ISOLATION AND CHARACTERIZATION OF EXOSOMES

BY

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LA TROBE INSTITUTE FOR MOLECULAR SCIENCE
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“It is the theory which decides what we can observe.”

Albert Einstein

“At the heart of science is an essential balance between two seemingly contradictory attitudes - an openness to new ideas, no matter how bizarre or counterintuitive they may be, and the most ruthless skeptical scrutiny of all ideas, old and new. This is how deep truths are winnowed from deep nonsense.”

Carl Sagan
Abstract

Cell-cell communication is an integral physiological process that relies on the sending and receiving of signals. Communication may involve direct contact between adjoining cells, or require the release of secreted molecules to facilitate the interaction. Recently, extracellular vesicles (EVs) secreted from cells have been recognized to be involved in cell-cell communication. EVs comprise shed microvesicles (sMVs), apoptotic bodies and exosomes which differ based on their mechanism of biogenesis and size; of these, exosomes have been most widely studied. Exosomes are ~40-100 nm EVs released from a multitude of cell types that perform pleiotropic extracellular functions within the cellular microenvironment. These functions include autocrine and paracrine signaling, immunological modulation, and horizontal transfer of proteins, lipids and genetic material (miRNA/mRNA) to recipient cells. When investigating exosome functionality, sample homogeneity is of critical importance, a caveat which, thus far, has been ostensibly overlooked. Although numerous functions have been ascribed to exosomes, the interpretation of the majority of these studies has been confounded by sample heterogeneity with other secreted vesicles.

This thesis aims to address the concern of exosome purity by evaluating widely-used exosome isolation procedures, using the human colorectal cancer (CRC) cell line LIM1863 as a model. The information gained from this comparative purification study will be used to provide insights into the isolation and characterization of two exosome sub-populations released from this highly-polarized CRC cell line. Additionally, using an epithelial-mesenchymal transition (EMT) cell model, exosomes will be isolated and characterized for the purpose of identifying their contribution to the EMT process.

The first experimental chapter compared and evaluated three strategies for exosome isolation: differential ultracentrifugation, density gradient centrifugation using iodixanol (OptiPrep™) and EpCAM immunoaffinity capture. All exosome preparations contained 40-100 nm diameter vesicles based on electron microscopy, and were positive for stereotypical exosome markers Alix, TSG101, HSP70 using Western blotting. Using MS–based proteomic profiling, the protein composition of exosomes isolated from each of these
procedures was investigated. The effectiveness of each method was assessed by quantitating the number of MS/MS spectra identified for exosome markers and proteins associated with key exosome processes including their biogenesis, intracellular trafficking, and exocytic release. This study revealed that proteins in all aforementioned categories were significantly enriched (at least 2-fold) in immunoaffinity isolated exosomes when compared to density gradient- or differential ultracentrifugation-derived exosomes. Overall, this study concluded that immunoaffinity capture was the most effective method for isolating exosomes.

The second experimental chapter investigated sub-populations of exosomes from highly-polarized LIM1863 cells. In this study, a sequential immunoaffinity capture strategy was developed using anti-A33- and anti-EpCAM-coupled magnetic beads to isolate A33- and EpCAM-Exos. A key finding of this study was the exclusive identification in EpCAM-Exos of the classical intracellular apical trafficking molecules CD63, mucin 13, and apical intestinal enzyme sucrase isomaltase. The increased expression of dipeptidyl peptidase IV and the apically-restricted pentaspan membrane glycoprotein prominin 1 was also observed. In comparison, A33-Exos were enriched with intracellular basolateral trafficking molecules including early endosome antigen 1 (EEA1), the Golgi membrane protein ADP-ribosylation factor (ARF1) and clathrin. Collectively, these data are consistent with EpCAM- and A33-Exos being released from the apical and basolateral surfaces, respectively. Intriguingly, several members of the MHC class I family of antigen presentation molecules were exclusively observed in A33-Exos. Additionally, EpCAM-Exos contained molecules known to complex together to promote tumor progression including, EpCAM, claudin-7 and CD44. This study is the first robust characterization of two distinct populations of exosomes from highly-polarized epithelial cells.

A proteome analysis of LIM1863-derived sMVs (also referred to as plasma membrane blebs, microvesicles and oncosomes) was also performed to reveal that sMVs are clearly distinguishable from A33- and EpCAM-Exos. Interestingly, sMVs were shown to be significantly enriched with members of the human ATP-binding cassette (ABC) transporter superfamily, which act to shuttle substrates (e.g., drugs) across cellular membranes.
The third experimental chapter analyzed exosomes released from cells following EMT. EMT is a highly conserved morphogenic process defined by the loss of epithelial characteristics and the acquisition of a mesenchymal phenotype. This process is associated with increased aggressiveness, invasiveness, and metastatic potential in carcinoma cells. Using an EMT cell model consisting of Madin-Darby canine kidney cells (MDCK) and its oncogenic H-Ras-induced variant (21D1) established by Dr. Zhu (The University of Melbourne), MDCK- and 21D1-exosomes (MDCK- and 21D1-Exos) were isolated using density gradient centrifugation (OptiPrep™). Proteomic profiling revealed that typical cellular EMT marker proteins are present in MDCK- and 21D1-Exos. These include reduction of a characteristic inhibitor of angiogenesis (e.g., thrombospondin-1) and epithelial markers (e.g., E-cadherin and EpCAM), with a concomitant upregulation of a mesenchymal marker (e.g., vimentin). Further, this study revealed that 21D1-Exos were enriched with several proteases and integrins that have been recently implicated in regulating the tumor microenvironment to promote metastatic progression. A salient finding, however, was the unique enrichment of key transcriptional regulators (e.g., the master transcriptional regulator YBX1) and core splicing complex components in mesenchymal 21D1 cell-derived exosomes. Overall, these findings demonstrate that exosomes from Ras-transformed MDCK cells are reprogrammed with factors which may be capable of inducing EMT in recipient cells.

In summary, this thesis evaluated three strategies for purifying exosomes. The findings of this study revealed that immunoaffinity capture was significantly more effective at enriching exosomes from cellular media than density gradient centrifugation or differential centrifugation. This purification knowledge was used to isolate and characterize two populations of exosomes secreted from the colon carcinoma cell line LIM1863 as well as to evaluate the contribution of exosomes in the epithelial-mesenchymal transition process.
Declaration

This is to certify that:

(i) this thesis comprises only my original work towards the PhD except where indicated in the Preface.

(ii) due acknowledgement has been made in the text to all other material used.

(iii) the thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies, appendices and footnotes.

Signed: _________________________

Bow Tauro
Preface

Publications during candidature not included in this thesis:


Publications during candidature included in this thesis:


The work described in the manuscripts included this thesis was performed entirely by myself except for the following collaborations: Chapters 2 and 3, Dr. David Greening and Dr. Rommel Mathias (assistance with manuscript editing), Dr. Suresh Mathivanan (performed initial bioinformatic analysis), Chapter 4, Dr. Rommel Mathias (assistance with manuscript writing), Dr. David Greening (assistance with manuscript editing), Mr. Shashi Kumar Gopal Krishnan (assistance with cell culture for Figure 2), Mr. Eugene Kapp (performed initial bioinformatic analysis), Dr. Bradley Coleman (cryo-transmission electron microscopy imaging), A/Prof. Robert Moritz (MS analysis of a second (replicate) dataset). I estimate that my contribution to Chapter 2 and 3 to be greater than 90% and my contribution to Chapter 4 to be greater than 80% therefore achieving an overall contribution of my work in the thesis to be greater than 85%.
Acknowledgements

This thesis is an account of several years of devoted work while enrolled through the Department of Biochemistry and Molecular Biology at The University of Melbourne. Experimental work was performed at the Ludwig Institute for Cancer Research (LICR) and the La Trobe Institute for Molecular Science (LIMS).

First, I would like to express my deep appreciation to my primary supervisor, Prof. Richard Simpson, for allowing me to undertake my PhD. He is incredibly accomplished within the scientific field and his level of success is something to aspire to. His ability to continually enthuse and inspire me, even in the toughest of times, has been amazing. I have genuinely enjoyed spending these years with him and value the friendship that has evolved further than a supervisor/student relationship. Attending conferences together, particularly the International Society for Extracellular Vesicles meeting in Sweden, have been a great highlight with many fond memories.

Next I would like to thank my co-supervisors, Dr. Hong Ji and Dr. Hong-Jian Zhu. From the first day I stepped into the laboratory, a friendship with Ji Hong grew like the orchids she would nurture; we were instantly a great team. Her skills within the laboratory are second to none. Her experimental design and execution are impeccable; I can only hope that my skills will continue to grow to one day reach her level. Hong-Jian’s guidance as an external supervisor has been amazing; his knowledge regarding key concepts including epithelial-mesenchymal transition has been invaluable.

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Abbreviations

ADAM       a disintegrin and metalloproteinase
A33-Exos   exosomes isolated using anti-A33 immunoaffinity beads
BMDC       bone marrow-derived hematopoietic progenitor cells
BMP        bone morphogenic protein
CCM        concentrated culture medium
CM         culture medium
CRC        colorectal cancer
DMEM       Dulbecco’s modified eagle medium
EGF        epidermal growth factor
EGFR       epidermal growth factor receptor
EM         electron microscopy
EMMPrin    extracellular matrix metalloproteinase inducer
EMT        epithelial-mesenchymal transition
eMVs       extracellular microvesicles
EpCAM      epithelial cell adhesion molecule
EpCAM-Exos exosomes isolated using anti-EpCAM immunoaffinity beads
ESCRT      endosomal sorting complex required for transport
FACS       fluorescence-activated cell sorting
FCS        fetal calf serum
FGF        fibroblast growth factor
FITC       fluorescein isothiocyanate
FOXC2      forkhead box protein C2
GI         gastrointestinal
GSK-3β     glycogen synthase kinase 3 β
HMGB       high mobility group box
HPLC       high-performance liquid chromatography
HSP        heat shock protein
HUVEC      human umbilical vein endothelial cells
ILV        intraluminal vesicle
ITS        insulin-transferrin-selenium
LDH        lactate dehydrogenase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>LEF</td>
<td>lymphoid enhancer factor</td>
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<tr>
<td>MACS</td>
<td>magnetic activated cell sorting</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide</td>
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<tr>
<td>MVB</td>
<td>multivesicular body</td>
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<td>MVs</td>
<td>microvesicles</td>
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<tr>
<td>NSF</td>
<td>N-Ethylmaleimide-sensitive fusion protein</td>
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<tr>
<td>PDCD6IP/Alix</td>
<td>programmed cell death 6 interacting protein</td>
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<tr>
<td>PI(3)K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PM</td>
<td>plasma membrane</td>
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<tr>
<td>Rsc</td>
<td>relative spectral count fold change ratios</td>
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<tr>
<td>SIP</td>
<td>smad interacting protein</td>
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<tr>
<td>SMART</td>
<td>simple modular architecture research tool</td>
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<tr>
<td>sMVs</td>
<td>shed microvesicles</td>
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<tr>
<td>SNARE</td>
<td>soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
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<tr>
<td>SSM</td>
<td>solid support magnet</td>
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<tr>
<td>TEM</td>
<td>tetraspanin enriched microdomains</td>
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<td>TGN</td>
<td>trans-Golgi network</td>
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<td>TMHMM</td>
<td>transmembrane hidden Markov model</td>
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<tr>
<td>TF</td>
<td>tissue factor</td>
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<td>TGFβ</td>
<td>transforming growth factor β</td>
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<td>tumor necrosis factor α</td>
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<td>trans-proteomic pipeline</td>
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<td>tunneling nanotubes</td>
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<td>tumor susceptibility gene 101</td>
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<td>vascular endothelial growth factor A</td>
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<td>vascular endothelial growth factor receptor 1</td>
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<td>VAMP</td>
<td>vesicle-associated membrane protein</td>
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<tr>
<td>WCL</td>
<td>whole cell lysate</td>
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<tr>
<td>YBX1</td>
<td>Y-box binding protein 1</td>
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<td>ZEB1</td>
<td>zinc finger E-box binding homeobox 1</td>
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<tr>
<td>2ID1</td>
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Chapter 1: Background and literature review

1.1 Preface

Exosomes are 40-100 nm diameter membraneous vesicles released from most cell types. They function in extracellular communication by transferring a variety of bioactive molecules (e.g., proteins, lipids, RNA species such as mRNA/miRNAs, and possibly DNA) between cells [1, 2]. Over the past decade they have gained much attention for their role in cancer progression [3]. Although exosomes were first described almost thirty years ago [4-6], their purification to homogeneity has proven a difficult task. This is due to their inherent biophysical properties (e.g., size and buoyant density) being similar to extracellular vesicles (EVs) (e.g., shed microvesicles and apoptotic blebs), the problem of co-purification with protein oligomers (e.g., proteasome complexes), and the possible contamination with cellular pathogens (e.g., adenoviruses and prions) [7]. Obtaining homogeneous exosomes is a pre-requisite for in-depth biophysical and functional analyses.

Using semi-quantitative MS-based proteomic techniques, such as label-free spectral counting [8], this thesis first compares widely used exosome isolation strategies to assess their capability for enriching several classes of proteins involved in exosome biogenesis, trafficking, release and uptake. Secondly, a sequential immunoaffinity capture technique was developed to isolate exosome sub-populations released from apical and basolateral surfaces of highly-polarized cells. Finally, in the absence of an immunoaffinity capture strategy, density gradient centrifugation (OptiPrep™) was used to isolate and characterize exosomes, for the first time, from an epithelial-mesenchymal transition (EMT) cell model.
1.2 Secretome

The term ‘secretome’ refers to all soluble proteins and extracellular membranous vesicles that are secreted or shed into the extracellular space [9-15]. The secretome is a tightly regulated and sensitive feature required for cell-cell communication and normal physiological function [9]. Secreted proteins can constitute structural components of the extracellular matrix (ECM), such as collagens and laminins, while others are able to trigger intercellular responses in target cells [16, 17]. Secreted proteins are of particular importance as aberrant protein secretion is associated with pathological events within diseases such as cancer [13, 14], including pre-metastatic niche formation and metastasis [18-21]. In addition to soluble-secreted proteins, the secretome contains several types of EVs including shed microvesicles (sMVs), apoptotic blebs and exosomes (Figure 1-1, Table 1-1) [1]. It is now recognized that these vesicles are also important regulators of cell-cell communication, particularly within the tumor microenvironment [19, 22].

1.3 Extracellular vesicles

The release of EVs was first described in 1967 when Wolf and colleagues recognized procoagulant particulate matter around activated platelets (‘platelet dust’) [23]. Platelets were later identified to release microvesicles and exosomes [24]. In addition to normal cellular functioning, EV release can be caused by diverse biological mechanisms that are triggered during oncogenic transformation, cellular activation by stimuli in the microenvironment, stress or apoptosis [25]. The formation of vesicles may occur as budding events at the plasma membrane (e.g., sMVs) or within late endosomal compartments such as multivesicular bodies (e.g., exosomes), or as a consequence of apoptosis (e.g., apoptotic blebs). Following release, different EVs may have distinct functions. It is understood that isolating EVs to homogeneity is the first step towards obtaining a clear understanding of their biophysical properties and biological functions. However, such an undertaking has been impeded by the inherent technical difficulties – due largely to the similarity of their physiochemical characteristics (Table 1-1) [26].
Figure 1-1. Extracellular membranous vesicles released from cells

Shed microvesicles (sMVs) range in size between 100-1000 nm in diameter and are formed by direct outward budding of the plasma membrane (PM). During endocytosis, a receptor is internalized and sorted in early endosomes, where cargo may be recycled to the PM via recycling endosomes [27, 28]. Intraluminal vesicles (ILVs) are generated by inward budding from the limiting membrane of late endosomes, forming multivesicular bodies (MVBs). MVBs may be (i) sorted to the lysosome and become degradative or (ii) fuse with the PM to release 40-100 nm ILVs, now termed exosomes [29]. Exosomes display the same surface topology as the cell with extracellular domains of proteins at the surface, and enclosing cytoplasmic contents (cargo) in the lumen. Apoptotic blebs (50-5000 nm in diameter) are formed from cells undergoing programmed cell death.
<table>
<thead>
<tr>
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<th>Exosomes</th>
<th>Shed Microvesicles (sMVs)</th>
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<td><strong>Size</strong></td>
<td>40-100 nm</td>
<td>100-1000 nm</td>
<td>50-5000 nm</td>
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<td><strong>Mechanism of biogenesis</strong></td>
<td>Exocytosis of MVBs</td>
<td>Outward budding of the plasma membrane</td>
<td>Release of fragments/blebs from cells undergoing apoptosis</td>
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<td><strong>Isolation techniques</strong></td>
<td>Differential centrifugation at 100,000 x g [35]</td>
<td>Differential centrifugation at 10,000 x g [24]</td>
<td>Induction of apoptosis in cells, isolate apoptotic bodies at 50,000 x g [50, 51]</td>
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<td>Sucrose and OptiPrep™ density gradient ultracentrifugation (1.08-1.22 g/mL) [35]</td>
<td>Sucrose density gradient ultracentrifugation (1.16 g/mL) [48]</td>
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<td>Immunoaffinity capture:</td>
<td></td>
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<tr>
<td><strong>Markers</strong></td>
<td>Alix, TSG101, CD9, CD63</td>
<td>Annexin V binding, ARF6, ABCG2</td>
<td>Annexin V binding, DNA content, phosphatidylserine</td>
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<td></td>
<td>CD45 positive exosomes from Jurkat T cells [39]</td>
<td></td>
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<tr>
<td></td>
<td><strong>MHC class II</strong> positive exosomes from antigen presenting cells [40]</td>
<td></td>
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<tr>
<td></td>
<td>CD63 positive exosome-like vesicles from blood [41]</td>
<td></td>
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<tr>
<td></td>
<td>EpCAM positive exosomes from human serum of lung and ovarian cancer [42, 43]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A33 positive exosomes from colorectal cancer cell line [44]</td>
<td>Fluorescence-activated cell sorting (FACS) 1-10 µm size gate [49]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Size-exclusion HPLC [45-47]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1-1. Properties of extracellular vesicles [1, 7, 26, 30-36]
A recent initiative to assist in the cataloguing of EVs is the database Vesiclepedia ([http://www.microvesicles.org/](http://www.microvesicles.org/)) [52]. Vesiclepedia is a manually-curated compendium of molecular information (protein, lipid and RNA) identified in different classes of EVs. Vesiclepedia comprises 35,264 protein, 18,718 mRNA, 1,772 miRNA and 342 lipid entries comprised from 341 independent studies. A summary of EVs (sMVs, apoptotic blebs and exosomes) and their biophysical properties is outlined below.

