in cancer.

Tumor	Analysis technique	Shown association with OS	Notes	References
Breast cancer	Immunostaining	Controversial	May be a strong prognostic marker when inherently expressed pre-chemotherapy	Atalay et al., 2006; Filipits et al., 2005; Larkin et al., 2004; Legrand et al., 1999; Leith et al., 1999; Nooter et al., 1997; van der Kolk et al., 2001
Non-small cell lung carcinoma	Immunostaining RT-PCR	No Yes	Expression declines with progression of disease	Berger et al., 2005; Diestra et al., 2003; Huang et al., 1998; Li et al., 2010; Ota et al., 1995
Ovarian cancer	Immunostaining RT-PCR	No Yes	May be a useful marker to determine tumor aggressiveness	Bagnoli et al., 2013; Faggad et al., 2009; Sedlakova et al., 2013
Soft-tissue sarcoma	Immunostaining RT-PCR	Yes Yes	Expression is correlated with higher grade/advanced tumors	Citti et al., 2012; Komdeur et al., 2003; Martin-Broto et al., 2014; Oda et al., 1996; Villar et al., 2013
Neuroblastoma	Immunostaining	Yes	May be the primary mode of MDR in neuroblastoma	Alisi et al., 2013; Bordow et al., 1994; Haber et al., 1999, 2006; Lu et al., 2004; Manohar et al., 2004
Acute leukemia	Immunostaining RT-PCR	Controversial Yes	May be a useful marker to determine tumor aggressiveness	Mahjoubi & Akbari, 2012; Dransfeld et al., 2013; Gurbuxani et al., 2001; Laupeze et al., 2002; Leith et al., 1999; Mahjoubi & van der Kolk et al., 2002; Plasschaert et al., 2005; Schaich et al., 2005; van der Kolk et al., 2001; Winter et al., 2013

survival (RFS). Conversely, MRP1 negative patients had a comparatively significant reduction in relapse rate following the same treatment (Filipits et al., 2005). Collectively, these findings suggest that in breast cancer, MRP1 may be a strong prognostic marker for OS and RFS when inherently expressed in high levels pre-chemotherapy.

Non-small cell lung carcinoma

In NSCLC, MRP1 was found to be consistently overexpressed in a large patient population prior to treatment (Berger et al., 2005). Although overexpression of MRP1 resulted in tumors being resistant to chemotherapy, it was observed that these tumors were less aggressive and tended to be more differentiated (Berger et al., 2005). Some studies have reported a better survival rate in NSCLC patients with highly expressed MRP1, especially in those not pretreated with chemotherapeutics before surgical resection (Berger et al., 2005; Diestra et al., 2003; Huang et al., 1998). It was found that MRP1 expression seems to be prevalent in the early stages of the disease but declines with progression with the upregulation of P-gp (Berger et al., 2005). This may possibly mean that in NSCLC, MRP1 is an inherent protective mechanism as the cell adapts during disease progression. A significant predictive value for high MRP1 expression has been reported in locally advanced NSCLC (Li et al., 2010). Using quantitative or semi-quantitative RT-PCR, the overexpression of MRP1 was associated with a reduced OS (Li et al., 2010; Ota et al., 1995). However, using immunohistochemical staining, another study showed expression of MRP1, as well as P-gp and MRP3, had no correlation with OS or response to chemotherapy in NSCLC (Yoh et al., 2004).

Ovarian cancer

MRP1 may be a marker for aggressiveness as its overexpression is associated with tumor grade in ovarian cancer (Bagnoli et al., 2013; Faggad et al., 2009; Sedlakova et al., 2013). However, unlike NSCLC, less differentiated cells were found to express elevated levels of MRP1 (Bagnoli et al., 2013). As less differentiated cells have the greatest proliferation potential, they tend to be more aggressive. The overexpression of MRP1 in ovarian cancer was not associated with a reduced OS (Bagnoli et al., 2013; Faggad et al., 2009; Sedlakova et al., 2013). However, MRP1 may be a useful marker to determine tumor aggressiveness in ovarian cancer.

Soft tissue sarcoma

In malignancies such as soft tissue sarcoma (STS), MRP1 expression was found to be a negative prognostic marker (Martin-Broto et al., 2014). Indeed, studies have found selective inhibition of MRP1 sensitized cellular models of STS to doxorubicin (Villar et al., 2013). The first evidence demonstrating a significant negative prognostic relationship in MRP1 overexpression between RFS and OS in high-risk sarcoma emerged from a phase III clinical trial in 2013 (Martin-Broto et al., 2014). Patients were treated with a chemotherapeutic regimen of epirubicin and ifosfamide, followed by surgical resection. Epirubicin is a substrate of both P-gp and MRP1; however, ifosfamide is only extruded by MRP1 upon conjugation to GSH via glutathione-S-transferase (GST). The study showed that overexpression of MRP1 and increased *ABCC1* transcripts led to a significantly reduced 5-year RFS rate and OS. The combined *ABCC1*/MRP1 transcript and protein overexpression was shown to be the only independent prognostic marker in high-risk STS patients (Martin-Broto et al., 2014).

MRP1 was also correlated with tumor grade and type in STS. Expression of MRP1 correlated with higher grade/more advanced tumors (Citti et al., 2012; Komdeur et al., 2003). Interestingly, these higher grade tumors were also found to co-express both MRP1 and P-gp, with increased expression of

both transporters after treatment (Citti et al., 2012; Komdeur et al., 2003; Oda et al., 1996). This suggests that in STS, a functional redundancy exists where chemotherapeutics may be effluxed by both MDR transporters. This may provide the tumor with an additional survival mechanism, for instance, in the event where one transporter is repressed or inhibited, the other may extrude drugs in its place.

Neuroblastoma

In neuroblastoma, there has been much compelling evidence indicating a strong association of MRP1 with a negative clinical outcome. A retrospective study showed that MRP1 expression was found in 209 neuroblastoma samples (Haber et al., 2006). Multivariate analysis revealed that a greater level of expression was highly predictive of a lower RFS and OS. The study provided evidence of MRP1 as a strong independent prognostic indicator in neuroblastoma. Interestingly, the same findings were not found with P-gp/*ABCB1*, where expression did not predict clinical outcome (Haber et al., 2006). This suggests that MRP1, not P-gp, is the primary mode of MDR in neuroblastoma.

The *MYCN* oncogene is the most well-known marker in neuroblastoma with prognostic values associated with tumor aggressiveness, advanced clinical stage and a negative outcome (Domingo-Fernandez et al., 2013; Kuss et al., 2002; Schulte et al., 2013). Much evidence has been gathered to establish the link between MRP1/*ABCC1* and *MYCN* expression (Alisi et al., 2013; Bordow et al., 1994; Haber et al., 1999,2006; Manohar et al., 2004). P-gp expression did not exhibit any correlation between *MYCN* expression nor showed any prognostic significance in neuroblastoma (Haber et al., 2006). Indeed, the induction of MYCN in a neuroblastoma cell line also caused an increase in *ABCC1* mRNA as well as MRP1 protein, while antisense oligonucleotides directed toward *MYCN* had also downregulated *ABCC1* (Manohar et al., 2004). This reinforces a regulatory coordination between *MYCN* and MRP1 expression, providing a mechanistic link between tumor aggressiveness and drug resistance. This indicates that MRP1 is an important factor in mediating the clinical behavior of the tumor. Another study examined MRP1 and P-gp in 70 untreated neuroblastoma patients and found only MRP1 to be a negative prognostic marker (Lu et al., 2004). Taken altogether, MRP1 may be the primary MDR mechanism in neuroblastoma, and overexpression at any stage would lead to a poor clinical outcome.

Acute leukemia

There is still much controversy as to whether MRP1 plays a role in acute leukemic MDR. Many studies show compelling evidence of a prognostic association (Schaich et al., 2005; Winter et al., 2013), while some show no influence of MRP1 expression before or after drug treatment (Leith et al., 1999). A number of clinical studies in AML and ALL have reported an increased probability of disease-free survival in MRP1 negative patients (Laupeze et al., 2002; Schaich et al., 2005; Winter et al., 2013). Indeed, MRP1 mRNA levels were found to be significantly higher in patients after relapse, indicating that MRP1 expression was an induced response to treatment (Gurbuxani et al., 2001; Mahjoubi & Akbari, 2012; Plasschaert et al., 2005; van der Kolk et al., 2002). To date, MRP1 overexpression is a significant independent prognostic indicator for reduced OS in acute leukemia.

The expression of MRP1 in acute leukemia patients appears to have a differential prognosis on a diverse range of patient cohorts. Three single nucleotide polymorphisms (SNPs) in MRP1, rs129081, rs212090, rs212091 have been reported to have a significant influence on OS in AML patients (Dransfeld et al., 2013; Laupeze et al., 2002). The SNPs may explain discrepancies in some clinical associations of MRP1 with AML.

Similarly with ovarian cancer, a strong association MRP1 expression and maturation stage of AML has been identified, with less differentiated cells displaying higher activity (van der Kolk et al., 2001). This finding would indicate that the expression of MRP1 would depend on the maturation stage of AML.

ABCC1 pharmacogenetics and clinical controversy

The variability that exists in the association of MRP1 and clinical outcomes has been proposed to be due to a lack of a ''gold standard'' in analytical methodology for the screening of ABC-transporter expression in clinical samples (Berger et al., 2005). Furthermore, differences in patient properties may affect individual studies, such as disease progression or patient age. In addition, the availability of quality tumor samples suitable for analysis may be limited. Another reason may be the impact of genetic variations. Polymorphisms in MRP1/*ABCC1* will contribute to the variability in patient response observed via a variety of mechanisms (Kerb et al., 2001). One study which supports this has identified the presence of an SNP (MRP1/R723Q) to be associated with a longer time to disease progression and OS in melanoma patients (Buda et al., 2010). Furthermore, another report identified three SNPs in MRP1/*ABCC1* which are predictive of severe adverse hematological events associated with fluorouracil, epirubicin, cyclophosphamide (FEC) chemotherapy (Vulsteke et al., 2013). Finally, in ALL, two of nine identified polymorphisms were found to be associated with lower left ventricular fractional shortening after chemotherapy, which results in left ventricular dysfunction owing to genetic variants in *ABCC1* (Semsei et al., 2012). In addition, knockout mice have revealed the disposition of the reactive electrophile 4-hydroxy-2-trans-nonenal (HNE) by MRP1 generated by doxorubicin treatment. HNE is one of the primary and highly toxic products in lipid peroxidation (Jungsuwadee et al., 2012). The *ABCC1* polymorphism (G671V; rs45511401) resulted in a decrease in MRP1-dependent HNE efflux and increased doxorubicin cardiotoxicity (Jungsuwadee et al., 2012). Consequently, these genetic variants which impact on the expression or function of MRP1 may construe its clinical relevance in some studies.

Nonetheless, the value of MRP1 as a prognostic marker should not be underestimated, given the strong evidence for its role in MDR (Li et al., 2010). MRP1 remains one of the most interesting molecular markers associated with drug resistance and is still a strong potential candidate for inhibition in circumvention of MDR in the range of malignancies.

Substrates

Despite the substantial functional redundancy between the P-gp and MRP1, there are also clear distinctions in their patterns of substrate specificity (Seelig et al., 2000). P-gp typically translocates neutral or positively charged, hydrophobic and amphipathic compounds (Sun et al., 2004). In contrast, MRP1 also shows specificity toward organic anions such as leukotriene C4 (LTC_4) and GSH-conjugated xenobiotics (Seelig et al., 2000). MRP1 has perhaps the widest variety of substrates of all the human ABC-transporters.

Cells that overexpress MRP1 are resistant to a wide variety of drugs. These antineoplastics include anthracyclines, camptothecins, antimetabolites, epipodophyllotoxins and vinca alkaloids. However, unlike P-gp, this list excludes taxanes. Additionally, unlike P-gp, MRP1 requires a physiological amount of GSH to function as a transporter (Loe et al., 1996). It was once thought that only GSH-conjugated substrates may be transported by MRP1 (Jedlitschky et al., 1996). However, the role of GSH in MRP1 has been established to be more dynamic and complex than originally thought. For some compounds, such as vincristine and daunomycin, it has been shown that GSH conjugation is not necessary, rather, the unmodified substrate may be co-transported with free GSH (Rothnie et al., 2006). In another scenario, some compounds, such as estron-3-sulfate and NNAL-*O*-gluc, may be transported without GSH being transported as a substrate, rather, an allosteric interaction with GSH is required for transport (Manciu et al., 2003). Conversely, some compounds such as verapamil or flavonoids, stimulate the transport of GSH, without being transported themselves (Leslie et al., 2003; Loe et al., 2000; Romermann et al., 2013; Salerno et al., 2004). GSH alone, has a high K_m value (>1 mm) when transported by MRP1 (Leslie et al., 2001a), while LTC_4 alone remains the highest affinity substrate known (K_m value, 100 nm)(Cole & Deeley, 1998). The mechanisms that lead to the requirement or transportation of one or both compounds still remain largely unknown. It has also been suggested that an increase in MRP1 expression without an increase in GSH biosynthesis can lead to cell death (Akan et al., 2005).

Inhibitors

P-gp was implicated in causing MDR more than 35 years ago and has been the primary focus in this field of study and the search for clinical P-gp modulators is currently in its third generation (Callaghan et al., 2014). Despite improvements to potency and toxicity, some inhibitors are still prone to poor solubility, adverse side-effects and undesirable pharmacokinetic interactions with chemotherapeutic agents (Falasca & Linton, 2012). Since MRP1 has only more variability in its clinical relevance, the search of its inhibitors remain in its infancy. To date, there are no clinically valuable transporter inhibitors, and efforts to develop an ideal candidate compound are still ongoing. Due to structural and functional similarities, this led early researchers to believe that P-gp inhibitors have the same effect on MRP1 (Boumendjel et al., 2005; Germann et al., 1997). The screening of P-gp inhibitors found some potential candidates such as the pipecolinate derivative, VX-710 (biricodar) (Loe et al., 2000) with dual activity. However, most P-gp chemosensitizers have no effect on MRP1, possibly owing to the different preference in substrates.

The search for small molecules that selectively and potently interact with MRP1 proves to be much more difficult than for P-gp. This may be due to the substrate preference of anionic compounds which do not enter cells efficiently (Bakos et al., 2000). As a result, higher dosages may be required to achieve necessary intracellular concentrations for inhibition, which in turn would result in unacceptable toxicity *in vivo*. Furthermore, their overall specificity has yet to be defined (Boumendjel et al., 2005). A variety of inhibitors have been described, however, none have entered into clinical trials to specifically and selectively inhibit MRP1. General organic anion inhibitors such as probenecid, sulfinpyrazone and indomethacin have been used *in vitro* to inhibit MRP1. However, their inhibition of all organic anion importers and exporters prevents their utility clinically as specific MRP1 inhibitors (Boumendjel et al., 2005; Liu et al., 2010).

One of the first specific inhibitors of MRP1 was MK571 discovered in 1995 (Gekeler et al., 1995). MK571 is a highly potent leukotriene (LTD_4) receptor antagonist with reported specificity toward transporters in the MRP family. However, toxic therapeutic concentrations restricted it from entering clinical trials to inhibit MRP1. It was later found that MK571 had inhibitory capacity against P-gp, BCRP and MRP1, which limits its utility as an MRP1-specific inhibitor (Matsson et al., 2009).

Flavonoids have been largely explored as P-gp modulators (Huang et al., 2007; Tran et al., 2011). Many flavonoids such as quercetin, biochanin A, apigenin, chrysoeriol, morin, naringenin, myricetin, tamarixetin, galangin, baicalein, genistein and silymarin have been shown to possess MRP1 inhibitory activity (Li & Paxton, 2012). These flavonoids have a diverse mechanism of action and can interact with many physiological pathways. Most of the flavonoids explored so far are all naturally occurring food or plant compounds with only a few synthetics investigated (Li & Paxton, 2012). They interact with MRP1 by docking with the binding site either allosterically, non-competitively, competitively or prevent/interfere with ATP hydrolysis (Li & Paxton, 2012). A number of flavonoids have been tested for MRP1 modulation and some have proven to be more potent than others. The synthetic compound flavopiridol has been introduced as an MRP1 inhibitor and has shown high potency against other (iso)flavonoids (Hooijberg et al., 1999; Versantvoort et al., 1993).

There is some evidence of indirect inhibition of MRP1 by the flavonoids by diminishing or depleting cellular GSH (Lu et al., 2013; Nguyen et al., 2003). The flavonoids, genistein, biochanin A, quercetin, silymarin, chalcone and phloretin, all reduce intracellular GSH and possibly explain their modes of action on MRP1 (Kachadourian & Day, 2006). Since GSH is not required for substrate transport by P-gp, the inhibitory effect may be restricted to MRP1 (Cole et al., 1990). However, these compounds may interfere with the physiological role of GSH as a detoxifier of reactive oxygen species (Deshmukh et al., 2013). It should be noted that flavonoids should be considered individually as opposed to a class of compounds as small changes in structure lead to an altered

mechanism of action and unique interactions with MRP1, or other ABC-transporters (Di Pietro et al., 2002; Leslie et al., 2001b; Morris & Zhang, 2006).

Among the most promising classes of inhibitors are isoxazole-based compounds (Norman et al., 2005). These compounds were first identified as MRP1 inhibitors in 2002 from a high-throughput screening program by Eli Lilly (Norman et al., 2002). The most potent and specific inhibitors identified were LY402913 and LY475776 (Norman et al., 2002). These two compounds were found to inhibit MRP1-mediated LTC_4 transport in a GSH-dependent manner and reverse MDR *in vitro.* However, these studies later revealed that LY475776 exhibited P-gp inhibition (Dantzig et al., 2004). LY402913 re-sensitized Hela-T5 cells to doxorubicin without any inherent cytotoxicity (Norman et al., 2002). In addition, LY402913 showed high selectivity (around 22 fold) for MRP1 over P-gp. Furthermore, the study also found that LY402913 delayed the growth of MRP1-overexpressing tumors *in vivo* (Norman et al., 2002). This compound may be the most selective and potent compound for MRP1 modulation. However, there currently remains no clinical report of its use to inhibit MRP1.

In some cancer types such as leukemia and STS, ABC-transporters may be co-expressed, leading to the requirement for the inhibition of multiple transporters simultaneously (Brock et al., 1995; Legrand et al., 1999; Tang et al., 2004; Wang et al., 2002). Some studies have reiterated the need for the inhibition of both MRP1 and P-gp clinically for successful treatment (Teodori et al., 2006; Wang et al., 2002). Although, this may be a double-edged sword, with inhibition of additional ABC-transporters at pharmacological barriers increasing the bioavailability of chemotherapeutics drugs to normal tissues, leading to increased toxicity and adverse effects.

Currently, there is no compound that represents a clinically viable candidate for the selective and potent modulation of MRP1. Pharmacophore mapping studies provide an insight into the potential of different chemotypes on MRP1 modulation (Pajeva et al., 2009). These studies provide valuable information for the design of better MRP1 analogs. However, further research is required to determine the practicality of MRP1 inhibition in a clinical setting.

Microparticles and cancer MDR

Microparticles (MPs) are small membranous vesicular structures shed from all eukaryotic cell types upon activation, stress, proliferation or apoptosis (Bebawy et al., 2013; Gong et al., 2012; Mause & Weber, 2010). Unlike circulating membranes derived from the endosomal pathway, such as exosomes, MPs originate from the plasma membrane (Mause & Weber, 2010). As MPs shed, they package cellular cytoplasm/membranes and as a result, their cargo consists of proteins, lipids, nucleic acids, antigens and bioactive molecules from their parental cell (Diehl et al., 2012; Jaiswal et al., 2012a; Lu et al., 2013). MP cargo may be horizontally transferred between cells at distal sites (Bebawy et al., 2009; Distler et al., 2006; Gong et al., 2012). Their dissemination of cargo into a target cell causes functional changes (Bebawy et al., 2009).

Indeed, MPs play an intricate and versatile role in cancer MDR and metastasis resulting in treatment failure. A recent study has found that innate immune cell-derived MPs may play a role in the induction of hepatocarcinoma metastasis and possibly immune escape (Ma et al., 2013). It was found that tumor cells acquiring immune-related integrins from shed MPs had acquired an immune cell migratory phenotype. This was due to the transfer of $\alpha_M\beta_2$ (CD11b/CD18) integrin's which normally mediate immune cell migration, as blocking these integrins led to a significant decrease in MP-mediated metastasis. Also, the facilitation of cancer immune escape is hypothesized by the authors as immune-suppressive mechanisms may also have been transferred. The findings provide further insight into tumor metastatic mechanisms and provide another facet for the role of MPs in cancer survival.

In 2009, we reported the transfer of P-gp mediated MDR in a cancer cell population via MPs (Bebawy et al., 2009). MPs shed from P-gp overexpressing cells were found to package functional P-gp and were able to disseminate it into drug-sensitive cells, conferring MDR. This study revealed for the first time a rapid, non-genetic modality for the transfer of MDR. The transfer of functional P-gp into recipient cells provided evidence for an initial survival mechanism whilst the cell adapts itself for more permanent mechanisms of survival.

We subsequently provided evidence of MRP1-mediated MDR being conferred in a similar manner (Lu et al., 2013) (Figure 3). However, we reported a kinetic difference in functionality between acquired MRP1 and P-gp when transferred onto recipient cells via the MP vectors. We established that MPs confer functional P-gp mediated MDR at 4 h post MP exposure, whilst MRP1 functionality was observed at 12 h post MP exposure (Lu et al., 2013). This reveals a mechanistic difference between the acquired ABC-transporters.

Regulatory proteins present in recipient cells may also play a functional role on the MP-mediated transfer of MRP1 and its transcripts. NOTCH1 is a transmembrane protein that regulates normal cell development (Schroeter et al., 1998; Weng et al., 2004). The expression of NOTCH1 has recently been found to play a role in the upregulation of MRP1/*ABCC1* transcript (Cho et al., 2011). Subsequent studies have also found a functional relationship between the two proteins (Lu et al., 2012). In fact, MRP1-mediated drug resistant prostate cancer cell lines have been chemosensitized by silencing of NOTCH1 by shRNA (Liu et al., 2014). The mechanistic as well as a regulatory association to this transmembrane protein may explain the time difference in functionality as well as identify it as a possible target for specific MRP1/*ABCC1* inhibition.

The MP-mediated transfer may also result in intracellular localized MRP1. As aforementioned, MRP1 may localize subcellularly in organelles to provide additional xenobiotic protection or as a reserve. One study has found that depletion of GSH enhanced drug permeability to the mitochondrion, resulting in cell death (Armstrong & Jones, 2002). Further investigations into the trafficking and translocation of acquired MRP1 may further explain the time discrepancy.

