The Role of Prostate Cancer Microvesicles in Epithelial Mesenchymal Transition

Presented by

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Abstract

Microvesicles (MVs) are shed to the extracellular environment upon activation by both normal and diseased cells. Increasing evidence has shown that cancer cell-derived MVs carry pathogenic components, such as proteins, messenger RNAs, microRNAs, long non-coding RNAs, DNAs, lipids, and transcriptional factors, which can mediate paracrine signaling in the tumor microenvironment, and that they are thus highly capable of altering the function of the recipient cells. MVs released from tumour cells are thought to play an important role in cancer metastasis. Most carcinomas are derived from epithelial cell lines, which normally adhere together tightly to form the structural foundations of many organs. Epithelial Mesenchymal Transition (EMT) has been linked to malignancy in many carcinomas of epithelial origin. In this study, I look at the role of PC3 (Prostate Cancer cell line) derived MVs in the induction of EMT in PNT2 (normal prostate) cells. Uptake of PKH26-labelled PC3-derived MVs by recipient cells was greater at a low pH of 5.4 and was dependent on MV-based protein and phosphatidylserine interaction with recipient cells. Biochemical and morphological changes, indicative of EMT, (elongation, increased expression of mesenchymal marker Vimentin and reduced expression of epithelial marker, E-cadherin) were observed using fluorescent microscopy and flow cytometry. To further investigate the protein cargo of PC3, PNT2 and the transformed PNT2 (tPNT2) cells and their respective MVs LC-MS/MS analysis was carried out. Around 1290 proteins were identified collectively in all the samples. Pathway analysis tools such as DAVID, PANTHER, KEGG and STRING were used to analyse the protein data. Importantly this study identified key proteins in PC3 MVs that may have contributed to the process of EMT, such as integrins which play a vital role in the biology of invasive carcinoma and setting up a pre-metastatic niche.

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Abbreviations

ANOVA	Analysis of Variance
APS	Ammonium Persulfate
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
BSA	Bovine Serum Albumin
BzATP	2'(3')-O_(4-Benzoylbenzoyl)adenosine 5'-triphosphate triethylammonium salt
$CaCl_2$	Calcium Chloride
Cell^M	Software for fluorescent microscope
CGM	Cellular Growth Medium
CO_2	Carbon dioxide
CSF	Cerebral Spinal Fluid
DAPI	(4',6-diamidino-2- phenylindole)
DAVID	Database for Annotation, Visualisation and Integrated Discovery
ddH_2O	double deionized water
DMSO	dimethyl sulfoxide
DNA	Deoxy Ribonucleic acid
DTT	Dithiothreitol
EBV	Epstein - Barr virus
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme- Linked Immunosorbent Assay
EMT	Epithelial Mesenchymal Transition
ERK	Extracellular signal-regulated kinases
EVs	Extracellular vesicles
FBS	foetal bovine serum
FITC	Fluorescein isothiocyanate
HCD	High Energy Collision Dissociation
HEPES	4-(2-hydroxyethly)- 1-piperazineethanesulphonic acid
HRP	Horseradish peroxidase
ICAM	Intercellular adhesion molecule

IgG	Immunoglobulin G
IV	Intravenous
KCl	potassium chloride
KEGG	Kyoto Encyclopedia of Genes and Genomes
KH_2PO_4	Dipotassium hydrogen phosphate
KOH	Potassium hydroxide
LC	Liquid chromatography
LC-MS/MS	Liquid Chromatography Mass Spectrometry
LN	Lupus Nephritis
m/z	mass/charge
mAb	monoclonal antibody
MET	mesenchymal epithelial transition
MFI	medium fluorescence intensity
Mgf files	mascot generic format files
MHC	Major Histocompatibility Complex
microRNA	micro Ribonucleic acid
mM	milli molar
MMP-14	Matrix metallopeptidase 14
MMP-2	Matrix metallopeptidase 2
MMP-9	Matrix metallopeptidase 9
mRNA	messenger Ribonucleic acid
MVBs	Multivesicular bodies
MVs	Microvesicles
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	sodium chloride
NaN_3	Sodium azide
NaOH	Sodium hydroxide
NCE	Normalised Collision Energy
NTA	nanoparticle tracking analysis
p38MAPK	mitogen activated protein kinase
PANTHER	Protein Analysis Through Evolutionary Relationships
PBS	Phosphate buffer saline
PBS-T	Phosphate buffered Saline Tween-20
PC	Phosphatidylcholine
PC12	cell line derived from rat adrenal medulla

PC3	Prostate Cancer cells
PC3-1E8	prostate cancer cell line (highly aggressive)
PC3-M	prostate cancer cell line (highly aggressive)
PE	Phosphotidylethanolamine
PFA	paraformaldehyde
PKH26	Fluorescent cell linker Kit
PMP	Platelet Microparticles
PNT2	Prostate cells (no cancer)
PS	phosphatidylserine
RhoA/ROCK	Rho associated protein kinase
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPMI	cell growth medium
SDS	Sodium Dodecyl Sulfate
SKOV3	Ovarian Carcinoma cells
SL	Sphingolipids
SLE	Systemic lupus erythematosus
Smads	proteins involved in cell signaling
Snail	transcription factor protein
STRING	Search Tool for the Retrieval of Interacting Genes/proteins
SV40	polyomavirus simian Virus 40
TBS-T	Tris buffered saline
TEMED	Tetramethylethylenediamine
TGF β	transforming growth factor beta
TIM	T cell Immunoglobulin domain
TIM1	T cell Immunoglobulin domain 1
TIM4	T cell Immunoglobulin domain 4
tPNT2	transformed PNT2 cells
Twist	transcription factor protein
w/v	(weight/volume)
WB	Western Blot
Zeb	transcription factor protein

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Chapter 1: Introduction

Introduction

1.1 Cellular communication

Cellular communication is an important process that allows cells to interact with one another by passing an array of signals. One means of intercellular communication that has been the mainstay of cell communication studies is cellular release into the extracellular environment of molecules including proteins, nucleotides and lipids, where these molecules are picked up by cognate receptors present on the surrounding recipient cells; engagement of receptors results in intracellular signalling and modification of the recipient cell (Bobrie *et al.*, 2011). However, the secretion of these biomolecules is a bit more complicated, in that extracellular membrane vesicles are also formed packed full of biomolecules from the parent cell. The extracellular vesicles' (EVs') biological function is determined by the type of cell it comes from and the compartment they are derived from. These EVs can contain numerous proteins, lipids and nucleic acids from the parent cell.