1.3.1 Shed microvesicles

Shed microvesicles (sMVs) range in diameter between 100-1000 nm and are formed by direct budding of cytoplasmic protrusions from the cell membrane upon activation of various internal and external stimuli (Figure 1-1) [7, 26]. Nomenclature of these vesicles has been defined depending on their parent cell; for example, microvesicles released by platelets are referred to as ‘microparticles’ while microvesicles released by leukocytes are called ‘ectosomes’ [31]. Prostate cancer xenograft released vesicles are termed ‘oncosomes’ [49]. They are collectively referred to as sMVs in this thesis. The biogenesis of sMVs is reported to involve phospholipid redistribution caused by flippases and floppases translocating phospholipids from the outer-to-inner and inner-to-outer membrane leaflets, respectively [36]. Following this rearrangement, contraction of cytoskeletal structures by actin-myosin interactions, caused by ADP-ribosylation factor 6 (ARF6), initiates a signaling cascade that culminates in the phosphorylation and activation of myosin light chain, facilitating membrane budding and sMV release [36]. Shedding of vesicles occurs in resting cells, however, Ca$^{2+}$ is known to increase the rate of the process dramatically [53]. Interestingly, despite being generated by the analogous process of budding from the cell surface, sMVs isolated at rest and following stimulation can be molecularly different. For example, it was shown that vesicles shed from stimulated neutrophils express increased levels of integrin αMβ2 [54].

sMVs are recognized to participate in important biological events including coagulation by mediating the contribution of platelets, macrophages and neutrophils, and horizontal
trafficking of protein and RNA between cells [26] (for an excellent review see [55]). It is well recognized that various classes of sMV s are rich in ATP-Binding Cassette (ABC) transporters which form a defense network against a range of chemotherapeutics [56]. This network can result in multidrug resistance, a major impediment to curative cancer chemotherapy [57-60]. Following release into the extracellular space, sMVs may be degraded locally or move by diffusion to appear in biological fluids including cerebrospinal fluid, blood and urine [26]. When investigating sMVs released from tumor cells, it was demonstrated that they may rapidly break down to release cargo. This may include extracellular matrix metalloproteinase inducer (EMMPRIN), a transmembrane glycoprotein expressed at high levels by tumor cells that can stimulate matrix metalloproteinase expression in fibroblasts to facilitate tumor invasion and metastasis [61]. Additionally, sMVs (oncosomes) isolated by FACS from prostate cancer xenograft mouse blood were shown to stimulate the migration of normal endothelial cells indicating their ability to modify the microvasculature [49]. Methods used to isolate sMVs from culture medium or platelet-poor plasma include density gradient centrifugation (buoyant density of 1.16 g/mL) on sucrose gradients [24, 48, 49, 62]. sMVs can also be isolated by FACS using size-gating determined by polymeric bead standards [49]. Although separation by size-exclusion chromatography has been performed to isolate sMVs of varying size from urine, this process resulted in contamination with other EVs and proteins [63]. To date, little is known about sMVs structure and specific information about their biogenesis is limited.

1.3.2 Apoptotic blebs

Apoptotic blebs (and larger ‘apoptotic bodies’) are heterogeneous fragments of the cell (50-5000 nm) formed during programmed cell death (Figure 1-1) [36]. The occurrence of cell shrinkage and condensation of nuclear chromatin, hallmarks of apoptosis, leads to the generation of apoptotic blebs. These blebs contain remnants of the cell degradation processes including cytoplasmic components and DNA [64]. It is understood that during apoptosis, phosphatidylserine (PS) is relocalized from the inner leaflet of the PM to the outer leaflet where it triggers recognition for phagocytosis [65]. In addition to PS, other
phagocytic surface signals including integrins $\alpha V\beta 3/\alpha V\beta 5$ and CD36 are displayed [33, 34, 66]. Interestingly, from a functional aspect, phagocytosis of apoptotic blebs by dendritic cells results in efficient processing and presentation of apoptotic cell antigens on CD4+ and CD8+ T lymphocytes [67]. Further, apoptotic bodies derived from H-RasV12 or human c-myc transfected cells were up taken by murine fibroblasts resulting in loss of contact inhibition in vitro and a tumorigenic phenotype in vivo [68]. Protocols for the biogenesis and isolation of apoptotic blebs involve induction of cell apoptosis, commonly by UV irradiation or ethanol induction, and subsequent cell culture for up to 24 h. At different times after apoptotic induction, cells are collected and stained with FITC-labeled annexin V, an early marker of apoptosis. Blebs are then isolated by centrifugation at $50,000 \times g$ [50, 51] or by sucrose density gradients at a buoyant density of 1.16-1.28 g/mL [32]. In contrast to sMVs, which are formed and released at the cell membrane, and apoptotic blebs which are formed by cell shrinkage during programmed cell death, exosomes are formed by inward budding of late endosomes. These MVBs subsequently traffic to and fuse with the plasma membrane to release exosomes into the extracellular environment.

### 1.4 Exosomes

Exosomes were first described in the 1980s by Johnstone and colleagues while culturing maturing reticulocytes and were originally thought to be a mechanism for removal of obsolete cellular proteins [5, 6]. Using transmission electron microscopy they observed the internalization and release of immunogold labeled transferrin receptors associated with small (50 nm diameter) vesicles [4]. Exosomes are now recognized to be released from most cell types including B lymphocytes [69], dendritic cells [70], neurons [71], intestinal epithelial cells [72] and tumor cell lines [37, 44, 73]. A current definition describes exosomes as a discrete population of small 40-100 nm diameter membranous vesicles that are formed within endosomes and released into the extracellular space following fusion of MVBs with the plasma membrane (Figure 1-1) [74, 75]. This mechanism of biogenesis differs from that of sMVs and apoptotic blebs and led to the hypothesis that exosomes might have specialized functions that differ from other EVs [76].
1.4.1 Exosome biogenesis and cargo selection

The biogenesis of exosomes occurs within the endosomal network, a membranous compartment used to sort various intraluminal vesicles and proteins within the cell [77]. Endosomes are separated into three distinct compartments including early, late and recycling endosomes, each of which are defined by the association of specific proteins on their cytosolic surface [27, 78]. Early endosomes are characterized by the presence of Rab5 together with its effector VPS34/p150, a phosphatidylinositol 3-kinase (PI(3)K) complex [78]. These endosomes patrol the peripheral cytoplasm close to the plasma membrane by movement along microtubules [79] and are the major entry site for endocytosed material that arrives by clathrin-mediated-, caveolar- and ARF6-dependent pathways [80]. It is reported that endosomes accept content for approximately 10 min and cargo destined for recycling is sorted, via syntenin and syndecan heparin sulphate proteoglycans, into recycling endosomes, which fuse with the plasma membrane [27, 28]. The remaining material accumulates and is retained in the early endosomes which then undergo maturation into late endosomes [27].

Maturation of early to late endosomes occurs to reveal a series of transformations including increased size, a decrease in pH from (6.8-5.9) to (6.0-4.9) and a gradual change in markers including the loss of Rab5 and acquisition of Rab7 [81, 82]. Following this transformation, inward budding occurs to form intraluminal vesicles (ILVs/exosomes) in the late endosome lumen (also termed MVB). This process is governed by endosomal sorting complex required for transport complexes (ESCRT-0, -I, -II, -III) which are also reported to be involved in cargo selection [83-85]. The molecular mechanisms regulating cargo selection into exosomes remain to be fully characterized, however, mono-ubiquitination of the cytoplasmic tail of membrane proteins has been reported as one of the methods for cargo selection and sorting [84, 85] (Figure. 1-2). This process requires at least 18 conserved ESCRT complex proteins that were originally identified in yeast *Saccharomyces cerevisiae* [85-87]. ESCRT components are individually recruited from the cytosol to the surface of endosomes to segregate cargo into domains within the limiting membrane. Specifically, ESCRT-0, -I, and -II complexes function in sorting of ubiquitin-tagged proteins into the
Figure 1-2. Exosomal-cargo selection involving ESCRT complexes

Plasma membrane proteins (e.g., Integrin, E-cadherin) are selected as cargo following ubiquitination (Ub). The ubiquitin- and lipid-binding domains are indicated (FYVE, UIM, UEV, GLUE). The ESCRT machinery components form ESCRT complexes -0, -I, -II, -III, which are recruited in a sequential manner to the limiting membrane of the multivesicular endosome (MVE). Following inward budding, ATPase VPS4 mediates the disassembly of ESCRT-III oligomers. Yeast nomenclature is used in this figure, with the exception of HRS, STAM, and TSG101 (mammalian). UIM, ubiquitin interacting motif; UEV, ubiquitin E2 variant; GLUE, GRAM-like ubiquitin-binding in Eap45; PI(3)P, phosphatidylinositol-3-phosphate; PI(3,5)P2, phosphatidylinositol-3,5-bisphosphate; DUBs, de-ubiquitinating enzymes. Adapted from [88].
endosomal delimiting membrane [84, 89]. After sorting, the ESCRT-III complex sequentially associates and recruits the de-ubiquitinating enzyme Doa4 to remove the ubiquitin tag from the protein cargo, prior to incorporation into ILVs [90]. The ESCRT-III complex is also responsible for membrane budding and scission in a ‘purse string’ model [84, 89, 91]. It is reported that depletion of ESCRT machinery components results in fewer ILVs, and accumulation of cargo in endosomes with abnormal morphology. This indicates their importance in this process [92].

Conversely, it has been reported that cargo incorporation into exosomes does not depend on ubiquitination and ESCRT machinery [1]. This is exemplified by the trafficking of pre-melanosome protein 17 (Pmel17), which through association with CD63, relies on a luminal domain enabling its sorting and inclusion into exosomes [93, 94]. Exosome biogenesis also appears to be dependent on cytoplasmic adaptors syntenin and syndecan [95] and sphingolipid ceramide [96]. In a study by Trajkovic and colleagues, ceramide was shown to be enriched in exosomes; they demonstrated that the release of exosomes was reduced following inhibition of neutral sphingomyelinase (nSMase), suggesting the lateral segregation of lipids contributes to exosome formation and subsequent release, a process devoid of ESCRT machinery [96].

Cargo selection is also dependent on adaptor proteins. For example, phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a tumor suppressor protein localized to the cytoplasm and nucleus, has been demonstrated to be secreted in exosomes [97]. It was found that PTEN secretion in exosomes requires Ndfip1, an adaptor protein for members of the Nedd4 family of E3 ubiquity ligases [97]. To clearly define the exosome formation pathway, further examination into ESCRT-dependent and ESCRT-independent formation mechanisms is required.
1.4.2 Endosome motility and exosome release

Following vesicle formation, motility of endosomes is dependent on dynein and kinesin motors that migrate endosomes in opposite directions along microtubules [98]. Rab GTPases are also integral in ensuring endosome cargo is delivered to the correct destination. Through sorting adaptors and tethering factors, Rab GTPases control membrane identity and vesicle budding, motility and fusion. Following endosome maturation (see section 1.4.1), late endosomes containing ILVs may be targeted to lysosomes for degradation via the Rab7 effector Rab-interacting lysosomal protein (RILP), which mediates minus-end-directed trafficking [99]. Late endosomes move to the perinuclear area of the cell, fuse together to form larger bodies, undergo transient ‘kiss-and-run’ interactions and eventually full fusions with lysosomes [100].

Alternative to the lysosome pathway, late endosomes may traffic to, and fuse with, the plasma membrane to release ILVs; ILVs released into the extracellular space are referred to as exosomes. This process requires various proteins involved in docking, tethering and fusion. Previous studies have reported the involvement of Rab35 in regulating exosome secretion by assisting docking and tethering of MVBs to the plasma membrane [101]. Additionally, Rab27a/b and their effectors Slp4 and Slac2b are reported to be involved exosome release [102, 103]. Soluble N-Ethylmaleimide-sensitive factor attachment protein receptor (SNARE) molecules are also involved in recognition and fusion of endosomal membranes to the plasma membrane by forming tetrahedral bundles on opposing membranes [104]. In a recent study, it was demonstrated that the SNARE Ykt6 was integral in the secretion of exosomes carrying the morphogen Wnt [105]. Further, vesicle-associated membrane proteins (VAMPs or synaptobrevins) are anchored in the vesicular membrane to mediate intracellular vesicle fusion [106, 107]. Finally, VAMP7 and ATPase NSF, a protein required for SNARE disassembly, are shown to mediate the fusion between MVBs with the plasma membrane to release exosomes into the extracellular space [108].
1.4.2.1 Exosome biogenesis and release from polarized cells

Investigation into exosome biogenesis and release in polarized cell systems has received little attention to date. In polarized cells, proteins are sorted into different sub-populations of carrier vesicles, a process mediated by sorting signals (e.g., tyrosine motifs, GPI anchors and N-glycans) and signal-decoding machinery (e.g., clathrin adaptors and lipid rafts) [109]. In these cells, endosomes are strategically located between the biosynthetic and endocytic routes. This allows newly synthesized and endocytosed proteins to be directed to the appropriate membrane for interaction with different extracellular environments at their apical and basolateral surfaces [110]. When considering the biogenesis, trafficking and release of exosomes in polarized cells, it is interesting to note that apical and basolateral endocytic routes have been shown to converge at the level of late endosomes in Madin-Darby canine kidney (MDCK) cells [111]. This has led to a hypothesis that exosomes destined for apical or basolateral membrane release might be generated within a common late endosomal pool [110]. However, it has also been suggested that epithelial cells could have distinct MVBs into which apical and basolateral proteins are differentially sorted and exosomes are formed [110].

Studies investigating these hypotheses have been quite limited. To date, only a single experimental study has investigated exosomes released from apical and basolateral surfaces. It was observed that polarized intestinal epithelial cells (T84), grown as a monolayer on microporous filters, allowed for collection of apical or basolateral exosomes. These exosomes contained proteins localized to corresponding plasma membrane surfaces including dipeptidyl peptidase IV on apical exosomes and glycoprotein A33 on basolateral exosomes [72]. Interestingly, using gel filtration, another study identified two populations of exosomes in human saliva, however it cannot be claimed that these are apical/basolateral exosomes due to the potential of multiple cell types contributing to the saliva sample [47]. Further investigation is required to isolate and characterize the contents of apical and basolateral exosomes. This may lead to an understanding of their formation and trafficking within polarized cells and potentially identify their varying functions following release.
1.4.3 Exosome sizing and morphology

Exosome are characterized by their size of 40-100 nm in diameter [74, 75], however some studies have reported the identification of larger vesicles up to 150 nm [112]. Various methods have been used to define vesicle size including transmission electron microscopy (TEM) which originally identified exosomes to have ‘cup shaped’ morphology [35]. It was later realized that this phenotype may be a consequence of fixation and dehydration during sample preparation [1]. In contrast, cryo-TEM preparations, where samples are snap frozen in liquid ethane, revealed spherical-shaped exosomes that visibly contained ultra-structural features [113, 114].

Another method used to characterize exosomes is atomic force microscopy (AFM). AFM provides topographical imaging of exosomes by scanning the surface of deposited samples with a tipped cantilever and translating tip deflection into a three-dimensional (lateral and vertical) image of the surface [115]. This method can reach sub-nanometer resolution of polydisperse samples. However, surface binding may affect the morphology of vesicles and can hamper the determination of their true diameter [116].

Size-based analysis may also be accomplished by examining the light-scattering properties of vesicles within a fluid medium that is based upon Brownian motion [117]. Dynamic light scattering (DLS) is a fast and accurate measurement tool for size distribution and electrokinetic potential (zeta potential) analysis of monosized particles in suspension. However, it does have limitations when analyzing polydisperse samples where large fluctuations in intensity of scattered light are measured [118, 119]. This limitation occurs as the size distribution analysis is highly influenced by the presence of small amounts of large particles which scatter more light than small vesicles, causing the distribution to be weighted towards larger vesicles [116]. Alternatively, it is reported that nanoparticle tracking analysis (NTA) can analyze polydisperse samples of vesicles between 50-1000 nm by measuring the absolute size distribution of particles in a mixture [116]. Particles in a fluid are illuminated by a laser and viewed as small bright spots through a conventional optical microscope. The Brownian motion of individual particles is recorded through live
imaging video and the mean velocity of each particle is calculated with image analysis software [116]. Although individual particles may be tracked, some limitations of NTA in comparison to DLS include a limited concentration range ($10^7$-$10^9$ vs. $10^8$-$10^{12}$ particles/mL) and increased acquisition time (2-5 min vs. 5-60 min). Additionally in NTA, large aggregates have been reported to mask smaller aggregates, which may enter and leave the viewing area, making size measurement impossible [120].

Finally, a novel high resolution flow cytometry technique has been developed to quantitate immunolabeled nano-sized vesicles. Using a new custom-made Influx™ flow cytometer, small-particle detection has been improved by a wider forward scatter angle and a photomultiplier tube to allow detection below the previously limiting 300 nm level [121]. This technological advance in the physical characterization of exosomes and other released vesicles awaits commercialization before becoming widely applicable.

1.4.4 Exosomal proteins

Exosomes have been extensively characterized with respect to their proteome content [35, 52, 122, 123]. Proteomics describes the unbiased and global analysis of all proteins in a given system [124]. Needless to say, for an accurate protein representation, a homogeneous exosome sample is essential. Several proteomic techniques, including 1-D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and 2-D differential in gel electrophoresis (DIGE) coupled with mass spectrometry (MS) have been used to identify between ~30 to ~900 proteins from exosome samples [114, 125]. In these workflows, SDS-PAGE bands/spots are cut, reduced, alkylated and trypsinised. Generated tryptic peptides are extracted and separated by reversed-phase high-performance liquid chromatography (HPLC) that is coupled online to a mass spectrometer for analysis [126]. MS tryptic peptide spectra are then searched against databases using programs including SEQUEST [127], PROWL [128], and MASCOT [129] to identify proteins. Additionally, Trans-Proteomic Pipeline (TPP) is an integrated software platform that encompasses most of the steps in a proteomic data analysis workflow [130]. In addition to identifying proteins, techniques can
be used to define the abundance of proteins between samples. Stable isotope labeling by amino acids in cell culture (SILAC) experimentally measures relative-abundance ratio of peptides by comparing heavy/light peptide pairs [131]. Other examples of chemical derivatization techniques for quantitative proteomics include isotope-coded affinity tags (ICAT), used for the labeling of free cysteine, and isobaric tags for relative and absolute quantification (iTRAQ), used for the labeling of free primary amine groups [132]. Label-free quantitation includes either spectral counting or peptide signal intensity to estimate abundance of proteins [133]. Additionally, semi-quantitative spectral counting using significant spectral count fold change ratios \( R_{SC} \) can also be used to quantitate protein levels between samples [8, 134]. Alternatively, absolute quantitation using selected reaction monitoring (SRM) [135], also referred to as multiple reaction monitoring (MRM), is a targeted approach requiring individually designed assays. This involves the selection of a ‘quantotypic’ peptide that is unique to the protein of interest and directly proportional to the amount of protein present [136]. These isotopically labeled peptides become the internal calibrant and therefore the unknown abundance of a specific protein can be determined by comparing its peptide signal intensity with the known calibrant standards [135, 137].