Acquisition of MP traits by cell population

Our recent studies have demonstrated a proteome and transcriptome re-templating effect of MP-transfer (Jaiswal

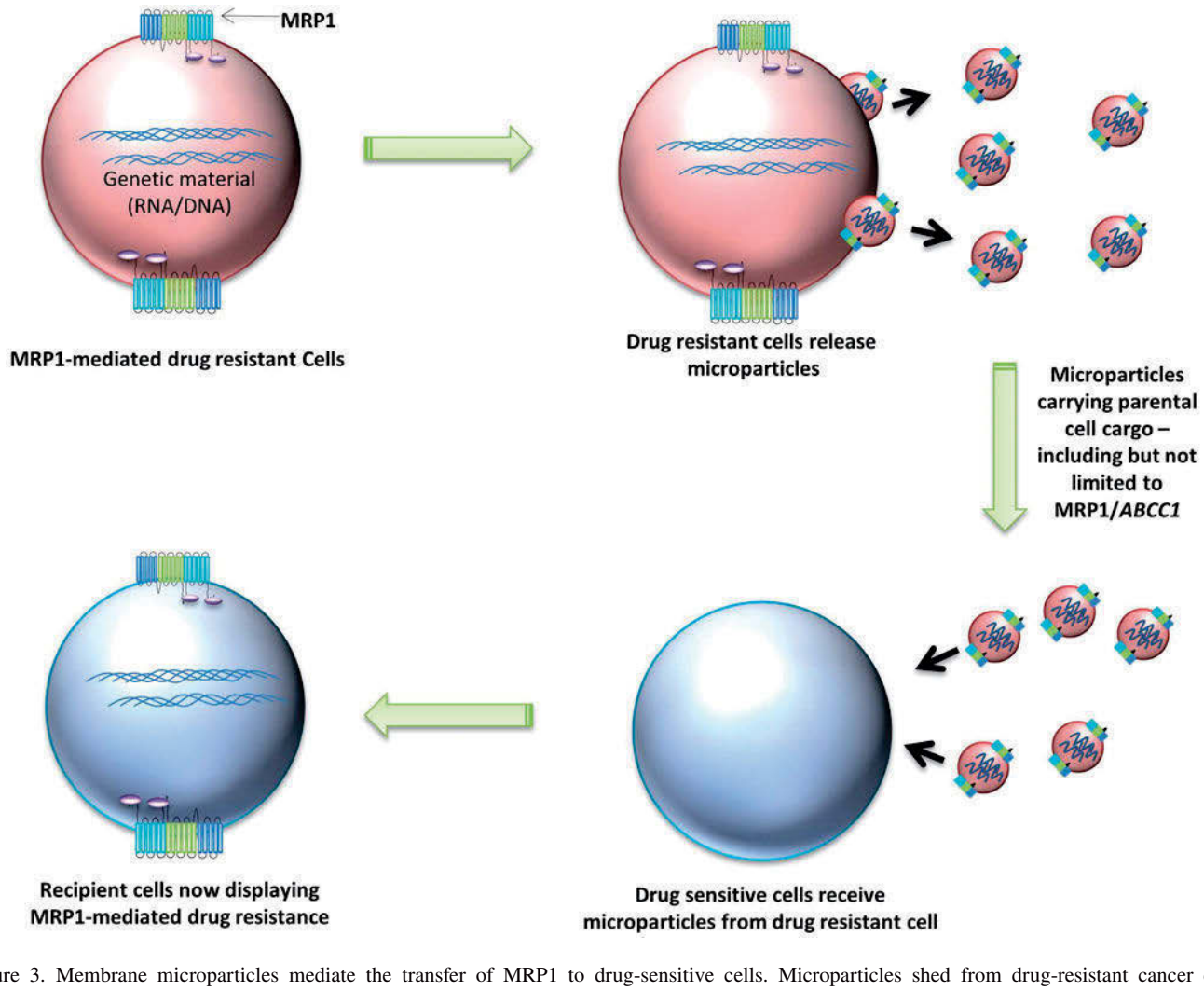

Figure 3. Membrane microparticles mediate the transfer of MRP1 to drug-sensitive cells. Microparticles shed from drug-resistant cancer cells incorporate cell surface MRP1 and transcripts from their donor cells. These membrane vesicles bind to drug-sensitive recipient cells and transfer functional MRP1, conferring MDR.

et al., 2012a, b; Lu et al., 2013). Upon exposure to MPs shed from a P-gp overexpressing ALL cell line, MRP1 overexpressing recipient ALL cells were re-templated to reflect donor-MP MDR phenotype (Lu et al., 2013). The resulting population after co-culture with MPs exhibited suppressed endogenous transporter transcript and an immediate increase in donor-MP transporter transcript. Although the cell phenotype is directed toward a single phenotype (donor-MP phenotype), the resultant cell population still possess a significant amount of mRNA coding for both phenotypes.

As mentioned above, some tumors express more than one MDR conferring ABC-transporter including the simultaneous expression of MRP1 and P-gp in some cancer cells (Brock et al., 1995; Legrand et al., 1999; Tang et al., 2004; Wang et al., 2002). This has prompted pharmacological attempts for the dual inhibition of transporters such as MRP1 and P-gp simultaneously to circumvent MDR. Given our recent findings, the expression of both transporters on a single tumor may also be the result of MP-transfer between two overexpressing phenotypes, resulting in a population expressing dual transporters in the immediate term.

Molecular mechanisms of phenotype alteration

The molecular mechanism for the re-templating of recipient cells following MP transfer still remains to be elucidated. One likely possibility is the packaging of functional transcripts, transcription factors and regulatory nucleic acids such as microRNAs (miRNAs), long non-coding RNAs and small nucleolar RNA into MP vectors. MPs are an emerging source of miRNAs and non-coding RNAs in blood circulation (Dinger et al., 2008; Jaiswal et al., 2012b; Kosaka et al., 2010). Degradation in the harsh circulatory environment is prevented by the encapsulation of RNAs in microvesicles (MVs) such as exosomes and MPs (Dinger et al., 2008).

Indeed, MPs have been established as a carrier of cancer-associated molecules and intact, functional oncogenic RNA species. The total RNA found in MPs represent a snapshot of the parental cell transcriptome (Diehl et al., 2012; Jaiswal et al., 2012b; Li et al., 2013; Lu et al., 2013). As a result, MPs play a fundamental role in homeostasis as well as aberrant biological functions through modulation of gene regulation (Gong et al., 2012; Laffont et al., 2013; Morel et al., 2008).

Translation and detection of a reporter mRNA found in glioblastoma-derived MVs in target human umbilical vein

endothelial cells (HUVEC) cells demonstrate a capacity for cancer cell-derived MVs to modulate the surrounding tumor microenvironment and alter the recipient cell proteome (Gonda et al., 2014). In fact, the same study had observed an MV-mediated stimulation of proliferation of the glioma cells, indicative of a self-promoting capacity. Indeed, these MVs have been identified to carry a unique and specially enriched profile of small RNAs, most of which currently possess unknown function (Li et al., 2013). The ability to carry cancer-associated molecules which promote proliferation, migration and survivability of surrounding tumor cells highlights the importance of shed MVs in cancer cell pathogenesis and biology.

miRNA as possible re-templating mechanisms

Mature miRNAs are 21–25 nucleotides in length and are completely or partially complementary to its target gene. They function to upregulate or suppress genes regulated by translational repression, mRNA cleavage or deadenylation. One of the first reports of miRNA encapsulation by exosomes was reported by Valadi et al. (Valadi et al., 2007). Their report had shown the existence of miRNA in exosomes released from human mast cells. Since then, reports have emerged detailing the regulatory effect of packaged miRNA in many physiological conditions (Brase et al., 2011). miRNA profiling has revealed their strong association with tumor metastasis, tumor suppression and MDR (Brase et al., 2011; Chan et al., 2005; Chen et al., 2008; Volinia et al., 2006).

Recent reports detected vesicle-free circulating miRNA and elucidated their potential to be a valuable minimally invasive biomarker for assessing disease states and characteristics (Cheng et al., 2013; Turchinovich et al., 2013; Williams et al., 2013). However, their release into circulation has been suggested to be due to a passive leak from apoptotic or broken cells, which mitigates their purpose as active messages (Chen et al., 2012). Also, unlike vesicular packaged miRNA, their integration or entry into target cells to affect their regulatory function has yet to be challenged. Currently, there is no evidence to suggest that non-vesicle-associated miRNA is readily absorbed by cells and is functional in its new location. Due to their proven integration and dissemination of cargo with target cells, membrane vesicles may still prove to be the main driving force for the transfer of miRNA, as well as other nucleic acids, between cells (Bebawy et al., 2009; Diehl et al., 2012; Gong et al., 2012; Lu et al., 2013; Valadi et al., 2007). It has been found that miRNAs can exist in the blood stream without association to membrane vesicles (Arroyo et al., 2011). Rather, free circulating miRNAs which were associated with Agonaute2 (ago2) were protected from nucleases and become susceptible when treated with proteinase K. This is indicative that ago2 has formed a nuclease protective complex with miRNA. However, the miRNA profiles differed significantly between free circulating miRNA and miRNA associated with vesicles (Arroyo et al., 2011; Laffont et al., 2013). This difference suggests that extracellular vesicles carry a preferentially packaged miRNA profile for a distinctive purpose.

The contribution of miRNAs to the overexpression or suppression of MRP1-mediated MDR has been explored in the past years. Currently, studies have identified miR-7 and miR-345 to have known specificity toward MRP1, with their expression resulting in the suppression of their target (Pogribny et al., 2010). Indeed, their down-regulation in tumor cells has been correlated with an MDR-phenotype mediated by MRP1 (Liang et al., 2010). These miRNAs have been identified as a possible target against MDR in cancer (Jaiswal et al., 2012a, b).

Conclusion

Studies on MPs have entered a phase of rapid progress, whilst progress on MRP1-mediated MDR circumvention still remains in its infancy. Indeed, its clinical prognostic impact still remains a debatable topic. Despite this, many approaches have been exploited to screen and identify new MRP1 inhibitors. To this end, none of which have been viable candidates in clinical oncology. However, given the amount of recent attention on this topic, one may expect more potent and specific compounds to emerge in the near future.

Current studies have revealed a sinister role of MPs as an additional mechanism of cancer cell survival via the transfer of MRP1. Given the known cargo and recipient cell response, MPs represent a useful reservoir of biological information on cancer-related processes. Exploitation of this information may lead to new oncological treatment targets, potent regulatory mechanisms, possible biomarkers and potential new paradigms in clinical oncology. Taken together, there is much progress already made and also progress yet to be made in the circumvention of MDR in cancer. These past few years have solidified the crucial and versatile role MPs play in cancer biology. It is only a matter of time and patience to improve our understanding and fully exploit this biological treasure trove.

Declaration of interest

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Author Contributions Statement – Chapter 1

J.F.L wrote the manuscript. D.P provided figures and revised the manuscript. M.B was responsible for overall project, conceptual advice and revised the manuscript. All authors have reviewed the manuscript.

Aims and Objectives

The four specific aims of this thesis include:

1. **To determine the transfer and functionality of MRP1 by MPs (Chapter 2).** Our group has previously reported the transfer of functional P-gp to recipient cells within 4 h, conferring MDR. This aim validates the transfer of functional MRP1 and its capacity to confer MDR in recipient cells.

2. **To establish that MP transfer confers dominance of donor-MP trait in recipient MDR cells (Chapter 2).** This aim investigates the capacity of MPs to re-template a pre-existing hyper-expressed MDR phenotype with donor-MP MDR traits.

3. **To verify transfer and translation of functional MP-mRNA in recipient cells (Chapter 3).** Having established the transfer and dominance of donor-MP transcripts, the functionality of these transcripts in its new cellular location is to be determined in this aim.

4. **To identify the transcript suppression mechanisms involved in MP-mediated trait dominance (Chapter 4)**. The objective of this aim is to elucidate the mechanisms involved in the suppression of recipient cell endogenous MDR transcripts upon transfer of MP cargo.

Hypothesis and Rationale

It was previously reported that large membrane spanning proteins P-gp can be transferred to drug sensitive recipient cells, causing MDR (1). This thesis builds on these findings by elucidating the phenotypic changes of recipient cells conferred by transferred nucleic acids. The mechanistic significance of this study is the discovery of a novel intercellular pathway regulating trait acquisition in cancer cells.

The overarching hypothesis proposes spontaneously shed cancer derived MPs serve as important mediators in the alteration of recipient cell phenotype by virtue of the horizontal transfer of various bioactive components. Specifically, MP transfer selects for the acquisition of the donor cell resistance phenotype, whilst suppressing alternate compensatory pathways endogenous to the recipient cells. As aforementioned, this may explain why in some cancers, the overexpression of ABC-transporters are correlated with clinical stage, with early stage tumours expressing one efflux transporter and substituted by another transporter at an advanced stage (2-4).

This study proposes to validate this hypothesis and identify and validate key MP mediators involved in the acquisition and suppression of the donor cell MDR trait in recipient cells. The study promises to lead to the discovery of a novel intercellular pathway regulating trait acquisition and dominance in cancer cells.

Of therapeutic significance is the potential prognostic application for circulating MPs to predict therapeutic response and guide individualized treatment strategies in cancer patients. This research has considerable potential for translation into clinical outcomes with the identification of new drug targets and alternate therapeutic strategies for the clinical prevention and circumvention of cancer multidrug resistance.

Chapter 2

2. Microparticles mediate MRP1 intercellular transfer and the re-templating of intrinsic resistance pathways

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Microparticles mediate MRP1 intercellular transfer and the re-templating of intrinsic resistance pathways

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ABSTRACT

Multidrug resistance (MDR) is a major impediment to the overall success of chemotherapy in clinical oncology. MDR has been primarily attributed by the ATP-dependent transmembrane proteins, P-glycoprotein (P-gp, *ABCB1*) and Multidrug Resistance-Associated Protein 1 (MRP1, *ABCC1*). These proteins maintain sublethal concentrations of intracellular chemotherapeutics by virtue of their drug efflux capacity. In this study, we report the acquisition and dissemination of functional MRP1 via microparticle (MP) mediated intercellular transfer. After we showed the transfer and functionality of P-gp in drug sensitive recipient cells, we report the transfer and time-dependent functionality of MRP1 in drug sensitive leukaemia cells following exposure to MPs shed by MRP1-overexpressing MDR cells. We also demonstrate a remarkable capacity for MPs shed from cells with a P-gp dominant resistance profile to re-template a pre-existing MRP1 dominant profile in recipient cells. These findings have significance in understanding the molecular basis for tumour dominant phenotypes and introduce potential new strategies and targets for the acquisition of MDR and other deleterious traits.

1. Introduction

Chemotherapy is the major treatment modality used in cancer management. Development of resistance to a range of structurally and functionally unrelated drugs in cancer remains a major obstacle to the success of chemotherapy [1]. More than 90% of patients with metastatic disease relapse and become unresponsive to treatment due to the development of multidrug resistance (MDR) [2,3].

MDR is multi-factorial, with pathways including activation of detoxifying agents, defective apoptotic pathways and decreased drug influx or increased efflux of drug from cells ultimately contributing to tumour cell evasion of the cytotoxic insult [2]. The development of MDR in cancer is clinically correlated with the overexpression of the efflux transporters P-glycoprotein (P-gp) or Multidrug Resistance-Associated Protein 1 (MRP1) in many malignancies such as lung, breast, neuroblastoma and prostate [4–6]. These transmembrane pumps belong to the ATP-binding cassette (ABC) super-family of membrane transporters. ABC-transporters hydrolyse ATP to drive the extrusion of chemotherapeutic drugs against a concentration gradient from otherwise drug sensitive cells. Consequently, there is a reduction in tumour cell death as a result of intracellular drug accumulation deficit [2,7–9]. MDR may be intrinsically present in tumours arising from epithelium with high constitutive P-gp expression [10,11]. Alternatively, MDR can be acquired after chemotherapy via pre- and post-transcriptional events, including gene amplification, increased transcription or increased trafficking [5,12,13]. P-gp and MRP1 mediated resistance can be induced by a similar repertoire of drugs [12,14,15]. However, the resulting acquired trait displays preference to one or the other [16]. Consequently there must exist a mechanism for the dominance of a given resistance trait. The dominance of a distinctive phenotype may provide an additional survival advantage to the tumour cell, whereby when one transporter fails due to inhibition or inability to efflux chemotherapeutics, a second transporter may be up-regulated as replacement.

Previously, we discovered a non-genetic modality for the acquisition of P-gp mediated MDR via microparticles (MPs) [1]. MPs are small membrane vesicles (0.1–1 μm in diameter) released from the plasma membranes of most cell types and tissues as a result of membrane budding [5]. MPs are now recognised as intercellular mediators of inflammation, coagulation, vascular homeostasis and resistance [1,5,17–20]. The MP cargo consists of surface antigens, proteins, adhesion molecules, nuclear acids (RNAs, DNA) and

Abbreviations: FCM, flow cytometry; MFI, mean fluorescence intensity; MPs, microparticles; MDR, multidrug resistance; MRP1, Multidrug Resistance-Associated Protein 1; P-gp, P-glycoprotein; PBC, probenecid; qRT-PCR, quantitative real-time PCR; *Ct*, cycle threshold; a.u., arbitrary units.

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nuclear regulators derived from the donor cell [20,21]. Consequently, MPs have emerged to play an important role in cell–cell communication in vitro and in vivo [22–24].

We have recently provided evidence that MPs shed from drug resistant cancer cells (the MP donor) re-template the transcriptional landscape of drug sensitive cells (the MP recipient) and impose the dominance deleterious cancer cell traits via the intercellular transfer of functional P-gp, transcripts and non-coding nucleic acids [20,21].

To expand on these findings, we now examine whether the donor cell can re-template recipient cells and confer an alternative dominant resistance mechanism in recipient cells. We investigate for the first time a molecular basis to the dominance of a given resistant trait within heterogeneous tumour cell populations. We discuss the implications in understanding the molecular basis for tumour dominant phenotypes and explore a new paradigm in the prevention of MDR.

2. Materials and methods

2.1. Cell lines

The drug sensitive human acute lymphoblastic leukaemia cell line CCRF-CEM [25] (designated CEM for simplicity) and its MDR derivatives, E_{1000} and VLB_{100} were used in this study. The CEM and VLB_{100} cell lines have been validated earlier by us as an appropriate model for study of P-gp mediated MDR in vitro [4,26]. The E_{1000} represents a validated model for the MRP1 overexpressing phenotype [12,27]. The drug resistant human breast adenocarcinoma cell line, MCF7/DX was also used in this study. MCF-7/DX cells were initially developed from drug sensitive human breast adenocarcinoma, MCF-7 cells by incremental exposure to doxorubicin and are known to express high levels of P-gp with strong resistance to doxorubicin [28]. The E_{1000} cell line was a kind gift from Prof. Ross Davey (Kolling Institute, Royal North Shore Hospital, NSW, Australia). All cell lines were cultured in RPMI-1640 media (Sigma-Aldrich, NSW, Australia), supplemented with 10% heat-inactivated foetal bovine serum (Invitrogen, Life Technologies, Victoria, Australia) and maintained at 37 °C and 5% CO_2. All cells lines were tested for mycoplasma contamination routinely.

2.2. MP isolation and validation

MPs were isolated from approximately 4×10^8 to 6×10^8 cells by differential centrifugation as described previously [1]. This amount of cells yield approximately 180 μg of MPs. Briefly, cell supernatant was collected and centrifuged at $500 \times g$ for 5 min to pellet the cell population or debris. The supernatant was further centrifuged at $15{,}000 \times g$ for 1 h at 15 °C and the pellet was resuspended in serum free RPMI-1640. The MP suspension was further centrifuged at $2000 \times g$ for 1 min to remove remaining debris. To concentrate the MP population, the supernatant was further centrifuged at $18{,}000 \times g$ for 30 min and resuspended in serum free RPMI-1640. MPs were validated by flow cytometry (FCM) (LSRII, BD Biosciences, CA, USA) by annexin V-V450 (560506; BD Biosciences, NSW, Australia) staining as described previously [1]. MP total protein content was quantified using Qubit® 2.0 Fluorometer protein assay (Invitrogen, Life Technologies) as per manufacturer's instructions.

Scanning electron microscopy (SEM) was used to visualise the MPs. Samples were mounted on poly-L-lysine (P4707, Sigma–Aldrich) coated 10 mm glass coverslides in 12-well plates. Samples were fixed in 2% glutaraldehyde in cacodylate buffer for 30 min, followed by 1% osmium tetroxide in cacodylate buffer post-fixation. Dehydration was performed in grading alcohols with a final step in hexamethyldisilazane (H4875; Sigma–Aldrich) for 3 min. Samples were imaged using the Zeiss Ultra Plus scanning electron microscope (Carl Zeiss, Oberkochen, Germany) following platinum coating.

2.3. SDS-PAGE and Western blotting

Briefly 180 μg of MPs isolated from CEM and E_{1000} cells were co-cultured with 1×10^5 CEM cells and maintained at 37 °C and 5% CO_2 in 96-well plates. At specified time intervals, the recipient cells were washed twice with complete culture medium to remove unbound MPs. The isolated MP pellet and cells were lysed in CelLytic M™ Cell Lysis reagent (C2978; Sigma-Aldrich) in the presence of 1% (v/v) protease inhibitor cocktail (P8340; Sigma–Aldrich) on ice for 30 min. The lysates were centrifuged at $10{,}000 \times g$ for 10 min at 4 °C to pellet the undissolved fraction. Total protein lysate was quantified using Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies) as per manufacturer's instructions. 15 μg of total protein was separated by electrophoresis using a 4–12% NuPAGE Bis-Tris gel (Invitrogen, Life Technologies) at a constant voltage of 150 V for 60 min and electroblotted onto a PVDF membrane at 30 V for 90 min. For detection of MRP1 and β-actin internal control, 1:2000 dilution of anti-MRP1 mAb (Clone QCRL-1, Sigma–Aldrich) and 1:10,000 dilution of anti-β-actin antibody (Clone AC-74, Sigma–Aldrich) was used respectively. Incubation of both antibodies was for 1 h after blocking overnight with 5% skim milk in PBS and 0.05% Tween 20 (TBST). After washing 3 times in TBST, the membranes were incubated for 1 h with anti-Mouse-HRP secondary antibody (Promega, NSW, Australia) at a 1:10,000 dilution. Protein expression was visualised using an ECL (enhanced chemoluminescence) system (Roche Applied Science, NSW, Australia). The membranes were imaged using the QuantityOne 4.6.1 software on the ChemiDoc XRS system (Bio-Rad Laboratories, Gladesville, NSW, Australia).

2.4. Calcein-AM functional accumulation assay

Calcein-AM dye exclusion has been extensively validated for MRP1 functionality [29,30]. The effects of increasing MP amounts and length of co-culture with recipient cells were examined. Briefly MPs isolated from CEM and E_{1000} cells were co-cultured with 1×10^5 CEM cells and maintained at 37 °C and 5% CO_2 in 96-well plates. At specified time intervals, the recipient cells were washed twice with complete culture medium to remove unbound MPs and treated with 0.1 μM of Calcein-AM for 1 h in the presence and absence of a 1 h pre-incubation with 2 mM probenecid (PBC) (validated MRP1 inhibitor) [27] at 37 °C and 5% CO_2. The cells were subsequently washed three times with PBS and re-suspended in 200 μL PBS for flow cytometric analysis.

2.5. MPs incorporate transporter transcripts and modulate recipient cell phenotype

The presence of *ABCC1* and *ABCB1* mRNA, coding for MRP1 and P-gp respectively, in isolated MPs and in drug sensitive cells with and without co-culture with MPs was assessed using qRT-PCR. Reactions conducted on resistant and sensitive cells served as positive and negative controls for identification of *ABCC1* and *ABCB1* overexpression and basal endogenous transporter expression.