1.2 Extracellular vesicles

Extracellular Vesicles (EVs) were first reported in 1949 when Charagaff *et al*. recognised a perceptible factor that accelerated the generation of thrombin to be present in platelet-free plasma (Hargett and Bauer, 2013). Using ultracentrifugation, platelet-derived fragments were successfully isolated from the platelet-free plasma and it was found that these fragments were capable of producing thrombin. Interestingly, a direct correlation was reported between the amounts of platelet microparticles (PMP), referred to as "platelet dust", and the platelet count present in the original blood sample (Wolf P, 1967). Consistently,

polythaemic patients with higher platelet counts had higher levels of PMP compared with thrombocytopenic patients who had lower levels of PMP, thus suggesting a role for PMP in blood clotting (Wolf P, 1967).

There are three different types of EVs; exosomes, microvesicles (MVs) and apoptotic bodies. Until recently, exosomes and MVs were thought to be the same and were called microparticles. However, recent studies have shown the difference between the two, which will be referred to later on in this thesis. In terms of the type of proteins carried by MVs, there may be a preponderance to select for proteins lacking a signal peptide; these proteins may be released in MVs by the so-called 'non-conventional or unconventional protein export' first described by Nickel and colleagues (Nickel and Rabouille *et al*, 2009 and Pompa *et al*, 2017).

1.2.1 Exosomes

Exosomes are formed by inward budding (intraluminal budding) of endosomal compartments called multivesicular bodies (MVBs). Exosomes have been known for decades however, they were characterised as cell debris and signs of cell death until Stahl and Johnstone reported that multivesicular endosomes could fuse with the plasma membrane releasing their content into the extracellular environment (Harding *et al*., 1983; Pan *et al*., 1985). In 1987 Johnstones's group demonstrated the fusion of multivesicular endosomes with plasma membrane by electron microscopy, also showing its contents to be released to the extracellular environment (Pan *et al*., 1985), which led them to propose the term 'exosome.' Nevertheless, this area was dormant for over a decade until Raposo *et al*. reported that exosomes secreted from Epstein-Barr Virus (EBV) transformed B

lymphocytes carry functional MHC molecules and could present MHC antigenic peptide complexes to specific T cells (Raposo *et al*., 1996). Two years later Zitvogel *et al* discovered that exosomes released from dendritic cells in mice carry functional MHC peptide molecules that are capable of inducing an anti-tumour immune response *in vivo* (Zitvogel *et al*., 1998).

1.2.2 Microvesicles

Microvesicles (MVs) are small, intact heterogeneous membrane vesicles (with a diameter of 0.1- 1μm) that contain vital cell components involved in cell signalling and intercellular communication. Different cell types such as platelets, monocytes and tumour cell lines are known to release MVs either constitutively or upon extracellular stimulation (Stratton *et al*, 2015). MVs also vary in size, composition and biological effects. During microvesiculation only selective cell components are released to the surrounding microenvironment. In addition, chemical and physical factors in the cell stimulate MVs release during cell activation, stress, apoptosis, malignant transformation, hypoxia and differentiation (Yuana *et al*, 2011). MVs also take up some cytoplasm during shedding through their membrane and as a result contain proteins, microRNA and mRNA derived from parental cell (Inal *et al*, 2012).

1.3 Membrane lipids and MV release

It is essential to understand the biochemistry of lipid composition in the membrane in order to understand the mechanisms by which MVs are released. The cell membrane is a lipid bilayer which has amino phospholipids

(Phosphatidylethanolamine and Phosphatidylserine) located at the inner leaflet and Phosphatidylcholine and Sphingomyelin present in the outer leaflet (Daleke, 2002). There are three different types of proteins that catalyse the movement of the above lipids across the plasma membrane in order to maintain and indulge the trans bilayer lipid asymmetry (Daleke, 2002).

1.3.1 Flippase

Flippase is an aminophospholipid translocase that is responsible for transporting Phosphotidylserine (PS) from the outer monolayer of the membrane to the inner membrane leaflet. The lipid transporter protein, Flippase can be ATP dependent or ATP independent and it can be selective or non-selective (Daleke, 2002).

1.3.2 Floppase

The lipid transporter protein Floppase is however responsible for transporting phosphotidylcholine and sphinogmyeline from the cytoplasmic inner leaflet to the outer leaflet of the cell membrane with the aid of ATP as shown in (Figure. 1). The activity of floppase depends on intercellular Ca^{2+} concentration, which also results in inhibiting the activity of Flippase (Daleke, 2002).

1.3.3 Scramblase

The activity of scramblase is also dependent on the concentration of intercellular Ca^{2+} leading to budding of the plasma membrane through exposure of PS from the inner leaflet to the outer leaflet of the cell membrane. The intense activity of all these transporter proteins contributes to cell membrane conformation (Daleke, 2002).

Increasing levels of intercellular Ca^{2+} cause the collapse of plasma membrane asymmetry due to the effect of floppase and scramblase activity on down regulating flippase. As a result, PS is exposed on the outer membrane followed by MV release. Therefore, as shown in Fig.1 due to the remodelling of the lipid bilayer and loss of phospholipid asymmetry MVs are released (Vega and Ridley 2008). Upon secretion of the MVs they can either be taken up by local recipient cells or transported in body fluids for cargo delivery into recipient cells at different sites (Hashimoto *et al*, 2004). Microvesicles can be detected in different body fluids such as: blood, urine, milk, sweat, semen, breast milk, saliva, ascites fluid and cerebral spinal fluid (CSF) (Muralidharan- Chari *et al* 2007).