Proteomic studies have been performed on exosomes derived from multiple cell lines and body fluid sources [122]. Exosomes derived from hematopoietic cells including mast [138], B and T cells [139, 140], and dendritic cells [141]. Tumor cell lines derived from breast adenocarcinoma [37], melanoma [142], mesothelioma [143], medulloblastoma [144], bladder [145], and colorectal cancer [114] are used to characterize cancer-related exosomes which may assist in defining cell specific markers for disease. Proteomic studies have also been performed on exosomes derived from body fluids including breast milk [146], malignant pleural effusions [147], semen [148], urine [149], saliva [47] and blood [41]. An extensive review of proteomic studies of exosomes isolated from cell lines and body fluids has been included in Appendix A1 and A2. For a comprehensive account of exosome studies, including unpublished datasets, see ExoCarta (http://www.exocarta.org/) [122, 123] and Vesiclepedia (http://www.microvesicles.org/) [52].
A conserved set of proteins are observed in exosomes including those involved in their biogenesis, these include, Alix, TSG101 and other ESCRT complex proteins. Tetrspanin superfamily proteins CD9, CD63, CD81 and CD82 are commonly found in exosomes; they are reported to contribute to the protein sorting pathway [74, 150, 151]. Cytosolic proteins actin and tubulin, and chaperone proteins HSP70 and HSP90 are also often identified in exosomes. Additionally, exosomes are enriched in proteins that associate with lipid rafts, including glycosylphosphatidylinositol (GPI)-anchored proteins and flotillin [139] and Rab GTPases involved in trafficking and endosome fusion events [152]. Exosomes also contain lipid and nucleic acid (mRNA/miRNA) species [138, 153] (see section 1.4.5 and 1.4.6).

In addition to a conserved set of proteins generally identified in exosomes, a seminal study comparing proteomic analyses of exosomes revealed that proteins harbored by exosomes may be cell or tissue specific. This was demonstrated by a comparison of urine, mast cell and colorectal carcinoma-derived exosomes by Mathivanan and colleagues [44]. A proteomic analysis was performed on exosomes isolated from the LIM1215 colorectal carcinoma cell model using immunoaffinity targeting anti-A33, a marker of the basolateral surface in intestinal epithelial cells [154-156]. This study identified 394 exosomal proteins. A comparative protein profiling analysis of this study with murine mast cell and human urine-derived exosomes revealed a common set of 31 proteins including Alix, Rab5 and annexins A6 and A11. Interestingly, a protein signature reflecting the colorectal cell origin (LIM1215) was identified. This signature included A33, cadherin-17, carcinoembryonic antigen (CEA) and epithelial cell adhesion molecule (EpCAM).

### 1.4.5 Exosomal lipids

In addition to the protein content of exosomes, the lipid components of exosomes have also been investigated. Characterizing the lipid content of exosomes may assist in our understanding of their biogenesis and cell-mediated uptake, and in the longer term, the development of exosome (liposome) encapsulated drugs [153, 157].
The process of inward budding of MVBs and exosome formation requires critical lipid sorting [153]. During inward budding, as two thirds of the lipids required for ILV formation are located within the inner leaflet of the endosomal membrane, careful rearrangement and equilibration of lipids from inner and outer leaflets is required [153]. It is reported that lysobisphosphatidic acid (LBPA) accumulates in the MVB membrane and interacts strongly with Alix during exosome biogenesis [153]. Interestingly, addition of LBPA to a lipid composition similar to that of MVBs caused internal budding of small vesicles within liposomes when adjusted to pH 5.5 (pH of MVBs) [158]. A lipidomic study of exosomes in comparison to parental cells revealed enrichment in sphingomyelins by 1.3 times in erythrocytes and 2.3 times in B lymphocytes. It was also observed that cholesterol was enriched in B-lymphocyte-derived exosomes while phosphatidylserine was enriched in dendritic cell-derived exosomes [153]. Further, it was recognized that phosphatidylcholines and phosphatidylethanolamines present in exosomes are enriched in saturated fatty acids comparatively to parental cells [153]. Interestingly, bioactive lipids such as prostaglandins, and lipid mediators that display multiple biological effects related to inflammation, are sorted into exosomes [159]. Lipid raft-like domains have also been reported in exosome membranes. These domains consist of glycerophospholipids with long saturated fatty acyl chains, cholesterol, flotillin, stomatin, high amounts of sphingolipids and low levels of phosphatidylcholine [160].

A recent comprehensive lipid profiling study of isogenic primary and metastatic colon cell lines (SW480 and SW620) revealed differential expression of lipids identified as being associated with cancer progression [161]. These included increased plasmanylcholine and triglyceride lipid levels, decreased plasmenylethanolamine and ceramide lipid levels, and a significant increase of total cholesterol ester and triglyceride lipids in the SW620 cells compared to those in SW480 cells [161]. It is interesting to hypothesize if similar lipid contributions would be observed in exosomes. Understanding the lipid content of exosomes, through comprehensive lipidomic studies of exosomes from various sources, may shed light on their fate and physiological function.
1.4.6 Exosomal RNA

A major breakthrough in characterizing exosomes was the finding of functional mRNAs and miRNAs, small 22-25 nucleotide non-coding RNAs that suppress the translation of target mRNAs [162]. This finding highlighted exosomes as prospective vehicles for the horizontal transfer of biologically important information [163, 164]. Valadi and colleagues demonstrated that mast cells secreted exosomes containing mRNAs from approximately 1300 genes and more than 100 different miRNAs [138]. Further, an in vitro translational assay demonstrated that exosomal mRNAs from mouse mast cell line MC/9 are functional in human mast cell line HMC-1 cells [138]. In a separate study, glioblastoma cell-derived exosomes containing a reporter gene were incubated with recipient human brain microvascular endothelial cells (HBMVECs) to reveal mRNA was delivered and translated [165]. A summary of miRNAs identified from in vitro studies of cancer cell exosomes is outlined (Table 1-2).

In-depth RNA analysis has been performed to characterize multiple RNA species contained in exosomes. For example, a small RNA deep sequencing study revealed RNA profiles in exosomes released from prion-infected neuronal cells [166]. Bellingham and colleagues demonstrated that neuronal exosomes contain a diverse range of RNA species including retroviral RNA repeat regions, messenger RNA fragments, transfer RNA fragments, non-coding RNA, small nuclear RNA, small nucleolar RNA, small cytoplasmic RNA, silencing RNA as well as known and novel candidate miRNAs. The authors concluded that exosomes released from prion-infected neuronal cells have a distinct mRNA/miRNA signature that can be utilized for diagnosis of prion disease [166]. Further work is required to establish a link between these exosomal RNA species and prion disease pathogenesis.

In another study, deep sequencing technology was used to characterize the RNA content of exosomes isolated from breast milk [167]. This study revealed 602 unique miRNAs. Of these, 59 were well characterized immune-relate miRNAs [167]. The longevity of miRNAs contained within exosomes was increased when compared with circulating RNAs. It was speculated that due to the protection and durability of exosomal miRNAs, they may assist
Table 1-2. A summary of *in vitro* studies of miRNAs identified in cancer cell exosomes. Modified from [168].

<table>
<thead>
<tr>
<th>Cell line model</th>
<th>Major findings</th>
<th>Predominant microRNAs</th>
<th>Target genes or pathways</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human and mouse mast cells</td>
<td>Identified small RNAs, including 121 microRNAs and 1,300 specific mRNAs. Detected mouse exosomal RNA and new mouse proteins in human mast cells after treatment with mouse mast cell exosomes. Coined the term ‘exosomal shuttle RNA (esRNA)’</td>
<td>let-7, miR-1, miR-15, miR-16, miR-181, miR-375</td>
<td>None tested</td>
<td>[138]</td>
</tr>
<tr>
<td>Metastatic gastric cell line</td>
<td>Profiled microRNA expression by microarray in exosomes isolated from gastric cancer cells. let-7 microRNA family was enriched in exosomes</td>
<td>let-7 family</td>
<td>None tested</td>
<td>[169]</td>
</tr>
<tr>
<td>Co-culture of IL-4-activated macrophages and breast cancer cells</td>
<td>miRNAs can be transferred from macrophages to breast cancer cells. miR-223 released by macrophages was found in MCF7 and MDA-MB-231 cells and promoted invasion</td>
<td>miR-223</td>
<td>Mef2c-β-catenin pathway</td>
<td>[170]</td>
</tr>
<tr>
<td>Mouse dendritic cells</td>
<td>Exosomal microRNA from dendritic cells can be transferred to a recipient dendritic cell and repress microRNA target mRNAs in the acceptor cell</td>
<td>miR-148a, miR-451</td>
<td>Luciferase reporter containing tandem microRNA target sequences</td>
<td>[171]</td>
</tr>
<tr>
<td>Leukemia cells and endothelial cells</td>
<td>Leukemia cells released microRNAs from the miR-17-92 cluster and were taken up by human umbilical vein endothelial cells (HUVECs) and repressed a target mRNA. Did not affect the growth of HUVEC cells, but did enhance cell migration and tube formation</td>
<td>miR-92a</td>
<td>Integrin α5</td>
<td>[172]</td>
</tr>
<tr>
<td>Metastatic rat adenocarcinoma cells</td>
<td>Exosomes were preferentially taken up by lymph node stroma cells and lung fibroblasts. The transferred microRNAs affected mRNA translation of many genes</td>
<td>miR-494, miR-542-3p</td>
<td>Cadherin-17, proteases, adhesion molecules, chemokine ligands, cell cycle- and angiogenesis-promoting and oxidative stress response genes.</td>
<td>[173]</td>
</tr>
<tr>
<td>Renal cancer stem cells</td>
<td>Microvesicles were secreted from human renal cell carcinoma that could trigger angiogenesis and pre-metastatic niche formation in a severe combined immunodeficient (SCID) mouse model</td>
<td>miR-92, miR-141, miR-29a, miR-650, miR-151, miR-19b, miR-29c</td>
<td>Increase in VEGFR1 and MMP-9 expression</td>
<td>[174]</td>
</tr>
</tbody>
</table>
the development of the infant immune system [167]. Further studies are required to
determine whether specific intracellular miRNAs are enriched in exosomes. Recent
evidence supporting specific miRNA loading of exosomes was the identification that
different miRNA content depended on the maturation stage of the DCs [171], and that cis-
acting elements may target specific RNAs into exosomes [175]. Interestingly, the
identification of a ‘zipcode’ contained within the 3’ region of mRNA has been identified
that leads to incorporation of specific mRNAs into EVs [176]. In-depth characterization of
mRNA and miRNA signatures within exosomes from cancer cells may lead to their use as
clinical biomarkers and give insight into their contribution in disease progression.

1.4.7 Exosome targeting and uptake

It is understood that exosomes are key mediators of intercellular communication and,
therefore, the molecular mechanisms governing exosome recognition and uptake by
recipient cells have gained much attention. An overview of possible modes of exosome
interaction and uptake by recipient cells is given in (Figure 1-3) [32].

Differences in exosomal tetraspanin complexes appear to influence target cell interaction in
vitro and in vivo, possibly by modulating the functions of associated integrin adhesion
molecules [177]. For example, exosome capture by dendritic cells was reduced by 5-30%
by co-incubation with blocking antibodies specific for various integrins, adhesion
molecules or tetraspanins [178]. Other membrane proteins also reported to be important in
targeting exosomes to recipient cells include intercellular adhesion molecule 1 (ICAM-1)
and milk fat globule-epidermal growth factor VIII protein (MFGE-8) [179, 180]. In
addition to membrane proteins, the delivery efficiency of exosomes to cells is reported to
be directly related to rigidity of exosomes that contain lipids including sphingomyelin and
N-acetylneuraminyl-galactosylglucosylceramide (GM3) [181].

In addition to receptor-mediated interactions leading to activation of cell signaling
pathways [25], exosomes may also be internalized. Escrevente and colleagues
demonstrated that fluorescently-labeled ovarian cancer cell-derived exosomes were
Figure 1-3. Exosome targeting and uptake
Following release by parent cells, exosomes are shown to communicate and elicit an effect with recipient cells by (i) receptor-mediated interactions involving tetraspanins and integrins [177], (ii) membrane fusion [181], and (iii) endocytic uptake [182] by phagocytosis [183] and macropinocytosis [184].
internalized by clathrin-mediated endocytosis in ovarian cancer cells [182], while Feng and colleagues reported exosomes being internalized via phagocytosis in monocyte-derived macrophages [183]. Fitzner and co-workers also showed that selective transfer of exosomes from oligodendrocytes to microglia could occur by macropinocytosis [184], a process whereby bending of single surface lamellipodia gives rise to circular curved ruffles which are finally sealed to form discrete endocytic vacuoles [184]. Exosome uptake has been reported to be a temperature dependent process. For example, exosomes from rat pheochromocytoma (PC12 cells) were internalized by resting PC12 cells by actin-dependent endocytosis [185]. Exosomes were shown to be internalized by lipid raft mediated endocytosis in HUVECs – this process was subsequently negatively regulated by caveolin-1 [186, 187].

In another study, exosomes were shown to fuse with the plasma membrane in a pH dependent manner [181]. For example, exosomes purified from metastatic melanoma cell culture medium were labeled with a self-quenching lipid fluorescent probe, R18, and shown to fuse at the plasma membrane of recipient melanoma cells [181]. Interestingly, fusion efficiency was higher with exosomes released from metastatic cells compared to those derived from primary melanoma cell tumors or normal peripheral blood mononuclear cells [181]. A mechanism of exosome uptake involving a combination of fusion and endocytosis has also been reported [171]. Montecalvo and colleagues demonstrated that dendritic cell-derived exosomes were shown to fuse in ‘2-step event’ consisting of exosome hemifusion with the cell membrane, followed by endocytosis and complete fusion of the exosomes with the limiting membrane of the phagosome [171].

Although there are numerous studies demonstrating exosome uptake by recipient cells, very little is known about the specific target recognition motifs in exosomes. This highlights the need for in-depth characterization of exosome membranes to identify barcode signatures that allow and signal for their recognition and uptake by specific recipient cells. Following exosome uptake there is limited data showing how exosomes traffic intracellularly to exert their biological effect.
1.4.8 Exosome function in the tumor microenvironment and cancer progression

In addition to cancer cells, the tumor microenvironment comprises endothelial cells, supporting pericytes, fibroblasts, and both innate and adaptive infiltrating immune cells [188]. Interestingly, as cancer cells accumulate genetic mutations, they can secrete molecules (and vesicles) that can reprogram normal stromal cells to serve the budding neoplasm; this process ultimately enables cancer cells to invade adjacent tissues and disseminate to distant loci [189]. Recently, there has been significant interest in understanding the involvement of exosomes in the tumor microenvironment and cancer progression [190]. For example, exosomes have been reported to be involved in multiple aspects of cancer progression including causing immune suppression [191], stimulating angiogenesis [192], modulating stromal cells [193], promoting metastatic ability [194], and establishing the pre-metastatic niche [195] through the transfer of oncogenic material in an intra- and inter-cellular circuitry [196] (Figure 1-4, Table 1-3).

It has been reported that tumor-derived exosomes bear immunosuppressive molecules and are implicated in immune suppression [191]. Further, it was demonstrated that exosomes can inactivate T lymphocytes, or inhibit the differentiation of precursors to mature antigen presenting cells to suppress immune surveillance, thereby assisting cancer progression [191]. Angiogenesis can also be influenced by exosomes [192]. For example, melanoma exosomes were observed to rapidly stimulate the production of endothelial spheroids and sprouts in a dose-dependent manner [192]. These exosomes elicited paracrine endothelial signaling by regulation of inflammatory cytokines, including IL-1α, TNFα and VEGF, suggesting that exosomes can promote endothelial angiogenic responses and contribute to tumor metastatic potential [192]. Tumor-derived exosomes were also shown to express membrane TGF-β and activate the SMAD-dependent signaling pathway in stromal fibroblasts, resulting in myofibroblast differentiation [193]. Myofibroblasts are enriched in solid tumors and represent an altered stroma that usually supports tumor growth, vascularization, and metastasis [193].
Figure 1-4. Extracellular vesicles (EVs) are components of the intra- and intercellular oncogenic signaling circuitry

Oncogenic mutations (onc) and the resulting synthesis of altered mRNA and oncoproteins (ONC) result in perturbations of the intracellular signaling circuitry (SIGNAL) in a primary tumor. EVs containing onc are released into the extracellular space, and sometimes further into circulation, enabling exposure of stromal or indolent cells to transforming onc molecules. Upon uptake of tumor-derived EVs, oncogenic mutations may affect cellular signaling which can contribute to increased proliferation [197] and invasiveness [198]. Within the circulation, EVs can educate bone marrow-derived cells (BMDC onc) resulting in mobilization of BMDC onc to lymph nodes to assist pre-metastatic niche formation [195]. EVs alone can also home to the lymph node for pre-metastatic niche formation [119]. Figure adapted from [196] using Sevier Medical Art.
Table 1-3. Examples of EV-mediated transfer of oncogenic/transforming molecules. Adapted and updated from [196].