Heterotypic and homotypic co-cultures were performed to assess the dominance of the donor cell phenotype. Briefly, MPs isolated from E_{1000} cells were homotypically co-cultured with CEM cells for 15 h. Similarly, MPs isolated from MCF7/DX and VLB_{100} cells were respectively heterotypically and homotypically co-cultured with E_{1000} cells for 4 h. The 4 h timepoint for the transfer of transporter and nucleic material from these MPs has been

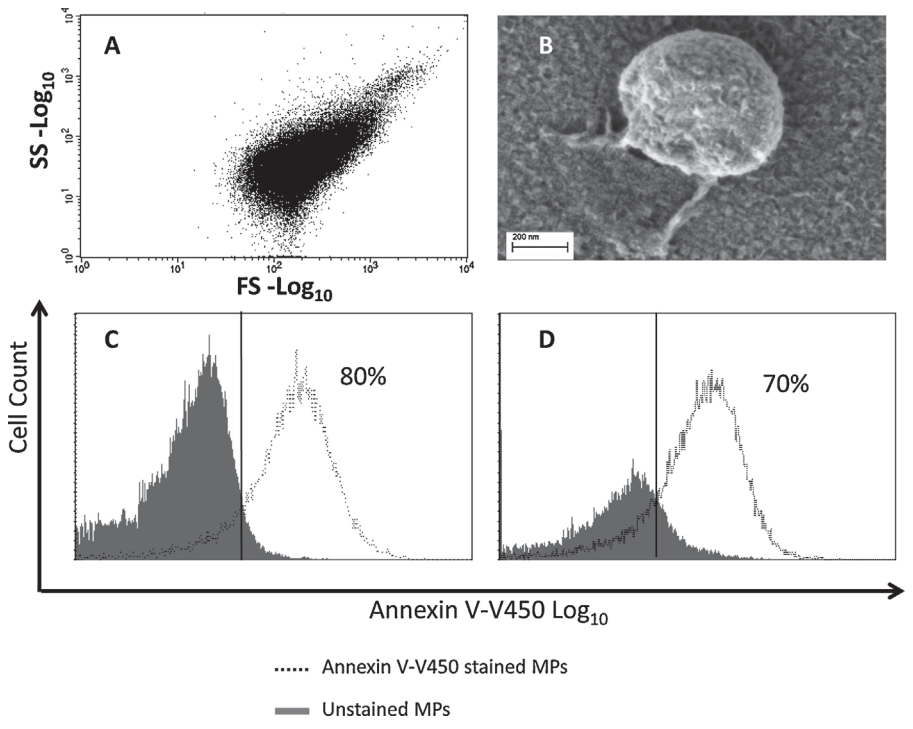

Fig. 1. Characterisation of MPs derived from E_{1000} and CEM cells. (A) MPs isolated from E_{1000} and CEM cells were analysed via flow cytometry and (B) SEM image of an E_{1000}MP, image taken using Zeiss Ultra Plus following fixation with osmium tetroxide and coating with platinum. MP population was identified as Annexin V-V450 positive events and represented (C) 80% of the population in E_{1000}MP and (D) 70% in CEMMP. All experiments were repeated 2–3 times with similar results. Data is representative of a typical experiment.

previously been validated by us [1,20,21]. All cells were maintained at 37 °C and 5% CO_2 in 96-well plates. The cells were subsequently washed three times with PBS and total RNA contents were extracted from the pellet as described below.

Total RNA contents were extracted from samples using the Trizol® (Molecular Research Center, OH, USA) reagent as per the manufacturer's recommendations. cDNA synthesis was performed using 1500 ng RNA from each sample with the Advantage RT-for-PCR Kit (Clontech Laboratories, Inc., CA, USA) on the Mastercycler® Gradient thermal cycler (Eppendorf AG, North Ryde, Australia). The following primers (Sigma–Aldrich) were used: *ABCC1* forward: 5′-TGT GGG AAA ACA CAT CTT TGA-3′, reverse: 5′-CTG TGC GTG ACC AAG ATCC-3′, and *ABCB1* forward: 5′-AAG GCA TTT ACT TCA AAC TTG TCA-3′, reverse: 5′-TGG ATT CAT CAG CTG CAT TTT-3′ for human MDR gene target; and *GAPDH* forward: 5′-TGC CAA ATA TGA TGA CAT CAA GAA-3′; reverse: 5′-GGA GTG GGT GTC GCT GTTG-3′ for housekeeping gene.

SYBR green qRT-PCR amplifications were performed using the Mastercycler® Gradient instrument (Eppendorf). Reactions were carried out in a 20 μL volume containing 10 μL of 2× SYBR Green Premix ExTaq (Takara Bio, Shiga, Japan) and specific primers (10 pmol/reaction). The thermal profile for the qRT-PCR was 95 °C for 5 min followed by 45 cycles of 95 °C for 5 s, 55 °C for 10 s and 72 °C for 15 s. The *Ct* data for each sample was collected automatically.

The ΔCt of each group was calculated using the following formula: ΔCt = MDR gene Ct − housekeeping gene Ct. Relative expression was calculated using $\Delta\Delta Ct = 2^{-\Delta Ct}$ and expressed as fold difference from the experimental control ($\Delta\Delta Ct$ of sample/$\Delta\Delta Ct$ of drug sensitive cells × 1) as arbitrary units (a.u.).

2.6. Statistical analysis

Means between groups were compared using an unpaired two-tailed students *t*-test. *P*-values less than 0.05 ($P < 0.05$) were accepted as statistically significant. All statistical analyses were performed using GraphPad Prism version 5.0 for Windows Software (Graphpad Software, CA, USA).

3. Results

3.1. Human acute lymphoblastic leukaemia cell lines spontaneously shed MPs

MRP1 overexpressing E_{1000} cells presented spontaneous shedding of MPs (Fig. 1A). High resolution imaging by SEM showed MPs as spherical in shape and ranging from 300 nm to 600 nm in diameter (Fig. 1B). The MP population stained positive for AnnexinV-V450 is indicative of phosphatidylserine exposure (Fig. 1C and D). This result is consistent with our previous findings which demonstrate the spontaneous shedding of MPs from the P-gp overexpressing cell lines, MCF-7/DX, and VLB_{100} [1,20].

3.2. MP-mediated transfer of MRP1 from overexpressed MDR cells to drug sensitive cells

MRP1 expression was detected by Western blot analysis using the anti-MRP1 monoclonal antibody; clone QCRL-1 (Fig. 2). Both E_{1000} cells and their MPs were positive for the 190 kDa MRP1. Upon co-culture with E_{1000}MPs for 4 h (Fig. 2B) and 15 h (Fig. 2C), drug sensitive CEM cells acquired MRP1 from the E_{1000}MP cargo. We did not detect MRP1 expression in CEM cells (Fig. 2B and C).

3.3. MP-mediated transfer of MRP1 is functional in drug sensitive recipient cells after 12 h of co-culture

To investigate whether the transferred MRP1 was functional in recipient CEM cells, calcein-AM dye exclusion assays were conducted on CEM, CEM + CEMMP, CEM + E_{1000}MP and E_{1000} over a 24 h period (Table 1). The extent of calcein accumulation is

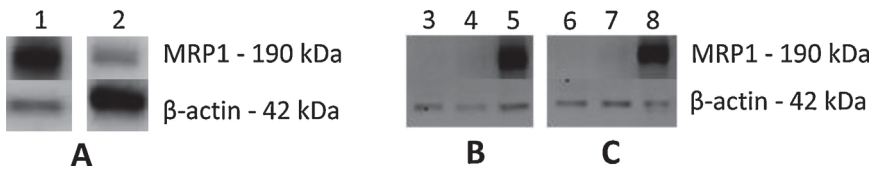

Fig. 2. Immunodetection of MRP1 in E_{1000} cells, their derived MPs and cells co-cultured with E_{1000}MPs. Western blot analysis of (A) E_{1000} cells and E_{1000}MPs, (B) CCRF-CEM cells alone as well as co-cultured with MPs for 4 h, (C) CCRF-CEM cells alone as well as co-cultured with MPs for 15 h. All co-cultures are performed with 180 μg of MPs. Lanes (1) E_{1000} Cells only, (2) E_{1000}MPs only, (3) 4 h CEM only, (4) 4 h CEM + CEMMP, (5) 4 h CEM + E_{1000}MP, (6) 15 h CEM only, (7) 15 h CEM + CEMMP, (8) 15 h CEM + E_{1000}MP. 190 kDa MRP1 was detected in both CEM + E_{1000}MP co-cultures and E_{1000}/E_{1000}MP samples using anti-MRP1 mAb (clone QCRL-1). Detection of β-actin using the anti-β-actin mAb (clone AC-74), was used as the internal control. All experiments were repeated 2–3 times. Data is representative of a typical experiment.

Table 1
Time-course of MRP1 functionality in recipient cells following MP co-culture.

Time Interval	4 h	6 h	12 h	15 h	23 h
Fold difference relative to CEM control	1.39 ± 0.10*	1.45 ± 0.04*	1.74 ± 0.14*	1.98 ± 0.29*	1.65 ± 0.35

Calcein-AM exclusion assay was used to assess the fold difference in mean fluorescence intensity of calcein in CEM + E_{1000}MP co-culture relative to CEM control. Intracellular accumulation of calcein was detected by FCM. Data represents mean ± S.D of 2–3 independent experiments.
* $P < 0.05$ compared to CEM control.

dependent on functionality of MRP1 protein and is commonly used in the assessment of the MRP1 mediated MDR phenotype [29]. There was no significant change between CEM and CEM + CEMMP drug accumulation (Fig. 3).

E_{1000} cells displayed a 6.69 ± 1.48-fold less drug accumulation than CEM cells, consistent with its MRP1 overexpressing phenotype. Although a co-culture period of 4 h has been shown to transfer MRP1 (Fig. 2B), only a modest shift in mean fluorescence intensity (MFI) was detected between CEM + E_{1000}MP and CEM (approximately 1.3–1.4-fold) (Table 1). However, with a MP co-culture period of 12 h and greater, an average of 1.8-fold reduction in fluorescent calcein accumulation was observed relative to parental CEMs and CEM + CEMMP (Table 1). This is consistent with the transfer of functional MRP1 to drug sensitive cells by E_{1000}MP.

To further confirm the functionality of MRP1 transferred to the recipient cells, the effects of the MRP1 inhibitor PBC, were examined on CEM cells co-cultured with E_{1000}MP for 15 h (Fig. 4). In the absence of PBC, 64.8% of the cell population displayed a reduction in calcein accumulation. When the co-cultured cells were pre-treated with PBC for 1 h prior to the addition of calcein-AM, we observe only 10.42% of cell population with calcein deficit. This equates to an average of 1.67 ± 0.09-fold reversal of the calcein-AM drug accumulation, consistent with inhibition of MRP1 functionality.

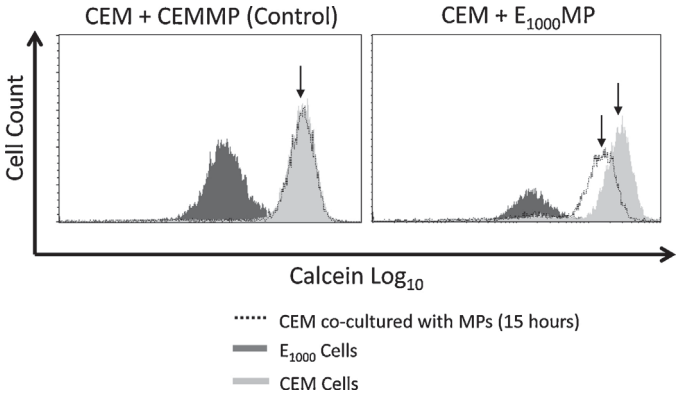

Fig. 3. MRP1 functionality in recipient cells following 15 h co-culture with E_{1000}MP. Calcein-AM dye exclusion assay on (A) CEM + CEMMP control (B) and CEM + E_{1000}MP co-culture. No significant change was observed between CEM + CEMMP and CEM cells. Shift in calcein accumulation detected between CEM + E_{1000}MP and CEM cells (black arrows). Intracellular calcein accumulation was detected using FCM. Data is representative of a typical experiment ($n = 3$).

3.4. MP dependent accumulation deficit

To investigate if the change in drug accumulation was dependent on MP amount, we performed the aforementioned calcein-AM dye exclusion studies with increasing amounts of MPs at the 15 h co-culture time point. We observed an MP dependent accumulation deficit relative to the drug sensitive CEM cells, with the effect greatest at 180 μg MP (Fig. 5).

3.5. MPs incorporate transporter transcript and selectively modulate recipient cell phenotype

ABCC1 and *ABCB1* mRNA transcript profiles were established using qPCR to determine the effect of MPs on recipient cell transcript levels (Fig. 6). Following a homotypic co-culture between CEM + E_{1000}MPs for 15 h, we observed a 30% suppression in basal endogenous *ABCB1* mRNA levels relative to that present in the CEM cells (Fig. 6A). This observation was parallel to the *ABCB1* suppression observed in the donor E_{1000} cells relative to the CEM cells (Fig. 6A). Additionally, we observed an increase in *ABCC1* transcript, with levels approaching the E_{1000} MP-donor cells following CEM + E_{1000}MP co-culture (Fig. 6B).

We assessed whether the donor cell phenotype was acquired upon MP exposure. To establish this we conducted heterotypic co-culture experiments whereby MPs isolated from the P-gp overexpressed leukemic cell line, VLB_{100}, designated VLBMPs, were co-cultured with the drug resistant E_{1000} cells for 4 h. Following

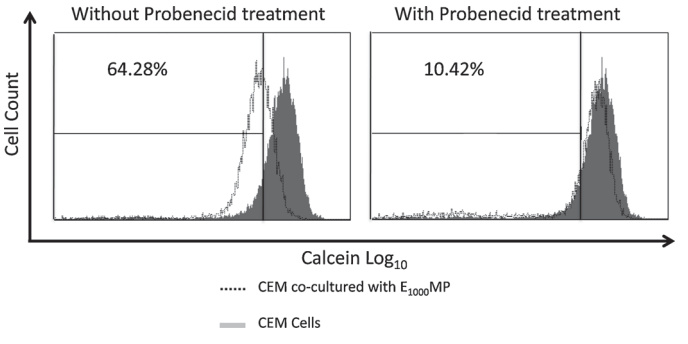

Fig. 4. MRP1 functionality in recipient cells is inhibited by PBC. Calcein-AM dye exclusion assay on co-cultured cells (A) without 2 mM PBC and (B) with 2 mM PBC (B). Calcein accumulation deficit observed in CEM + E_{1000}MP relative to CEM cells was reversed following treatment with PBC. Intracellular calcein accumulation was detected using FCM. Data is representative of a typical experiment ($n = 3$).

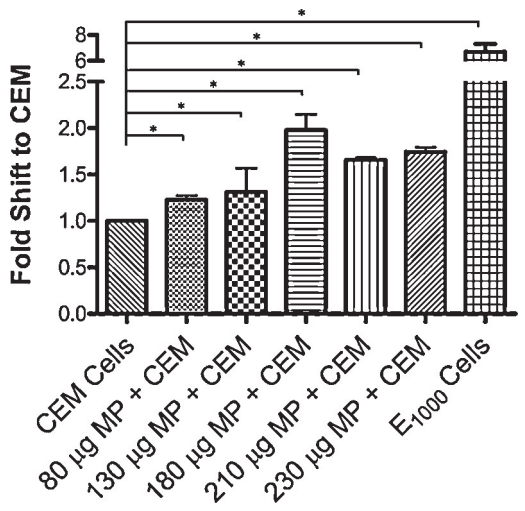

Fig. 5. MP dependent MDR transfer to recipient cells. Calcein-AM dye exclusion assays were performed and whole cell accumulation of intracellular calcein was analysed by FCM. Increasing amounts of E_{1000}MPs co-cultured with CEM cells for 15 h. Fold deficit in intracellular calcein accumulation in CEM + E_{1000}MP and CEM co-cultured with increasing MP relative to CEM cells. Data represents mean ± SE of 2–3 independent experiments $^*P < 0.05$.

co-culture we observed a significant increase in *ABCB1* transcript (Fig. 6C), with levels approaching the donor cells relative to the drug sensitive CEM cells. We also observed 30% suppression in *ABCC1* mRNA (Fig. 6D), consistent with the VLB donor cell relative to the drug sensitive CEM cells. Likewise, there was a significant increase in *ABCB1* (Fig. 6E) and a 30% suppression of *ABCC1* relative to E_{1000} cells following a heterotypic co-culture with breast cancer MPs (DXMPs) (Fig. 6F). In both instances, the recipient MRP1/*ABCC1* overexpressed phenotype was suppressed, with transcript levels approaching the donor P-gp/*ABCB1* overexpressed phenotype. This finding is consistent with our earlier work and supports the acquisition of the donor cell trait upon MP co-culture [21].

4. Discussion

Development of MDR in cancer patients remains a major obstacle to the success of chemotherapy. Previously, the discovery of a non-genetic modality for the acquisition of P-gp mediated MDR via MPs released from the plasma membranes of drug resistant cells demonstrated the important role of MPs in cell–cell communication [1]. The current study shows that the MRP1 overexpressing human acute lymphoblastic leukaemia cell line (E_{1000}) spontaneously shed MPs, which are capable of transferring functional MRP1 to its drug sensitive counterpart (CEM).

Recent evidence has shown that MPs disseminate cargo into recipient cells via active endocytosis, involving mechanisms consistent with both phagocytosis and macropinocytosis [31]. This is the first demonstration that MPs confer MRP1 mediated resistance to drug sensitive recipient cells. We further describe the heterotypic re-templating properties of MPs to reflect traits present in the donor cell. These results expand on our knowledge on MP function and lend further support for a capacity by MPs to re-template the transcriptional landscape of recipient cells to reflect the profiles present in the donor cells.

Here we show MPs spontaneously shed from MRP1 overexpressing cells (E_{1000}) and bear phenotypical and morphological characteristics of the submicron sized vesicles (Fig. 1). Items that are larger than 1 μM have been excluded from the analysis by size gating, as previously described [1].

The presence of functional MRP1 in recipient cells (Figs. 2 and 3) is indicative of MRP1 transfer into recipient cells and is consistent with our earlier work showing the transfer of functional P-gp into recipient cells by MPs [1,21,24]. Unlike our previous studies which demonstrated P-gp functionality following MP transfer at 4 h [1], the kinetics for acquired MRP1 differ significantly. We show that MRP1 following MP transfer to recipient cells displays optimal functionality after 12 h (Table 1) of MP co-culture. Currently, the reasons for this time difference remain unclear. It may be attributed to the presence of the additional N-terminal transmembrane region and the effects of the insertion within the membrane of the recipient cell [32]. Another reason for this delayed functionality may relate to the endogenous molecular environment of the recipient cell. A recent study showed that Casein Kinase 2α, a regulator of cell growth, proliferation, death and survival, regulates MRP1 function via phosphorylation of conserved regions [33]. Expression of this key regulator and others may vary between recipient and donor cells used in this study, leading to changes in MRP1 activity. Furthermore, as MRP1 transporter activity is dependent on intracellular glutathione (GSH) concentrations [34], fluctuations in GSH levels upon MP binding may also explain the time discrepancy.

Having established that MPs confer MRP1-mediated MDR to recipient cells, we subsequently investigated the molecular basis for this phenomenon. We have previously reported the packaging and transfer of *ABCB1* transcripts to drug sensitive cells [20,21]. Herein, we examine if this effect takes place with *ABCC1* transcripts. E_{1000}MPs imposed a donor dominant *ABCC1* trait on drug sensitive cells. Surprisingly, we also observed a suppression of endogenous *ABCB1* mRNA levels, consistent with that observed in the donor cell relative to the drug sensitive counterparts. This is the first demonstration of *ABCC1* phenotype dominance onto recipient cells via MP vectors.

In examining this phenomenon further, a homotypic and heterotypic co-culture between cells overexpressing two distinct resistance pathways was used to ascertain MP-mediated donor MDR trait dominance in recipient cells. We observed MP-mediated dominance of overexpressing *ABCB1* cells of haematological and non-haematological origin onto *ABCC1* overexpressing leukaemia cells. We demonstrated that the dominance of ABC-transporters in tumour populations are reflective of the donor cell trait and provide evidence that this phenomenon occurs between different cancer types, regardless of their origin. This adds to the complexity of cancer MDR, whereby the "re-templating" of recipient cell trait may provide an additional survival advantage against xenobiotics. For instance, in the event of P-gp failure to extrude certain chemotherapeutics due to transporter inhibition or otherwise, it may be possible for the expression of a second transporter to take its place. A study has suggested the requirement for the simultaneous inhibition of both P-gp and MRP1 to help circumvent MDR [35]. One may suggest the expression of both these proteins may be a result of re-templating due to MPs. The instance of this MP-mediated "fail-safe" mechanism emphasizes the complexity of MPs and the versatile role they play in cancer cell survival, identifying them as an oncological treatment target.

Vesicle cargo contains a wide array of constituents such as cells adhesion molecules, growth factors, signalling molecules and nucleic acids, many of which may be implicated in intercellular transfer and ABC-transcript suppression in recipient cancer cells [20]. These results lend further support to our previous findings that MPs, in the transfer of their cargo, effectively ensure the dissemination and acquisition of the donor cell trait within cancer [21].

The molecular mechanisms governing ABC-transporter regulation and trait acquisition via MPs are largely unknown. Identification and characterisation of the regulatory mediators packaged in MPs are currently under investigation. Transcriptional regulators such as microRNAs (miRNAs) have been shown previously by us to play a role in MP-mediated selective trait dominance

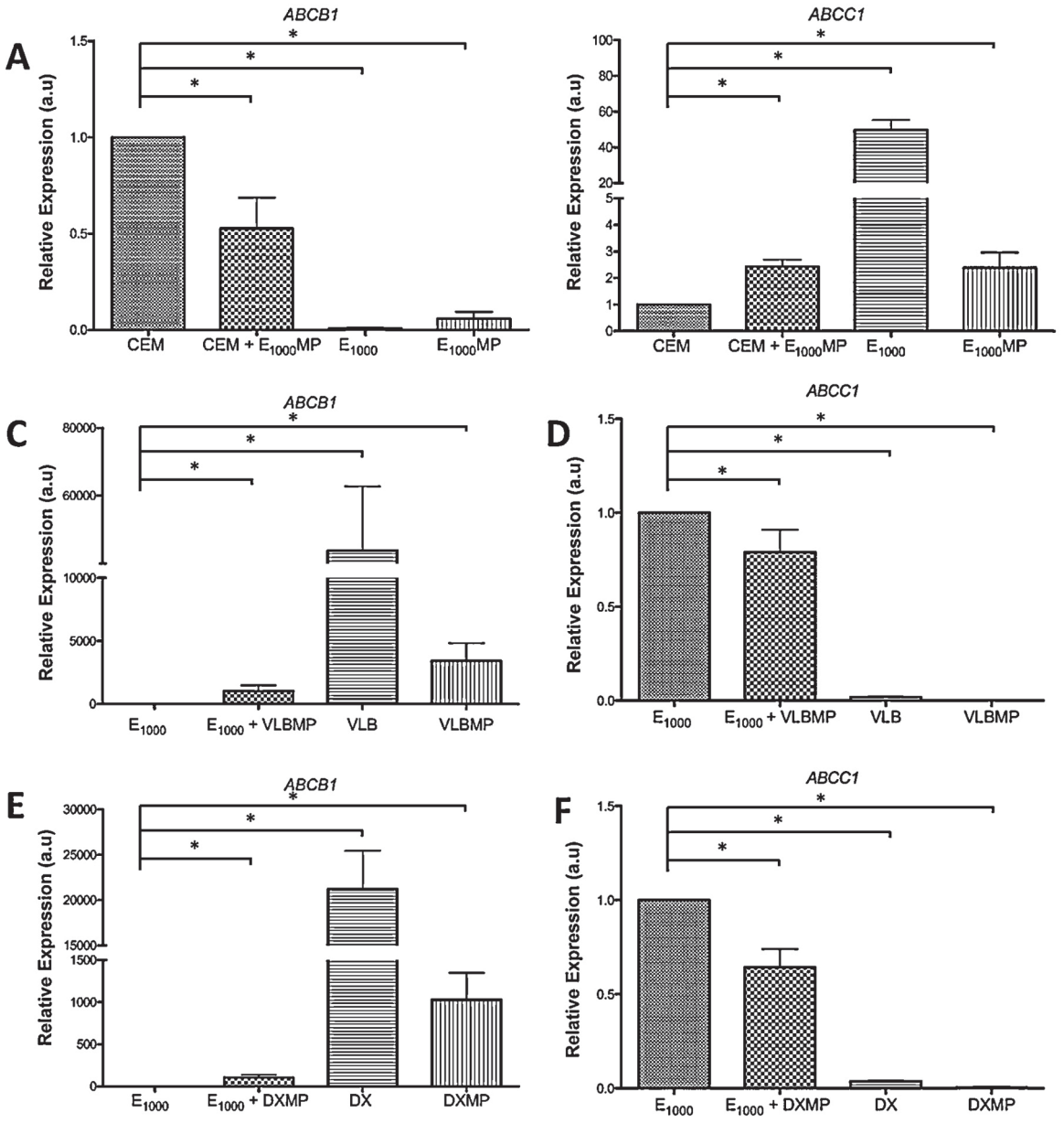

Fig. 6. MP mediated re-templating of recipient cell transcriptional environment. *ABCC1* and *ABCB1* profiles of cells and MPs were determined with qPCR: (A and B) CEM + E_{1000}MP, (C and D) E_{1000} + VLBMP, (E and F) E_{1000} + DXMP. Values are expressed as relative expression of *ABCB1* and *ABCC1* with respect to recipient cell control. Data represents mean ± SE of 2–3 independent experiments conducted in triplicate $^{*}P < 0.05$.