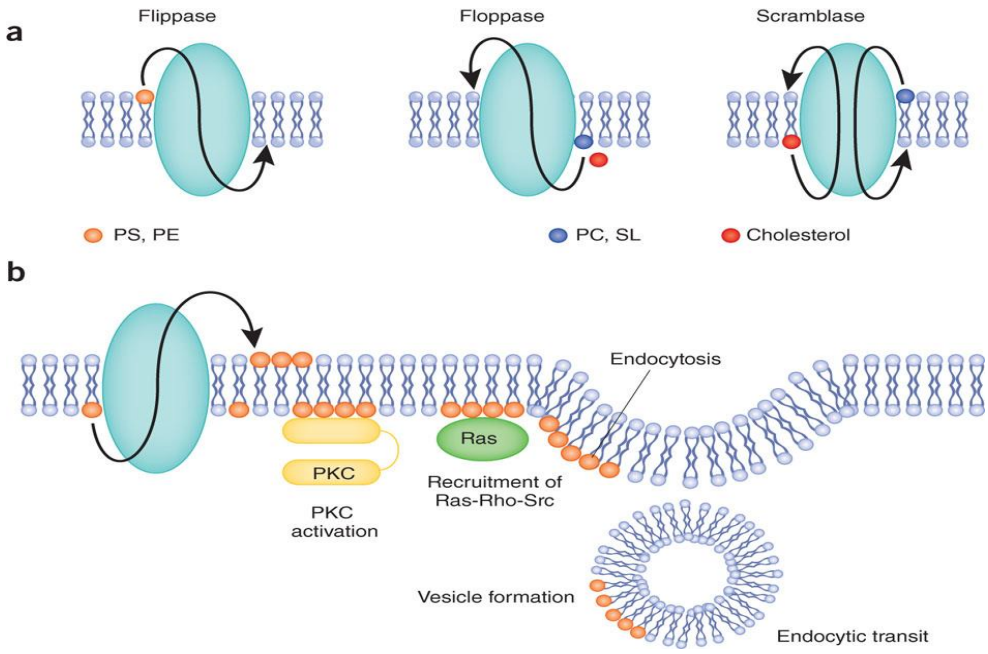


Figure 1: The role of lipid transporter proteins in cell membrane asymmetry and the formation of Microvesicles. (a) Shows the functions of the lipid transporter proteins; flippase, floppase and scramblase moving PS, PE, PC, SL and cholesterol. **(b)** Shows activation of scramblase causing PS exposure on outer leaflet of the cell membrane during apoptosis and the release of microvesicles as a result. Source: Marcus R Clark. Flippin' lipids Nature Immunology 12, 373–375 (2011).

Recent studies have shown the role of vesicles in cell to cell communication. Thery *et al* was one of the first groups to investigate such roles and demonstrated that vesicles could help in improving immune responses in cells, by carrying antigens to increase the number of antigens presenting cells (Thery *et al*., 2002). Researchers started paying attention to the vesicle field after Valadi *et al*., reported that exosomes can transport mRNA and miRNA (Valadi *et al*., 2007). The group showed that mRNA delivered by exosomes could be translated into functional protein in the recipient cells, indicating that exosomes are capable of directly transferring genetic material from cell to cell and modifying recipient cells. In other early work it was shown that EVs could transfer surface receptor proteins, in this case platelet and

megakaryocyte- derived EVs can transfer the HIV receptor CXCR4 to cells lacking the receptor and rendering them infectible by HIV (Rozmyslowicz et al., 2003).

1.4 Microvesicles interaction with the recipient cells

It is relatively unknown as to how an individual MV interacts with the recipient cell however, but currently there are believed to be three possible ways; MVs via the proteins they carry on their surface can bind to specific receptors on the cell surface, enter the recipient cell via endocytosis (which may be receptor-mediated or by macropinocytosis) or fuse in a process that may be involved in complete fusion (lipid mixing) or hemi-fusion with the plasma membrane (Mulcahy *et al.*, 2014).

1.4.1 Entry through endocytosis

One of the most effective and common ways of exosome uptake is phagocytosis. In work performed by Tian *et al*., exosomes from PC12 cells (a rat pheochromocytoma) were internalised through endocytosis. They were actively transported by the cytoskeleton to the perinuclear region (Tian *et al*., 2010).

Escrevente *et al*. showed that an energy dependant mechanism is used by the recipient cell to internalise exosomes secreted from the cell (SKOV3), an ovarian carcinoma cell line. They then inhibited the internalisation of the exosomes by treating the cells or exosomes with proteinase K, which showed a requirement for protein on exososmes and on cells to mediate such internalisation. The research group suggested that clathrin-independent endocytosis is associated with uptake of exosomes by SKOV3 cells, however the group also suggested that other endocytic pathways might be involved (Escrevente *et al*., 2011). It has been reported by Feng

and colleagues that phagocytic cells have a greater uptake of exosomes than non-phagocytic cells (Feng *et al*., 2010).

1.4.2 Entry through ligand-/receptor-binding

It is generally thought that EVs may enter the recipient cell through ligand receptor binding. The membrane of EVs contains phosphatidylserine and protein on the surface that can be captured by phosphatidylserine receptors such as the T cell immunoglobulin domain (TIM) and mucin domain protein. There are two types of phosphatidylserine binding molecules; TIM1 is expressed on the surface of activated lymphocytes and TIM4 are expressed on the surface of activated phagocytes. Both TIM1 and TIM4 have been shown to mediate exosome uptake (Miyanishi *et al*., 2007).

The intercellular adhesion molecule 1 (ICAM), also involved in EV-cell interaction is present on exosomes secreted from mature dendritic cells (Segura *et al*., 2007) and activated T cells (Nolte-'t Hoen *et al*., 2009).

1.4.3 Entry through direct fusion

Extracellular vesicles can directly fuse with plasma membrane and release their internal contents into the cell cytoplasm. Parolini *et al* showed that exosomes could fuse with the plasma membrane by demonstrating that exosomes undergo a lipid dependent fusion process with plasma membrane, and that this fusion is more efficient in an acidic microenvironment (Parolini *et al*., 2009). This finding suggested that fusion of exosomes with cells is more frequent in the acidic environment found in a tumour compared to that in the surrounding normal tissue. It should be noted that for intravesicular (RNA) cargo to transfer to recipient cells, direct fusion of EVs with

recipient cells and/or attachment of EVs is important for the transfer of intravesicular RNA.

1.5 Normal function of MVs

Microvesicles are released by almost all cells in the body and they are present in all body fluids: plasma, urine, cerebrospinal fluid, tears, saliva, breast milk, semen (Santiago-Dieppa *et al*., 2014). Their role in normal cell physiology is much less studied than that in disease pathology (Fleissner *et al*, 2011). Microvesiculation may be considered a survival mechanism adapted by cells however, it also plays a pivotal role in intercellular communication as mentioned above. Therefore, if MVs are released from healthy cells such as antigen presenting cells then their release can help in carrying antigens to other cells, and so improving immunity in the body (Thery *et al*, 2002). Similarly, if the MVs are released from an unhealthy cell or a tumour cell then it can have an adverse effect on the recipient cells (Theiry *et al*, 2002).