<table>
<thead>
<tr>
<th>Transforming molecule</th>
<th>Vesicle</th>
<th>Observations</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein/EGFRvIII</td>
<td>EVs</td>
<td>Glioblastoma EVs transfer EGFRvIII protein to indolent cancer cells causing oncogenic activity including activation of transforming signaling pathways MAPK and Akt</td>
<td>[199]</td>
</tr>
<tr>
<td>mRNA/EGFRvIII</td>
<td>EVs</td>
<td>Glioblastoma EVs containing EGFRvIII oncoprotein transfer functional mRNA causing stimulation of tubule formation by endothelial cells</td>
<td>[165]</td>
</tr>
<tr>
<td>Protein/EGFR</td>
<td>EVs</td>
<td>Cancer EVs transfer oncogenic EGFR to endothelial cells causing autocrine reprogramming of VEGF</td>
<td>[200]</td>
</tr>
<tr>
<td>Protein/MyrAkt1</td>
<td>EVs</td>
<td>Cancer EVs transfer oncogenic Akt to recipient cells causing rapid phosphor-tyrosine and Akt pathway signaling stimulating proliferation and migration</td>
<td>[62]</td>
</tr>
<tr>
<td>Protein/tTG and FN$^a$</td>
<td>EVs</td>
<td>Cancer EVs transfer tissue transglutaminase and fibronectin to NIH3T3 fibroblasts causing tumorigenic conversion</td>
<td>[201]</td>
</tr>
<tr>
<td>DNA/retrotransposon$^a$</td>
<td>EVs</td>
<td>Cancer cell-derived EVs transfer retrotransposons to endothelial cells</td>
<td>[202]</td>
</tr>
<tr>
<td>Protein/LMP1</td>
<td>Exosomes</td>
<td>Cancer exosomes transfer Epstein-Barr virus latent membrane protein 1 causing activation of ERK and AKT signaling pathways in recipient epithelial cells</td>
<td>[203]</td>
</tr>
<tr>
<td>Mutant KRAS</td>
<td>Exosomes</td>
<td>Exosomes containing mutant KRAS can cause enhanced three-dimensional growth of wild-type KRAS-expressing non-transformed cells</td>
<td>[197]</td>
</tr>
<tr>
<td>Wnt11</td>
<td>Exosomes</td>
<td>Fibroblast-derived exosomes mobilize autocrine Wnt-PCP signaling in breast cancer cells to stimulate invasive behavior and metastasis in animal models</td>
<td>[198]</td>
</tr>
<tr>
<td>Wnt3a</td>
<td>Exosomes</td>
<td>Exosomes containing Wnt3a can induce Wnt signaling activity in target cells</td>
<td>[105]</td>
</tr>
<tr>
<td>Tyrosine kinase MET</td>
<td>Exosomes</td>
<td>Exosomes expressing tyrosine kinase MET increased the metastatic capability of bone marrow cells</td>
<td>[195]</td>
</tr>
</tbody>
</table>

$^a$ Oncogenic activity not directly demonstrated
The transfer of metastatic activity has also been attributed to exosomes. Melanoma-derived exosomes were shown to promote epigenetic transfer of metastatic activity in murine models. Highly metastatic BL6-10-derived exosomes were transferred to poorly metastatic melanoma F1 cells to express Met 72 tumor antigen [194]. The subsequent Met 72-positive F1 tumor cells showed increased metastatic activity by growing numerous lung tumor colonies following their intravenous injection into mice [194]. In addition to exosomes, microvesicles (MVs) derived from platelets are also capable of enhancing the invasive potential of breast cancer and induce angiogenesis and metastasis in lung cancer [204, 205]. In a further study, transfer of oncogenic potential to a recipient cell through activation of MAPK and Akt signaling pathways highlights new mechanisms of intercellular communication of oncogenic material via MVs in the tumor microenvironment [165, 200].

It is well recognized that tumors preferentially home to specific metastatic sites. For example, metastasis of melanoma often homes to the lung and brain [206], while colorectal cancer to the liver [207]. Interestingly, it has recently been shown that malignant cancers use secreted signals to effectively prime a specific site for metastasis in a ‘seed-soil’ manner by preparing a fertile environment [19, 208]. Melanoma-derived exosomes injected into the foot pads of mice preferentially colonized and conditioned sentinel lymph nodes by the introduction of genetic molecular signals for cell recruitment, extracellular matrix deposition, and vascular proliferation [119]. Further, studies by Peinado and colleagues have demonstrated that tumor exosomes can horizontally transfer genetic material to bone marrow-derived cells (BMDCs) resulting in upregulation of the MET oncoprotein. This led to mobilization of BMDCs to home to the lung and lymph node to support tumor vasculogenesis, invasion and metastasis [195] (Figure 1-4).

Interestingly, the ability to interfere with molecular pathways responsible for exosome formation and release may in turn lessen their contribution to disease progression [209]. This could potentially involve the modulation of a key protein required for their formation (i.e., Rab27a) [103, 210]. Additionally, it has previously been demonstrated that regulation of Rab GTPase-activating proteins (GAPs) can alter Rab35 activity which leads to the accumulation of endosomal vesicles and impairment of exosome secretion [101].
Attenuation of exosome release from cancer cells can also be accomplished by administration of neutral sphingomyelinase inhibitors [96], or treatment with the blood-pressure-lowering drug amiloride [211]. Alternatively, the inhibition of EV uptake by recipient cells can be altered using diannexin to block phosphatidylserine, which is an important molecule required for cell adhesion [200, 212]. This experimental process was shown to reduce the growth rate of human glioma xenografts in mice [200].

1.4.9 Exosome therapeutics

The ability of exosomes to transport bioactive molecules has driven research to improve their large-scale isolation and characterization for use in translational nano-medicine [213] (Figure 1-5). Current drug delivery vehicles, including viral and bacterial vectors, have limited efficacy following repeat administration. These vectors may also elicit cellular toxicity by causing insertional mutagenesis in host cells [214] (Figure 1-6). Alternatively, exosomes may have advantageous properties over other drug delivery vehicles as they have limited immunogenicity when derived from ‘self’, increased stability and efficient delivery of cargo delivery abilities.

A groundbreaking discovery in exosome therapeutics was the ability of exosomes to function as natural cell-derived nano-carriers that can cross biological barriers [215, 216]. For example, exosomes may be used to encapsulate and protect exogenous RNA interference (RNAi) and miRNA regulatory molecules in addition to other single stranded oligonucleotides [216]. Exciting new work by Alvarez-Erviti and colleagues have demonstrated that following systemic injections, neuron-specific peptide targeting allowed effective delivery of exosomes loaded with siRNA into the mouse brain [216]. As a result of siRNA contained within exosomes, they observed the knock-down of BACE-1, a therapeutic target in Alzheimer’s disease. Additionally, injections of exosomes (150 µg) appear to be well-tolerated with little or no immunogenicity or toxicity, even following repeat (n=3) administration [210, 216].
Figure 1-5. Functional roles and therapeutic targeting of extracellular vesicles (EVs)

EVs containing protein and RNA may be exploited for functional roles in therapeutics. The presence of MHC molecules on exosomes enables efficient antigen presentation to suppress tumor growth [218]. Further, EVs can cause immune modulation by protecting T cells from activation-induced cell death [219], or cause immune suppression by EV/FASL-mediated T cell apoptosis [220]. Regenerative medicine applications of EVs have also been demonstrated. For example, EVs derived from mesenchymal stem cells are shown to reduce myocardial ischemia/reperfusion injury by mediating cardioprotection and reducing infarct size in mouse models [46, 221]. EVs may also be endogenously loaded using recombinant expression of targeting ligands or overexpressing therapeutic RNAs [210, 216]. Alternatively, EVs may be exogenously loaded with siRNA by electroporation prior to being used in therapeutic treatments [222]. Adapted from [210].
Figure 1-6. Advantages and drawbacks of siRNA delivery by exosomes, viruses and lipid nanoparticles

Major advantages are indicated in green, disadvantages in red. (a) Dendritic cell-derived exosomes can directly fuse with the plasma membrane through CD9 tetraspanin interactions with surface glycoproteins on the target cell to ensure direct cytosolic delivery of siRNA [223]. (b) Viruses mediate direct delivery of vectors encoding short hairpin (sh) RNA to the cytosol. However, viruses are prone to clearance by opsonins, complement, coagulation factors and virus-specific antibodies in the systemic circulation. Although genomic insertion of the shRNA-encoding vectors allows long-term RNAi, a potential risk of this strategy is genetic deregulation and oncogene activation by insertional mutagenesis [214]. (c) Internalization of lipid nanoparticles occurs by endocytosis. Endosomal escape is required for cytoplasmic delivery by fusion with, or rupture of, the endosomal membrane using viral or bacterial proteins. This escape is often hampered by the man-made nature of lipid nanoparticles. The direct delivery of RNA into endosomes can lead to unwanted TLR7/8 activation [224]. Adapted from [225].
The ability of exosomes to activate T cells has prompted their potential use as vaccines [217]. Chaput and colleagues demonstrated that peptide loaded dendritic cell-derived exosomes could mediate tumor rejection in mice [217]. Further, exosomes secreted from Gm-CSF/IL-4 bone marrow dendritic cells were shown to eradicate established murine tumors in mammary TSA carcinoma, P815 mastocytoma and MCA205 sarcoma models [217]. These immunogenic properties have allowed the first phase I clinical trial of dendritic cell-derived exosomes to treat metastatic-tumor-bearing patients [226]. Good manufacturing practice (GMP) was used to isolate large-scale pharmaceutical-grade exosomes and directly load peptides on functional MHC complexes. Autologous exosomes were inserted into patients to observe an increase in NK cells, thus highlighting their potential use as a vaccine [226]. It was also observed that increased levels of hsa-let-7b and hsa-let-7g miRNAs in mesenchymal stem cell-derived exosomes were able to treat myocardial infarction [227, 228] and provide protection against myocardial ischemia [46]. A list of other therapeutic applications of exosomes is outlined in (Table 1-4).

An alternative to tailored biological exosomes are artificial exosome ‘mimetics’, which are being developed for clinical immunology [229]. It is hypothesized that the incorporation of only key exosomal lipids, protein and nucleic acids will lead to the creation of a viable therapeutic vehicle [229]. Using nanotechnology-based approaches, targeted and traceable in vivo super paramagnetic artificial exosomes (termed liposomes) have been developed [230, 231]. Liposomes coated with a MHC class I/peptide complexes and a range of ligands for adhesion were tracked in vivo using fluorescence and magnetic resonance imaging and shown to activate and expand functional T cells [232]. Interestingly, Delcayre and colleagues have coined ‘exosome display technology’ which utilizes the fusion of antigens onto the C1C2 domain of lactadherin [233], allowing for presentation of antigens to the immune system. Further, this technology could also be incorporated into liposomes. Inclusion of tetraspanin proteins in exosome mimetics has been proposed due to their ability to functionally mediate fusion and cell-cell adhesion [234]. Challenges associated with liposome creation and usability include their sensitiveness to clearance by opsonins, complement and coagulation factors in the blood [225] (Figure 1-6). Additionally, although lipid assembly is understood, incorporation of functional membrane proteins into
<table>
<thead>
<tr>
<th>Application</th>
<th>Therapeutic observation</th>
<th>Ref.</th>
</tr>
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<tbody>
<tr>
<td>Antigen presentation</td>
<td>Dendritic cells pulsed with tumor-related peptides are release on exosomes to suppress tumor growth</td>
<td>[218]</td>
</tr>
<tr>
<td>Immune modulation</td>
<td>Exosomes cause immune activation by protecting T cells from activation-induced cell death</td>
<td>[219]</td>
</tr>
<tr>
<td>Tissue repair</td>
<td>Manufacturing of therapeutic exosomes following immortalizing of mesenchymal stem cells</td>
<td>[236]</td>
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<tr>
<td></td>
<td>Exosomes secreted by mesenchymal stem cells reduces myocardial ischemia/reperfusion injury</td>
<td>[46, 221]</td>
</tr>
<tr>
<td>Gene therapy</td>
<td>Transfection of miR-150 in monocytes released miR-150 containing functional exosomes</td>
<td>[237]</td>
</tr>
<tr>
<td></td>
<td>siRNA loaded into exosomes by electroporation is shown to be functional by knocking down</td>
<td>[210, 216]</td>
</tr>
<tr>
<td></td>
<td>BACE1 in mouse brain, a therapeutic target in Alzheimer’s disease</td>
<td>216</td>
</tr>
<tr>
<td>Gene therapy</td>
<td>Human plasma-derived exosomes were electroporated with siRNA to deliver a functional RNAi</td>
<td>[222]</td>
</tr>
<tr>
<td></td>
<td>response in human monocytes and lymphocytes <em>in vitro</em></td>
<td></td>
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<tr>
<td></td>
<td>Adendo-associated viruses (AAVs) displaying viral caspid proteins contained within ‘vexosomes’ can deliver cargo to recipient cells</td>
<td>[238]</td>
</tr>
<tr>
<td>Tissue targeting</td>
<td>Plasmid expressing exosomal proteins fused to brain specific peptide (rabies virus glycoprotein, RVG) is displayed on exosomes and delivered across blood brain barrier</td>
<td>[216]</td>
</tr>
<tr>
<td></td>
<td>Exosomes containing the transmembrane domain of platelet-derived growth factor receptor fused to the GE11 peptide homed to EGFR-expressing tumor cells to deliver let-7a anti-tumor miRNA</td>
<td>[239]</td>
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</table>
liposomes has proven to be a more difficult process [235]. Overall, it is hypothesized that these challenges can be overcome, and that liposomes may be produced on a large scale to act as pharmaceutical-grade delivery vehicles that carry similar therapeutic properties to exosomes [229].

1.4.10 Exosome and microvesicle diagnostics

The capacity of exosomes and other EVs to protect genomic material makes them a promising source of potential biomarkers. One assay reaching clinical utility is the use of mRNA profiling of serum-derived EVs to identify glioblastoma-bearing patients [240]. It has also been reported by Mitchell and colleagues that miR-141 levels from serum, proposed to be contained within exosomes, can robustly distinguish prostate cancer patients from healthy controls [241]. Additionally, there are several companies that have begun development of exosome-based diagnostics. New York based biotechnology company, Exosome Diagnostics (http://www.exosomedx.com/), is performing the characterization of mRNA and miRNA contained within exosomes (and other EVs) to develop biofluid-based molecular diagnostic tests for use in personalized medicine. Caris Life Sciences (http://www.carislifesciences.com/) is also developing methods for isolating and characterizing blood-based circulating microvesicles. Finally, HansaBioMed (http://www.hansabiomed.eu/) has developed an ELISA-based platform called Exotest which utilizes CD63, CD9 and CD81 antibodies immobilized on 96-well plates in an effort to capture, quantify and characterize exosomes. It is evident that there is significant interest in this field of research, with several companies having initiated programs that exploit the diagnostic potential of exosomes.

1.4.11 Exosome isolation strategies

A major challenge in the field is to improve and standardize methods for exosome isolation and characterization. Current strategies for isolating exosomes differ significantly based
upon their various physical attributes. These strategies include ultracentrifugation [242], sucrose or OptiPrep™ gradients relying on vesicle density [35, 243] and immunoaffinity approaches relying on a specific exosome surface protein target (Table 1-1). This diversity in purification methodology has led to variations in sample homogeneity and confusion with respect to nomenclature within the field (e.g., exosomes vs. exosome-like vesicles). As different isolation strategies may have varying fractionation capabilities, it is critical to evaluate these techniques and their abilities to isolate a homogeneous population exosomes.

These isolation strategies are described in turn:

Differential ultracentrifugation involves removal of intact cells and bulky cell debris by low speed centrifugation (e.g., 500 × g, 2,000 × g) followed by high-speed centrifugation (e.g., 100,000 × g) to sediment exosomes [242]. This widely-used methodology, however, can potentially result in co-sedimentation of protein aggregates and/or other membranous vesicles which may be pelleted during ultracentrifugation [7].

Ultracentrifugation using a linear sucrose gradient has been developed to isolate exosomes based on their density of 1.08-1.22 g/cm³ [35, 242]. For clinical purposes, the preparation of GMP-grade exosomes can be prepared using a combination of ultrafiltration, ultracentrifugation and a 30% sucrose/deuterium (D₂O) (98%) cushion (1.21 g/cm³) [244]. Interestingly, sucrose gradients have been inefficient in separating exosomes from HIV-1 particles due to similarities in their size and buoyant density [245]. To overcome this problem, Cantin and colleagues describe the use of iodixanol (OptiPrep™) 6-18% gradients to separate HIV-1 particles and apoptotic vesicles from exosomes [243]. Iodixanol is a non-ionic, water-soluble radiographic contrast medium [246] that may be beneficial for future clinical use with exosomes due to its physical tolerance and low incidence of adverse effects when injected and used as an x-ray contrast medium [247].

Immuoaffinity capture (IAC) is another widely used method for exosome isolation. This strategy is based on the use of specific exosome-surface protein directed antibodies coupled to magnetic beads (Table 1-1). IAC can be a powerful tool for exosome isolation, provided a specific exosomal cell surface protein can be identified that discriminates the exosome of
interest from other EVs present in the biological matrix. Immuno-isolation of exosomes has been performed to purifying antigen presenting cell-derived exosomes [40]. CD45-exosomes from Jurkat T cells [39], HER2-positive exosomes from breast adenocarcinoma cell lines and ovarian cancer patient-derived ascites [37], A33-positive exosomes released from colon carcinoma cancer cells [44], and EpCAM-positive exosomes from the sera of lung cancer [42] and ovarian cancer [43] patients. This technique is limited by the number of known cell, and thus, specific exosome membrane proteins. A final strategy, size-exclusion HPLC, has also been successfully employed for the large-scale production of microparticles from human mesenchymal stem cells [45, 46], and human saliva [47].

1.5 Research models used in this thesis

Cell lines that are readily cultured in vitro are often used as surrogates for tumors as they can reflect their tissue of origin [248]. Cell lines have an advantage over cancer biopsy tissue in that they are homogeneous as compared to heterogenic solid tumors which, in addition to cancer cells, contain multiple other cell types [249]. Cell lines also provide an unlimited auto-replicative source that can be easily handled, may contain specific known mutations, or can be genetically manipulated [250]. In this thesis, two different cell line models were utilized: the colorectal cancer (CRC) cell line LIM1863 [251] and an EMT cell model created from epithelial Madin-Darby canine kidney (MDCK) cells [250].

1.5.1 Colorectal cancer model

1.5.1.1 Colorectal cancer

The structure of the adult colon, or large intestine, is composed of three concentric tissue layers. The outer layer consists of several sheets of smooth muscle that execute the rhythmic peristaltic movements of the intestine [252]. The space between the outer muscle and the inner epithelial layer is filled by connective stromal tissue that contains numerous
blood and lymph vessels, nerve fibers, and various immune cells. The inner luminal surface consists of a single-cell layer comprising differentiated epithelial cells that mediate functions of the intestinal epithelium [252]. Finally, finger like invaginations of this epithelial sheet into the underlying connective tissue form ‘colonic crypts’, the basic functional unit of the colon [253]. CRC is defined as the development of malignant tissue in the wall of the colon or rectum and is caused by a series of stepwise genetic changes (Figure 1-7) [254].

1.5.1.2 CRC statistics and subtypes

About 608,000 deaths from colorectal cancer are estimated to occur worldwide each year. This accounts for 8% of all cancer deaths, making it the fourth most common cause of cancer-related death [255]. According to the Australian Institute of Health and Welfare, CRC was the most commonly registered form of cancer in Australia in 2009 with 14,410 new cases and 3,982 deaths, making up 9.3% of all cancer deaths in Australia [256]. The Dukes’ staging system (A-D) is commonly employed to stage progression and it has been shown that CRC can be effectively treated if detected early [257, 258]. Based on 5-year survival rates, patients presenting with Dukes’ stage A CRC have >90% chance of survival, while more commonly, patients presenting with late stage Dukes’ stage D CRC have 5-year survival rates <10% [259]. A lack of early detection occurs due to low patient compliance with current testing modalities coupled with the limited sensitivity of current detection techniques [260].