[20,21]. MPs are an emerging source of miRNA in circulation of cancer patients [36]. Certain miRNAs have potent capacity to regulate ABC-transporters such as miR-27a, miR-326 and miR-451 [5,20,21,37,38]. Packaging and transfer of such miRNA's in MPs shed by MDR cells may heavily influence the MDR phenotype of recipient cancer cells. This may explain the observed phenomenon whereby MRP1 or P-gp is suppressed upon co-culturing with MPs shed from distinctive phenotypes (Fig. 6).

Also, of interest is evidence of long non-coding RNA (lncRNA) as an emerging transcriptional regulator in a wide range of biological processes [39]. Recent studies have found cisplatin resistance in non-small-cell lung cancer cells relate to the differential expression of lncRNA [40]. Indeed there is a very few studies confirming the specific function of lncRNA and their mechanisms still remain unclear. Further miRNA and lncRNA profiling of MPs and their recipient cells may shed light on novel molecular mechanisms leading to trait dominance and in further our understanding on ABC-transporter regulation.

As MPs circulate through the vasculature, they are not only capable of affecting their local environment, but also tissues at a remarkable distance. The transfer of molecular constituents via MPs may assist in cancer cell survival in physiological sites where cell–cell contact is not possible. The transfer of ABC-transporter protein established in this process, will provide a drug sensitive cell with an immediate survival advantage, an initial barrier to extreme chemotherapeutic exposure prior to the cells modification of its intracellular machinery for more permanent or sustained mechanisms of survival.

In summary, we show a kinetic difference between P-gp and MRP1 becoming functional following their MP-mediated transfer to recipient cells. The elucidation of this discrepancy provides a mechanistic understanding between the functionality of the two well-known transporter proteins. More importantly, we provide evidence of a potent capacity of MPs to alter traits and transcriptional environment of cells to which they are exposed. This work elucidates the significant and versatile role MP-transfer plays on

the dynamic acquisition of MDR in cancer and is likely to have significant potential for the translation into alternative oncological treatment strategies and circumvention of MDR. One may envision the use of phenotype dominance mechanisms to steer traits towards a state that may be easily treated. Furthermore, re-templating of resistance profiles may allow one to pre-emptively tailor treatment strategies towards an impending dominant resistance profile. The intrinsic mechanisms of MP-mediated donor trait dominance need to be deciphered to expand our armamentarium and treatment strategies for MDR in cancer. We further iterate the role MPs play in altering the transcriptional landscape of target cells and its contribution to cancer cell survival. Finally, the current findings of MP mediated MDR further supports the potential of MPs as clinical targets for inhibition to prevent the spread of MDR in cancer as well as other MP-related afflictions.

Conflicts of interest

None.

Acknowledgements

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Author Contributions Statement – Chapter 2

J.F.L performed all experiments, analyzed the data and wrote the manuscript. F.L provided technical support and advice and helped with manuscript preparation. J.G and R.J provided scanning electron microscope image and helped with manuscript preparation. G.E.R.G edited and approved the manuscript. M.B was responsible for overall project and experimental design, supervision of the project, conceptual advice and revised the manuscript. All authors have reviewed the manuscript.

Chapter 3

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3. A novel method to detect translation of membrane proteins following microparticle intercellular transfer of nucleic acids

Running Title: Novel method to detect nucleic acid translation.

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3.1. Abstract

Background: Extracellular vesicles or microparticles (MPs) serve as vectors of nucleic acid dissemination and are important mediators of intercellular communication. However, the effect and functionality of packaged nucleic acids on recipient cells following transfer of MP cargo has not been clearly elucidated. This limitation is attributed to a lack of methodology available in assessing protein translation following homotypic intercellular transfer of nucleic acids. **Methods**: We describe a novel methodology to confirm the functionality of MP contained native nucleic acids transferred from human donor to human recipient cells. Using surface peptide shaving we have demonstrated that MPs derived from leukaemic cells transfer functional P-glycoprotein transcripts, conferring drug-efflux capacity to recipient cells. We demonstrate expression of newly synthesized protein using Western blot. Furthermore, we show functionality of translated P-gp protein in recipient cells using Calcein-AM dye exclusion assays on flow cytometry. **Results:** Newly synthesized 170 kDa P-gp was detected in recipient cells after co-culture with shaven MPs and these proteins were functional, conferring drug-efflux. **Conclusions**: This is the first demonstration of functionality of transferred nucleic acids between human homotypic cells as well as the translation of the cancer multidrug-resistance protein in recipient cells following intercellular transfer of its transcript. This study supports the significant role of MPs in the transfer of deleterious traits in cancer populations and describes a new paradigm in mechanisms governing the acquisition of traits in cancer cell populations.

3.2. Introduction

Microparticles (MPs) are shed from a diverse range of eukaryotic and prokaryotic cells either spontaneously or following cellular activation or stress (5). They selectively transfer the phenotypic and genotypic characteristics of their parental cell by packaging proteins and nucleic acids upon vesiculation (1, 5-7). MPs have been shown to participate in a variety of processes involving the development of immune tolerance, immune activation, phenotype modification, cell-cell signalling, drug-sequestration and reprogramming of cell function (1, 5, 7-9). Numerous studies have demonstrated the presence of RNA, mRNA and miRNA in MPs (5, 7, 9-11), with some nucleic acid species being either selectively packaged or completely absent during the MP vesiculation and transfer processes (5, 9). Consequently, MPs have emerged as important vehicles for nucleic acid based communication between cells.

In assessing functionality of the transferred nucleic acid cargo, previous studies have used interspecies methodologies to establish nucleic acid functionality following intercellular transfer by MPs (11). However, this approach is not physiologically relevant due to interspecies differences, which may impact on protein translation of the foreign transcript in recipient cells. Furthermore, there have been no technical means to distinguish between the translation of conferred transcript and the transfer of endogenous protein contained within the MP cargo, hence confounding results. Other attempts to detect protein translation from MP-RNA have utilised labelled probes such as luciferase reporter genes (10). Once again this does not reflect the native nucleic acid cargo of MPs. Consequently, the functionality of transferred native MP-RNA between homotypic cells has remained unestablished.

Herein, we demonstrate for the first time, the translation of native human MP-RNA in recipient human cells following intercellular transfer by MPs. This study establishes the functionality of human MP-nucleic acids encoding transmembrane proteins by employing MP surface peptide shaving. We anticipate this methodology will be used to assess the expression of membrane bound proteins in the context of MP-transfer. This study demonstrates for the first time that MPs confer phenotypic changes in recipient cells following the transfer of functional nucleic acids in addition to the transfer of functional proteins and reinforces the significance of MPs in the acquisition of cellular traits.

3.3. Materials and Methods

3.3.1. Cell Lines

The drug sensitive human acute lymphoblastic leukaemia cell line CCRF-CEM (designated CEM for simplicity) and its MDR derivative, VLB_{100} was used in this study. VLB_{100} cells hyper-express functional cell surface P-gp relative to the parental drug sensitive CCRF-CEM cells. The CEM and VLB_{100} cell lines have been validated earlier as an appropriate model for study of P-gp mediated MDR *in vitro* (1, 12, 13). All cell lines were cultured in RPMI-1640 media (Sigma-Aldrich, NSW, Australia), supplemented with 10% heat-inactivated foetal bovine serum (Invitrogen, Life Technologies, Victoria, Australia) and maintained at 37^0C and 5% CO_2. All cells lines were tested for mycoplasma contamination routinely.

3.3.2. MP isolation

MPs were isolated from 5 x 10^9 – 7 x 10^9 cells by differential centrifugation, as described previously (1, 7, 14). The integrity and purity of the isolated MP fraction have been previously extensively validated (1, 7). This amount yields approximately 2 mg of total MP protein in 100 µL final volume. Briefly, cell supernatant was collected and centrifuged at 500 × *g* for 5 min to pellet the cell population or debris. The supernatant was further centrifuged at 15,000 × *g* for 1 h at 15^0C and the pellet was resuspended in serum free RPMI-1640. The MP fraction was further centrifuged at 2000 × *g* for 1 min to remove remaining debris. To concentrate the MP fraction, the supernatant was further centrifuged at 18,000 × *g* for 30 min and resuspended in serum free RPMI-1640. MP total protein content was quantified using Qubit®2.0 Fluorometer protein assay (Invitrogen, Life Technologies) as per the manufacturer's instructions.

3.3.3. Flow Cytometric analysis

To confirm the presence of nucleic acids in MPs, flow cytometric analysis (LSRII, BD Biosciences, CA, USA) was used following acridine orange (AO) (Sigma-Aldrich) staining. Cytograms were acquired using BD FACSDiva software (BD Biosciences) and analyzed using BD Cell-Quest Pro (BDBiosciences). Approximately 50 µg of MPs were treated with RNAse A (Invitrogen, Life technologies) and resuspended in 200 µL of $RPMI^-$ and incubated with AO to a final concentration of 20 µmol/L. Unstained MPs were used as the control.

MP integrity was assessed using the calcein-AM (Invitrogen, Life technologies drug exclusion assay. Calcein-AM is a non-fluorescent membrane permeable drug.

Upon entering MPs, calcein-AM is cleaved by endogenous esterases to its fluorescent non-membrane permeable form, calcein (7). Approximately 50 µg of MPs were stained with 0.1 µM calcein-AM for 1 h at 37^{o}C and 5% CO_2. MPs were washed twice with PBS to remove unincorporated calcein-AM and resuspended in 200 µL of PBS for flow cytometric analysis.

For cell-MP binding studies, MPs are stained with the cell membrane dye PKH26 (Sigma-Aldrich) for 1 min according to the manufacturer's instructions, immediately followed by two washes with PBS at 18,000 x *g*. MPs are then immediately analysed on the flow cytometer or used in co-culture experiments with recipient CEM cells.

In assessing cell binding of MPs to recipient cells, 1 x 10^5 CEM cells were co-cultured in a 96 well plate at 37^{o}C, 5% CO_2 for 4 h with 180 µg of MPs preloaded and labelled with calcein-AM, AO and PKH26 MPs respectively. The 4h time point has been previously validated to be sufficient time for the transfer of functional protein and nucleic material from MPs to recipient cells (1, 7, 9, 15). Cells are subsequently washed thrice with PBS at 500 x g for 5 min at room temperature to remove unbound MPs. Cells were resuspended in 200 µL of PBS before analysis on the flow cytometer.

3.3.4. In vitro rabbit reticulocyte translation

The *in vitro* rabbit reticulocyte translation assay (RRTA) (Promega, NSW, Australia) was used according to the manufacturers instruction with the exception that a total of 400 µg/mL final concentration of total MP RNA was used to obtain efficient translation of P-gp/*ABCB1* from MPs. A rabbit reticulocyte lysate sample without RNA was used as the control. Samples were immediately stored at -20^{o}C until required.

Total RNA was isolated from MPs and cells using the RNeasy mini kit with genomic DNA eliminator (Qiagen, NSW, Australia). 300 µg – 1 mg MPs were pelleted at 18,000 x g and disrupted and homogenized using 350-600 µL of buffer RLT plus (Qiagen, NSW, Australia). Purified total RNA was isolated from the homogenate according to the manufacturer's protocol. The RNA isolate was resuspended in 30 µL of RNAse free H_2O and quantified using the Nanodrop 1000 spectrophotometer (Nanodrop technologies, DE, USA). Purified total RNA was stored at -80^{o}C until required.

3.3.5. SDS-PAGE and Trypsin in-gel digestion

4 µL of rabbit reticulocyte lysate, as prepared above, was mixed with 2x SDS sample buffer containing Tris-HCl, glycerol, SDS, bromophenol blue and water (pH 8.8) at a ratio of 2:1 (protein : SDS buffer). The sample was sonicated in a waterbath for 5 min followed by centrifugation for 5 min at 16,500 x *g* at ambient temperature. Proteins were separated using 4-10% SDS-PAGE at a voltage of 150 V in MES SDS running buffer (Invitrogen, Life Technologies). The gel was fixed with 40% Methanol / 10% Acetic acid for 30 min with gentle shaking before staining with Coomassie blue overnight. In-gel trypsin digestion was performed as previously described (6). Briefly, the gel was sectioned into 12 pieces, according to the MW markers and the sections diced into 1 x 1 mm pieces which were de-stained by adding 50% acetonitrile (ACN)/50 mM NH4HCO3 and incubated for 10 min at ambient temperature. The process was repeated until the Coomassie stain disappeared. 100% ACN was added to dehydrate the gel pieces before rehydration with 12.5 ng/µL of trypsin solution (Trypsin Gold-MS grade, Promega) and overnight incubation at 37°C. After incubation, the supernatant

was collected after sonication in a water-bath for 10 min, followed by another sonication following the addition of 30 µl of 50% ACN/2% formic acid. The solution was added to the previously collected peptides and the volume reduced to 15 µL by rotary evaporation. The peptide solution was centrifuged at 14,000 x *g* for 10 min to remove any insoluble material prior to liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis.

3.3.6. LC/MS/MS

Using an Eksigent AS-1 autosampler connected to a Tempo nanoLC system (Eksigent, USA), 10 µL of the sample was loaded at 20 µl/min with MS loading solvent (2% ACN + 0.2% Trifluoroacetic Acid) onto a C8 trap column (CapTrap. Michrom Biosciences, USA). After washing the trap for three minutes, the peptides were washed off the trap at 300 nL/min with MS solvent A (2% ACN + 0.2% Formic Acid) onto a PicoFrit column (75 mm x 150 mm, New Objective) packed with Magic C18AQ resin (Michrom Biosciences). Peptides were eluted from the column and into the source of a QSTAR Elite hybrid quadrupole-time-of-flight mass spectrometer (ABSciex) using the following program: 5-50% MS solvent B (98% ACN + 0.2% Formic Acid) over 30 min, 50-80% MS solvent B over 5 min, 80% MS solvent B for 2 min, 80-5% for 3 min. The eluting peptides were ionised from the PicoFrit column at 2,300 V. An Intelligent Data Acquisition (IDA) experiment was performed, with a mass range of 350-1,500 Da continuously scanned for peptides of charge state 2+-5+ with an intensity of more than 30 counts/sec. Selected peptides were fragmented and the product ion fragment masses measured over a mass range of 100-1,500 Da. The mass of the precursor peptide was then excluded for 15 sec.

3.3.7. Data Analysis

Three biological replicates of total proteins from RRTA and its background control were analysed by LC/MS/MS. The data files produced by QSTAR Elite were searched using Mascot Daemon (version 2.4 provided by the Walter and Eliza Hall Institute) and searched against the LudwigNR database (comprised of the UniProt, plasmoDB and Ensembl databases (vQ213)) with the following parameter settings. Taxonomy: Homo sapiens. Fixed modifications: none. Variable modifications: propionamide, oxidised methionine, deamidated asparagine. Enzyme: semi-trypsin. MPNumber of allowed missed cleavages: 3. Peptide mass tolerance: 100 ppm. MS/MS mass tolerance: 0.2 Da. Charge state: 2+, 3+ and 4+. Scaffold (v4.2.1, Proteome Software, Portland, OR) was used to validate and compare MS/MS-based peptide and protein identifications as previously described (6). Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (16) and contained at least 2 identified peptides in 2 replicates. Proteins identified from RRTA were considered unique if not present in RRTA without RNA background control. Protein probabilities were assigned by the Protein Prophet algorithm (16). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were clustered by Scaffold to satisfy the principles of parsimony.

3.3.8. MP surface shaving

To determine whether *ABCB1* transcript packaged in MPs could be transferred and translated in human recipient cells, we used surface shaving methodology to eliminate endogenously packaged transmembrane proteins on the MP membrane

using proteinase K digestion prior to co-culture with recipient cells. Our approach allows for the detection of newly synthesized transmembrane protein in recipient cells and eliminates endogenous protein present in MPs, which would confound the results. Briefly, the MP pellet was resuspended in PBS, pH 7.8 at room temperature. Samples, including proteinase K were pre-warmed for 15 min in 37^{O}C. 20-200 ng/mL of proteinase K was added to the MP sample and incubated for 5 min at 37^{O}C. To stop the reaction, protease cocktail (1%) (Sigma-Aldrich) was immediately added to the sample. MPs were washed twice with ice cold PBS by centrifugation at 18, 000 x g at 4^{O}C and the pellet resuspended in serum free RPM-1640.

3.3.9. Translation of MP derived nucleic acid in CEM cells

Shaved and non-shaved VLB_{100}MPs (180 µg) were co-cultured with 1 x 10^5 CEM cells to a final volume of 200 µL in complete RPMI medium supplemented with 10% FCS and maintained at 37^{O}C, 5% CO_2 in 96 well plates. At specific time intervals, cells were washed twice with pre-warmed (37^{O}C) PBS to remove unbound MPs before Western blot analysis. CEM cells alone were used as the control. The newly translated P-gp was detected using the anti-P-gp antibody (clone F4, Sigma-Aldrich).

3.3.10. SDS-PAGE and Western Blotting

The isolated MP pellet and cells were lysed in CelLytic M^{TM} Cell Lysis reagent (C2978; Sigma-Aldrich) in the presence of 1% (v/v) protease inhibitor cocktail (P8340, Sigma–Aldrich) on ice for 30 min. The lysates were centrifuged at 10,000 × g for 10 min at 4^{O}C to pellet the debris. Total protein lysate was quantified using Qubit®2.0 Fluorometer (Invitrogen, Life Technologies) as per the manufacturer's instructions.

30-60 µg of total protein was separated by electrophoresis using a 4–12% NuPAGE Bis-Tris gel (Invitrogen, Life Technologies) at a constant voltage of 150 V for 60 min and transferred onto a PVDF membrane at 30 V for 90 min. For detection of P-gp and β-actin control, 1:1000 dilution of anti-P-gp mAb (Clone C219, Amersham Biosciences, Piscataway, NJ), 1:5000 dilution of anti-P-gp (Clone F4, Sigma-aldrich, NSW, Australia) and 1:10,000 dilution of anti-β-actin antibody (Clone AC-74, Sigma–Aldrich) was used. Incubation of antibodies was overnight at 4^{o}C after blocking for 1 h with 5% skim milk in TBS and 0.05% Tween 20 (TBST). After washing 3 times in TBST, the membranes were incubated for 1 h with anti-Mouse-HRP secondary antibody (Promega, NSW, Australia) at 1:10,000 dilution. Protein expression was visualised using an ECL (enhanced chemoluminescence) system (Roche Applied Science, NSW, Australia). The membranes were imaged using the luminescent image analyser LAS-3000 (Fujifilms, Brookvale, NSW, Australia).

3.3.11. Calcein-AM dye exclusion assay

Calcein-AM dye exclusion has been extensively used to measure P-gp and MRP1 functionality (7, 14, 17). Briefly MPs isolated from VLB_{100} cells were co-cultured with 1× 10^5 CEM cells and maintained at 37^{o}C and 5% CO_2 in 96-well plates. After 4 h, the recipient cells were washed twice with complete culture medium to remove unbound MPs and treated with 0.1 µM of Calcein-AM for 1 h in the presence and absence of a 1 h pre-incubation with 10 µM cyclosporin A (CsA) (14) at 37^{o}C and 5% CO_2. The cells were subsequently washed three times with PBS and re-suspended in 200 µL PBS for flow cytometric analysis.

3.3.12. Statistical Analysis

Means between groups were compared using an unpaired two-tailed students t-test. P-values less than 0.05 ($P < 0.05$) were accepted as statistically significant. All statistical analyses were performed using GraphPad Prism version 5.0 for Windows Software (Graphpad Software, CA, USA).

3.4. Results

3.4.1. MPs package functional ABCB1 transcript

We previously showed the packaging and transfer of mRNA encoding the ATP Binding Cassette superfamily of drug transporters (*ABCB1* and *ABCC1*) by MPs into recipient cells (7, 9, 14). Herein, we sought to establish the functionality of these transferred transcripts in recipient cells following MP transfer. The functionality of MP packaged mRNA was first confirmed *in vitro* using a rabbit reticulocyte translation assay (RRTA). The expression of the cancer multidrug resistant protein, P-gp was used as the reporter protein for the mRNA translation studies. Using RRTA and the anti-P-gp clone F4 antibody, we observed a 170 kDa band (Figure 3.1) after the addition of total RNA purified from VLB_{100}MPs (final 400 µg/mL) supporting in vitro translation of RNA into protein. We also detected a 42 kDa β-actin band in both the RRTA with and without RNA. We subsequently sought to examine the full repertoire of proteins translated using the RRTA system using shotgun proteomics (Table 3.1). We identified more than 200 proteins (Supplementary data S3.1) in our RRTA, of which 5 were unique when compared to RRTA in absence of RNA (Table 3.1).

Table 3.1. List of *homo sapiens* proteins translated from VLB_{100}MP RNA using rabbit lysate translation assay as analysed by LC/MS/MS.

No.	Name of protein	No of unique peptides detected in 3 independent replicates		Function
		Translation	Background	
1	P-glycoprotein (P-gp)	8-5-5	0-0-0	ATP-binding cassette transporter protein. Overexpression in cancer cells is strongly associated with MDR (1, 18).
2	60S ribosomal protein L26 (RPL26)	2-2-0	0-0-0	Binding of RPL26 to the 5'UTR of p53 gene. Found to modulate p53 protein levels and affect p53 induction after DNA damage. P53 gene is commonly mutated in human cancer and lead to cancer-predisposing Li-Fraumeni syndrome (19). Ribosomal proteins are reported to be increased in human cancer and in maintaining cancer phenotype (20, 21).
3	Isoform 2 of 4F2 cell-surface antigen heavy chain (SLC3A2)	3-1-3	0-0-0	SLC3A2 is the protein that is encoded by SLC3A2 gene, which binds to a highly conserved C-terminal domain of integrin β1A and β3 cytoplasmic subunits, thereby affecting the integrin signalling cascade (22). These proteins influence malignant tumour cell behaviour and are overexpressed in variety of cancers by contributing to tumour growth (23).
4	Proteasome subunit beta type-6 (PSMB6)	2-2-1	0-0-0	*PSMB6* is a member of 20S proteasome subunit family, which forms the proteolytic core of 26S proteasome. Enhanced proteasome activity has been demonstrated as a mediator of resistance to chemotherapy (24). Overexpression of the *PSMB1* proteasome subunit is associated with resistance to cisplatin in cancer cell lines (25) as well as anthracycline-resistance in breast cancer (26).
5	Sodium/Potassium-transporting ATPase subunit alpha-4 (ATP1A4)	2-0-2	0-0-0	ATP1A4 was reported to be overexpressed in pancreas tumour cells compared with normal cells (27).