1.6 Pathological function of MVs

High levels of MVs are observed in the body fluids of individuals with pathological conditions. For example, patients suffering from thrombotic diseases have high numbers of platelet derived MVs in their peripheral blood. It has been suggested that MVs take part in the activation of the coagulation cascade through the presence of phosphatidylserine (Owens & Mackman, 2011). Similarly, patients suffering from rheumatoid arthritis also show high levels of MVs in their synovial fluid (Yuana *et al*. 2013). An increased number of MVs has been observed in the body fluids of patients with prostate and ovarian cancer, suggesting the role of MVs in human malignancies and metastasis (Yuana *et al*. 2013). Raised EVs levels have also been observed in SLE (Systemic Lupus Erythematosus) patients, where the EVs act as

auto-adjuvants, maintaining and spreading inflammation by initiating and perpetuating auto antibody production (Perez-Hernandez and Cortes 2015). The presence of immune complex molecules is an indication of an early event in glomerulus of Lupus Nephritis (LN) (Perez-Hernandez *et al*, 2017). Correspondingly, Inal's group showed that high plasma MV levels were indicative of SLE and the proteins expressed on these EVs show specific patterns that could be used as biomarkers for disease progression (Inal *et al*, 2010).

1.7 MVs and angiogenesis

Angiogenesis is defined as a process that involves the formation and growth of new blood vessels to expand tissues and organs by providing oxygen and nutrients and removing metabolic waste. MVs have pro- and anti-angiogenic properties. Platelet-derived MVs have been shown to induce and support tumour angiogenesis and metastasis (Slomka *et al*, 2018). For example, binding platelet-derived MVs to metastatic lung cancer cells triggers the expression of matrix metalloproteinases (MMP-9, MMP-2 and MMP-14) on the cells. Cancer cell derived vesicles have been shown to promote tumour angiogenesis (Vader *et al*, 2014). Although, MVs contribute to promoting angiogenesis, they also play a role in anti-angiogenesis. Microvesicles inhibit angiogenesis by stimulating the production of endothelial reactive oxygen species (ROS) (Yang *et al*, 2008). In an experiment carried out *in vitro,* Yang *et al* (2008) showed that lymphocyte derived MVs released after actinomycin D treatment decreased nitrite oxide production and increased ROS production by stimulating phosphatidylinositol 3 kinase, xanthine oxidase and nicotinamide adenine dinucleotide phosphate oxidase pathways (Yang *et al*, 2008).

1.8 MVs as diagnostic and therapeutic tools

Microvesicles can be very easily isolated from body fluids, and because they carry proteins, lipids and genetic materials from the host cells, they serve as a very useful diagnostic tool for many diseases (Vader *et al*, 2014). MVs as biomarkers is an emerging field in the pharmaceutical sciences. Their use as therapeutic vehicles is also first developing, due to the fact that EVs constitute bioactive cargoes, and so can be successfully used in tissue regeneration (Vader *et al*, 2014). Microvesicles derived from stem cells have the ability to induce angiogenic programmes in resting endothelial cells by suppressing apoptosis, stimulating proliferation, delivering immunomodulatory signals and recruiting cells required for tissue regeneration (El Andaloussi *et al*, 2013).

1.9 MVs and Cancer

Cancer patients have elevated numbers of MVs in their body compared to healthy individuals (Mathivanan and Simpson 2010). However, not all MVs found in cancer patients are shed by cancer cells, the majority of shed MVs being secreted by erythrocytes, lymphocytes, macrophages and activated platelets (Mathivanan and Simpson 2010). Owing to the rich biomolecular composition of MVs and their ability to interact with other cells, MVs play a functional role in many pathophysiological processes. Different cancer cell populations already exchange EVs between them as reported by Al-Nedawi *et al*. (2008). Microvesicles can enhance migration of cancer cells, due to the vesicles bearing MMP2 and 9, which digest the extracellular matrix causing tumour cells to migrate to distant sites resulting in tumour metastasis (Aga *et al*, 2014). The ability of extracellular vesicles to induce epithelial mesenchymal

transition (EMT) was confirmed by showing reduction in the expression of E-cadherin by the transformed cancer cells (Aga *et al*, 2014).

1.10 Epithelial Mesenchymal Transition

1.10.1 Definition

Epithelial mesenchymal transition (EMT) is a normal physiological process that plays a vital role in the formation of tissues and organs during development. An additional key role of EMT is wound healing and tissue regeneration following injury and inflammation (Kalluri and Weinberg, 2009; Thery *et al*., 2009). A research group suggested that EMT contributes to the progression of cancer by promoting invasion and metastasis of neoplastic cells (Thery, 2002). Because of the link between EMT and cancer metastasis, there is a vast interest in elucidating the factors involved in the regulation of EMT, particularly in the field of extracellular vesicles.

The process of EMT involves the conversion of tightly packed epithelial cells to mesenchymal cells and some of the main characteristics of EMT include loss of cell-to-cell junctions, changes in cell morphology, enhanced cell matrix adhesion and dramatic remodelling of the cytoskeleton (Yilmaz and Christofori, 2009; Lamouille *et al*., 2014). Some transcription factors are expressed on cells during EMT including Snail, Twist and Zeb that promote downregulation of E-cadherin, an epithelial marker, and upregulation of vimentin, a mesenchymal marker (Kalluri and Weinberg, 2009). These changes promote cell migration and invasion into the extracellular environment and hence cancer metastasis.

EMT can be initiated by a variety of factors the main one being Tumour Growth Factor β. TGF β activates a variety of intracellular signalling pathways within cells

that regulate cytoskeletal organization and signalling and ultimately the progression of EMT. Activation of Smads, RhoA/ROCK, p38MAPK, and ERK regulates cytoskeletal reorganization and the expression of cytoskeletal associated proteins. Changes in the cytoskeleton can then impact cell shape, contractility, and motility.

There are three different types of epithelial to mesenchymal transition processes in the human body, but the end result for all is the generation of motile cells of a mesenchymal phenotype. However, the mechanisms for EMT induction and progression vary largely from one type to the other.