Typically there are two forms of CRC, termed sporadic and hereditary. Sporadic CRC refers to random occurrences in which family history is absent [261]. This accounts for approximately 70% of all CRCs [262]. Sporadic CRC arises from the accumulation of multiple genetic mutations in somatic cells over an extended period of time and therefore a late age onset is commonly observed [263]. Hereditary CRC is divided into two classes, familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer
Figure 1-7. Model of intestinal cancer initiation and progression

Crypt base columnar cells (green) and adenoma cell (red) are located at the base of the crypt. Loss of *adenomatous polyposis coli* (*APC*) is known to be an initiating event behind early adenoma formation (red-hued) where cells begin to migrate up the transit amplifying compartment (blue). The accumulation of additional oncogenic mutations (shown in italics) drives tumor progression from a benign intermediate and late adenoma to an invasive carcinoma. Other cell types include entero-endocrine cell (yellow), goblet cell (orange), and tuft cell (purple). Adapted from [254].
(HNPCC), which together make up ~10% of all colon cancers [262]. FAP is characterized by germline mutations in the *adenomatous polyposis coli* (*APC*) gene, resulting in the presence of numerous adenomatous polyps [264]. The *APC* gene encodes a ~300 kDa protein which selectively localizes to the nucleus and cytoplasm [265]. Under normal circumstances, *APC* functions as a tumor suppressor by mediating the downregulation of β-catenin. When *APC* is mutated, β-catenin accumulates in the nucleus and binds to transcription factors to promote cellular proliferation [266]. HNPCC is caused by mutations in one or more DNA mismatch repair genes, most commonly hMSH2 and hMLH1 [267]. Other less frequently mutated genes are hMSH6 and hPMS2 [267]. These mutations are autosomal dominant, resulting in aggressive tumor growth in the right side colon in 70% of cases [268].

1.5.1.3 Colorectal cancer cell line LIM1863

The human LIM1863 (Ludwig Institute Melbourne) cell line was derived from a tumor sample taken from the ileocecal valve, between the ileum of the small intestine and the caecum of the large intestine, from a 74-year-old Caucasian female [251]. This sporadic carcinoma extended through the full thickness of the muscle wall and mutational analysis revealed truncated *APC* (stop at aa 1506) and mutated *p53* (aa 234 Y to H) genes. *In vitro*, the LIM1863 cell line grows as a suspension culture of floating organoids, clusters of cells resembling crypt like structures, which are morphologically and functionally organized. LIM1863 organoids comprise two polarized cell types, columnar and goblet cells surrounding a central lumen (Appendix A-3). Along with continuous cell proliferation and organoid formation, dead cells are continuously shed. This organization is very similar to that of *in vivo*, where intestinal epithelial cells form intestinal crypts [251]. Significantly, single cell cloning was used to isolate 29 subclones of the LIM1863 cell line, all of which grew to display the same phenotype and percentage of morphological cell types as the parent line, thus indicating ‘stemness’ like properties of the cells [269].
Isolating exosomes from CRC cell lines may allow insights into their contribution to the disease. As the LIM1863 cell line grows as floating organoids in suspension, it can be cultured on a large scale, this is beneficial for characterization studies as large amounts of exosomes can be isolated. Further, as LIM1863 cells are polarized, the cell model can be used to understand exosomes in the context of apical and basolateral trafficking and release.

1.5.2 Epithelial-mesenchymal transition (EMT) model

Epithelial-mesenchymal transition is a process by which epithelial cells lose polarity and cell adhesion characteristics and acquire migratory and invasive properties of mesenchymal cells [270, 271]. For many decades, EMT has been recognized as a cell phenotypic switch in developmental biology, embryogenesis, tissue remodeling and wound healing [272, 273]. During these processes, a series of molecular and cellular changes occur allowing epithelial cells to escape from their rigid structural constraints to accommodate a phenotype more amenable to cell migration and invasion (Figure 1-8) [274]. These changes include reduced cell-cell adhesion, loss of cell polarity, elongated spindle-shaped morphology, an invasive phenotype, induction of mesenchymal-specific proteins, ECM alterations, and increased migratory capacity (Appendix A-4) [275]. The activation of an EMT program has also been proposed as the critical mechanism for the acquisition of malignant phenotypes by epithelial cancer cells [189, 276, 277] (Figure 1-9).

1.5.2.1 EMT during cancer progression

Early growth of primary epithelial cancers is characterized by excessive cell proliferation and angiogenesis [189]. Following these changes, local invasiveness, initially through the basement membrane, a dense specialized ECM barrier between the epithelial cell layer and underlying stroma, is thought to begin the multi-step process that leads to metastatic dissemination [270]. The ability of cells to penetrate through the basement membrane may be attributed to the increased expression of secreted proteases [278].
Epithelial-mesenchymal transition (EMT) is a morphogenetic process whereby immotile polarized epithelial cells become migratory mesenchymal cells. This highly conserved cellular process involves dissolution of cell-cell junctions, *i.e.*, tight junctions (dark blue), adherens junctions (light blue) and desmosomes (green), and loss of apical-basolateral polarity. Cells will then acquire a mesenchymal phenotype, this is characterized by actin reorganization, stress fiber formation (red), a ‘leading-edge’ polarity, migration and invasion phenotypes. Modified from [274].
Figure 1-9. EMT during tumor progression and metastasis

Normal epithelia proliferate to form a local adenoma. Genetic alterations and epigenetic events produce a carcinoma in situ. Loss of cell-cell adhesion and increased invasiveness, possibly by EMT, drives cells through a fragmented basement membrane where they can intravasate into lymph or blood vessels. It is proposed that the invasive tumor cells are passaged to distant sites where they repopulate and form secondary metastases. Modified from [276].
These proteases include matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases (ADAMs) that function at cells invasive leading edge to proteolyse collagens and laminins to assist cell migration [278, 279]. Cells expressing a mesenchymal phenotype are typically at the invasive tumor front and are considered to ultimately follow an invasion-metastasis cascade [270]. Interestingly, at distant sites colonized mesenchymal cells are reported to revert back and resemble their originating tumor phenotype. This process is known as mesenchymal-epithelial transition (MET), whereby mesenchymal characteristics are lost in favor of epithelial traits [270]. It is proposed that this may occur as signals originally required for driving and maintaining the mesenchymal phenotype in the primary tumor environment are no longer apparent [270, 280].

1.5.2.2 Molecular markers of EMT

The architecture of epithelial cells is controlled by the expression of various proteins including E-Cadherin, which are associated with cell-cell contact [281]. E-cadherin is a transmembrane protein required for the establishment and maintenance of adherens junctions and is fundamental to the epithelial phenotype [282-284]. The suppression of E-cadherin diminishes epithelial cell adhesion, alters polarity and is associated with increased cell invasiveness during cancer metastasis [285]. Following E-cadherin suppression, the mesenchymal cell-cell adhesion molecule N-cadherin becomes highly expressed, a process also known as the ‘cadherin switch’ [284].

In addition to E-cadherin, the modulation of many other components has been reported during EMT [286]. These alterations include downregulation of epithelial adhesion molecules (e.g., claudins, occludin and the ZO-family) and upregulation of mesenchymal molecules (e.g., vimentin, fibronectin and α5β1 integrin) [287]. Additionally, during EMT cytokeratin networks are replaced by vimentin-rich intermediate filaments, leading to enhanced cell migration and invasiveness [288].
1.5.2.3 Signaling pathways and inducers of EMT

The induction of EMT can occur via a complex array of extracellular signaling components, cytoplasmic molecules and nuclear effectors of transcription [289]. The EMT process may be initiated by growth factors such as fibroblast growth factor (FGF), epidermal growth factor (EGF), TGF-β, TNF-α, and bone morphogenic protein (BMP) [270, 290, 291]. These initiating events can activate signaling pathways of cytoplasmic effectors including SMADs, glycogen synthase kinase 3 β (GSK-3β), mitogen-activated protein kinase (MAPK), and reactive oxygen species leading to activation of transcriptional regulators [289]. It is understood that once expressed and activated, EMT-inducing transcription factors, notably, smad interacting protein (SIP-1), Lymphoid enhancer factor (LEF), Snail, Slug, Zinc finger E-box binding homeobox 1 (ZEB1), Twist, Goosecoid, and Forkhead box protein C2 (FOXC2) can act pleiotropically to choreograph the complex EMT program [276, 289, 292].

In addition to extracellular signaling components, the transfection of oncogenes into epithelial cells can also induce EMT [293]. Ras is a small GTPase that is involved in many signaling pathways that regulate cellular processes including cell proliferation, differentiation, and cell motility [294]. Active Ras-GTP initiates a MAPK phosphorylation cascade that facilitates the altered expression of transcription factors including Jun, Fos and Slug, which can induce EMT [282]. Additionally, it was found that oncogenic Ras signaling downregulated Rac activity and upregulated Rho activity, leading to rearrangement of the actin cytoskeleton during EMT [295]. Further, the Ras effector pathway, Raf/MAPK, was shown to transform Madin-Darby canine kidney (MDCK) cells towards a mesenchymal phenotype [296]. Examination of MDCK cells following transformation with oncogenic H-RasV12 demonstrated a loss of polarity, detachment from the substratum, and multilayer growth [297].
1.5.2.4 Madin-Darby canine kidney (MDCK) and 21D1 cell lines

The MDCK cell line was derived from the kidney of a normal male cocker spaniel in 1958 [298] and is one of the most-widely used and best characterized epithelial cell lines available [299]. MDCK cells appear round and resemble cobble-stone like structures. They grow as a polarized monolayer, with strong cell-cell contact and show expression of epithelial cell junction markers E-cadherin and ZO-1 [250].

MDCK cells and oncogenic H-Ras-transformed MDCK cells, termed 21D1 cells, are together recognized as an epithelial-mesenchymal cell model [250]. 21D1 cells were generated by transfecting pcDNA3 containing v-Ha-Ras under the control of a CMV promoter and a SV40-driven neomycin resistance plasmid gene into MDCK cells [250]. In this study, 21D1 cells were shown to have reduced cell-cell contact and display spindle shaped morphology following transformation [250] (Figure 1-10). Confocal microscopy images of mesenchymal 21D1 cells showed reduced expression of E-cadherin and ZO-1 between cell junctions, and increased expression of the mesenchymal marker vimentin. Wound healing and migration assays also revealed that 21D1 cells have enhanced motility and invasive capabilities when compared to MDCK cells [300].

1.5.2.5 Proteomic analysis of the secretome from MDCK cells following EMT

Using the EMT model previously described, the secretome of MDCK cells following Ras-induced EMT was analyzed in the Simpson laboratory to identify differentially expressed secreted proteins which may influence tumor cell state and invasive potential [250, 300]. Using a semi-quantitative label-free spectral counting method, common constituents of the basement membrane including laminins and collagens were downregulated, indicating ECM remodeling following EMT. Several proteases such as MMP-1 and kallikrein 6 were identified to be highly upregulated after EMT (Appendix A5). The precise function of MMP-1 in EMT was further studied by an MMP-1 siRNA knock-down assay in 21D1 cells which led to reduced cell migration. Collectively, it is hypothesized that secreted effectors
Figure 1-10. MDCK and H-Ras-transformed MDCK cells (21D1) morphology

(A) MDCK cells grow as round cobblestone-like sheets. (B) 21D1 cells exhibit elongated and spindle shaped cell morphology.
might co-ordinate cell migration and EMT in a hierarchical manner [300].

1.5.2.6 Exosomes and EMT

While the direct involvement of exosomes in the process of EMT has not yet been studied, pioneering work investigating exosome-like vesicles following EMT has been undertaken [301]. It was found that A431 epithelial cancer cells adopt mesenchymal-like features upon activation of epidermal growth factor receptor (EGFR) and repression of E-cadherin. Exosomes were isolated from these mesenchymal-like cells and shown to contain increased levels of tissue factor (TF) that caused pro-coagulant conversion of endothelial cells. This indicated that cells with mesenchymal-like features release vesicles capable of altering cell interactions within the vasculature [301]. Further, there are a significant number of studies identifying that exosomes are able to transfer ‘oncogenic’ material to assist cancer progression (see section 1.4.8) [196]. Although exosomes are identified to be released from mesenchymal cells [301], a detailed proteomic characterization has not yet been performed. It is therefore hypothesized that an in-depth proteomic analysis of exosomes isolated from MDCK and 21D1 cell lines may define extracellular modulators contained in exosomes and lead to a better understanding of their functional roles.

1.6 Thesis aims

Exosomes are 40-100 nm diameter membrane-derived nanovesicles of endocytic origin that are released by most cell types. They are reported to have pleiotropic biological roles including involvement in cell-cell communication. The majority of functional studies performed on exosomes to date have involved heterogeneous or crude vesicle populations. It is anticipated that by developing robust exosome purification strategies, and studying precise protein profiles in homogeneous exosome populations, valuable biological insights will be gained.
The overarching aim of this thesis is to undertake in-depth proteomic analyses on highly-purified exosomes in order to gain insights into the role of exosomes in cancer biology and the epithelial-mesenchymal transition process.

The specific aims of this thesis are:

1. *To evaluate exosome purification strategies*

Our capacity to gain meaningful biochemical insights from exosomes is impeded by the fundamental difficulty in isolating exosomes to homogeneity. Chapter 2 describes a comparative evaluation of three widely-used methods for isolating exosomes: differential ultracentrifugation, OptiPrep™ density gradient centrifugation and immunoaffinity capture using EpCAM monoclonal antibody bound to magnetic beads. In this study the human colorectal cancer cell line LIM1863 [251] was used as a model. In order to compare proteome profiles between the three isolation methods, GeLC-MS/MS and label-free MS spectral counting was employed. Following isolation of exosomes, sample homogeneity was based on the expression of defined stereotypical exosomal proteins reported in the literature.


2. *To analyze exosome sub-populations released from LIM1863 cells*

The colon cancer cell line LIM1863 [251] grows as crypt-like ‘organoid’ structures with highly-polarized columnar and goblet cells organized around a central lumen. Chapter 3 describes a strategy that involves the sequential use of two immunoaffinity target reagents
(anti-A33 antibody and anti-EpCAM antibody coated magnetic beads) for isolating different sub-populations of exosomes. This study was directed at characterizing protein cargo contained in apical- (e.g., positive for epithelial cell marker EpCAM) and basolateral- (e.g., positive for basolateral marker A33) derived exosome populations. A proteomic analysis of sMVs was also undertaken to assess whether these vesicles differ from exosomes.

This aim is addressed in Chapter 3 and incorporates - Tauro, B. J., Greening, D. W., Mathias, R. A., Mathivanan, S., Ji, H., and Simpson, R. J. (2013) Two distinct populations of exosomes are released from LIM1863 colon carcinoma cell-derived organoids. *Molecular & Cellular Proteomics* 12, 587-598.

3. To analyze exosomes following epithelial-mesenchymal transition

While it is becoming increasingly evident that epithelial-mesenchymal transition (EMT) plays a critical role in cancer cell plasticity [277], it is not known whether exosomes contribute to the EMT process. Chapter 4 describes the in-depth proteome analysis of exosomes released from Madin-Darby canine kidney (MDCK) cells and upon oncogenic H-Ras induced EMT (21D1 cells). This study identified exosomal proteins that were significantly dysregulated following oncogenic H-Ras induced EMT. This finding indicates potential functions of exosomes associated with the EMT process.

Chapter 2: Comparison of methods for isolating exosomes

2.1 Overview

The secretome, a rich complex subset of macromolecules secreted by a cell, is a vital aspect of cell-cell communication in eukaryotes [9]. There has been much interest in the secretome from cancer cells due to the recognition that secreted molecules could spawn new therapies [9, 13, 14, 302]. In addition to soluble proteins, the secretome also contains a multitude of extracellular nano-membraneous vesicles (EVs) [303] (see section 1.2); of these, 40-100 nm diameter exosomes have been the most widely studied from a functional perspective. However, one of the setbacks in the exosome field is that most functional studies have been conducted using impure preparations, typically containing a mixture of EVs. Since this situation confounds the interpretation of such studies there is a fundamental need to purify exosomes to homogeneity. For these reasons, I have performed, and describe in this chapter, a detailed comparison of typical exosome isolation methods currently used in the field. Due to the scope of this chapter and instrument limitations, we were unable to include HPLC purification of microparticles in this comparison. Size-exclusion HPLC has been successfully employed for the large-scale production of cardioprotective microparticles from human fetal mesenchymal stem cells for clinical purposes [45, 46], and for isolating two distinct exosome populations from human saliva [47].

Commonly used exosome isolation methods include: (i) differential ultracentrifugation to ‘pellet’ exosomes, (ii) density-gradient centrifugation to fractionate exosomes from other EVs by exploiting their buoyant density, and (iii) immunoaffinity capture on magnetic beads using antibodies directed towards known exosomal membrane proteins (see section 1.4.11) [35].
Differential centrifugation utilizes multiple centrifugation and filtration stages to separate organelles [304], intracellular vesicles [305], and exosomes [50] based on differences in density. Typically, this method involves serial low speed centrifugation (500 × g, 2000 × g) to remove cells and cellular debris, followed by 0.1 µm [306] or 0.22 µm [50] membrane filtration, and ultracentrifugation between 60-100,000 × g to pellet exosomes [307]. Due to its limited resolving ability, the differential ultracentrifugation procedure can result in exosome preparations being significantly contaminated by other classes of EVs, protein oligomers and pathogens (e.g., adenoviruses, prions etc) [7]. For example, it has been shown that shed microvesicles (sMVs, also referred to as oncosomes) and exosomes are morphologically distinct, the former being heterogeneous in size (100 nm-1000 nm) and sedimenting at lower speeds relative to exosomes [308]. Thus it is likely that EVs that sediment at 100,000 × g may contain a mixture of sMVs and exosomes.

The biogenesis of sMVs and exosomes are quite distinct. sMVs are formed by outward budding of the plasma membrane, however, it should be noted that not all plasma membrane proteins are incorporated into shed microvesicles, yet the topology of the membrane remains intact. Oncogenic membrane proteins and other growth factor receptor proteins, integrin receptors and soluble proteins such as proteases, cytokines and nucleic acids are found in microvesicles, however, it is not clear how specific cargo proteins are trafficked to microvesicles [49]. In contrast, exosomes are formed by endocytic invagination of late endosomes to form multivesicular bodies (MVBs) which then traffic to, and fuse with, the plasma membrane, releasing their cargo of exosomes (see section 1.4.1 – 1.4.2). Like sMVs, exosomes contain bioactive molecules such as oncogenic proteins, mRNAs and miRNAs that can be horizontally transferred to recipient cells and are thought to be implicated in tumor invasion and metastasis [165]. Whether biological differences exist between sMVs and exosomes remains to be determined. For these reasons it is important to purify exosomes to homogeneity (free of sMVs) in order to understand their biological function [49, 308].

Isolation of exosomes by density gradient centrifugation involves their fractionation across a continuous or discontinuous density gradient, i.e., sucrose or iodixanol (OptiPrepTM).
Exosomes can be purified by floatation on linear sucrose gradients (2.0-0.25 M) at densities of 1.08-1.22 g/cm³ [35, 242]. However, sucrose gradients have been shown to be inefficient in separating exosomes and HIV-1 particles due to their similarity in size and buoyant densities [245]. Recently, OptiPrep™ gradients have been shown to be an alternative to sucrose gradients due to their ability to separate exosomes from HIV-1 particles [243]. Interestingly, iodixanol (sold under the FDA approved Visipaque™ UCM198414) is commonly used as a radiopaque contrast agent for intravascular use to diagnose coronary angiopathy, brain, blood vessel and kidney disorders. For this reason, OptiPrep™ prepared exosomes are compatible with in vivo functional studies.