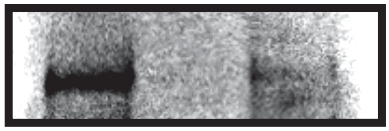

***Figure 3.1**. **MPs package functional ABCB1 transcript.** Total RNA purified from VLB_{100}MPs were translated to protein using the rabbit reticulocyte in vitro translation kit (Promega, NSW, Australia). All lanes were loaded equally by volume. A rabbit lysate sample in the absence of RNA was treated equally and used as a background control. Detection of newly synthesized P-gp was by Western blot analysis using the anti-P-gp antibody (C219, Amersham Bioscience, NJ, USA). A 170 kDa band was detected with addition of total VLB_{100}MP RNA. Data is representative of 2 independent experiments*.

3.4.2. MP-RNA is translated in recipient cells

To assess functionality of the transcripts in recipient cells following MP transfer of cargo, we first needed to eliminate detection of the endogenous transmembrane P-gp on the surface of MPs prior to co-culture with recipient cells. Doing so would ensure that any protein detected in recipient cells was a function of translation of cargo transcript rather than the transfer of functional P-gp contained within the MPs. In approaching this, we developed a novel approach using the non-specific protease, proteinase K, to cleave overhanging transmembrane segments of P-gp on the surface

of MPs. Following which, we co-cultured the surface 'shaved' MPs with CEM cells to detect translation of full length protein from donor transcripts in the recipient cells.

i. MP integrity is maintained following surface shaving

MPs were isolated and validated as previously described (1, 5, 7, 9, 14). Using 1.1 µM latex size beads (Sigma-Aldrich, NSW, Australia), events greater than 1.1 µM in size were excluded from flow cytometry (FCM) analysis by size gating (Figure 3.2 A). VLB_{100}MPs were identified as Annexin-V450 positive events and represented 61.95% of the gated population (1, 7, 9). This result is consistent with our previous findings, which demonstrate spontaneous shedding of MPs from the P-gp overexpressing cell line, VLB_{100} (1, 7, 9).

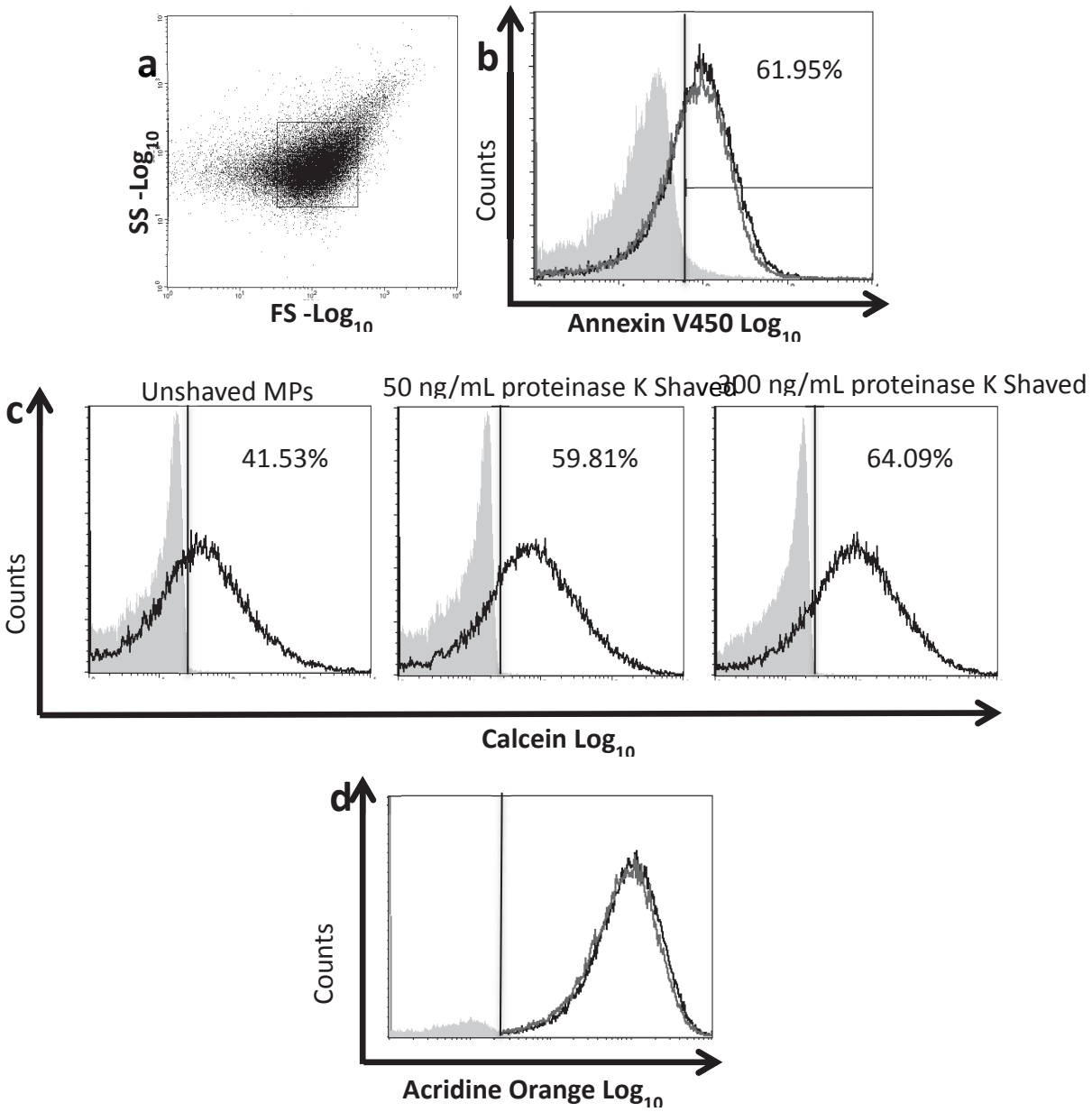

Figure 3.2. MP integrity is maintained following proteinase K shaving. (a) *MPs isolated from VLB_{100} were analysed by FCM. Latex beads (BD Bioscience) of 0.3 and 1.1 µM were used to define the upper and lower limit of the forward and side scatter (data not shown).* ***(b)*** *(▬) Shaved and (▬) unshaved MPs were stained for phosphatidylserine exposure using Annexin V450; (▬) unstained background control.* ***(c)*** *Intravesicular calcein accumulation in shaved and unshaved VLB_{100}MPs with increasing proteinase K concentration.* ***(d)*** *Acridine orange stained (▬) shaved , (▬) unshaved VLB_{100}MPs and (▬) unstained background control. Data is representative of three independent experiments*

To confirm the membrane integrity of MPs following surface shaving, we assessed the retention of intravesicular acridine orange (AO) and calcein-AM stains in MPs by FCM following dye loading as previously described (7) (Figure 3.2 C,D). Calcein-AM is a membrane permeable non-fluorescent drug. Upon entering MPs, calcein-AM is cleaved by endogenous esterases to its fluorescent non-membrane permeable form, calcein, which remains trapped in the intravesicular space (7). We observed a small but significant 1.36 ± 0.07 fold, ($P < 0.05$) increase in fluorescent calcein accumulation in MPs following surface shaving using 50 ng/mL Proteinase K. Despite this increase in calcein-AM permeability, we confirm the RNA content of shaved vesicles was not compromised as assessed by AO staining (Figure 3.2). We observed no significant difference in the AO fluorescence intensities when we compared shaved and non-shaved MPs, consistent with the maintenance of MP integrity and no leakage of the intra-vesicular nucleic acid material (Figure 3.2).

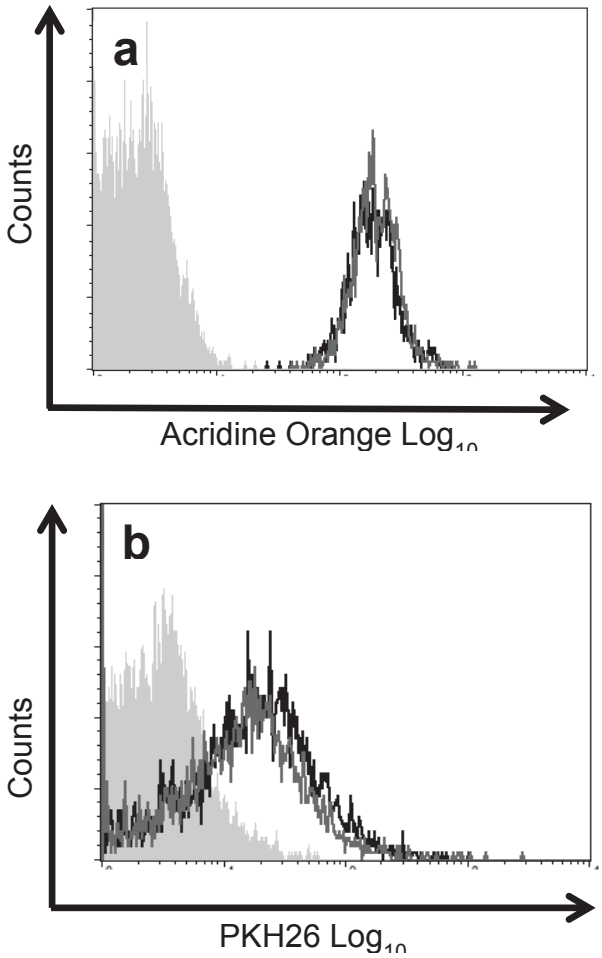

Figure 3.3. RNA content and MP binding retained following MP shaving. *VLB_{100}MPs are shaved using 50 ng/mL of proteinase K for 5 mins at 37^{o}C. Shaved MPs and non shaved MPs were stained with* ***(a)*** *acridine orange and* ***(b)*** *PKH26 prior to co-culture with CEM cells (4 h). Recipient cells are analysed by FCM to determine extent of RNA transfer and MP binding from (* ▬ *) shaved relative to (* ▬ *) unshaved MPs. (* ▬ *) Unlabelled CEM cells. Data is representative of 3 independent experiments.*

ii. Surface peptide shaving does not compromise MP binding to recipient cells

In assessing whether surface shaving impacted on cell binding of MPs to recipient cells we co-cultured CEM cells with MPs preloaded and labelled with calcein-AM, AO and PKH26 (Figure 3.3). Whole cells were washed and analysed by FCM to determine the uptake of stained MPs following co-culture. Enhanced fluorescence was detected in recipient CEM cells following co-culture with shaved and non-shaved MPs relative to the unstained control. We observed no significant difference in mean fluorescent intensity (MFI) in recipient CEM cells after co-culture with either shaved or non-shaved MPs (Figure 3.3). This suggests that the binding of MPs shed from VLB_{100} cells does not require exposed surface proteins, with the process relying on the physical interaction between the two membranes (Figure 3.3). This is contrary to some previous reports that show that recognition between MPs and their target cells involves the specific interaction between proteins (eg. ICAM-1, CD44, LFA-1, H-2Kb and CD81) at the surface of both cells and MP (16, 28-30). Here, the surface shaving of MPs with proteinase K, did not significantly affect binding of MPs to recipient CEM cells.

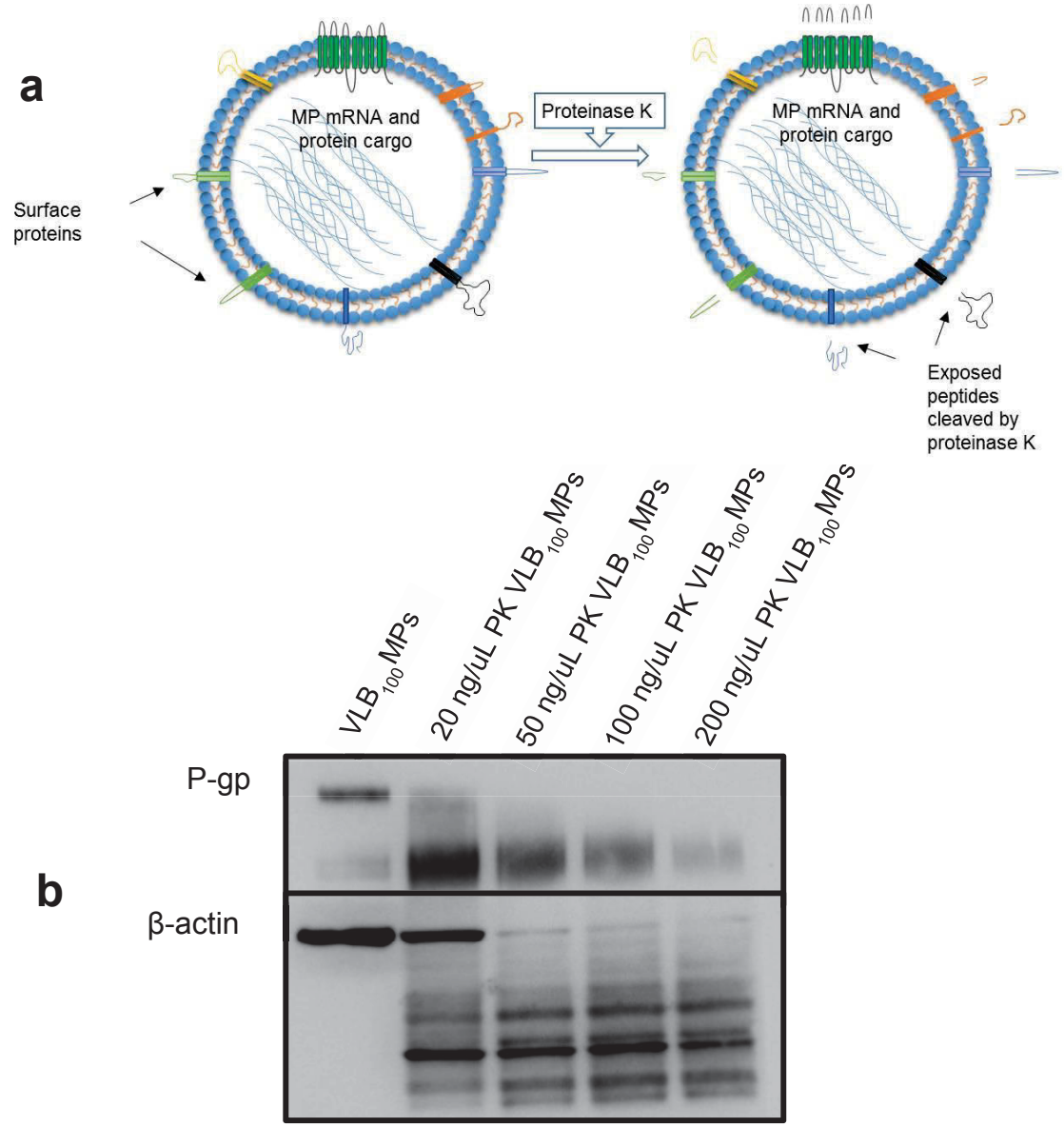

Figure 3.4. Surface shaving of P-gp fragments by proteinase K. *(a) Graphical representation of MP surface shaving methodology. Exposed surface peptides on P-gp are cleaved following treatment with proteinase K, reducing the overall molecular weight of membrane bound MP proteins. (b) Western blot detection of P-gp after surface shaving with increasing amounts of proteinase K. P-gp was detected using the anti-P-gp antibody, clone F4, Sigma-Aldrich, NSW, Australia). β-actin was detected on proteinase-K treatments below 100 ng/mL (clone AC-74, Sigma-Aldrich). Data is representative of 2-3 independent experiments.*

iii. The molecular weight of P-gp changes following surface shaving

Following proteinase K surface shaving we detected a change in the molecular weight of endogenous P-gp contained within the MPs. Specifically we observed a reduction of the proteins molecular weight from 170 kDa to the detection of multiple fragments around the 42 KDa size range (Figure 3.4). We observed a concentration dependent effect on the proteolytic digestion of P-gp. Treatment with 20 ng/mL of proteinase K for 5 min, resulted in a smearing around the 170 kDa region, consistent with incomplete shaving of surface peptides. Upon treatment with 50 ng/mL or more of proteinase K, we observed a complete loss of the 170 kDa band consistent with the proteolytic digestion of the protein (Figure 3.4).

iv. Newly synthesized full length P-gp is detectable in recipient cells following MP co-culture

In detecting functionality of the transferred nucleic acid transcript in recipient cells, shaved VLB_{100}MPs were co-cultured with recipient CEM cells, the latter inherently devoid of P-gp expression (Figure 3.5). Considering that human P-gp has a turnover of approximately 18.3 h (31), we co-cultured for periods of 19 h and 24 h. We observed a 170 kDa P-gp band in recipient CEM cells by Western blot analysis at both time points (Figure 3.5). The presence of the P-gp band is consistent with the transfer and translation of the *ABCB1* transcript in recipient cells following MP co-culture.

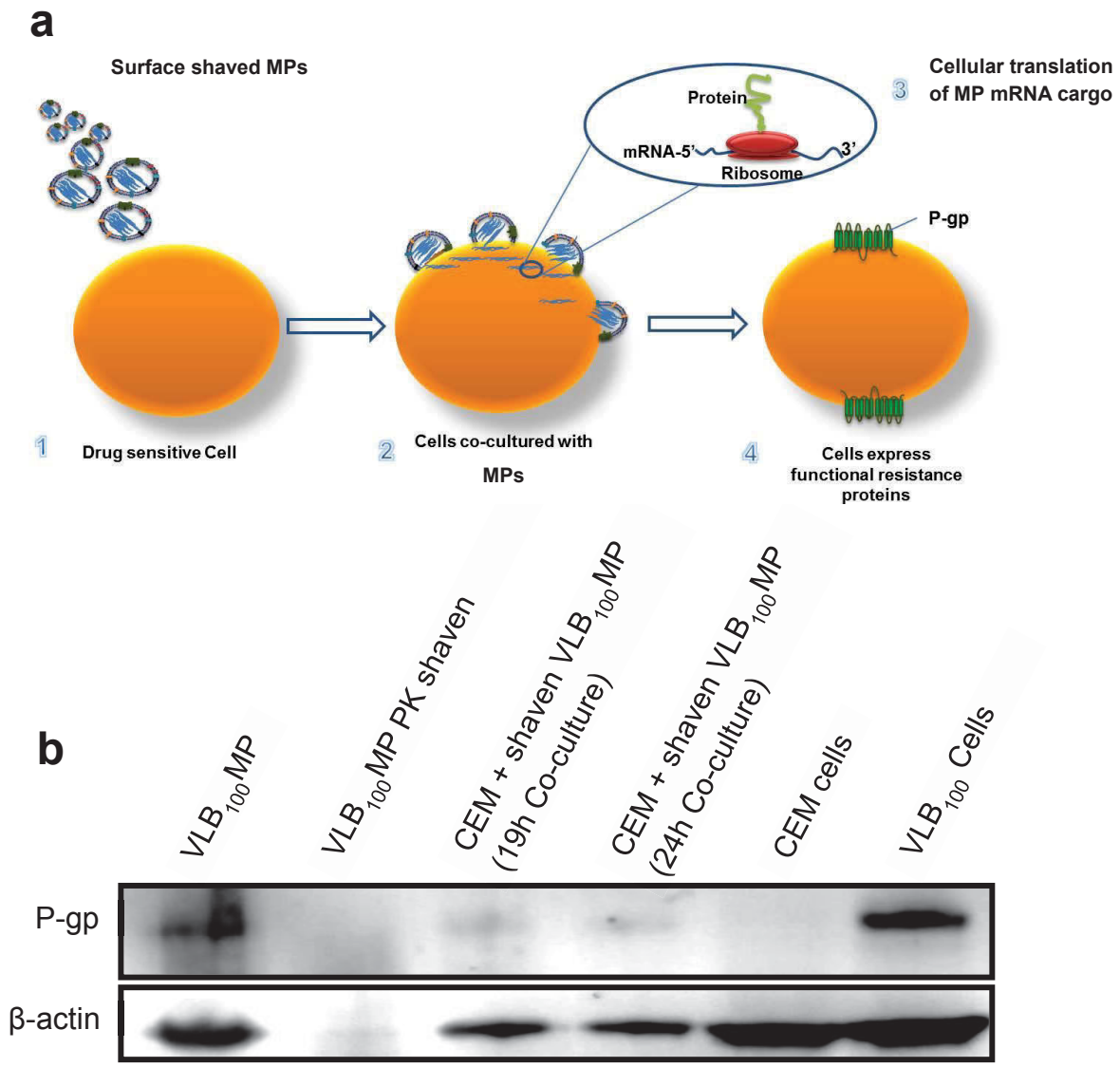

Figure 3.5. Translation of MP contained ABCB1 transcript into recipient cells. *(a) Graphical representation of translation of transferred MP RNA in recipient cells. Surface shaved VLB_{100}MPs were co-cultured with drug sensitive CEM cells to allow transfer of MP mRNA cargo. Transferred ABCB1 transcript translates to a 170 kDa surface protein in recipient cells. (b) Translated P-gp/ABCB1 were detected using Western blot analysis. P-gp was detected in CEM cells after 19 h of co-culture with shaved VLB_{100}MPs using anti-P-gp mAb (Clone F4, Sigma-Aldrich, NSW, Australia). All MPs were shaven with 50 ng/uL for 5 mins at 37^{o}C with gentle shaking. Data is representative of a typical experiment (n=2-3).*

3.4.3. Newly synthesised P-gp is functional in the recipient cells.

To investigate the drug transport functionality of the newly translated P-gp in the recipient cells, the calcein-AM dye exclusion assay was used following a 4 h and 24 h co-culture with shaven MPs (Figure 3.6). The exclusion of calcein-AM in P-gp expressing cells is dependent on the functionality of the protein and is commonly used in the assessment of P-gp mediated MDR (7, 14, 32). We previously reported the transfer and functionality of P-gp in recipient CEM cells following a 4 h co-culture period and attributed this to the transfer of functional P-gp contained within the MPs (1). Following 4 h co-culture with shaven VLB_{100}MPs we observed no significant difference in calcein accumulation (Figure 3.6), consistent with the loss of functionality following surface shaving. Upon 24 h co-culture with shaven MPs, we observed a significant 1.311 (± 0.072) fold decrease in calcein accumulation in recipient cells (Figure 3.6), consistent with the presence of translated and functional protein at this time (Figure 3.5).

To further validate the functionality of the newly translated protein in recipient cells, the effects of the P-gp inhibitor Cyclosporin A (CsA) were examined. In the presence of 10 μM CsA, after 24 hours co-culture, we observed a significant 1.218 (± 0.172)*, $P < 0.05$, fold reversal of calcein accumulation relative to control CEM + VLBMPs, consistent with the pharmacological inhibition of P-gp mediated transport of calcein in the recipient cells. Similarly, we observed a significant 2.37 (± 0.68) fold reversal in CsA treated control VLB_{100} cells compared to VLB_{100} cells alone, demonstrating the inhibition of P-gp mediated drug efflux in our controls. These

results evidence the presence of functional P-gp following MP transfer of functional nucleic acid in recipient cells.