Type 1: EMT during embryogenesis

EMT takes place in the earliest stages of embryogenesis such as the implantation of the embryo and the initiation of placenta formation (Vicovac and Aplin 1996). Once the epithelial cells generate EMT these then become capable of undergoing a reverse EMT known as MET (mesenchymal to epithelial transition) which generates secondary epithelia such as the formation of placenta mentioned above and the three germ layers during embryogenesis (Kalluri and Weinberg 2009). During embryonic development the epithelial cells of the neuroectoderm transformed by EMT gives rise to migratory neural crest cells which disperse to the different parts of the embryo undergoing further differentiation into different cell types, one of them being the melanocytes that provide pigment to the skin (Duband and Theiry 1982).

1.10.2 Type 2: EMT during tissue regeneration

During wound healing EMT naturally occurs to generate new tissue as a response to inflammation. EMT then ceases once the wound is healed. However, EMT can respond to ongoing inflammation eventually causing organ destruction in the setting

of organ fibrosis (Kalluri and Weinberg 2009). Organ fibrosis occurs in a number of epithelial tissues which is mediated by inflammatory cells and fibroblasts that release many inflammatory signals initiating EMT (Okada *et al,* 1997). The research group also found that epithelial cells that had undergone EMT gave rise to important precursors of fibroblasts during organ fibrosis (Okada *et al*, 1997).

1.10.3 Type 3: EMT during cancer metastasis

The third type of EMT differs from type 1 and 2, in that it occurs in epithelial cancer cells. It is estimated that more than 80% of tumours are of epithelial carcinomas. During EMT the transformed cells are capable of invading and metastasising to other locations via the human circulation. The invasion of transformed cells starts initially by invasion through the basement membrane, leading to metastatic dissemination and localising tumours elsewhere in the body.

Although much is known about the signalling pathways involved in type 1 and type 2 EMT (Skromne and Stern 2001) and (Vicovac and Aplin 1996), it is still unclear what specific signals induce type 3 EMT in epithelial carcinoma cells.

In summary, EMT occurs in three distinct biological processes and the main purpose of EMT in all three processes is to generate motile cells of mesenchymal phenotype.

1.10.4 Molecular mechanisms of EMT

The induction of EMT involves activation of transcription factors, expression of specific cell surface proteins, expression and recognition of skeletal proteins, changes in the expression of microRNA and degrading enzymes. Different assays are used to

assess the progression of metastasis in patients, such as loss of cell adhesion and cytoskeletal rearrangement.

1.10.5 Cell surface markers of EMT

E-cadherin is an adhesion molecule present in epithelial cells, the down regulation of which is a prototypical epithelial cell marker of EMT in embryonic development, tissue fibrosis and cancer.

1.10.6 Cytoskeletal markers of EMT

The intermediate filament vimentin is a controversial marker of EMT. It is expressed in various cells including, fibroblasts, endothelial cells, cells of the haematopoietic lineages, and glial cells. During embryonic development in mouse, vimentin is expressed, and it is used as a marker for type 1 EMT specifically during gastrulation. However, because adult epithelial cells transiently express vimentin in response to various insults, vimentin should not be considered a marker of type 2 EMT in fibrosis. Based on the positive correlation of vimentin expression with increased invasiveness and metastasis, vimentin is commonly used to identify cells undergoing Type3 EMT (Zeisberg and Neilson, 2009).

1.10.7 Transcription factors of EMT

It has been shown that several transcription factors induce EMT. Transcription factor proteins such as Snail1, Slug, Zeb and Twist bind to the promoter region of genes associated with cell- cell adhesion and as result repress their transcription, thereby initiating EMT. Due to the involvement of many transcription factors in EMT initiation, it is important to regulate them by allowing for multiple layers of control.

Evolving evidence suggests that many of the transcription factors involved in EMT induction act synergistically with one another and use common pathways. However, inhibiting a single transcription factor is sufficient to block MET, also shown in a recent study (Gonzalez and Medici, 2014).

1.11 Prostate cancer and EMT

Prostate cells are epithelial cells, and as they undergo EMT during prostate cancer progression the stroma is forced to undergo structural rearrangement in order to accommodate the tumour cell. As a result, the stroma is not only responsible for assisting in EMT but also contributes to tumour vascularization (Rennebeck, 2005).

1.12 Prostate cancer and E-cadherin

Mammalian organs are abundant with epithelial tissues, and around 80% of carcinomas being of epithelial origin. One of the key differences between a normal epithelial and a mesenchymal epithelial tissue is loss of cell-to-cell adhesion. E-cadherin plays a significant role in the maintenance of intercellular adhesion and cell polarity. In epithelial cancer cells, E-cadherin expression is downregulated. The actual molecular mechanism is not yet known.

1.13 Prostate cancer and Vimentin

Vimentin is a well-established marker for Type 3 EMT in cancers, based on the positive correlation of vimentin expression with increased invasiveness and metastasis (Burch et al., 2013). Vimentin is highly expressed in cells undergoing EMT particularly in highly aggressive types of prostate cancer (PC3-M and PC3-1E8).

EMT progression involves many signalling pathways that could be targeted clinically. However, the use of MVs as therapeutics especially MVs derived from stem cells remains the most efficient and least invasive (Lindoso et al., 2015). As this is a new emerging, and sometimes contradicting field a lot of research needs to be done to understand the exact mechanism of MV interaction, uptake and subsequent effect on the recipient cells such as PC3 and PNT2.

1.14 Methods used to isolate and determine MVs

There are two main processes used by researchers in the field of EVs for the detection of MVs. These are the nanoparticle tracking analysis NTA using NanoSight and flow cytometric analysis using the flow cytometer. The NanoSight measure measures the MVs particles based on the light scattering properties of the particles within a fluid medium. All particles including the MVs in a fluid are subject to Brownian motion, when illuminated by a laser, suspended particles appearing as bright spots under a microscope. The machine is automated to track the Brownian movement of the bright spots through time lapse video frames (D'Souza- Schorey and Clancy, 2012). This method of analysis is used to size and enumerate exosomes as well as MVs.

Flow cytometers are the standard method for MV sorting and counting and used by many researchers in the EV field. MVs are detected based on both physical characteristics (size) and affinity (using fluorescently conjugated antibodies). The distribution of the MVs is determined by a combination of both forward and side scatter measurements. Polystyrene beads with defined size are used to calibrate the equipment and the experimental population is gated in the analysis. The only drawback is that the flow cytometer only detects MVs bigger than 0.2 µm. It is a

concern for many researchers, as exosomes and smaller MVs that are smaller than 0.2 μm cannot be analysed directly by this method (D'Souza- Schorey and Clancy, 2012) unless fluorescently labelled antibodies are used.