Imunoaffinity capture can be used to isolate exosomes from a variety of cellular sources (Table 1-1), however, this method is dependent on the availability of monoclonal antibodies directed towards tissue-specific exosomal membrane markers [44]. For example, immunoaffinity capture has been used to isolate exosomes containing A33 from epithelial colorectal cancer cells [44], HER2-tumor exosomes from breast adenocarcinoma cell lines [37] and ascites of ovarian cancer patients [37], Rab5b-positive exosomes from melanoma cell lines [38], CD45-positive exosomes from Jurkat T cells [39], MHC class II-exosomes from antigen presenting cells [40], CD63-positive exosome-like vesicles from blood [41], and EpCAM-positive exosomes from human sera of lung cancer [42] and ovarian cancer patients [43].

In this chapter, label-free MS spectral counting [8, 134] was used to quantitate the relative levels of proteins in exosomes prepared by the three commonly-used exosomal isolation strategies described above. Using the LIM1863 colorectal carcinoma cell line as a model, exosomes were isolated from concentrated culture medium by differential ultracentrifugation (UC-Exos), OptiPrep™ density gradient centrifugation (DG-Exos), and EpCAM-magnetic microbead immunoaffinity capture (IAC-Exos). This study revealed that stereotypical exosome markers Alix, TSG101, and HSP70 were significantly enriched in the IAC-Exos dataset when compared with differential centrifugation and density gradient centrifugation (OptiPrep™) prepared samples. Detailed examination of the IAC-Exos dataset also revealed the enrichment of other ESCRT complex subunit proteins involved in
exosome biogenesis, and Rab proteins involved in exosome trafficking and release. Taken together, the findings from this study indicate that EpCAM-based immunoaffinity capture is more effective than density gradient centrifugation, which in turn is more effective than differential centrifugation, for enriching exosomes.

For IAC to be more generally applicable there is a need, first, to identify tissue- and cell-type specific membrane proteins and, secondly, to generate mAbs directed to such proteins that allow for the selective capture of exosomes using mAb-loaded magnetic beads. In addition, there is a need to fully evaluate the effectiveness of different immunoaffinity capture beads, since in my studies (used in Chapter 3), the background contaminants associated with Protein G Dynabeads® and magnetic assisted cell sorting (MACS) microbeads varied significantly. Further studies need to be performed to discriminate whether this is due to mAb isotype or the chemistry of the magnetic beads employed. Advances in the identification of tissue-specific exosome surface proteins, the development of corresponding mAbs directed toward these proteins, and improvement of magnetic bead chemistry will lead to better understanding of exosome biochemistry and may also allow them to be exploited for clinical use (e.g., biomarker diagnostic), vaccines [309], and delivery vehicles for therapeutic cargo [215].

Chapter 3: Use of sequential immunoaffinity capture to isolate and characterize two distinct populations of exosomes released from LIM1863 colon carcinoma cells

3.1 Overview

To date, the content and function of exosomes released from highly-polarized epithelial cells has received limited attention. In polarized cells, the apical and basolateral surfaces are separated by adherens and tight junctions [310], and endosomes are used to recycle apical and basolateral proteins to their respective plasma membrane surfaces in order to maintain cell polarity. It has been reported that apical and basolateral exosomes, each containing different cargos, may traffic to and be released from apical and basolateral plasma membranes, respectively [110]. Van Neil and colleagues have hypothesized that basolateral exosomes and their cargo may be able to cross the basement membrane and convey immune information to non-contiguous immune cells [72]. To test this hypothesis, polarized T84 colorectal cancer cells were grown as a monolayer on microporous filters to allow for apical and basolateral exosome collection. This group subsequently performed a limited proteomic analysis of exosomes to identify 31 proteins. A key finding was the differential protein expression of apical trafficking molecule syntaxin 3 in apical exosomes and basolateral transmembrane protein A33 in basolateral exosomes [72].

To further investigate apical and basolateral trafficking of exosomes in the context of colon cancer biology, I performed an in-depth proteomic analysis of these exosome sub-populations from a polarized colon cancer cell line. Having established in Chapter 2 that immunoaffinity capture (IAC) is the preferred method to enrich exosomes from cell culture medium, I used this approach to isolate highly-purified apical and basolateral exosomes using the human colon carcinoma cell line LIM1863 as an experimental model (see section 1.5.1.3). I designed a sequential immunoaffinity method utilizing magnetic beads coated
with mAbs directed towards A33 antigen, a definitive marker of basolateral surfaces in intestinal epithelial cells [154-156], followed by anti-EpCAM loaded magnetic beads, a ubiquitously expressed epithelial cancer marker [311]. This strategy allowed for the isolation of two sub-populations of exosomes, A33-Exos and EpCAM-Exos. Proteomic-profiling revealed the unique identification in EpCAM-Exos of classical apical trafficking molecules, apical intestinal enzymes and the apically-restricted membrane proteins. By comparison, A33-Exos were selectively enriched with basolateral trafficking molecules, such as early endosome antigen 1 (EEA1), ADP-ribosylation factor (ARF1) and clathrin. Intriguingly, several members of the MHC class I family of antigen presentation molecules were exclusively observed in A33-Exos, while neither MHC class I or II molecules were observed in EpCAM-Exos. Findings from this study also showed the co-localization of EpCAM, claudin-7 and CD44 in EpCAM-Exos. When complexed together, but not alone, these proteins promote tumor progression [312]. Further investigation is required to ascertain whether EpCAM/claudin-7/CD44 do in fact complex together in this study, and whether this complex confers tumor promoting activity in recipient cells.

The findings in this chapter also shed light on the membrane composition of A33- and EpCAM-Exos. For example, a total of 119 transmembrane-containing proteins were identified, of which 13 have been previously implicated in colon cancer (e.g., ADAM10 and tumor-associated calcium signal transducer 2). An interesting finding was the expression of three cell surface proteins specifically localized on normal colon epithelium, MUC13, TSPAN8 and A33, as indicated following examination of the Human Protein Atlas (http://www.proteinatlas.org/). Although these three membrane proteins are specifically expressed in normal colon epithelium, they are over-expressed in many other cancer tissues. Ongoing studies are required to identify specific membrane proteins in colon cancer cell-derived exosomes that will morphologically distinguish these exosomes in blood and other body fluids from exosomes generated from other tissue/cell types. In addition, two other integral membrane proteins (CD46 and CD59) identified in EpCAM-Exos, but not A33-Exos, may have the potential to increase the half-life of these exosomes by protecting them from complement mediated lysis. CD59 prevents activation of the membrane attack complex by inhibition of C9 incorporation in C5b-9 [313], whereas CD46
has cofactor activity for inactivation of complement components C3b and C46 by serum factor 1, which protects cells from damage by the complement system [314]. Further, EpCAM-Exos also uniquely expressed high-mobility group box proteins HMGB2 and HMGB3 which act as sensors to distinct types of nucleic acid and activate innate immune responses [315]. Studies aimed at determining the functional significance of apical and basolateral exosomes in colon cancer biology were limited due to time constraints.

In order to eliminate the possibility that A33- and EpCAM-Exos were actually shed microvesicles (sMVs) (see section 1.3.1), I isolated sMVs and examined their protein content. This analysis identified 1392 proteins, of which 426 were unique to sMVs. This showed that A33- and EpCAM-Exos were clearly distinguishable from sMVs. A key finding was the enrichment of ATP binding cassette transporter proteins, which suggests that sMVs may be implicated in multidrug resistance [316, 317]. Ongoing studies are underway in the Simpson laboratory to establish whether LIM1863-derived sMVs are capable of transferring resistance to drug sensitive colon cancer cells.

Following is the manuscript: Tauro, B. J., Greening, D. W., Mathias, R. A., Mathivanan, S., Ji, H., and Simpson, R. J. (2013) Two distinct populations of exosomes are released from LIM1863 colon carcinoma cell-derived organoids. Molecular & Cellular Proteomics 12, 587-598. Supplemental Information located in Appendix A-6 and online at http://www.mcponline.org/content/suppl/2013/07/11/M112.021303.DC1.
Chapter 4: Characterization of Madin-Darby canine kidney (MDCK) cell-derived exosomal proteins following oncogenic H-Ras induced epithelial-mesenchymal transition

4.1 Overview

Epithelial-mesenchymal transition (EMT) occurs during embryonic morphogenesis and has recently been implicated in cancer invasion and metastasis [277, 318-320]. In previous studies, Mathias and colleagues in the Simpson laboratory examined the components of the secretome in EMT using oncogenic H-Ras transformed Madin-Darby canine kidney (MDCK) cells as a model (see sections 1.5.2.4 and 1.5.2.5). These studies, which focused on the proteomic profiles of soluble-secreted proteins, revealed that following EMT, secreted components mediating cell-cell and cell-matrix adhesion (e.g., collagen IV, XVII, and laminin 5) were decreased in abundance, with the concordant upregulation of proteases and ECM constituents promoting cell motility and invasion (e.g., MMP-1, TIMP-1 kallikrein-6, -7) [250, 300].

In this chapter I extended the secretome studies of Mathias and colleagues [250, 300] by examining the contribution of exosomes in oncogenic H-Ras-induced EMT in MDCK cells. As this was the first study of the role of exosomes in EMT using the MDCK model, and given that there are no known specific MDCK-derived exosomal membrane marker proteins, immunoaffinity capture isolation was not a viable purification option. Instead I employed density gradient centrifugation using iodixanol (OptiPrep™) described in Chapter 2. Proteomic profiling of MDCK-derived exosomes (MDCK-Exos) and oncogenic H-Ras transformed MDCK-derived exosomes (21D1-Exos) was performed using GeLC-MS/MS as described in Chapter 2 and 3.

Findings from this study showed that purified MDCK- and 21D1-Exos were morphologically similar by cryo-electron microscopy and contained stereotypical exosome
marker proteins such as TSG101, Alix and CD63. Typical cellular EMT hallmark protein changes are also mirrored in MDCK and 21D1-Exos, including decreased expression of E-Cadherin and increased expression of vimentin. This study also revealed that 21D1-Exos are enriched with several metalloproteinases (e.g., MMP-1, -14, -19, ADAM10, ADAMTS1), and integrins (e.g., ITGB1, ITGA3, and ITGA6) that have been recently implicated in regulating the tumor microenvironment to promote metastatic progression [321-323]. A significant finding of this study was the observation that upon EMT, mesenchymal 21D1-Exos are extensively reprogrammed to be enriched with key transcriptional regulators (e.g., master transcriptional regulator YBX1) and core splicing complex components (e.g., SF3B1, SF3B3, and SFRS1). These findings suggest that Ras-transformed MDCK cell-derived exosomes may be reprogrammed with factors capable of inducing EMT in recipient cells. In this regard, preliminary experiments showed that 21D1-Exos were capable of maintaining the mesenchymal morphology of 21D1 cells. Further experiments are required to reveal whether 21D1-Exos are capable of inducing EMT in other recipient cell types, and whether over-expression of YBX1 alone in epithelial cells, or in conjunction with soluble secreted proteins, is capable of inducing tumorigenesis in ‘normal’ epithelial cells.

Chapter 5: Summary and future directions

5.1 Evaluation of exosome isolation methods and the importance of purity

As microvesicles, 100-1000 nm in diameter, and exosomes, 40-100 nm in diameter, are morphologically distinct and may have different biological functions, there is a pressing need to evaluate methods for obtaining homogeneous vesicle preparations in order to characterise their bioactive content (e.g., proteins, lipids and RNA species). This is particularly important when considering exosomes for use as clinical agents in therapeutics (see section 1.4.9). A clear understanding of all exosome components may also lead to the design of exosome mimetics for personalized medicine [229]. Recently, as circulating exosomes have been identified to contain disease-specific miRNA signatures, there is also evidence for the use of exosomes as diagnostic biomarkers [241]. Exosomes also display the capabilities to be therapeutic agents, these include immune modulation, tissue repair, and drug delivery due to their biocompatibility, immunological inertness, and ability to cross the blood brain barrier [209, 210]. Before the full potential of exosomes use in drug delivery can be realized, large-scale production protocols must be standardized [209].

Currently, detailed biochemical and functional analyses of exosomes are confounded by technical difficulties in their isolation from cell culture medium and body fluids. Without stringent purification, exosome samples are typically contaminated with other membranous vesicles including shed plasma membrane vesicles (e.g., microparticles and oncosomes) and apoptotic blebs. As these other secreted vesicles share similar physiochemical characteristics (size, buoyant density) to exosomes, Chapter 2 compared the effectiveness of three widely-used isolation strategies (ultracentrifugation, density gradient separation and immunoaffinity capture) to obtain exosomes from colorectal cancer cells. Further, an in-depth proteomic analysis was performed to establish the purity of secreted exosomes. The findings from this chapter showed that immunoaffinity capture (IAC) using anti-
EpCAM antibody was the optimum method for purifying exosomes. In this study the assessment of exosome sample homogeneity was based upon selective enrichment of proteins involved in exosome biogenesis, trafficking, and release. To further this research, investigation into the isolation of exosomes from other cell-derived sources and biofluids (e.g., blood, urine, and malignant ascites) needs to be performed to validate the compatibility of the IAC approach.

Further, it is noted that the potential for exosomes to act as a blood-based biomarker diagnostic source (e.g., using specific disease miRNA signatures) is confounded by the challenge in isolating exosomes that are characteristic of specific disease cell types, such as cancer. Such cancer-derived exosomes may be in low abundance when compared to the general population of exosomes and other microvesicles in circulation. Additionally, because these disease specific miRNA signatures may be masked by analyzing low abundance disease-derived exosomes in blood, there is a need to isolate highly pure exosomes (e.g., using IAC) for diagnostic purposes. In future studies it is therefore essential to identify tissue-specific membrane protein markers for IAC isolation of disease-related exosomes.

It remains to be seen whether a large-scale IAC approach will emerge as an alternative to the filtration-based/size-exclusion HPLC approach currently used for preparing clinical grade exosomes for regenerative medical purposes [45, 221]. Needless to say, such evaluation of exosome purification methods is required, as pre-clinical and clinical trials (http://www.australianclinicaltrials.gov.au/) require large-scale cost-effective standardized protocols for the production of GMP exosomes.

5.2 Analysis of two exosome sub-populations released from LIM1863 cells

In Chapter 3, a sequential immunoaffinity capture strategy using magnetic beads coupled with antibodies towards A33 and EpCAM was developed to isolate exosome sub-populations released from apical and basolateral surfaces of polarized human colon
carcinoma cells. An in-depth proteomic characterization of exosome sub-populations revealed distinct apical and basolateral trafficking proteins, consistent with release of exosomes from the respective surfaces of polarized epithelial cells. Proteomic analysis revealed 684 proteins were common to both exosomal datasets, while 340 and 214 proteins were unique to A33- and EpCAM-Exos, respectively. Interestingly, the key components of a metastasis-promoting complex, EpCAM/claudin-7/CD44, were uniquely identified in apically-derived exosomes, while MHC-I components were unique to basolaterally-derived exosomes.

The finding of selectively enriched proteins in apical and basolateral exosomes raises questions into the functional roles of these two distinct exosome populations, especially in the context of tumor biology. To examine their biological function, studies investigating the abilities of the metastasis-promoting complex, EpCAM/claudin-7/CD44, may be performed. Although all three components were uniquely identified in EpCAM-Exos, it could not be claimed that they form a functional complex within exosomes. Further studies are required to determine whether exosomes contain active signaling complexes involving oncogenic proteins and, if so, whether such complexes may remain intact upon exosome uptake in recipient cells and contribute to functional signaling pathways in target cells. Ongoing efforts using Blue-Native PAGE and co-immunoprecipitation are underway in the Simpson laboratory to assess the presence of this protein complex and functional validation of its constituents. Following identification of this complex, experiments involving its modification (e.g., the knock-down of one or more of the components from the originating cell line) could be performed. To assess the ability of the complex to act as a metastasis promoter, experiments involving the application of exosomes, with or without the intact complex, to recipient cells may be performed. Functional readouts assessing cell proliferation, migration, invasion or resistance to apoptosis may indicate the hypothesized metastatic effect of exosomes on recipient cells.

Interestingly, the presence of MHC-I components were only observed in A33-Exos. Further experiments are required to establish whether these components also form a functional complex in exosomes. It would be exciting to determine whether the MHC complexes carry
peptides, or are capable of carrying peptides, that can elicit a stimulatory or inhibitory immune cell response.

To further differentiate apical and basolateral exosomes and understand their biological functions, characterization of their RNA content (miRNA and mRNA) may be investigated. The first report of exosome-mediated transfer of exogenous nucleic acids was published in 2010, when it was shown that monocytic miR-150 was functionally delivered to endothelial cells [237]. Since then, numerous other studies demonstrating the RNA transporting capabilities of exosomes have been reported [171, 173, 324, 325]. Although at an early stage, these findings collectively illustrate the potential of exosomes for therapeutic delivery of nucleic acids – for reviews see El-Andaloussi et al., Kooijmans et al., and Rak et al. [196, 210, 229].

In addition to the characterization of apical and basolateral-derived exosomes, shed microvesicles (sMVs) were also isolated using differential centrifugation (10,000 × g) to differentiate these vesicles from exosomes. A proteomic characterization revealed 1392 proteins in sMVs with 462 proteins unique to these vesicles. This characterization revealed another distinct vesicle population and therefore, the potential of different functional capabilities to that of exosomes. One intriguing finding was the enrichment of ATP binding cassette transporter proteins, which suggests that sMVs may be involved in multidrug resistance [316, 317]. Functional studies are planned to establish whether LIM1863-derived sMVs are capable of transferring resistance to drug sensitive colon cancer cells. Further investigation into the isolation of sMVs is also being performed in the Simpson laboratory to identify if sup-populations of sMVs exist within their large size range of 100-1000 nm diameter.