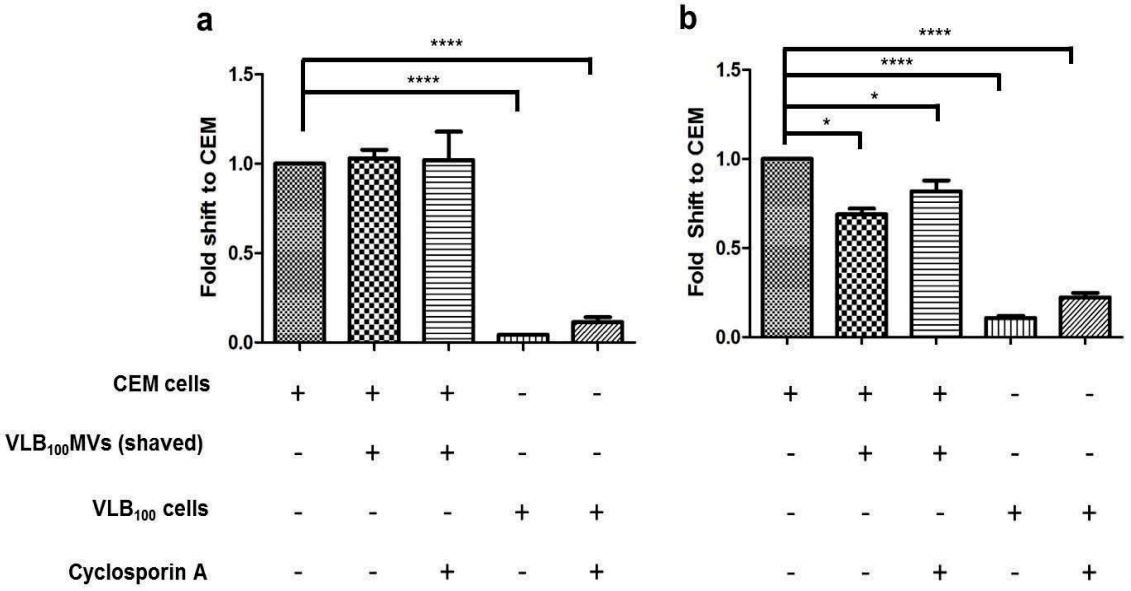

Figure 3.6. Newly translated P-gp is functional. *Calcein accumulation in CEM cells after* ***(a)*** *4 h or* ***(b)*** *24 h co-culture with shaved VLB_{100}MPs. Data represents the fold decrease in calcein accumulation relative to CEM cells. A decrease in calcein accumulation in CEM cells was observed after a 24 h co-culture with shaved VLB_{100}MPs. The accumulation deficit was reversed following CsA exposure. No significant shift in fluorescence was detected in CEM cells co-cultured with VLB_{100}MPs after 4 h. Data represents the mean ± S.E.M of at least 3 independent experiments. *P < 0.05, ****P <0.0001*

3.5. Discussion

Currently there is no methodology that allows for the detection of newly synthesized proteins in their native form as a result of homotypic nucleic acid transfer by MPs. Previous studies that utilize cross-species co-culture models (11) or reporter gene methodologies to demonstrate mRNA functionality (10) are not reflective of the native endogenous state. We report for the first time the translation of native, unlabelled human MP-RNA in human recipient cells following intercellular transfer by MPs.

We confirm the packaging of functional transcripts in MPs using a RRTA. The observation of a 170 kDa P-gp band on Western blot after addition of VLB_{100}MP total RNA into a RRTA system establish that MPs carry intact functional transcripts (Figure 3.1). We further identify additional proteins translated from VLB_{100}MP RNA using tandem mass spectrometry (Table 3.1). The reason for the large overlap between sample and background, we believe is due to the conservation of proteins between mammalian species (33). We identify 5 unique proteins from the VLB_{100}MP translation lysate including P-gp, 60S ribosomal protein L26, Isoform 2 of 4F2 cell-surface antigen heavy chain, proteasome subunit beta type 6 and sodium/potassium-transporting ATPase subunit alpha-4. These proteins are reported to play a significant role in human cancer tumorigenesis and multidrug resistance (Table 3.1).

Using surface shaving methodology we demonstrate for the first time functionality of MP nucleic acid cargo through the translation of exogenously acquired *ABCB1* transcripts from VLB_{100} MPs in recipient CEM cells. We first confirm the integrity of intravesicular content in MPs using Calcein-AM and AO. After treatment of

shaved MPs with calcein-AM, we detect a modest increase in fluorescent calcein accumulation 1.36 ± 0.07 fold, ($P < 0.05$) (Figure 3.2). This increase may be attributed to the increased permeability as a result of surface shaving. However, our observation of an increase, rather than a decrease of calcein accumulation, is indicative that MP contents remains trapped in the intravesicular space as calcein fluorescence is dependent on the presence of cleavage of calcein AM by intravesicular esterases (Figure 3.2)(7, 14). We further confirm the integrity of the nucleic acid content within MPs using AO staining and observe no significant changes in fluorescence intensity in response to proteinase K treatment once again consistent with maintenance of integrity of MP content following shaving (Figure 3.2).

After co-culturing AO or calcein-AM stained MPs with cells, there was no significant difference in MFI with either shaved or non-shaved MPs (Figure 3.3). One possible reason for this difference is the cell type in question with the immunological origin of VLB_{100} MPs and their reliance to interact with numerous cell types in the context of their immunological role.

In determining the optimal concentration of proteinase K for surface peptide shaving, we performed a Western blot analysis on MPs (Figure 3.4). We determined that a minimum of 50 ng/mL was required to completely remove detection of full length 170 kDa P-gp by Western blot by cleavage of its extracellular domains. Interestingly, we also observed a decrease in the 42 kDa β-actin detection. This data certainly may support penetration of the proteinase K within the intravesicular space, however it is also consistent with our previous finding, where we detected two distinct vesicle orientation with respect to proteins present on shed MPs (14). The inside-out

vesicle orientation may also explain the exposure of otherwise internalised β-actin to proteinase K shaving.

For complete surface protein digestion, other studies have performed proteinase K shaving experiments with longer time frames (30 min – 3 h) and significantly increased concentrations of enzyme (μg amounts) (34-36). It may be possible that due to our low proteinase K concentrations (50 ng/uL) and incubation period (5 min), that other membrane protein may remain intact. To adapt this method for detection of other unique proteins, this amount of proteinase K treatment may require additional proteinase K treatment optimization.

We have previously reported that MPs isolated from VLB_{100}MPs contain on average 2.2 ng of total RNA for every 1 x 10^5 MPs(14). On this basis, we do not expect to observe a large amount of translated protein relative to parental cells. It may be possible to use fluorescent or infrared secondary antibodies to improve sensitivity in this detection method. Our results are consistent with this as we detect a modest 170 kDa P-gp band in cells after co-culture with shaven MPs with Western blot analysis (Figure 3.5, Lane 3 and 4). This is the first demonstration of nucleic acid functionality using a human homotypic cell system. Our findings also demonstrate for the first time that MP nucleic acids are functional. In the context of cancer MDR, these nucleic acids translate into functional P-gp in recipient cancer cells and hence confer a functional drug efflux capacity (Figure 3.6). We also previously established that co-culture with CEM-MPs for more than 15 h did not induce expression of P-gp (37). Hence MPs or MP proteins do not indirectly induce transcription / translation of P-gp.

We also establish that drug efflux capacity is not evident at 4 h MP co-culture as we observed following a simple cell to cell transfer of functional proteins (1). Rather, functionality, as presented in the data here, required a longer period consistent with the additional time required for mRNA translation. This data again confirms that endogenous P-gp is no longer functional, nor detectable, after proteinase K shaving (Figure 3.4, 3.6). However, after 24 h of MP co-culture we observe a decrease in calcein accumulation in recipient cells, indicative of the acquisition of a functional drug efflux capacity following nucleic acid translation. This accumulation deficit was reversible with the addition of Cyclosporin A, consistent with the inhibition of newly translated functional P-gp (Figure 3.6).

Conclusions

These results expand on our previous findings where we observed a "re-templating" of the recipient cell transcriptional landscape (7). We demonstrate the capacity of MPs to alter the recipient cell proteome and subsequently alter cellular phenotypes through the intercellular transfer of functional nucleic acids.

Our method allows for the observation of translated membrane proteins in a physiologically relevant host cell and eliminates the use of interspecies models and reporter markers. This provides a physiologically relevant analysis of membrane protein expression from MP cargo. Although our experiments have focused on P-gp/*ABCB1* transmembrane protein expression, this methodology can be adapted for the study of various membrane spanning proteins and their transcripts. This method has potential to be expanded for provide further possibilities in this regard.

Chapter 3: Author Contributions Statement

J.F.L performed all experiments, analyzed the data and wrote the manuscript. D.P performed mass spectrometry experiments, provided technical expertise and analyzed the data. M.P and M.B devised experiments and provided technical expertise. All authors have reviewed the manuscript.

Chapter 4

(Chapter Submitted for publication in *Journal of Biological Chemistry*)

4. A Novel mechanism governing the transcriptional regulation of ABC transporters in MDR cancer cells

RUNNING TITLE: Transcriptional regulation of ABC transporters

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4.1. Abstract

P-glycoprotein (P-gp/*ACB1*) and Multidrug resistance-associated protein 1 (MRP1/*ABCC1*) are the main drug efflux transporters associated with treatment failure in cancer. Much attention has been focused on the molecular mechanisms regulating the expression of these transporters as a viable approach for identifying novel drug targets in circumventing cancer multidrug resistance (MDR) clinically. In this paper, we examine the role of miR-326 in the context of its intercellular transfer between cancer cells by extracellular membrane vesicles called microparticles (MPs). We observe that cellular suppression of *ABCC1* by miR-326 is modulated by the presence of *ABCB1* transcript. Specifically we show that siRNA silencing of MP-transferred *ABCB1* transcript reverses the knockdown effects of miRNA-326 on target MRP1/*ABCC1* transcripts. We also demonstrate, a dominance of *ABCB1* transcripts when co-localised with *ABCC1* transcripts, which is consistent with the facilitation of miR-326 function by *ABCB1*. This study identifies a novel pathway regulating the expression of ABC transporters and positions *ABCB1* mRNA as a transcriptional regulator of other members of this superfamily in multidrug resistant cells through its actions on miRNAs.

4.2. Introduction

Cancer Multidrug resistance (MDR) is a significant obstacle in clinical oncology and is a leading cause of treatment failure and relapse in cancer patients (37). The overexpression of P-gp and MRP1 have been correlated with poor prognosis and survival in cancers such as acute and chronic leukaemia, pancreatic cancer, lung cancer, hepatic carcinoma and neuroblastoma (3, 37-44) . Interestingly, in cancers such as non-small-cell lung and laryngeal carcinoma, expression of MRP1 is prevalent at the early stages of disease but declines with disease progression being replaced by the overexpression of P-gp (2, 3). Although found co-localised at numerous pharmacological sites within the body, very rarely are both proteins overexpressed simultaneously in the context of malignancy and the molecular pathways regulating their selective expression, remains unclear (2, 44-48).

MDR can either be inherent at the time of diagnosis or acquired following an initial positive response to chemotherapy. Both P-gp/*ABCB1* and MRP1/*ABCC1* mediated MDR can be induced by a similar yet a distinctly unique repertoire of drugs (49, 50). There are many transcriptional regulators governing the expression of these transporters including pregnane-X-receptor (PXR), Notch1, NF-κB and Heat-shock transcription factor 1 (HSF1) (51-53). Each of these molecular regulators target specific transporter, as a result; the acquired trait displays bias towards a single phenotype (3, 54, 55). However, as aforementioned, the MDR phenotype has also been shown to switch with disease progression. The mechanism governing the dominance and the steering of one MDR phenotype from one to another remains unknown.

We have previously reported on the transfer and acquisition of MDR via the intercellular exchange of functional resistance proteins as well as nucleic acids by a class of extracellular vesicles called microparticles (MPs) (7, 9). MPs are important vectors in intercellular communication processes across a number of different pathologies, through their capacity to incorporate and transfer bioactive cargo both locally and to distant sites (1, 7, 9, 10, 14, 56-58). MPs have a remarkable capacity to re-template recipient cell phenotypes to reflect that of their donor cells (7, 9, 58). The mechanism for MP-mediated trait dominance was proposed to arise from the transfer of functional proteins, transcripts, regulatory nucleic acids and functional intermediates (1, 7, 9, 58). These regulatory mediators can either be enriched or depleted in MPs relative to their parental cells, broadening the scope of MP capabilities (9).

One of the proposed mechanisms governing MP-mediated trait dominance is the packaging of discrete microRNAs (miRNAs)(9, 58, 59). miRNAs are small non-coding RNAs (19-23 nucleotides) that are important regulators of gene expression at the post-transcriptional level (59, 60). These regulatory nucleic acids bind to the 3'UTR regions of target transcripts and cause repression of translation, cleavage of mRNA or induction of translation (59-63). These small non-coding nucleic acids have attracted a lot of interest due to their key roles in a wide range of biological processes such as proliferation, differentiation, carcinogenesis, chemoresistance and embryogenesis (7, 9, 58, 59, 62, 64-68)

The suppressive function of miR-326 on *ABCC1* has been firmly established via luciferase reporter assays and transfection of mimics/inhibitors (9, 61, 62). The

transfer of miR-326 from P-gp mediated MDR breast cancer and leukaemia cells to recipient cells was previously reported (9). Relative to leukaemia MPs, breast cancer MPs were shown to package 4-times more *ABCB1* transcripts, whilst miR-326 levels were similar in both samples. Despite the similar levels of miR-326, there was no suppression of *ABCC1* when co-cultured with MPs carrying less *ABCB1* (9). Herein, we expand on our previous findings and show that miR-326 is a significant player in the MP-mediated suppression of *ABCC1* in recipient leukaemia cells and its function is regulated by the presence of *ABCB1* transcript.

Furthermore, by simultaneously co-culturing MPs isolated from P-gp and MRP1 overexpressed cell lines, we demonstrate the dominance of the former in otherwise drug sensitive recipient cells. The cellular capacity to interchange between distinct and functionally redundant drug transporters equips cancer cells with an additional survival advantage against xenobiotic exposure. This study introduces a mechanistic relationship between miR-326 and *ABCB1* in the suppression of *ABCC1* and describes the existence of a novel pathway regulating ABC-transporter expression and sheds light on the mechanisms relating the acquisition of MP transferred traits in cancer cells.

4.3. Materials and Methods

4.3.1. Cell lines

The drug sensitive human acute lymphoblastic leukaemia cell line CCRF-CEM [25] (designated CEM for simplicity) and its MDR derivatives, E_{1000} and VLB_{100} were used in this study. The CEM and VLB_{100} cell lines have been validated earlier as an appropriate model for the study of P-gp mediated MDR *in vitro* (1, 12). The E_{1000} cells represent a validated model for the MRP1 overexpressed phenotype (69, 70). The E_{1000} cell line was a kind gift from Prof. Ross Davey (Kolling Institute, Royal North Shore Hospital, NSW, Australia). All cell lines were cultured in RPMI-1640 media (Sigma-Aldrich, NSW, Australia), supplemented with 10% heat-inactivated foetal bovine serum (Life Technologies, Victoria, Australia) and maintained at 37◦C and 5% CO_2. All cells lines were tested for mycoplasma contamination routinely.

4.3.2. MP isolation

MPs were isolated from approximately $4 \times 10^8 - 6 \times 10^8$ cells/100 mL of VLB_{100} and E_{1000} cells by differential centrifugation as described previously (1, 6, 7, 9, 14, 58, 71). Briefly, the cell supernatant was collected and centrifuged at 500 x g for 5 min to pellet the cell population or debris. The supernatant was further centrifuged at 15,000 x g for 1 h at 15 ^oC and the pellet was resuspended in serum free RPMI-1640. The MP suspension was further centrifuged at 2,000 x g for 1 min to remove remaining debris. To concentrate the MP fraction, the supernatant was further centrifuged at 18,000 x g for 30 mins and resuspended in serum free RPMI-1640.

MPs isolated from VLB_{100} and E_{1000} cells (VLB_{100}MPs and E_{1000}MPs, respectively) were previously characterized and validated using flow cytometry, scanning electron microscopy and mass spectrometry (1, 6, 7, 9, 71). MP total protein content was quantified using Qubit® 2.0 Fluorometer protein assay (Invitrogen, Life Technologies, Victoria, Australia) as per manufacturer's instructions.

4.3.3. ABCB1 and ABCC1 profiling by qRT-PCR

The presence of *ABCC1* and *ABCB1* transcripts encoding for MRP1 and P-gp respectively, in isolated MPs and in recipient cells prior to and after co-culture with VLB_{100} and/or E_{1000} MPs was assessed using qRT-PCR. Reactions conducted on MRP1 overexpressing E_{1000} cells, E_{1000}MPs, P-gp overexpressing VLB_{100}MPs and drug sensitive CEM cells served as positive and negative controls for the expression of *ABCC1* and *ABCB1*.

4.3.3.1. ABCB1 Silencing and miR-326 Inhibiton of E_{1000} recipient cells after VLB_{100}MP co-culture

For silencing of *ABCB1* and inhibition of miR-326, experiments were performed using lipofectamine® 3000 (Life Technologies, Victoria, Australia) according to manufacturer's instructions. For P-gp siRNA experiments (SI00018718; Qiagen, VIC, Australia) and miR-326 inhibitor experiments (4464084; Invitrogen, Life Technologies, VIC, Australia), a final concentration of 50 nM of nucleic acid was used. A siRNA scrambled control (SI03650318; Qiagen, VIC, Australia) and miRNA inhibitor negative control (4464076; Invitrogen, Life Technologies, VIC, Australia) was used. Briefly, 1 x E1000 cells were seeded in a 96-well U-bottom plate and allowed to incubate for 24 h

before transfection. 200 µg of VLB_{100}MPs were to the added to the E_{1000} cells immediately before transfection of cells and allowed to incubate for 24 h. After 24 h of culture, cells were washed at 500 x g and resuspended in pre-warmed RPMI media supplemented 10% FCS. Transfection was repeated and cells incubated for another 24 h at 37◦C and 5% CO_2 in 96-well plates. The cells were washed three times with PBS at room temperature at 500 x g, and total cellular RNA extracted from cell or MP the pellet as described below.

4.3.3.2. Competitive transfer studies

For competitive transfer studies using qRT-PCR, 100 µg of MPs isolated from VLB_{100} and E_{1000} cells were co-cultured with 1×10^5 CEM cells. For the single MP transfer studies, 100 µg of MPs isolated from VLB_{100} or E_{1000} cells were co-cultured with 1×10^5 CEM cells for 15 h, as described previously above. All cells were maintained at 37◦C and 5% CO_2 in 96-well plates. The cells were washed three times with PBS at room temperature at 500 x g, and total cellular RNA extracted from cell or MP the pellet as described below.

4.3.3.3. Total RNA isolation and qRT-PCR of samples

Total cellular RNA was extracted from samples using the Trizol®(Molecular Research Center, OH, USA) reagent as per the manufacturer's recommendations. cDNA synthesis was performed using 1000-1500 ng RNA from each sample using the Advantage RT-for-PCR Kit (Clontech Laboratories, Inc., CA, USA) on the Mastercycler®Gradient thermal cycler (Eppendorf, North Ryde, Australia).The following primers (Sigma–Aldrich, NSW, Australia) were used: *ABCC1* forward: 5'-TGT GGG AAA

ACA CAT CTT TGA-3', reverse: 5'-CTG TGC GTGACC AAG ATCC-3', and *ABCB1* forward: 5'-AAG GCA TTT ACT TCAAAC TTG TCA-3', reverse: 5'-TGG ATT CAT CAG CTG CAT TTT-3'; and *GAPDH* forward: 5'-TGC CAA ATA TGATGA CAT CAA GAA-3'; reverse: 5'-GGA GTG GGT GTC GCT GTTG-3'. Each 20 µL reaction was diluted to 50 µL with RNAse free H_2O and 3 µL of cDNA was used for each qRT-PCR reaction. SYBR green qRT-PCR amplifications were performed in triplicates using the Mastercycler®Gradient instrument (Eppendorf, North Ryde, Australia). Reactions were carried out in a 20 µL volume containing 10 µL of 2× SYBR Green Premix ExTaq (Takara Bio, Shiga, Japan) and specific primers (10 pmol/reaction). The thermal profile for the qRT-PCR was 95◦Cfor 5 min followed by 45 cycles of 95◦C for 5 s, 55◦C for 10 s and72◦C for 15 s. The Ct data for each sample was collected automatically. The ΔCt of each group was calculated using the following formula: Δ*Ct* = MDR gene *Ct* − housekeeping gene *Ct*. Relative expression was calculated using ΔΔ*Ct* = 2−ΔCt and expressed as fold difference relative to the drug sensitive control (ΔΔ*Ct* of sample/ΔΔ*Ct* of drug sensitive cells × 1) as arbitrary units (a.u.)

4.3.4. miR-326 profile of cells and MPs by qRT-PCR

Total RNA from isolated MPs and whole cells was extracted as described above. cDNA for miRNA was synthesized using the NCode® miRNA First Strand cDNA Module kit (Life Technologies, Victoria, Australia) using the Mastercycler®Gradient thermal cycler (Eppendorf, North Ryde, Australia). miR-326 specific primers (10 pmole/reaction) were used for the PCR reaction for the detection of miRNA using miR-U6 as the housekeeping primer (Sigma-Aldrich, NSW, Australia). The nucleotide

sequences used were: miR-326, 5'-CCU CUG GGC CCU UCC UCC AG-3', miR-U6 forward, 5'-CTC GCT TCG GCA GCA CA-3', and miR-U6 reverse, 5'-AAC GCT TCA CGA ATT TGC GT-3'. SYBR Green qRT-PCR amplifications were performed using the Mastercycler® Realplex (Eppendorf, NY, USA). Reactions were carried out in a 20 μL volume containing 10 μL of 2 × SYBR Green Premix ExTaq (Takara Bio, Shiga, Japan). The thermal profile for the qRT-PCR was 91°C for 5 min followed by 45 cycles of 91°C for 15 sec, 60°C for 30 sec, followed by melting curve detection. The C*t* data of each sample was collected automatically and data analyzed as described above.

4.3.5. SDS-PAGE and Western blotting

Briefly, 100 μg of MPs isolated from either VLB_{100} and / or E_{1000} cells were co-cultured with 1×10^5 CEM cells for 4 h and maintained at 37◦C and 5% CO_2 in 96-well plates. We have previously demonstrated the MP-transfer of both P-gp and MRP1 after 4 h of co-culture (1, 7). The recipient cells were washed twice with complete culture medium to remove unbound MPs. MPs were isolated as described above and the MP pellet and cells were lysed using CelLytic M™Cell Lysis reagent (C2978; Sigma-Aldrich, NSW, Australia) in the presence of 1% (v/v) protease inhibitor cocktail (P8340; Sigma–Aldrich, NSW, Australia) on ice for 30 min. The lysates were centrifuged at 10,000 × g for 10 min at 4◦C to pellet the insolubilized fraction. Total protein lysate was quantified using a Qubit®2.0 Fluorometer (Life Technologies, Victoria, Australia) as per the manufacturer's instructions. 30 μg of total protein was separated by electrophoresis using a 4–12% NuPAGE Bis-Tris gel (Invitrogen, Life Technologies, Victoria, Australia) at a constant voltage of 150 V for 110 min and electro-blotted onto

a PVDF membrane at 30 V for 90 min. For detection of P-gp, MRP1 and β-actin loading control, 1:5000 dilution of anti-P-gp mAb (Clone F4, Sigma-aldrich, NSW, Australia), 1:2000 dilution of anti-MRP1 mAb (Clone QCRL-1, Sigma–Aldrich, NSW, Australia) and 1:10,000 dilution of anti- β-actin antibody (Clone AC-74, Sigma–Aldrich, NSW, Australia) were used, respectively. Incubation of the antibodies was for 1 h after blocking overnight with 5% skim milk in Tris buffered saline and 0.05% Tween 20 (TBST). After washing 3 times in TBST, the membranes were incubated for 1 h with anti-Mouse-HRP secondary antibody (Promega, NSW, Australia) at 1:10,000 dilution. Protein expression was visualised using the Lumi-light Western Blotting Substrate (Roche Applied Science, NSW, Australia). The membranes were imaged using the luminescent image analyser LAS-3000 (Fujifilms, Brookvale, NSW, Australia).