1.15 Proteomics

Proteomics is the analysis of proteins in biological samples. The word Proteomics made its first appearance in 1997 much like the genome. However, in 1986 Mark Wilkins invented the term proteome by combining protein and genome (Gupta *et al*, 2015). The analysis of proteomics involves a set of analytical techniques to analyse the proteins. The term "proteome" refers to all the proteins present in a given cell at a specific time (Delmotte *et al*, 2010). As the dynamics and metabolic activity of the cell changes, the proteome of that cell changes too. Proteomics is used to investigate; expressed proteins' location and time, the rates of protein production and degradation, the movement of proteins between cell compartments, involvement of proteins in metabolic pathways and protein-protein interaction (Gupta et al, 2015).

1.15.1 Techniques used in proteomics

Proteomics is based on techniques used to fractionate and separate large number of cells or tissue derived proteins that are analysed by mass spectrometry. After the proteins have been analysed with mass spectrometry, identification and characterization of the protein is necessary to help analyse the proteins.

1.15.2 Sample preparation

Sample preparation is dependent on the type of sample to be analysed and the analysis method used to analyse the sample. For example, preparation procedures are different when preparing tissue samples compared to cells sample. As clinical or vivo tissue samples require mechanical or chemical breakdown to access and solubilise

proteins. Extra care is taken when preparing a heterogeneous tissue sample, where isolation of homogenous tissue samples is required, particularly when tissues are contaminated with blood serum the high abundant protein such as albumin can mask detection of lower abundant proteins (Liu et al, 2011).

Although, for cultured cell, sample preparation is less of a concern from homogeneity point of view however, obtaining high concentrations of protein for analysis can be quite challenging. Experimental triplicates as well as biological triplicates samples can be used to ensure high concentrations of proteins for analysis. Different methods are used to achieve homogenous and solubilised sample. Sonication and Sodium Dodecyl sulphate (SDS) are commonly used to disrupt the cell membrane and solubilise proteins (Xu and Keiderling 2004). Other solvents such as urea and (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) (CHAPS) Detergent is used to solubilise proteins (Fountoulakis and Takacs 2008).

1.15.3 Sample separation

Sample preparation involves separation of the very large number of proteins in cell or tissues prior to analysis on mass spectrometry. However, the challenge remains in that the protein identifications are often masked by the most abundant proteins in the sample. To avoid abundant proteins masking the proteins present in small concentrations, one or a combination of these three different sample separation methods are used (Gupta *et al*, 2015). Separation methods such as; one and two dimensional gel electrophoresis, one or two dimensional chromatographic separation or ion exchange and reversed phase resins can be used prior to mass spectrometry (Van den Bergh and Arkens, 2004).

1.15.4 Mass spectrometry

Mass spectrometry is a modern technique used to identify the proteoforms (proteins and their biologically modified variants) from limited biological samples. This technique relies on determining the mass to charge (m/z) ratios of ionized chemicals. Quantitative proteomics studies provide absolute or relative quantification of protein amounts in the sample analysed. This is particularly useful in studying the proteome response to stimuli and can be comparable to the healthy control. There are now two broad groups of quantitative proteomic analysis; the label free and label-based analysis. Label free does not require additional sample treatment, the quantification is derived from spectral counts or intensity of a recorded counts. However, label-based analysis requires chemical labelling of analysed proteins that can be used to quantify the unknown proteins.

1.15.5 LC-MS/MS

LC-MS/MS is a technique that combines the separating power of High-Performance Liquid Chromatography (HPLC) and the detection power of mass spectrometry. Generally, liquid chromatography starts by pumping mobile phase from the solvent reservoir. The sample is injected into the autosampler through the sample loop. The solvent in the system pushes the sample from the injection port into the analytical column where analytes are separated by chromatography. The analytical column outlet is connected to the ionisation source, where the ions are produced and then detected using MS (Downard, K. 2006).

The Mass spectrometer device is made of five components, an ion source, vacuum system, mass analyser, detector and computer system for acquiring the digitalised

data. In this thesis, the sample from liquid chromatography was sprayed into the mass spectrometer by Easy-Spray source (Thermo Fisher Scientific Inc.). Orbitrap mass analyser was set to 70000 in resolution and was scanned between 380-1500 to measure m/z values. The results were uploaded on to scaffold software for data analysis.

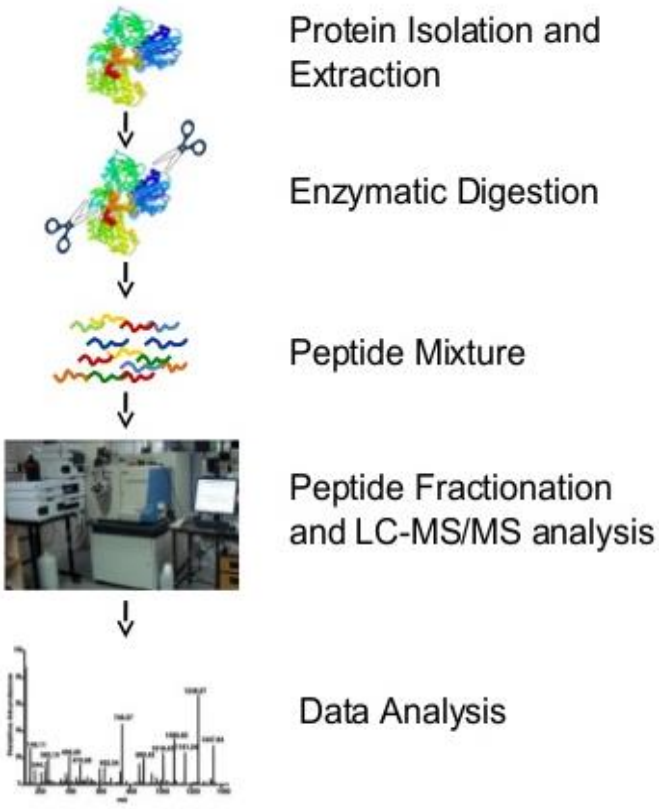


Figure 2: Mass Spectrometry based proteomics workflow.

1.16 Pathway analysis

Proteomics and other "omics" studies produces large amounts of data that is identified with the samples analysed. These large lists of proteins identified are then to be contextualised, by using pathway analysis tools. The proteins are searched and mapped on to the databases already available for genes and proteins and their relationships to each other. Different pathway tools determines different relationship between proteins.