5.3 Analysis of exosomes following epithelial-mesenchymal transition (EMT)

In Chapter 4 of this thesis, I investigated whether exosomes are modified during the EMT process. I studied the EMT process using the epithelial MDCK cell line and its oncogenic
H-Ras-transformed mesenchymal variant (21D1) as an experimental model. Exosomes released into the culture media from both cell lines were isolated by density gradient centrifugation (as described in Chapter 2) and their protein content characterized by MS-based profiling, ultimately to gain insight into any functional roles of exosomes in EMT. Proteomic data revealed profound changes in 21D1 cell-derived exosomes. Foremost, these studies revealed that cellular hallmarks typically identified in the epithelial-mesenchymal transition (e.g., downregulation of E-cadherin and upregulation of vimentin in mesenchymal cells) were mirrored in exosomes. These studies also showed that oncogenic H-Ras reprograms MDCK cell-derived exosomal proteins. For example, mesenchymal exosomes were enriched with several proteases (e.g., MMP-1, -14, -19, ADAM10 and ADAMTS1) and integrins (e.g., ITGB1, ITGA3 and ITGA6) which are implicated in promoting metastasis. A key finding in mesenchymal exosomes was the unique expression of numerous splicing factors (e.g., SF3B1, SF3B3, and SFRS1) and master transcriptional regulator, YBX1. Whether these proteins are functional in recipient cells remains to be investigated.

It is interesting to speculate that exosomes from 21D1 cells may induce a mesenchymal phenotype in recipient cells. If so, it would be important to identify the specific cargo (e.g., proteins, transcription factors or RNAs) that can cause such a transformation. To assess this, initial experiments involving the addition of exosomes to recipient cells are planned to be performed to assess changes in proliferation, invasion and migration abilities. Following an observed morphological change, identification of the specific factors contributing to these effects could be undertaken. For example, using an RNAi based system to create stable knock-down cell lines; the expression of selected target genes that govern key cellular processes may be altered in 21D1 cells. For these studies, target gene selection will be based on proteomic data identified in 21D1-Exos. For example, highly upregulated protein and inducer of EMT (e.g., transcription factor YBX1), or characteristic proteins of the mesenchymal phenotype (e.g., MMP-1) will be selected first for generating knock-down lines from 21D1 cells. Following gene knock-down, exosomes will be isolated and characterized to assess that the corresponding protein expression is also reduced. Exosomes isolated from knock-down and wild-type cell lines will then be applied to recipient cells to
identify the contribution of these modified constituents (i.e., YBX1 and MMP-1) in exosome functionality and recipient cell modulation.

Additionally, the characterization of exosomal miRNA may also allow functional insights into epithelial and mesenchymal cell-derived exosomes. Rana and colleagues have recently demonstrated that miRNA contained within exosomes from metastatic rat adenocarcinoma cells can alter mRNA transcription of proteases, adhesion molecules, chemokine ligands, cell cycle- and angiogenesis-promoting genes, and genes engaged in oxidative stress responses [173]. It is hypothesized that similarly to the proteomic analysis of exosomes, miRNA content may also be dysregulated following EMT and therefore cause different mRNA transcription in target cells which may result in altered biological functions. To further support this theory, it has been demonstrated that exosomes can also deliver functional miRNA to dysregulate mRNA in dendritic cells [171, 324]. Additionally it is reported that mRNA and miRNA within melanoma exosomes may be actively transported into normal melanocytes enabling increased invasive properties of these cells [325]. Based on these recent observations, it would be exciting to examine the RNA species that are contained within exosomes derived from MDCK to 21D1 cells. This may allow the identification of bioactive molecules (RNAs) that have functional capabilities associated with the EMT process.

5.4 Conclusion

Exosomes are important extracellular conveyors of information between cells. This may occur through the transmission of bioactive molecules such as oncogenic proteins, tumor suppressors, transcription factors, bioactive lipids and genetic information such as mRNAs/miRNAs that alter the phenotypic and biological function of target cells. One of the key obstacles in ascribing any biochemical or functional characteristics to exosomes is that of sample homogeneity. To date, the majority of functional exosome studies reported in the literature have been performed on impure samples that are significantly contaminated with other EV types such as microvesicles and possibly apoptotic bodies.
In this thesis I carefully evaluated three commonly-used exosome purification protocols (differential ultracentrifugation, density gradient centrifugation and immunoaffinity capture) to establish that immunoaffinity capture was the superior method for the enrichment of exosomes. With this knowledge, I then developed a sequential immunoaffinity capture method to isolate and characterize, for the first time, two distinct populations of exosomes from LIM1863 organoids. In a further application, I used density gradient centrifugation (OptiPrep™) to investigate, for the first time, exosomes in oncogenic H-Ras MDCK induced EMT. This study showed that exosomes were significantly reprogrammed during the EMT process, being selectively enriched with the master transcriptional regulator, transcription factor YBX1, and components of protein splicing machinery.

In summary, the findings of this thesis are the first step towards standardizing protocols for the purification of exosomes. Already, the applications of these purification strategies have paved the way for a better understanding of the roles of exosomes in colon cancer biology and their contribution to the oncogenic H-Ras induced EMT process.
References


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Appendix
## Appendix A-1

### Isolation and characterization of cell line-derived exosomes

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Isolation strategies&lt;sup&gt;a)&lt;/sup&gt;</th>
<th>Validation&lt;sup&gt;b)&lt;/sup&gt;</th>
<th>Proteomic strategies</th>
<th>Comments&lt;sup&gt;c)&lt;/sup&gt;</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Haematopoietic cells</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>B cells (RN HLA-DR15&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>DC, DG and Immunobeads (MHC-II)</td>
<td>WB (MHC-II)</td>
<td>1-DE, MALDI-TOF MS and nESI/MS/MS</td>
<td>21 proteins identified including MHC-I and MHC-II, CD45, integrin α4, hsc70, hsp90, Gia2, actin, tubulin, moesin, clathrin, GAPDH, enolase and EF1α</td>
<td>[139]</td>
</tr>
<tr>
<td>B cells (RN HLA-DR15&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>DC, DG</td>
<td>IEM (MHC-II, CD53 and CD82), WB (MHC-II, HLA-DM, CD37, CD63, CD81, CD82 and CD86)</td>
<td>-</td>
<td>Identified proteins CD37, CD53, CD63, CD81, CD82 and CD86</td>
<td>[326]</td>
</tr>
<tr>
<td>Dendritic cells (D1)</td>
<td>DC, F</td>
<td>EM, WB (MHC-II and Lamp2), FACS (MHC-I and II, CD9, Mac-1 and CD86)</td>
<td>1-DE, MALDI-TOF MS and nESI/MS/MS</td>
<td>37 proteins identified including actin, tubulin, cofilin, MFG-E8, annexins, rabs, CD9, hsp90β, TSG101, syntenin, histones, Alix, 14-3-3 proteins, galectin-3, gag, reverse transcriptase/pol and Mac-1 αβ</td>
<td>[50]</td>
</tr>
<tr>
<td>Dendritic cells (MD-DC)</td>
<td>DC, DG</td>
<td>EM, WB</td>
<td>1-DE, MALDI-TOF MS</td>
<td>35 proteins identified including Alix, annexins, ICAM-1 and cofilin</td>
<td>[327]</td>
</tr>
<tr>
<td>Dendritic cells (D1 and BMDC)</td>
<td>F, UC, DG</td>
<td>EM, WB (clathrin, hsc70, annexin-II, CD9, flotillin 1, ICAM-1, MHC I, MHC II, TSG101, and MFG-E8), FACS</td>
<td>1-DE, LC-MS/MS</td>
<td>150 proteins identified including CD9, annexin II, ICAM-1 and TSG101</td>
<td>[141]</td>
</tr>
<tr>
<td>Dendritic cells (D1 and BMDC)</td>
<td>DC, DG</td>
<td>EM, IEM (MHC-II), WB (MHC-II)</td>
<td>1-DE, MALDI-TOF MS</td>
<td>9 proteins identified including CD9, gag, Mac-1, MFG-E8, hsc73 and annexin-II</td>
<td>[223]</td>
</tr>
<tr>
<td>Tumor cells</td>
<td>Methodology</td>
<td>Identification</td>
<td>Identifiable Proteins and Functions</td>
<td></td>
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<td>----------------------------------------------------------------------------</td>
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<tr>
<td><strong>Mast cell (MC/9, HMC-1, BMMC)</strong></td>
<td>DC, F, DG</td>
<td>EM, FACS (CD63)</td>
<td>1-DE, LC-MS/MS 271 proteins identified including mast carboxypeptidase A, tubulins, TCP proteins, ezrin, moesin, 40S ribosomal proteins, 14-3-3 proteins, CD43, CD63, CD97, annexins, MHC-I, histones, hsc70 and integrin-α6</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mast cell (MC/9 and BMMC) and mastocytoma (P815)</strong></td>
<td>DC</td>
<td>EM, IEM</td>
<td>1-DE, MALDI-TOF MS, ELISA 7 proteins identified including MHC-II, CD40, CD40L, CD86, LFA-1, ICAM-1, CD13, annexin-VI, actins and CDC25</td>
<td></td>
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<tr>
<td><strong>T cells (Jurkat cells, T cell blasts, E+ cells and MART-1+ T cell)</strong></td>
<td>F, UC</td>
<td>EM, IEM (TCR β and CD3ε), WB (TCR β and CD3ε), FACS (CD63, TCR β, CD3ε, MHC-I and -II)</td>
<td>WB, FACS Proteins identified including TCR β, CD3ε and ζ (phosphorylated ζ), MHC-I and -II, CD2, CD18, chemokine receptor CXCR4, c-Cbl, tyrosine kinases Fyn and Lck</td>
<td></td>
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<tr>
<td><strong>Brain tumor (EGFRvIII-transfected SMA560)</strong></td>
<td>DC, DG</td>
<td>EM, WB (Alix, GAPDH, α1-anti-trypsin, CD9, PDI, CRT, transferrin, GPNMB, TGF-β1 and EGFRvIII), acetylcholinesterase assay</td>
<td>2-DE, MALDI-TOF/TOF 36 proteins identified including HSP70, α-enolase, MVP, ARRDC2, CENP-P, GAPDH, syntenin, PCNA, CRMP-2 and sialic acid synthase</td>
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<tr>
<td><strong>Breast adenocarcinoma (BT-474 and MDA-MB-231)</strong></td>
<td>DC, F, DG</td>
<td>EM, FACS (HER2), WB (HER2 and actin)</td>
<td>WB, FACS Protein HER2 identified</td>
<td></td>
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<tr>
<td><strong>Colorectal cancer (HT29)</strong></td>
<td>DC, diafiltration (100K), DG</td>
<td>EM, WB (CD63 and CD81)</td>
<td>1-DE, LC-MS/MS 547 proteins identified including annexins, ARFs, rabs, ADAM10, CD44, NG2, ephrin-B1, MIF, β-catenin, junction plakoglobin, galectin-4, RACK1 and tetraspanin-8</td>
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<tr>
<td>Tissue Type</td>
<td>Methodology</td>
<td>Identified Proteins</td>
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<tr>
<td>Colon carcinoma cell lines (SW403, 1869col and CRC28462)</td>
<td>DC, EM, IEM (CD63, FasL and TRAIL), WB and FACS (CD63, FasL, TRAIL, CEA and MHC-I)</td>
<td>WB, FasL and TRAIL identified</td>
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<tr>
<td>Colorectal cancer (LIM1215)</td>
<td>F, diafiltration (5K), UC, EM, IEM (A33), WB (CD9, A33, TSG101, Rab7 and hsc70)</td>
<td>1-DE, LC-MS/MS, 400 proteins identified including A33, CEA, EGFR, ADAM10, dipeptidase 1, ephrin-B1, hsc70, tetraspanins, ESCRT proteins, integrins, annexins, rabs and GTPases</td>
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<tr>
<td>Colorectal carcinoma cell lines SW480 and SW620</td>
<td>DG (OptiPrep™), Cryo-EM, WB (Alix, TSG101, CD9, HSP70, Flotillin-1, and EGFR)</td>
<td>1-DE, LC-MS/MS, 941 and 796 proteins identified in SW480 and SW620 exosomes, respectively, including MET, S100A8, S100A9, TNC, EFNB2, EGFR, JAG1, SRC and TNIK</td>
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<tr>
<td>Colorectal carcinoma cell lines SW480 and SW480APC</td>
<td>F, DC, EM, WB (Alix, TSG101, CD9 and HSP70)</td>
<td>1-DE, LC-MS/MS, 563 and 497 proteins were identified in SW480 and SW480APC exosomes respectively including DKK4, Alix, TSG101, VPS28, VPS25, CHMP2A and tetraspanins CD9, CD63, CD81 and CD82</td>
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<tr>
<td>Mammary adenocarcinoma (TS/A, H-24), P815 mastocytoma (H-2a), melanoma (Fon and Mel-888)</td>
<td>DC, DG, EM, WB (hsc70 and MHC-I)</td>
<td>WB, IEM, Proteins MHC-I, hsp70, MART-1 and TRP identified</td>
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<tr>
<td>Melanoma (MeWo and SK-MEL-28)</td>
<td>F, UC, WB (MHC-I, MART-1, Mel-CAM and annexin II), DG</td>
<td>2-DE, MALDI-TOF/TOF MS, 41 proteins identified including Alix, hsp70, Gifβ2, Gita, moesin, GAPDH, malate dehydrogenase, p120 catenin, PGRL, syntaxin-binding protein 1 and 2, septin-2 and WD repeat-containing protein 1</td>
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</tr>
<tr>
<td>Tissue/Cell Type</td>
<td>DC, EQ</td>
<td>EM, IEM, NTA, WB (CD9 and HSP70)</td>
<td>1-DE, MALDI-TOF MS</td>
<td>30 proteins identified including annexins, actins, actinin-4 tubulins, hsc70, hsp90, integrins, fibronectin, GAPDH, MHC-I, PLVAP and DEL-1 [143]</td>
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<tr>
<td>Medulloblastoma cells (D283MED)</td>
<td>F, DG</td>
<td>NTA, WB (CD9 and HSP70)</td>
<td>1-DE, LC-MS/MS</td>
<td>148 proteins identified including PDI, HPX, HNF4A and GPNMB [144]</td>
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<td></td>
<td></td>
<td>acetylcholinesterase assay</td>
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<tr>
<td><strong>Primary and normal cells</strong></td>
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<tr>
<td>Cardiac myocytes (Sprague-Dawly rats)</td>
<td>DC, EQ</td>
<td>EM</td>
<td>1-DE LC-MS/MS</td>
<td>57 proteins identified including tropomyosin-1α, myomesin 2 (M-band), α-crystallin B, cardiac α-actin, GAPDH and long-chain specific acyl-CoA dehydrogenase. [331]</td>
<td></td>
</tr>
<tr>
<td>Cortical neurons (8-day primary culture)</td>
<td>DC, DG</td>
<td>EM, WB (Alix, TSG101 and flotillin)</td>
<td>1-DE, LC-MS/MS</td>
<td>19 proteins identified including GLAST1, brain-specific ceruloplasmin, L1 cell adhesion molecule, GPI-anchored prion protein and GluR2/3 [332]</td>
<td></td>
</tr>
<tr>
<td>Intestinal epithelial (HT29-19A and T84-DRB1*0401/CIITA)</td>
<td>DC, DG</td>
<td>EM, IEM (CD26, CD63, MHC-I and -IIα), WB (MHC-I and -IIα, CD26, CD63, TIR and Na⁺K⁺-ATPase)</td>
<td>1-DE, MALDI-TOF MS</td>
<td>28 proteins identified including syntaxin-3, syntaxin-binding protein 2, EPS8, microsomal dipeptidase in AM and A33, epithelial cell surface antigen and major vault protein [72]</td>
<td></td>
</tr>
<tr>
<td>Microglia (N9 and primary culture from SJL/J mice)</td>
<td>DC, DG</td>
<td>EM, WB (CD9, CD63, syntaxin-8, Rab7, Rab11, clathrin, Lamp-1 and -2, Vti-1A and -1B)</td>
<td>1-DE, LC-MS/MS</td>
<td>59 proteins identified including cathepsin S, aminopeptidase N (CD13), MCT-1, MHC class II-associated chaperone li, CD14, NAP-22, FcR for IgE and GP42 [333]</td>
<td></td>
</tr>
<tr>
<td>Oligodendrocytes (primary culture and Oli-neu)</td>
<td>DC, DG</td>
<td>EM, WB (Alix, PLP, CNP and TSG101)</td>
<td>LC-MS/MS</td>
<td>143 proteins identified including CD81, 14-3-3 proteins, actins, tubulins, histones, EF1 and 2, hsp90, hsc70, Na⁺K⁺ATPase α chains, PLP, CNP, MBP and MOG [334]</td>
<td></td>
</tr>
<tr>
<td>Source/Cell Type</td>
<td>Workflow</td>
<td>Immunostaining</td>
<td>Method</td>
<td>Protein Identified</td>
<td>Notes</td>
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<tr>
<td>Human tracheobronchial epithelial cell</td>
<td>DC, F, DG</td>
<td>EM, WB (Muc1, EB50, CD133, Annexin II, TSG101 and CD63)</td>
<td>1-DE, LC-MS/MS</td>
<td>40 proteins identified including CD63, TSG101, mucins, actins and tubulins</td>
<td>[335]</td>
</tr>
<tr>
<td>Platelets</td>
<td>DC, DG</td>
<td>EM, IEM (CD63), WB (CD63)</td>
<td>WB, FACS</td>
<td>CD63 identified</td>
<td>[24]</td>
</tr>
<tr>
<td>Hepatocytes (MLP-29 and livers from 9-week old Sprague-Dawley rats)</td>
<td>DC, F, DG</td>
<td>EM, WB (TSG101, Alix, integrin-β1, CD63, CD81, ICAM-1 and lactadherin)</td>
<td>1-DE, LC-MS/MS</td>
<td>251 proteins identified including tetraspanins, ASGR, cytochromes P450, cytoskeletal proteins, apolipoprotein-E and -AV, paraoxonase-1 and -3, regucalcin, UDP-glucuronosyltransferases [336]</td>
<td></td>
</tr>
<tr>
<td>Keratinocytes (2-day primary culture from foreskin)</td>
<td>DC, diafiltration (100K), DG</td>
<td>EM, WB (Hsc70 and LAMP2)</td>
<td>WB</td>
<td>14-3-3σ (stratifin) identified</td>
<td>[337]</td>
</tr>
<tr>
<td>Lymphoblasts</td>
<td>F, DC</td>
<td>EM, NTA</td>
<td>LC-MS/MS</td>
<td>489 proteins identified including MHC isoforms, CD4, CD8, ADAM10, CD44, CD98, integrins, ERM proteins and syntenin</td>
<td>[338]</td>
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</table>