4.3.6. Calcein-AM dye exclusion assay

To assess the functionality of transferred MRP1 and P-gp, we performed the flow cytometric calcein-AM dye exclusion assay as previous described (7, 14, 72). Calcein-AM dye exclusion has been extensively validated for MRP1 and P-gp functionality previously (7, 14, 72). We sought to assess the dominance of *ABCB1* or *ABCC1* after co-culture with drug sensitive CEM cells. For conventional MP transfer studies (E_{1000}MPs or VLB_{100}MPs co-cultured with recipient cells), 200 μg of MPs isolated from VLB_{100} or E_{1000} cells were co-cultured with 1×10^5 CEM cells as described previously (7, 9, 58). For competitive transfer studies (both VLB_{100}MP and E_{1000}MPs in co-culture with recipient cells), 100 μg of MPs isolated from VLB_{100} and E_{1000} cells were co-cultured with 1×10^5 CEM cells. All cells were maintained at 37◦C and 5% CO_2 in 96-

well plates. The 15 h time-point required for the transfer of transporter and nucleic acid material from these MPs has been previously established (7). At 15 h post MP exposure, the recipient cells were washed twice with complete culture medium to remove unbound MPs and treated with 0.1 µM of Calcein-AM (Life Technologies, Victoria, Australia) for 1 h in the presence or absence of a 1 h pre-incubation with 2 mM probenecid (PBC) (MRP inhibitor)(7, 72) at 37◦C and 5% CO_2. The cells were subsequently washed three times with PBS and re-suspended in 200 µL PBS for flow cytometric analysis as previously described (1, 6, 7, 9, 71). Data is expressed as MFI fold change, where MFI fold change = MFI sample / MFI control.

4.3.7. Statistical Analysis

Means between groups were compared using an unpaired two-tailed students t-test. P-values less than 0.05 ($P < 0.05$) were accepted as statistically significant. Fold shift or percentage change results were expressed as mean ± standard deviation (SD) and were representative of 2-3 independent experiments. All statistical analyses were performed using GraphPad Prism version 5.0 for Windows Software (Graphpad Software, CA, USA).

4.4. Results

4.4.1. miR-326, ABCC1 and ABCB1 transcripts are packaged in VLB_{100}MPs and E_{1000}MPs and are transferred to recipient cells

We have previously reported on the presence of miR-326 in VLB_{100} cells and their MPs (9). In this paper, we reconfirm the presence of miR-326 in VLB_{100}MPs and its transfer to E_{1000} recipient cells by qRT-PCR (Figure 4.1). We observe a similar large amount of endogenous miR-326 in E_{1000} cells compared to VLB_{100}. VLB_{100}MPs were shown to package 1.59 ± 0.20-fold more miR-326 compared to E_{1000}MPs. The amount in E_{1000} cells was increased by 2.78 ± 0.59-fold after co-culture with VLB_{100}MPs for 4 h, consistent with MP-transfer of the cargo.

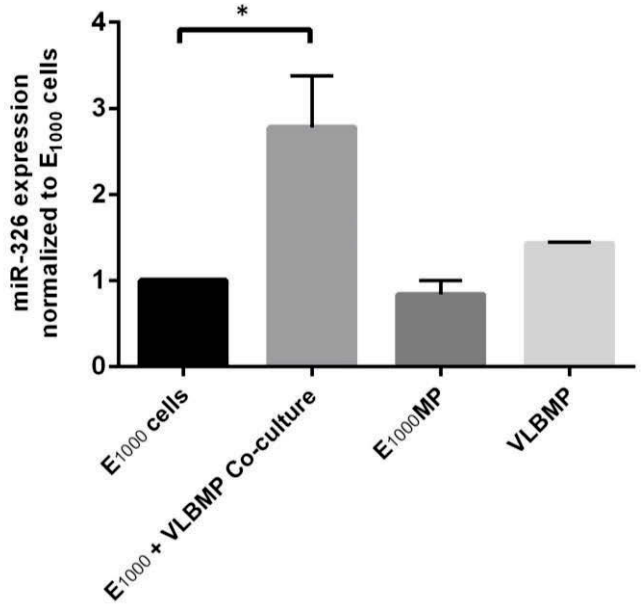

Figure 4.1. VLB_{100}MPs transfer miR-326 to recipient E_{1000} cells. *miR-326 is present in E_{1000}MPs, VLB_{100}MPs and E_{1000}Cells before and after 4 h co-culture with VLB_{100}MPs. miRNA326 levels are significantly increased in E_{1000} cells following co-culture with*

*VLB_{100}MPs. Data was normalised to the levels of expression in E_{1000} cells. Data represents mean ± S.E of 2-3 independent experiments conducted in triplicate. *P < 0.05*

4.4.2. ABCB1 regulates ABCC1 suppression through miR-326 function

To confirm the role of miR-326 in *ABCC1* suppression, we inhibited VLB_{100}MP-transferred miR-326 immediately after co-culture using miRNA inhibitors in recipient cells and examined the effects on *ABCB1* and *ABCC1* expression (Figure 4.2). VLB_{100}MPs were shown to package relatively large amounts of *ABCB1* (48987.74 ± 10206.00-fold compared to E_{1000} cells) and lacked *ABCC1* transcript.

Relative to E_{1000} cells alone, E_{1000} + VLB_{100}MPs showed a significant 66 ± 4 % suppression in *ABCC1* transcript levels (Figure 4.2 A) and a significant 3647.78 ± 321.40-fold increase in *ABCB1* transcript levels (Figure 4.2 B) consistent with our previous findings with this cell and MP pair (7)(Figure 4.2). We attribute the suppression of *ABCC1* to the transfer of miRNA 326 and the increase in *ABCB1* to the transfer of vast amounts of *ABCB1* transcript present in these (9). Upon inhibition of miR-326, we observe a 36 ± 16 % ($P < 0.05$) suppression *ABCC1* transcript after inhibition of transferred miR-326 transcript when compared to E_{1000} + VLB_{100}MP negative control. This represents a significant reversal in MP-mediated suppression of *ABCC1* in E_{1000} + VLB_{100}MP co-cultured cells (Figure 4.2 A).

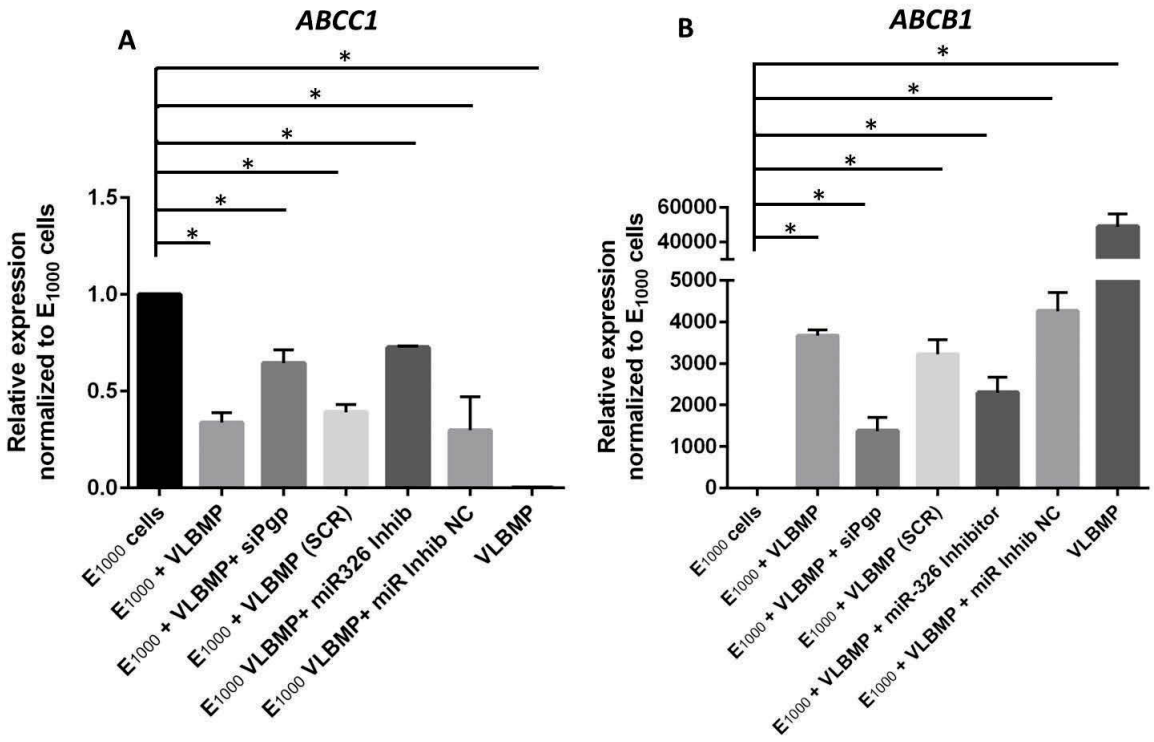

Figure 4.2. ABCB1 regulates ABCC1 suppression through miR-326 function*. Relative ABCB1 and ABCC1 expression were determined using qRT-PCR. Co-culture of recipient E_{1000} cells to VLB_{100}MPs resulted in a suppression of ABCC1 and an increase in ABCB1. The suppression of ABCC1 was reversed upon inhibition of miRNA-326 or silencing transferred ABCB1. Data is normalised to E_{1000} cells. Data represents mean ± S.E of 2-3 independent experiments conducted in triplicate. *P ≤ 0.05.*

To examine the relationship between the transfer of *ABCB1* transcripts by MPs and miR-326 function in recipient cells, we silenced the *ABCB1* in E_{1000} cells acquired following its transfer by VLB_{100}MPs (Figure 4.2A). In doing so, we observed a 27 ± 07 % suppression of *ABCC1* transcript relative to E_{1000} cells. Interestingly, the level of suppression observed was consistent with the level of *ABCC1* suppression observed following miR-326 inhibition (Figure 4.2 A). We observed no significant difference with the scrambled controls in both *ABCB1* and *ABCC1* profiles (Figure 4.2). Likewise there

was no significant difference in ABC expression profiles in miR-326 inhibitor and miRNA inhibitor negative control. These results strongly support a role for both *ABCB1* and miR-326 in the suppression of *ABCC1*.

4.4.3. ABCB1 expression dominates ABCC1 expression in context of MP-transfer of nucleic acids

We sought to examine the dominance of transcript acquired in recipient cells by MP transfer by conducting competitive transfer studies (Figure 4.3). Specifically, we co-cultured recipient drug sensitive cells CEM cells with MPs isolated from both P-gp overexpressing cells (VLB_{100}) and MRP1 overexpressing cells (E_{1000}). We compared the results of the competitive transfer study with cells co-cultured with a single MP type (E_{1000}MPs or VLB_{100}MPs).

Relative to CEM cells alone, we observed a significant 91 ± 8% suppression of *ABCC1* levels and a 35.83 ± 3.51-fold increase in *ABCB1*, when CEM cells are co-cultured with VLB_{100}MPs and is consistent with our previous reported findings for a P-gp dominant phenotype of the cells from which the MPs were derived (Figure 4.3 B). Contrary to this, when CEM cells were co-cultured with E_{1000}MPs, specifically; there was a significant 33 ± 13 % suppression of *ABCB1* and a 5.34 ± 3.58-fold increase in *ABCC1,* consistent with the MRP1 dominant phenotype of the cells from which the MPs were derived (Figure 4.3 A). These results are also consistent with our previous findings (7, 9, 58, 59). In our competitive transfer study however, we observe a significant 48 ± 6 % suppression of *ABCC1* transcript and a 37.74 ± 7.17-fold increase in

ABCB1 transcript (Figure 4.3 A, B). This demonstrates the dominance of the *ABCB1* transcript relative to the *ABCC1* transcript conferred by MPs.

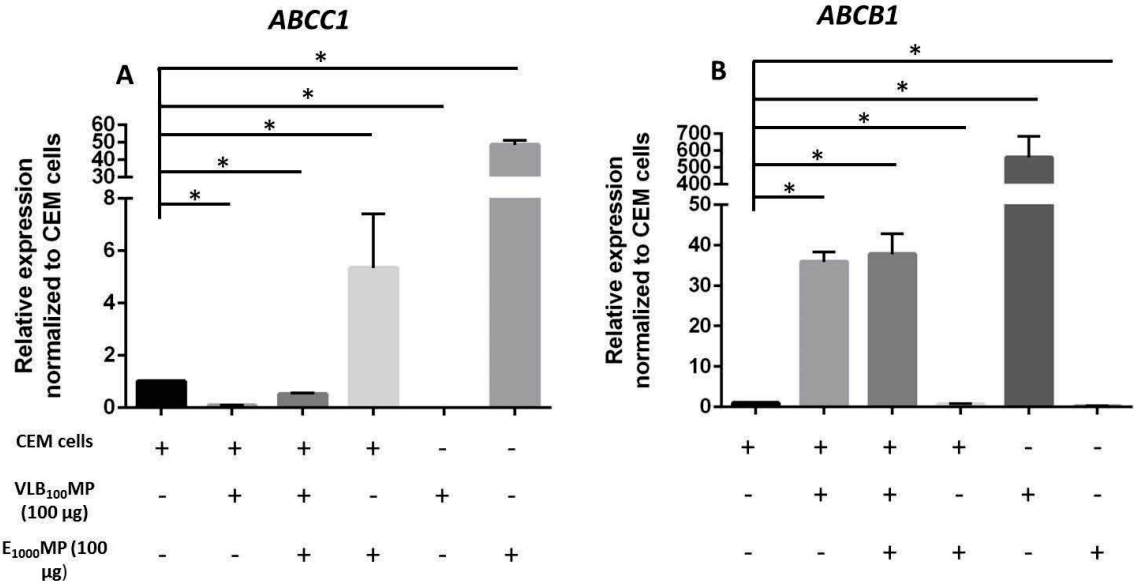

Figure 4.3. Dominance of ABCB1 transcripts in competitively co-cultured CEM cells. ***(A):*** *ABCB1 and* ***(B):*** *ABCC1 profiles following both competitive MP transfer and single MP-transfer studies, are determined using qRT-PCR. MP-mediated trait dominance is shown with conventional MP-transfer studies. CEM cells co-cultured with VLB_{100}MPs display suppression in ABCC1 transcript and increase in ABCB1 transcript. Contrary to this, CEM cells co-cultured with E_{1000}MPs displayed an increase in ABCC1 transcript and suppression of ABCB1 transcript. Competitive transfer shows suppression of ABCC1 and increase in ABCB1, despite transcript transfer by E_{1000}MPs. Data was normalised relative to CEM cells. Data represents mean ± S.E of 2-3 independent experiments conducted in triplicate.* * $P \leq 0.05$

4.4.4. MP-transfer of packaged MRP1 and P-gp is functional in recipient cells

Upon demonstrating that cells had been primed towards a single dominant transcript following MP transfer, we sought to determine the immediate phenotype conferred in the presence of both VLB_{100} and E_{1000} MPs in recipient CEM cells. Our findings confirm that both MRP1 and P-gp proteins co-exist in recipient CEM cells after MP transfer (Figure 4.4). Western blot analysis shows that E_{1000}MPs, E_{1000} cells and CEM + E_{1000}MPs detected positive for the 190 kDa MRP1. Similarly, VLB_{100}MP, VLB_{100} cells and CEM + VLB_{100}MPs detected positive for the 170 kDa P-gp. When both VLB_{100}MP and E_{1000}MPs were co-cultured with CEM cells, we detected the expression of both MRP1 and P-gp in recipient cells following a 4 hr co-culture period. We did not detect P-gp or MRP1 expression in the drug sensitive recipient CEM cells alone. These findings demonstrate that, in the first instance, recipient cells have the capacity to acquire multiple ABC transporters through the process of MP transfer.

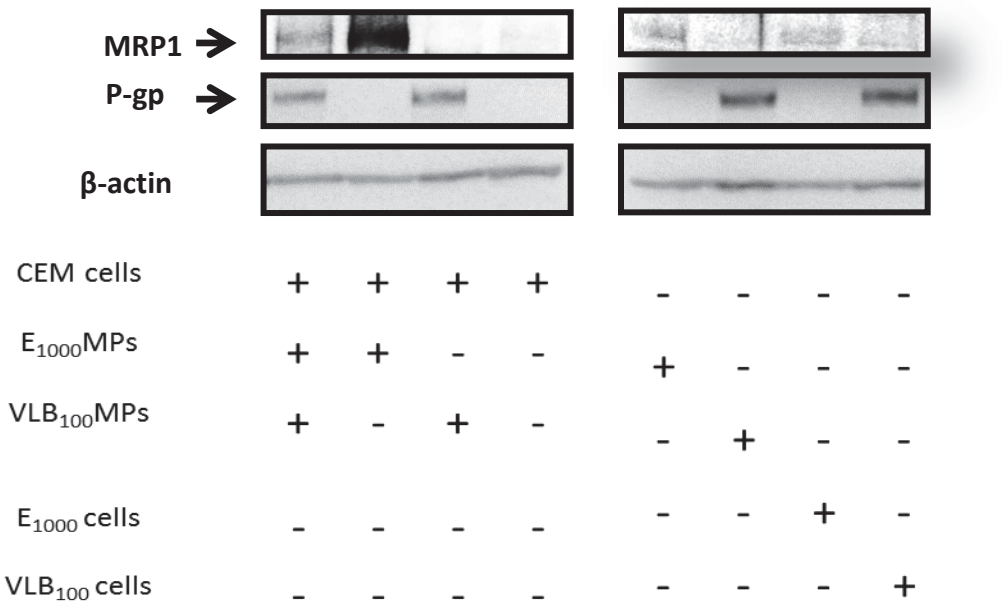

Figure 4.4. Immunodetection of MRP1 and P-gp in CEM cells and MPs*. 190 kDa MRP1 was detected in E_{1000}MPs, E_{1000} cells, CEM + E_{1000}MP and CEM + E_{1000}MP/VLB_{100}MPs using anti-MRP1 antibody (clone QCRL-1, Sigma-Aldrich). 170 kDa P-gp was detected in VLB_{100}MPs, VLB_{100} cells, CEM + VLB_{100}MP and CEM + E_{1000}MP/VLB_{100}MPs using anti-P-gp antibody (Clone F4, Sigma-Aldrich). Detection of β-actin using the anti-β-actin mAb (clone AC-74, Sigma-Aldrich), was used as the internal control. Data is representative of a typical experiment (n=3).*

4.4.5. The acquired transporters are functional in recipient cells and provide an additional immediate fail-safe modality in context of MDR

To investigate whether the co-transferred proteins were functional in drug sensitive recipient cells, the calcein-AM dye exclusion assay was used as previously described (7). The extent of calcein-AM accumulation is dependent on the functionality of both MRP1 and P-gp proteins and is commonly used to assess MRP1/P-gp mediated MDR (7, 14, 32, 72)(Figure 4.5).

For MDR cell controls, VLB_{100} cells and E_{1000} cells displayed a significant fold-shift in mean fluorescent intensity (MFI) of 6.25 ± 1.25 and 9.85 ± 1.85 (100% drug resistant population, $P < 0.05$) less mean fluorescent intensity in calcein accumulation, respectively, compared to CEM cells (Data not shown). After treatment with PBC, this fold shift was 5.94 ± 1.22 and 3.23 ± 1.31 ($P < 0.05$), fold less drug accumulation in VLB_{100} cells and E_{1000} cells compared to CEM cells, respectively, consistent with the inhibition of MRP1 in recipient CEM cells (Data not shown).

A drug resistant positive gate was established based on the calcein fluorescence of drug sensitive CEM cells. Cells which have reduced fluorescent calcein accumulation falling below this gate fail to accumulate calcein and hence have functional ABC-transporters (Figure 4.5). When co-cultured with VLB_{100}MPs or E_{1000}MPs, 75.37 ± 16.14% and 70.83 ± 18.14% of the CEM cell population displayed reduced calcein accumulation, respectively. This is consistent with our earlier findings (7, 14). When CEM cells were co-cultured with both VLB_{100}MPs and E_{1000}MPs, 65.15 ± 7.23% of cells displayed reduced calcein-accumulation (Figure 4.5 A, C, E).

We used a MRP-specific inhibitor to demonstrate the functional redundancy present following MP-transferred of both P-gp and MRP. As expected, there was no significant shift in fluorescence in CEM + VLB_{100}MPs with or without PBC treatment. When CEM + E_{1000}MPs were pre-treated with PBC for 1h, we observed only 5.24 ± 2.4% of the CEM population positive for calcein efflux, consistent with inhibition of MRP1 functionality (Figure 4.5 D). The competitive transfer study displayed 39.37 ± 5.34% of CEM cells positive for calcein efflux after PBC treatment, consistent with simultaneous functionality of P-gp with inhibiton of MRP1 (Figure 4.5 E,F).