Due to this data variability in proteomics measurements Wu and the team believe that statistical significance alone is not sufficient to evaluate proteomics results (Wu *et al*, 2014). They consider that both functional information and topological information for each pathway models should be integrated to relate the proteomics data to relevant biological mechanisms (Wu *et al*, 2014). There were four analysis tools that were used in this thesis; DAVID, PANTHER, KEGG and STRING. Before using these analysis tools, list of proteins was pasted on to UniProt obtaining accession number and gene name.

1.17 Proteomics in the Extracellular Vesicles field

Proteomics studies have been widely used in the field of EVs to investigate the protein cargo in EVs. The first such proteomic analysis was carried out by Thery and co-workers in 1999, who analysed the most abundant proteins in EVs (Thery *et al*, 1999). Over the years improvements have been made in proteomic techniques and researchers have been interested in identifying proteins in EVs from cells and various body fluids including urine, semen, plasma, saliva and breast milk, which helps in identifying potential biomarkers for diseases.

Also, studying EVs proteomics together with bioinformatics analysis can help with understanding EV biogenesis, formation, assembly and trafficking of EV cargo. There are now several publicly available internet databases developed to gather knowledge obtained from proteomics studies and make it accessible to many users globally. Exocarta was first established to collect protein and RNA data on exosomes (Simpson *et al*, 2012), later vesiclepedia was developed when several research groups provided proteomics analysis on other complex EVs such as MVs (Kalra *et al*, 2012).

1.18 Aim

The aim of this study was to elucidate whether PC3 derived Microvesicles carry bioactive molecules such as proteins, mRNA, miRNA or RNAs, which are able to induce an epithelial to mesenchymal transition (EMT) in normal prostate cell line (PNT2). The fundamental elements of EMT includes reduction of cell-cell adherence, delocalisation of adherent junction and tight junction (Nauseef and Henry 2011). However, to understand the mechanism by which MVs induce EMT and the factors carried by MVs aiding the process of EMT, further tests including immunohistochemistry, angiogenesis and Western blotting as well as proteomic analyses will be considered.

In this thesis, the aim was to investigate MV interaction with recipient cells and to understand the effect of various physical parameters including temperature and pH levels on these interactions. Upon establishing factors affecting the interaction between MVs and recipient cells, MVs would then be further studied for their vital role in EMT; biomarkers and morphological characteristics of the process of EMT will be investigated.

After the immortal (PNT2) cells have been transformed into (tPNT2) via PC3 MVs, the aim was to study the proteome of MVs to look at the differentially expressed proteins that play important role(s) in this transition process.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Cell lines used

Human Prostate Cancer cell line (PC3) and normal benign prostate epithelial cell line (PNT2) was used throughout the experiments. PC3 microvesicles (MVs) were isolated from the PC3 cell line.

2.1.2 Cellular Growth Medium (CGM)

Cellular growth medium was made by supplementing RPMI 1640 with 10% FBS to cultivate PC3 and PNT2 cells. It was always stored at 4°C. CGM used for experiments was centrifuged to remove the MVs from the FBS that was added. After the centrifugation the CGM was also filtered with 0.2µm filter to remove MVs.

2.1.3 Experimental buffers and solutions

Cell freezing medium

20 %	FBS
10 %	DMSO
70 %	RPMI

Lysis Buffer pH 7.4

100 mM	HEPES-KOH
2 mM	$CaCl_2$
0.2 %	Triton X-100 (v/v)
	Protease inhibitor (AEBSF)
	Millipore water

(X4) SDS Sample Buffer

200 mM	Tris-HCl
25 %	Glycine (w/v)
2 %	SDS (w/v)
0.2 %	Bromophenol blue (w/v)
20 mM	DTT (added fresh on the day of use)
	Millipore water

(1.5 M) Resolving Buffer pH 8.8

18.17 g Tris base

Dissolved in 100 ml deionised water and pH adjusted to 8.8

(0.5 M) Stacking Buffer pH 6.8

6.06 g Tris base

Dissolved in 100 ml deionised water and pH adjusted to 6.8

Resolving Gel Solution (12%)

2 ml	ddH_2O
1.25 ml	1.5M Tris-HCl (PH 8.8)
0.050 ml	10% SDS (w/v)
1.66 ml	Acrylamide/Bis 30% (w/v)
0.025 ml	10% APS (w/v)
0.0025 ml	TEMED

Stacking Gel Solution

1.53 ml	ddH_2O
0.625 ml	0.5M Tris-HCl (PH 6.8)
0.025 ml	10% SDS (w/v)
0.335 ml	Acrylamide/Bis 30% (w/v)
0.0125 ml	10% APS (w/v)
0.0025 ml	TEMED

Electrophoresis Running Buffer (1L)

30 g	Tris-HCl (PH 8.3)
144 g	Glycine
50 ml	20% SDS (w/v)
950 ml	ddH_2O

Coomassie Brilliant Blue G-250

0.025 %	Coomassie Blue (w/v)
10 %	Acetic acid (v/v)
90 %	ddH2O (v/v)

Mixed and filtered (Whatman number 1 paper)

De-Staining Solution (500 ml)

35 ml	Acetic acid
25 ml	Methanol
440 ml	ddH_2O

Transfer Buffer (10X)

250 mM	Tris base
1925 mM	Glycine
500 ml	ddH_2O

1.17.1.1 Sartoblot buffer (500 ml)

40 ml	Transfer buffer (1X)
100 ml	Methanol
360 ml	ddH_2O

Ponceau Solution

0.25 %	Ponceau S (w/v)
3 %	Trichloroacetic acid (v/v)
	ddH_2O

Phosphate Buffer Saline (PBS) Solution - 1L

140 mM	NaCl
2.7 mM	KCl
10 mM	Na_2HPO_4
1.8 mM	KH_2PO_4
	ddH_2O

Phosphate Buffer Saline – Tween 20 (PBS-T)

1 L	PBS
1 ml	Tween 20

Blocking Buffer

5 %	Milk powder (w/v)
100 ml	PBS-T

Antibody Dilution Buffer (WB)

3 %	Milk powder
100 ml	PBS-T

Permeabilisation Buffer (PB)