**Virus-infected cells**

<table>
<thead>
<tr>
<th>Source/Cell Type</th>
<th>Workflow</th>
<th>Immunostaining</th>
<th>Method</th>
<th>Protein Identified</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rov epithelial and Mov neuroglial cells infected with PrP</td>
<td>DC, DG</td>
<td>IEM (PrP, flotillin, TSG101 and TfR), WB (PrP, flotillin, TfR, TSG101 and hsc70)</td>
<td>1-DE, LC-MS/MS</td>
<td>93 proteins identified including 14-3-3 proteins, annexins, hsc70, integrins, rabs, actin, tubulins, MFG-E8, Gi2α, histones, PrP and PrPsc</td>
<td>[339]</td>
</tr>
<tr>
<td>EBV-positive lymphoblastoid cells (JY and B95-8)</td>
<td>DC</td>
<td>WB (HLA-DR and CD86)</td>
<td>WB</td>
<td>LMP1</td>
<td>[340]</td>
</tr>
</tbody>
</table>
a) DC: Differential centrifugation; DG: Sucrose density gradient or otherwise stated; EQ: Exoquick; F: Filtration; GF: Gel filtration; SC: Sucrose cushion; UC: Ultracentrifugation

b) CRT: Calreticulin; EM: Electron microscopy; EGFRvIII: Epidermal growth factor receptor variant III; FACS: Fluorescence–activated cell sorting; GPNMB: Glycoprotein nonmetastatic B; IEM: Immunoelectron microscopy; NTA: Nanoparticle tracking analysis; PDI: Protein disulfide isomerase; TGF-β1: Transforming growth factor beta 1; TfR: Transferrin receptor; WB: Western blot.

c) ADAM10: A Disintegrin and metalloproteinase domain-containing protein 10; AM: Apical membrane; ARF: ADP-ribosylation factor; ARRDC2: Arrestin domain-containing protein 2; ASGR: Asiaglycoprotein receptor; BM: Basolateral membrane; CENP-P: Centromere protein P; CHMP2A: Charged multivesicular body protein 2A; CNP: 2′,3′-cyclic-nucleotide 3′-phosphodiesterase; CRMP-2: Collapsing response mediator protein 2; DEL-1: developmental endothelial locus-1; DKK4: Dickkopf 4; Di EBV: Epstein-Barr virus; EFNB2: Ephrin-B2; EGFR: Epidermal growth factor receptor; ERM proteins (ezrin, radixin, and moesin); ESCRT: Endosomal sorting complex required for transport; FasL: Fas Ligand; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GPNMB: glycoprotein non-metastatic B; HNF4A: hepatocyte nuclear factor alpha; HPX: Hemopexin; JAG1: Jagged 1; LAMP1: Lysosome associated membrane protein 1; LMP1: Latent membrane protein 1; MART-1: Melanoma antigen recognized by T cells 1; MBP: Myelin basic protein; MET: Met proto-oncogene (hepatocyte growth factor receptor); MIF: Macrophage migration inhibitory factor; MOG: Myelin oligodendrocyte glycoprotein; MHC: Major histocompatibility complex; MVP: Major vault protein; NG2: Chondroitin sulfate proteoglycan; PDI: Protein disulfide isomerase; PLP: Myelin proteolipid protein; PrP: Prion protein; PrPsc: Prion protein scrapie; RACK1: Lung cancer oncogene 7; S100A8: S100 calcium binding protein A8; S100A9: S100 calcium binding protein A9; SRC: v-sr sarcoma; TNC: tenascin C; TRAIL: TNF-related apoptosis-inducing ligand; TNIK; TRAF2 and NCK interacting kinase; TRP: 5,6-dihydroxyindole-2-carboxylic acid oxidase; VPS proteins: Class E Vacuolar protein-sorting proteins. Reproduced and updated from [74].
## Appendix A-2

### Isolation and characterization of body fluid-derived exosomes

<table>
<thead>
<tr>
<th>Body fluid</th>
<th>Isolation strategies&lt;sup&gt;a)&lt;/sup&gt;</th>
<th>Validation&lt;sup&gt;b)&lt;/sup&gt;</th>
<th>Proteomic strategies</th>
<th>Comments&lt;sup&gt;c)&lt;/sup&gt;</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast milk</td>
<td>DC, F, DG</td>
<td>IEM (CD63 and HLA-DR), WB (HLA-DR, CD81 and HSC70), FACS (HLA-DR, CD63, CD81 and mucin-1)</td>
<td>In-solution trypsinization, scx-LC-MS/MS</td>
<td>73 proteins identified including MFG-E8, MUC1, hsp70, ARF-1, EH domain-containing protein 1, CD36, butyrophilin and polymeric-Ig receptor</td>
<td>[146]</td>
</tr>
<tr>
<td>Bronchoalveolar lavage fluid</td>
<td>DC, immuno-beads (MHC-II)</td>
<td>IEM (HLA-DR and CD63), FACS (HLA-DR, CD54, CD63 and CD86)</td>
<td>-</td>
<td>-</td>
<td>[341]</td>
</tr>
<tr>
<td>Malignant pleural effusions</td>
<td>DC, DG</td>
<td>EM</td>
<td>1-DE, MALDI-TOF MS</td>
<td>50 proteins identified including MHC-I, actin, G protein, hsp90, BTG1, Bamacan, PEDF, BTG-1, TSG14 and TSP2</td>
<td>[147]</td>
</tr>
<tr>
<td>Malignant pleural effusions and malignant ascites</td>
<td>DC, DG</td>
<td>EM, IEM (MHC-I and -II, TRP, gp100 and CD81), WB (MHC-I and -II ,MART-1, HER2 and hsc70)</td>
<td>WB</td>
<td>Proteins including MART-1, TRP, gp100 and HER2 identified</td>
<td>[342]</td>
</tr>
<tr>
<td>Saliva</td>
<td>GF</td>
<td>EM WB (Alix, TSG101, HSP70 and CD63)</td>
<td>2-DE LC-MS/MS</td>
<td>68 proteins were identified including GW182, immunoglobulin A, polymeric immunoglobulin receptor, Mucin-5B, Alpha –amylase 1, Dipeptidyl peptidase 4 (CD26).</td>
<td>[47]</td>
</tr>
<tr>
<td>Saliva</td>
<td>F, diafiltration (100K), GF</td>
<td>EM</td>
<td>N-terminal amino acid sequencing, WB</td>
<td>Dipeptidyl peptidase IV (CD26), polymeric-Ig receptor, actin, galectin-3 and Ig chains</td>
<td>[343]</td>
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<tr>
<td>Semen</td>
<td>DG</td>
<td>EM</td>
<td>1-DE, LC-MS/MS</td>
<td>440 proteins identified</td>
<td>[148]</td>
</tr>
<tr>
<td>Synovial fluid (RA, OA and reactive arthritis)</td>
<td>DC, DG</td>
<td>EM, IEM (IgG)</td>
<td>2-DE, WB, MALDI-TOF MS</td>
<td>Citrullinated fibrin α-chain, CD5 antigen-like, fibrinogen fragment D and β-chain</td>
<td>[344]</td>
</tr>
<tr>
<td>Urine and amniotic fluid</td>
<td>DC, DG</td>
<td>EM, WB (hsp70, AQP2, annexin-1 and CD9)</td>
<td>WB</td>
<td>CD24</td>
<td>[345]</td>
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</tr>
<tr>
<td>Urine</td>
<td>UC</td>
<td>EM, IEM (APN, AQP2, CD9 and NCC), WB (TSG101, Alix, CD9, Rab-4, -5B and -11, SNX18)</td>
<td>1-DE, LC-MS/MS</td>
<td>295 proteins identified including VPS proteins, AQP2, polycystin-1, carbonic anhydrase II and IV</td>
<td>[346]</td>
</tr>
<tr>
<td>Urine</td>
<td>DC</td>
<td>Not performed</td>
<td>1-DE, LC-MS/MS</td>
<td>1132 proteins identified including AQP2, vacuolar H+ -ATPase subunits and ESCRTs. 14 phosphorylated proteins including NCC, GPRC5B and GPRC5C</td>
<td>[347]</td>
</tr>
<tr>
<td>Urine (Prostatic secretions)</td>
<td>DC</td>
<td>Not Performed</td>
<td>1-DE LC-MS/MS</td>
<td>877 proteins identified including markers CD9, CD63, CD81, Alix, TSG101, FLOT1, FLOT2, ANXA2 and ANXA5. Prostate-enriched proteins PSA, ACPP, TGM4 and PSMA</td>
<td>[348]</td>
</tr>
<tr>
<td>Urine (Renal cell carcinoma, 9 patients)</td>
<td>DC, DG (OptiPrep™)</td>
<td>EM, WB (Flotillin-1, TSG101 and CD9)</td>
<td>1-DE LC-MS/MS</td>
<td>186 proteins identified including matrix metalloproteinase 9, ceruloplasmin, podocalyxin, dickkopf related protein 4 and carbonic anhydrase IX</td>
<td>[349]</td>
</tr>
<tr>
<td>Urine</td>
<td>DC</td>
<td>WB (Alix, TSG101 and AQP1)</td>
<td>In-solution trypsinization LC-MS/MS</td>
<td>280 proteins identified including major urinary protein 1, CD10</td>
<td>[149]</td>
</tr>
<tr>
<td>Urine</td>
<td>DC</td>
<td>EM</td>
<td>1-DE LC-MS/MS and In-solution trypsinization LC-MS/MS</td>
<td>1025 proteins identified including 12 annexin proteins, 32 RAB proteins, 5 RAP proteins, aquaporin-1, aquaporin-2, aquaporin-7, solute carrier family 12 member 1, Kidney specific Na-K-Cl symporter and solute carrier family 12 member 3</td>
<td>[350]</td>
</tr>
</tbody>
</table>
a) DC: Differential centrifugations; DG: Sucrose density gradient or otherwise stated; F: Filtration; GF: Gel filtration; UC: Ultracentrifugation.
c) ACPP: Prostatic Acid Phosphatase; ANXA2: Annexin A2; ANXA5: Annexin A5; AQP1: Aquaporin-1; AQP2: Aquaporin-2; ARF: ADP-ribosylation factor; Bamacan: Basement membrane chondroitin sulfate proteoglycan; BTG1: B-cell translocation gene 1; FLOT: Flotillin GP100; Melanocyte protein Pmel 17; GPRC5B and GPRC5C: G-protein coupled receptor family C group 5 member B and C; MART-1: Melanoma antigen recognized by T cells 1; NCC: Na-Cl Cotransporter; OA: osteoarthritis; PEDF: Pigment epithelium-derived factor; PSA: Prostate specific antigen; PSMA: Prostate specific membrane antigen; RA: Rheumatoid arthritis; TGM4; Transglutaminase 4; TRP: 5,6-dihydroxyindole-2-carboxylic acid oxidase; TSG14: Tumor necrosis factor-stimulated gene 14; TSP2: Thrombospondin-2; VPS proteins: Class E Vacuolar protein-sorting proteins.
Reproduced and updated from [74].
Appendix A-3  Human colon cancer LIM1863 cells

(A) Phase contrast microscopy of LIM1863 organoids Scale 20 μm. (B) A confocal microscopy cross-section through a LIM1863 organoid highlights concentrated syntaxin 3 staining of the apical ring (red), and A33 staining at the basolateral cell periphery (green). Scale 20 μm. (C) LIM1863 cell metabolic activity was measured using the MTT assay following 24 h culture in RPMI containing either 5% FCS or 0.6% ITS. (D) Cell viability of LIM1863 cells was measured using the lactate dehydrogenase (LDH) assay following 24 h culture in RPMI containing either 5% FCS or 0.6% ITS. Data represents the mean ± S.D of three independent experiments performed in triplicate.
**Appendix A-4  Distinguishing features of EMT**

<table>
<thead>
<tr>
<th>Feature observed</th>
<th>Epithelial cells</th>
<th>Mesenchymal cells</th>
</tr>
</thead>
</table>
| **Morphology**   | • Round cobblestone growth in culture  
                    • Strong cell-cell contact | • Elongated, spindle shaped, scattered growth in culture  
                        • Weak cell-cell contact |
| **Polarity**     | • Well maintained apical basolateral polarity  
                    • Intact tight junctions (ZO-1), adherens junctions (E-cadherin) and desmosomes | • Leading edge front-back polarity  
                        • No tight junction, adherens junction or desmosomes. Diminished E-cadherin and ZO-1 expression |
| **Motility**     | • Little cell migration due to intact epithelial sheets | • Increased cell migration of individual cells |
| **Invasiveness** | • Minimal, spherical cyst growth in collagen gel | • Increased, penetrating growth into collagen gel |
| **Cytoskeleton** | • Apical actin ring and actin stress fibers  
                    • Cytokeratin intermediate filament network inside cell border  
                    • No vimentin intermediate filaments | • No apical actin ring and dissolved actin stress fibers  
                        • Loss of cytokeratin intermediate filaments  
                        • Vimentin intermediate filaments across cell body |

References [270, 286, 289].
Appendix A-5  
Secreted extracellular modulators of EMT

Secretome analysis of MDCK cells reduced expression of cell-cell and cell-matrix attachment proteins following oncogenic Ras-transformed EMT, possibly to enable greater mobility. Basement membrane constituents were downregulated, and increased protease expression was observed following transformation. Production of factors associated with migration and invasion were also elevated in the 21D1 cell secretome. Adapted from [250].

Supplemental data associated with this article can be found at doi:10.1016/j.ymeth.2012.01.002.

Supplemental Table 1 - Proteins identified in UC-Exos, DG-Exos, and IAC-Exos.

Supplemental Table 2 - Relative quantification of cancer related proteins using label-free-based spectral counting.

Supplemental Figure 1 - Human colon cancer LIM1863 cells (Appendix A-3).

Supplemental Figure 2 - Distribution of proteins identified in UC-, DG-, and IAC-Exos (Appendix A-7).

Supplemental Figure 3 - Plasma membrane proteins identified in the exosome datasets (Appendix A-8).


Supplemental data associated with this article can be found at http://www.mcponline.org/content/suppl/2013/07/11/M112.021303.DC1.

Supplemental Table 1 - A33- and EpCAM-Exos Protein Datasets.

Supplemental Table 2 - sMVs Protein Dataset.
Supplemental Table 3 - A33-Exos Peptide Identifications.

Supplemental Table 4 - EpCAM-Exos Peptide Identifications.

Supplemental Table 5 - sMVs Peptide Identifications.


Supplemental data associated with this article can be found at http://www.mcponline.org/content/early/2013/05/03/mcp.M112.027086/suppl/DC1.

Supplemental Table 1 - Proteins identified in exosome proteome profiling of H-Ras-induced EMT (21D1) in MDCK cells.

Supplemental Table 2 - Peptides identified in MDCK-Exos.

Supplemental Table 3 - Peptides identified in 21D1-Exos.

Supplemental Table 4 - 139 proteins identified in MDCK- and 21D1-Exos not reported in ExoCarta database.

Supplemental Figure 1 - 21D1 cells require their own culture medium to maintain a mesenchymal phenotype (Appendix A-9).

Supplemental Figure 2 - Exosome characterization following OptiPrep™ density gradient separation (Appendix A-10).

Supplemental Data - Experimental procedures for replicate GeLC-MS/MS analysis of MDCK- and 21D1-Exos (Appendix A-11).
Appendix A-7  Distribution of proteins identified in UC-, DG-, and IAC-Exos

A three-way Venn diagram depicting number of proteins commonly observed in all three datasets (265), and those that are unique to each purification strategy; ultracentrifugation (UC-Exos), density gradient centrifugation (DG-Exos), and immunoaffinity capture (IAC-Exos).
Appendix A-8 Plasma membrane proteins identified in the exosome datasets

Proteins were annotated as plasma membrane based on HPRD classification (GO: 0005886). Proteins containing one or more transmembrane domains were predicted using TMHMM (v2.0). IAC-Exos contained the highest proportion of proteins annotated as plasma membrane (32.1%), and predicted to contain at least one TM spanning domain (19.8%).
Appendix A-9 21D1 cells require their own culture medium to maintain a mesenchymal phenotype

21D1 cells were grown as described in Chapter 4 Experimental Procedures with growth media changed daily and supplemented with either 10% (A) or without (B) their own culture medium. After a period of 5 days, phase contrast images of 21D1 cells supplemented with 10% culture medium display an elongated mesenchymal-like spindle shape (A), while 21D1 cells not supplemented with 10% culture medium revert back to an epithelial-like cobblestone morphology (B).
Appendix A-10  Exosome characterization following OptiPrep™ density gradient separation

Concentrated culture medium from MDCK and 21D1 cells was isolated as described in Chapter 4 Experimental Procedures (Cell culture and CCM preparation) and separated using OptiPrep™ density gradient. Protein fractions (1.01-1.30 g/mL) from MDCK (A) and 21D1 (B) were stained with SYPRO® Ruby protein stain. Western blot analysis of MDCK (C) and 21D1 (D) OptiPrep™ density gradient fractions (10 µg) revealed the exosomal marker Alix predominantly detected in fraction 1.09 g/mL. This fraction was selected for proteomic analysis.
Appendix A-11 Experimental procedures for replicate GeLC-MS/MS analysis of MDCK- and 21D1-Exos

MDCK- and 21D1-Exos samples (20 μg) were lysed in SDS sample buffer, and proteins separated by SDS-PAGE and visualized by Imperial™ Protein Stain (Thermo Fisher Scientific), according to manufacturer’s instructions. Gel lanes were cut into 20 × 2 mm bands using a GridCutter (The Gel Company, San Francisco, CA) and individual bands subjected to automated in-gel reduction, alkylation and trypsinization using a Tecan EVO liquid handler robotic system. Briefly, gel bands were reduced with 10 mM DTT (Calbiochem, San Diego, USA) for 20 min, washed and alkylated for 30 min with 50 mM iodoacetic acid (Fluka, St. Louis, USA), and digested with 100 ng trypsin (Promega Sequencing Grade Product V5111X, Wisconsin, USA) for 8 h at 35 °C with automated agitation. Tryptic peptides were extracted with 3 × 50 μL 50% (v/v) acetonitrile, 50 mM ammonium bicarbonate, concentrated to ~10 μL by centrifugal lyophilisation, pooled (2 bands pooled as 1 fraction) and analyzed by LC-MS/MS. RP-HPLC was performed on a Dr. Maisch 3 μm ReproSil-Pur C18 AQ (Dr Maisch GmbH, Germany) 150 × 0.075-mm internal diameter reversed phase column with an integrated KASIL Frit using an Agilent 1100 nanoHPLC coupled online to an LTQ-Orbitrap mass spectrometer equipped with a nanoelectrospray ion source (Thermo Fisher Scientific, San Jose, USA). The column was developed with a linear 60 min gradient with a flow rate of 0.3 μL/min from 0-60% solvent B, 60-80% solvent B in 2 min and held for 10 min and then returned to 2% solvent B for 15 min equilibration where solvent A was 0.1% (v/v) aqueous formic acid and solvent B was 0.1% (v/v) aqueous formic acid/100% acetonitrile. Survey MS scans were acquired with the resolution set to a value of 30,000. Real-time recalibration was performed using a background ion from ambient air in the C-trap. Up to 10 selected target ions were fragmented and then were dynamically excluded from further analysis for 120 secs from sample analysis [351]. Raw mass spectrometry data is deposited in the PeptideAtlas and can be accessed at http://www.peptideatlas.org/PASS/PASS00225 [352-354].
Author/s:
Tauro, Bow Jesse

Title:
The isolation and characterization of exosomes

Date:
2013

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