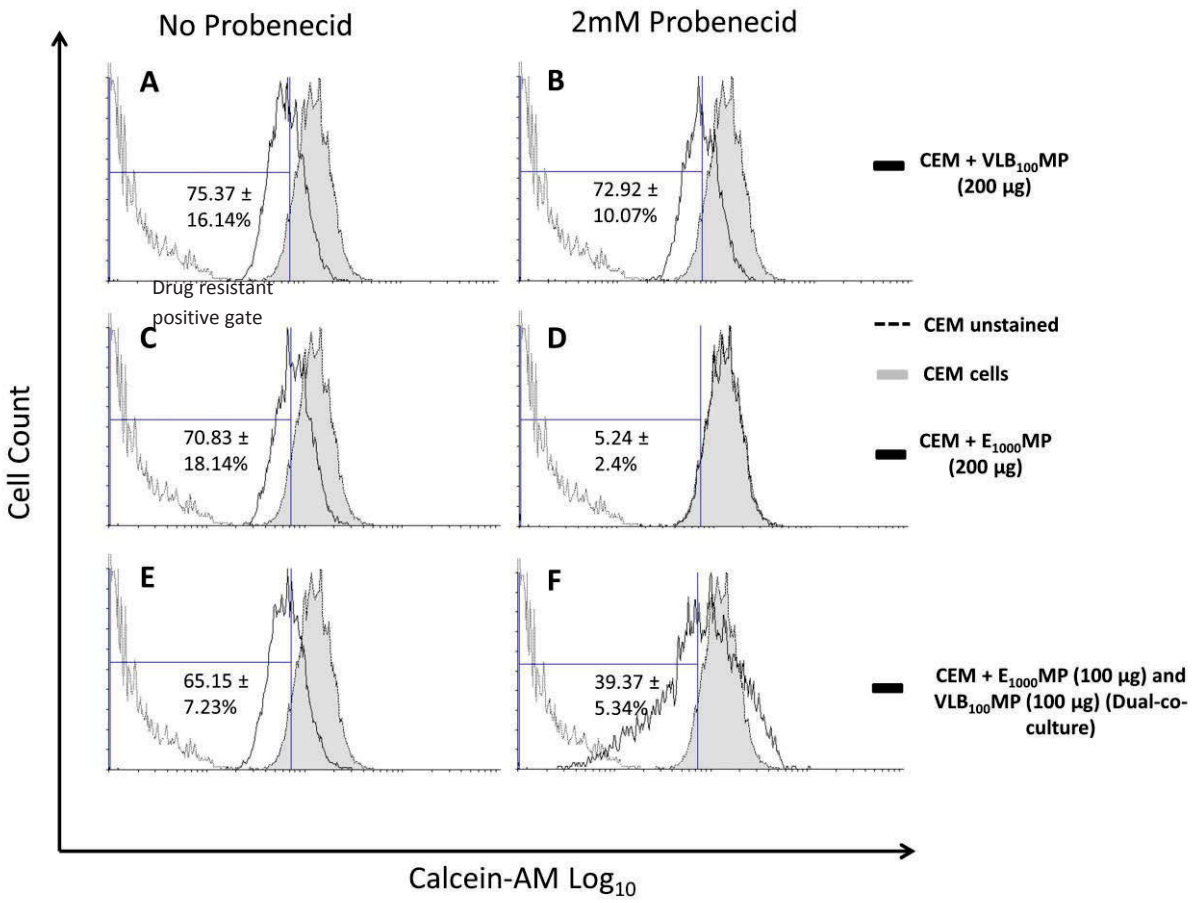

Figure 4.5. MRP1 and P-gp transferred from E_{1000}MPs and VLB_{100}MPs is functional in recipient CEM cells*. Calcein-AM dye exclusion assay with and without PBC treatment on CEM cells co-cultured with (**A, B**): VLB_{100}MPs, (**C, D**): E_{1000}MPs, and (**E, F**): both VLB_{100}MP and E_{1000}MP. Calcein accumulation deficit was observed when CEM cells were co-cultured with VLB_{100}MPs and/or E_{1000}MPs (**A, C, E**). No change was observed on CEM + VLB_{100}MPs before and after pre-treatment with PBC (**A, B**). However, calcein accumulation deficit was reversed in CEM + E_{1000}MPs (**D**) and CEM + VLB_{100}MP + E_{1000}MP cells (**F**) after pre-treatment with PBC. Percentages (%) refer to population of CEM cells positive for calcein deficit. Intracellular calcein accumulation was detected using FCM. Data is representative of a typical experiment (n = 3).*

4.5. Discussion

This is the first demonstration that the function of miR-326 is facilitated with the presence of *ABCB1*. miRNA function has been commonly known to be modulated through a variety of mechanisms such as, miRNA amount, presence of RISC associated protein, quantity of target mRNA and RNA editing through adenosine deaminases (ADARs)(73, 74). We report for the first time an additional mechanism for the regulation of miRNA suppressive function through non-target transcripts.

Our group has previously reported on the transfer and functionality of large membrane spanning proteins, P-gp and MRP1, which are responsible for conferring MDR (1, 7). In addition, we also reported that MPs serve as intercellular communication vehicles through the transfer of discrete miRNA and mRNA species (9, 58, 59). We previously established the transfer of miR-326 from P-gp mediated MDR breast cancer (MCF7/Dx) and VLB_{100} cells to recipient cells via MPs (9). In breast cancer MPs, we detect less than 1/4 *ABCB1* compared to VLB_{100}MPs whilst miR-326 levels were similar in both samples. Similar levels of miR-326 were detected in both breast cancer MPs and VLB_{100} MPs, however, we did not detect similar levels of ABCC1 suppression in elicited by both MPs. MPs carrying less *ABCB1* had less *ABCC1* suppressive effect (9). Therefore, we sought to identify the regulatory role of *ABCB1* in regulating *ABCC1*.

We first established the presence of miR-326 in VLB_{100}MPs and cells via qRT-PCR (Figure 4.1). This is consistent with the transfer of miR-326 from VLB_{100}MPs to drug sensitive CEM cells previously reported (9). Herein, we also confirm the MP-transfer of miR-326 to MRP1/*ABCC1* overexpressing cells, E_{1000} (Figure 4.1). After co-

culture with VLB_{100} cells for 4 h, there is a 48 ± 24% increase in miR-326 levels in E_{1000} cells. Surprisingly, we detect a large amount of miR-326 in recipient E_{1000} cells prior to co-culture, despite its overexpression of MRP1/*ABCC1*. It may be possible that miR-326 exists as an inactive form within these cells, where they are not actively engaged in mRNA target repression. A recent study has observed the abundance of inactive miRNA, where they pool in a reservoir and were regulated by intracellular signalling of the RISC complex (75). This allows for resting cells to dynamically regulate gene expression and respond rapidly to the environment. One possibility exists where miR-326 is dormant in recipient cells prior to the co-culture with VLB_{100}MPs, hence we achieve rapid suppression of *ABCC1* after 4-24 h of co-culture with MPs (Figure 4.2) (7, 9).

We show here that the suppression of *ABCC1* via VLB_{100}MPs is facilitated by the presence and transfer of *ABCB1* transcript by MPs. In silencing *ABCB1* upon MP transfer, we observe a reversal of the MP-mediated suppression of *ABCC1* previously observed (Figure 4.2). This result reveals a possible mechanism for the preference towards a given MDR phenotype. Furthermore, the presence of transferred *ABCB1* may also subsequently induce the activation of the dormant endogenous miR-326 in recipient cells, this, however remains to be determined. Using miRNA inhibitors, we confirm the *ABCC1* suppressive activity of miR-326 in recipient cells. VLB_{100}MPs induce 66 ± 4% suppression of *ABCC1* in E_{1000} cells (Figure 4.2). However, with the inhibition of miR-326 directly after co-culture, we observe a suppression of 35% of *ABCC1* compared to E_{1000} cells alone (Figure 4.3). The significant reversal of *ABCC1*

suppression suggests that miR-326 expression and the presence of *ABCB1* are significant players in the MP-mediated *ABCC1* knockdown observed in these cells.

To further assure the link between miR326 and ABCB1 transcript, it may be possible to perform co-transfection experiments of these nucleic acid species. Also, the act of co-culturing MPs with cells may constitute a form of "stress". In this study, we cannot exclude the effects of stress from MP co-culture or any additional co-transferred biological material from MPs.

Following competitive transfer studies, *ABCB1/C1* profiling showed that resistance traits are steered towards an *ABCB1* dominant state in recipient cells, despite the co-transfer of *ABCC1* by E_{1000}MPs (Figure 4.3). This is consistent with the facilitation of miR-326 suppressive function by *ABCB1* transcripts we observed (Figure 4.2). Although there are no putative binding sites shared between *ABCB1* and miR-326, there must be either a direct or indirect relationship between these two nucleic species in the regulation of *ABCC1*.

We demonstrate the simultaneous transfer MP-packaged P-gp and MRP1 transporters by Western blot analysis and the calcein-AM dye exclusion assay (Figure 4.4 and 4.5). Phenotypes that are acquired in this manner may be transient; however, the dominance of the transferred transcripts appeared to be priming the recipient cells towards a stable and given MDR phenotype in the long term (Figure 4.3).

However, given the varying amounts of MPs between competitive and non-competitive transfer studies in Western blot (100 μg vs 200 μg) (Figure 4.3), we cannot

exclude the additional mediators co-transferred or stress from co-culture with MPs which may play a role in the upregulation of either P-gp or MRP1.

The molecular mechanisms allowing the interchangeability between these two distinct but functionally redundant drug transporters provides an additional means of cancer cell survival. Despite the likely-transient co-expression of the transferred protein, we demonstrate that these transporters can function simultaneously in cancer cells (Figure 4.5). This provides evidence of an additional fail-safe mechanism, whereby the inhibition of a single transporter may not be sufficient to achieve successful treatment.

In conclusion, we show that VLB_{100}MP-regulation of *ABCC1* in recipient cells is governed by the transfer of both miR-326 and *ABCB1* from donor cells. We introduce a novel mechanistic association between miR-326 function and the presence of *ABCB1* transcript, of which there are no known putative binding sites. We provide evidence that although both transporters may co-exist briefly due to horizontal transfer by MPs, recipient cells are primed to express a single phenotype for a sustained MDR phenotype in the long term through the regulation of transferred nucleic acids. This mechanism has the potential to provide cells with a rapid survival advantage to xenobiotic exposure whilst the cells adapt for long-term resistance and suggests the presence of a dynamic process of ABC-transporter expression in cancer cells. This novel pathway driving ABC-transporter regulation presents a new paradigm in which resistance phenotypes are acquired in malignancy over time. Interrogating the nature and implications of this pathway has significant potential in identifying alternative approaches for the management of MDR in clinical practice.

Chapter 4: Author Contributions Statement

J.F.L performed all experiments, analyzed the data and wrote the manuscript. D.P helped devise experiments and revised the manuscript. M.B devised experiments and provided technical expertise. All authors have reviewed the manuscript.

Chapter 5

5. Conclusions and Future Directions

Multidrug resistance (MDR) represents a major obstacle in clinical oncology. MDR is mainly attributed to the overexpression of P-glycoprotein (*ABCB1*/P-gp) and Multidrug Resistance-associated protein 1 (*ABCC1*/MRP1) in cancer cells (76, 77). For many years, the most logical approach to circumvent MDR was to target these transporters by chemomodulators. Chemotherapy involving the co-administration of specific transporter inhibitors have proven disappointing (37, 78). Drug development efforts to reverse or inhibit these proteins in the clinical context have been limited by dose limiting toxicity and pharmacokinetic issues (78). Despite advancements in specificity and potency of inhibitors, this therapeutic strategy is still confounded by adverse side effects, undesired pharmacokinetic interactions and functional redundancy among this group of drug transporters (37, 78). For this reason, this study directs investigations to the molecular basis of the acquisition of ABC-transporter mediated MDR.

Our group was the first to confirm the intercellular transfer of P-gp mediated MDR via microparticles (MPs) in cancer (1). This novel pathway for the transfer and acquisition of MDR has widespread oncological implications, with cells rapidly acquiring MDR within 4 h of MP exposure. Furthermore, our group demonstrated recipient tissue selectivity by MPs, meaning discrete MP cargo is purposefully transferred to a selected cell target. As a result, MPs are not unloading their cargo onto indiscriminate cell targets, but rather performing a transfer of relevant bioactive

cargo to pre-determined recipient cells (58, 59, 79). Furthermore, after the transfer of MP cargo, recipient cells were shown to be re-templated to reflect donor cell phenotype (7, 9). MPs provide a cellular basis for the intercellular transfer and dominance of deleterious phenotypes in recipient cells (1, 7, 9, 56-59, 79, 80). The therapeutic potential of microparticles (MPs) in circulation is seemingly limitless, with infinite combinations of biologically relevant material packaged from parental cells. Therefore, the study aims to investigate the molecular pathways involved after microparticle (MP) transfer in context of MDR in cancer.

The data presented in this thesis expands on these earlier findings and presents novel mechanisms and methodologies relating to the transfer of nucleic acids and their regulatory mediators associated with MP mediated MDR in cancer. In this thesis, one shows that MPs can alter the recipient cell phenotype for drug resistance via the transfer of various bioactive components, proteins, functional transcripts and miRNA regulatory mediators.

Chapter 2 confirms the transfer of functional MRP1, a highly promiscuous drug transporter with enormous functional redundancy with P-gp (7). The study determined that MRP1 overexpressing leukaemic cells can package and transfer functional MRP1 and its related transcripts to otherwise drug sensitive CEM cells (7). However, unlike our previous findings for P-gp, a kinetic difference was shown in the acquisition of MRP1 upon MP transfer (1). Instead of functionality of drug efflux after an expected 4 h co-culture, more than 15 h was required for MRP1 to confer drug resistance. This kinetic difference may possibly be a result of the larger size of MRP1 or extra transmembrane domains.

A calcein-AM functional assay was used to determine drug efflux capacity in this thesis. Calcein-AM has been used in numerous studies as a measure of P-gp or MRP1 drug efflux capacity (32, 81-84). On this basis, our group has used this in many peer reviewed articles and we are confident that we are measuring drug efflux functionality which is strongly correlated with MDR (7, 14, 32, 81-85).

In addition to demonstrating the transfer of functional protein, chapter 2 shows the transfer and dominance of transferred *ABCB1/ABCC1* transcripts (7). Previously our lab group has observed the trait dominance of MPs in drug sensitive recipient cells (9). Here it is demonstrated a dominance of the transferred MDR phenotype over a pre-existing recipient cell MDR trait. The resulting recipient cell begins to reflect donor-MP phenotype. The transfer of bioactive regulatory mediators and associated *ABCB1* transcripts result in the re-templating of recipient cell overexpressed *ABCC1* phenotypes (7, 9). In E_{1000} cells co-cultured with VLB_{100}MPs, it is observed around 30-40% suppression of endogenous *ABCC1/*MRP1 transcripts and a significant increase (up to 3000-fold) in *ABCB1*/P-gp transcripts. Due to this trait-steering phenomenon, there are widespread implications and clinical practicality for MPs. For instance, if evaluated by their phenotype in the vasculature, MPs can be used as a prognostic tool to predict impending MDR traits. This would allow clinicians to pre-emptively tailor and administer drugs appropriately to counter-act the spread of drug resistance by MPs. However cancer MPs circulating in the vasculature may need to reach a certain level for phenotype transfer. An *in vivo* study has shown that a P-gp MDR phenotype conferred by MPs was stable for up to two weeks after subcutaneous

injection of 100 µg DXMPs into mice (79). Furthermore, MPs isolated from human patients with metabolic syndrome (20 mL of peripheral blood), was found to stimulate endothelial dysfunction in mice (86). Clinically, MP-mediated re-templating may be dependent on tumour MP shed rate and packaged cargo. Future studies may determine the minimum quantity of MPs or packaged mediators for phenotype transfer *in vivo*.

Indeed membrane vesicles have emerged as powerful predictive markers in cancer therapy (10, 87, 88). There has been much recent attention to utilizing MVs as a new avenue for cancer diagnostics (89, 90). For instance, a recent study has developed a sensitive and rapid analytical method to analyse primary tumour mutations in shed glioblastoma MVs and use them as a predictive metric of drug response in patients (90). Using a micro nuclear magnetic resonance system (µNMR), the study assessed EGFR expression in MVs and used it as a prognostic marker for drug treatment. In this thesis, it is shown that P-gp and MRP1 in shed vesicles may be used as a marker in µNMR for prognostic information and individualised treatment.

Having established that MPs can transfer transcripts to recipient cells, this study aimed to confirm the functionality of transferred transcripts in chapter 3. Therefore, the translatable potential of MP mRNA into its new cellular environment is investigated. In doing so, a novel methodology is developed, which eliminates the requirement of exogenous labels/probes and interspecies models. This methodology demonstrates a more biologically relevant approach to understanding the functionality of packaged nucleic acids in MPs. Western blot detection of 170 kDa P-gp in MPs is eliminated by surface proteinase K shaving. However, upon co-culture with drug

sensitive recipient cells for more than 19 hrs, 170 kDa band is observed, indicative of translation of MP-transferred nucleic acid cargo. This methodology has widespread applications in determining transcript function of membrane bound proteins in context of MP transfer. For instance, MVs released by Epstein-Barr virus (EBV) infected nasopharyngeeal carocinoma cells (NPC) cells are known to transfer immunomodulatory proteins, such as latent membrane protein 1 (LMP1) and galectin 9 (91). By surface shaving and co-culturing of these exosomes with their target cells, the effect of these membrane proteins and their associated transcript may be studied.

It is demonstrated that up to 300 ng/mL of proteinase K for 5 mins does not compromise the integrity of MPs. Therefore, this methodology may encompass the detection of more difficult to digest membrane proteins. In addition to assessing the functionality of MP cargo, protein surface shaving of MPs may be used to determine the orientation of membrane proteins if peptide fragments are collected from the MP supernatant immediately after shaving and sequences analysed.

However, there are some limitations to our methodology. The transcript of interest must be hyperexpressed in cells and must encode membrane proteins that are susceptible to proteinase K treatment. Secondly, the study cannot exclude additional mediators co-transferred with MPs that may play a role in the up-regulation of P-gp. For instance, the activation of receptors during co-culture and transfer of regulatory non-coding RNA species (miRNA, lncRNA) and other mediators may confuse detection of translation of transcripts from MPs with cellular upregulation. Previous studies have used an *in vitro* translation system to confirm the translatable potential of packaged mRNA in exosomes (11). To our knowledge, this chapter is the first to confirm this in

cancer derived MPs using a similar kit and confirm the functionality of packaged *ABCB1* transcripts.

In the previous chapters the concept of MP-mediated trait dominance is introduced, then the functionality of packaged transcripts in recipient cells is validated. However, the mechanistic pathways leading to the MP-mediated suppression of recipient cell MDR traits were still unclear. Chapter 4 investigates the molecular pathways leading to the suppression of hyper-expressed *ABCC1* in recipient cells. The study validates that miR-326 is a contributing mechanism of *ABCC1* suppression mediated by P-gp overexpressed MPs. Remarkably; it is found that miR-326 function is correlated with the presence of *ABCB1* transcripts. By inhibiting miR-326 immediately after MP-transfer, a significant reversal in *ABCC1* transcript suppression is shown. This demonstrates that miR-326 may be a contributing mechanism in MP-mediated *ABCC1* suppression. Similarly, the same reversal of *ABCC1* transcript suppression is observed with silencing of *ABCB1* transcript directly after MP-transfer, indicative of the requirement of *ABCB1* transcripts for suppression. These results introduce for the first time a novel mechanistic link between microRNA and a transcript of no known putative binding sites. Consistent with these findings, a dominance of *ABCB1* transcripts when recipient CEM cells are simultaneously co-localized with *ABCC1* is observed. This result provides a molecular understanding on the regulation and dominance of ABC-transporter MDR in cancer.

The expression of both transporters which are conferred by protein transfer from distinct MP types (VLB_{100}MP and E_{1000}MP), results in an immediate survival mechanism against xenobiotic exposure, whilst cells adapt for more long-term MDR traits. Future

studies may involve surface peptide shaving to inhibit function of transferred proteins for calcein-accumulation experiments. This would reflect phenotypic changes conferred by transferred RNA rather than transferred MDR proteins. The functional redundancy that exists between the transporters represents an additional MDR fail safe against chemotherapeutics, in the event one transporter fails, the other may substitute for MDR.

Future Directions

This thesis defines MPs as veritable mediators of important biological cargo which are capable of influencing or altering cellular proteomes and molecular pathways at distant sites. The translational potential for this work is enormous, with future directions in disease prognosis and circumvention in drug resistance. The dominance of MP-MDR traits can be utilized in a clinical setting as a relatively less invasive prognostic tool. As MPs are “messengers” between cells, oncologists may intercept messages to predict impending traits. Further investigation may be required to develop improved quantification methods to reduce time and costs for each MP sample. In addition, miRNA, mRNA and protein expression profiles of circulating MPs may be required for more definitive estimates.

Having confirmed both MDR proteins and associated transcripts are functional, it is likely both a transient and sustained MDR phenotype may be conferred by MPs. This identifies MPs as potential drug targets in clinical oncology. The shedding of MPs only occurs upon cell activation or growth, thus in cancer patients, the majority of circulating MPs may originate from aggressive tumours (92, 93). The implications of MP inhibition in targeted therapy may also extend to other MP related pathologies

such as metastasis, hypercoagulation, HIV and cerebral malaria (94-97). As MPs represent an ideal modality for intercellular transfer of biological material, it is not surprising that viruses and other pathogens have exploited this modality to assist in disease transmission. One example is the transfer of prions which lead to many neurogenerative diseases such as Alzheimers (98, 99). MVs from prion infected cells can effectively initiate prion propagation in uninfected cells (100). Indeed the stimulation of MV release by ionophore and monensin has increased prion infectivity (101). Furthermore, the package and transfer of chemokines receptors aid in the spread of HIV to cells which are not usually susceptible to the virus (102). Given the MV related pathologies, there are currently investigations into the development of drugs for MP inhibition (71, 103, 104). A recent screening of drugs has found that a calpain inhibitor II (N-Acetyl-L-leucyl-L-leucyl-L-methioninal), significantly inhibited MP formation from cancer cells (71). However, to date, there are no MP inhibitors used in a clinical setting. With therapeutic implications in various diseases, the use of MP inhibitors in a clinical setting is quite promising

Conversely, as MPs are comprised of non-synthetic, non-viral components isolated from the host, they may prove to be ideal therapeutic agents or carriers. Some groups have investigated ways they may exploit this therapeutic potential. For instance, MVs or MPs isolated from endothelial progenitor cells were found to re-template hypoxic resident renal cells to regenerate and protect them from ischaemia-reperfusion injury (105), or activate an angiogenic program in islet endothelium to sustain revascularization and β-cell function, which may be result in increasing efficacy of insulin production after islet transplantation (106). Also, it has been found that MPs

isolated from mesenchymal stem cells were as effective as their parental cells in treating severe bacterial pneumonia in mice (107), demonstrating the biological potential of MV cargo. These exciting findings have established a proof of concept for developing extracellular vesicles as intelligent targeted gene therapy agents.

The cargo within the intravesicular space of MPs is heterogeneous and dynamic, with cytosolic components selectively packaged from its parental cell (9, 59). For instance, the identification of enriched miRNA, proteins, transcripts and regulatory components would lead to the discovery of novel molecular pathways, as such reported in this thesis. For instance, MVs released from lung cells induced epigenetic modifications in the recipient bone marrow cells, subsequently causing these cells to express pulmonary epithelial cell specific genes and pro-surfactant B protein (34, 108). It was found through microarray analysis that enrichment and transfer of specific miRNAs and mRNAs in MVs were responsible for the phenotype change in recipient cells (109). Furthermore, extracellular vesicles isolated from human breast milk were found to be highly enriched in miR-148a-3p and let-7 family miRNAs, which are important for immune system development. The enrichment of these miRNAs have led to hypothesis that MVs are imperative for normal healthy immune system and development in a newborn infant (110-112). Future lncRNA or miRNA profiling of drug resistant MPs vs drug sensitive MPs may help identify nucleic acid species which both directly or indirectly contribute to chemoresistance in cancer (9).

Determining the targets of miRNA remains a major challenge to this date. The interaction between miRNA and mRNA can occur with as few as 6 nucleotides of miRNA seed sequence and can regulate expression of large groups of genes by a

variety of mechanisms. The mechanisms of gene regulation include cleavage, degradation, cell-cycle control, translational inhibition, and mRNA transport. Therefore, any given miRNA can regulate a broad range of mRNA targets. As a result, miRNA-mRNA predictive bioinformatics analysis produce divergent results with high false-positive rates and do not account for indirect regulation by miRNAs. Hence, much effort has gone into the development of methods for mapping miRNA functionality (113, 114). Given the complexity and biological significance of packaged miRNA in MPs, as such presented in this thesis, the functional mapping of MP miRNA may shed light on novel pathways leading to phenotypic changes in recipient cells.

Collectively, this thesis provides a novel understanding for the acquisition and alteration of MDR phenotypes in context of cancer. There is substantial potential for clinical translation of this work to provide diagnostic/prognostic information and aid in therapeutic decisions in patients afflicted with cancer. Finally, the molecular pathways identified in this work substantially advances our understanding in the critical role MPs play in various biological pathways and provide further basis for the circumvention of MDR in cancer.

6. References

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7. Appendix

The following supplementary information is provided in electronic format

Chapter 3: Electronic Supporting Information

Supplementary file S3.1: All proteins identified from in vitro translation of $VLB_{100}MP$ total RNA