0.5 %	Tween 20 (w/v)
	PBS

Immunofluorescence Antibody Dilution Buffers

3 %	Milk powder (w/v)
100 ml	PBS-T

Primary and Secondary Antibody Dilution Buffer

3 %	BSA (w/v)
	PBS

Flow Cytometry Analysis Buffer

3 %	BSA (w/v)
1 %	NaN_3 (w/v)

PBS Annexin V Binding Buffer pH 7.4

10 mM	HEPES/NaOH
140 mM	NaCl
2.5 mM	$CaCl_2$

All components dissolved in 100ml of deionised water

2.1.4 Reagents

Annexin V Alexa Fluor 488	Invitrogen
Annexin V reagent	R&D Systems
BCA protein assay kit	Fisher Scientific
BSA (Bovine Serum Albumin)	Sigma- Aldrich
BzATP	Sigma -Aldrich
Calcium Chloride	Sigma- Aldrick
DAPI- VECTASHIELD	Vector Laboratories Inc, CA, USA
DMSO (Dimethyl sulfoxide)	Sigma- Aldrich
Ethanol	Fisher Scientific
FBS (Foetal Bovine Serum)	Fisher Scientific
Guava Nexin reagent (100 tests)	Guava Technologies, UK
Guava ViaCount reagent (100 tests)	Guava technologies, UK
Halt Protease Cocktail	Pierce, Thermo- Scientific
HCL (Hydrochloric acid)	Fisher Scientific
Kenacycin	Sigma- Aldrich
Magnesium chloride	Sigma _ Aldrich
Methanol	Fisher Scientific
Paraformaldehyde	Agar Scientific
PBS (Phosphate Buffer Saline)	Sigma- Aldrich
Penicillin/ Streptomycin	Fisher Scientific
Potassium Chloride	Sigma- Aldrich
Propidium iodide	Sigma- Aldrich
RPMI	Sigma- Aldrich
Sodium Azide	Sigma- Aldrich
Sodium Chloride	Sigma- Aldrich
Sodium Hydroxide	Sigma- Aldrich
Trichloracetic acid	Fisher- Scientific
Tris base	Sigma- Aldrich
Triton X-100	Sigma- Aldrich
Trypsin/ EDTA	Sigma- Aldrich

2.1.5 Technical devices

Cell culture flasks (75cm^2)	Fisher Scientific
Centrifuge 5804 R	Eppendorf
Centrifuge 5810 R	Eppendorf
F-20 micron rotor	Sorvall
Fluorescence microscope (1X81)	Guava Technologies, UK
FLUOstar Omega plate reader	Olympus Corporation, Germany
Gel loading tips	Corning
Guava EasyCyte 8HT cytometer	Millipore
Incubator Heraeus CO2- Auto- Zero	BMG Labtech, UK
Microcentrifuge 5417 R	Thermo Electron Corporation
Microplate (12- well)	Sigma Aldrich
Microplate (24- well)	Sigma Aldrich
Microplate (96- well)	Sigma Aldrich
PH- Meter 766 Calimatic	Jenway
Roto- Shake Genie	Denley
SE-12 rotor	Sorvall
Semi- dry transfer system	BioRad
Small volume tips	Sigmal Aldrich
Sorvall T-865 rotor	Sorvall
Sorvall ultracentrifuge RC6	Thermo electron Corporation

2.1.6 Antibodies

Anti- Annexin V AlexaFlour 488	Biosciences
Anti- E- cadherin	Cell Signalling Technology
Anti- rabbit IgG	Cell Signalling Technology
Anti- Vimentin	Cell Signalling Technology
Mouse anti- IgG AlexaFlour 488	Biosciences
Mouse anti- IgG FITC	Abcam
Mouse anti Vimentin	Abcam
Rabbit IgG anti-Human FITC	Abcam

2.2 Methods

2.2.1 Cell culture

Human prostate cancer cells and epithelial non-cancer cells were used in experiments in this thesis.

Adherent cells such as PC3 and PNT2 were maintained in growth medium containing RPMI and 10% FBS at 37°C with 5% CO_2 atmospheric conditions. Cells were split according to confluency every 3 to 5 days. The supernatant was collected for MVs isolation and was kept at -80°C and the cells were detached from the surface of the T75 flask by adding 3-4ml of 0.25% (v/v) trypsin EDTA. The T75 flask was incubated at 37°C for 5-10 minutes. The flask was tapped gently to detach all the cells and 5ml of complete growth medium (CGM) was added to inactivate trypsin. The detached cells were then transferred into a 15ml centrifuge tube and centrifuged at 200*g* for 5 minutes to remove the trypsin. Cells were further washed twice with PBS also by centrifuging at 200*g* for 5mins. The resulting cell pellet was dissolved by flicking the end of the tube to disrupt the pellet which was then resuspended in complete growth medium. The cell mixture was divided into new T75 flasks depending on the desired dilutions and cell density.

The number of cells and viability were determined before every experiment with the flow cytometer (ViaCount assay, Guava Technology) and occasionally with the haemocytometer. Cells with viability of 95% or higher were used in every experiment.

2.2.1.1 PC3 cell line

PC3 cell line is the most metastatic of all the prostate cancer cell lines. It was established in 1979, from a 62-year-old Caucasian male who had developed a Grade IV bone metastasis from prostate cancer (Jones *et* al, 1979).

2.2.1.2 PNT2 (non-cancer) cell line

PNT2 cells are normal prostate epithelial cells that were obtained from the prostate of a 33-year-old male at post mortem. The cells were immortalised by transfection with a plasmid containing SV40 genome with a defective origin of replication. The cells have been tested in nude mice and are non-tumorigenic (Sigma- Aldridge 2018).

2.2.1.3 Cryopreservation of eukaryotic cells

For long-term storage, cells were kept at -80°C in liquid nitrogen. Non-adherent, 98% confluent cells were transferred into a 15ml tube and centrifuged at 160*g* for 5 minutes. The supernatant was discarded, and cell pellet was washed twice with PBS. The cell pellet was dissolved and resuspended in PBS. The cells density was determined using Guava ViaCount as mentioned above. Freezing medium was prepared by adding 10% DMSO to CGM and was placed on ice. PC3/PNT2 (1x10) cells were added to 1ml of freezing medium and the cells were then transferred into labelled cryovials and were placed on ice immediately. The cryovials were kept at -80°C overnight in a polystyrene box to ensure a temperature decrease of 1°C per minute. For a long-term storage cells were plunged into and then stored in a liquid nitrogen tank.

Adherent cells were frozen following the same method however, adherent cells were detached first with trypsin and were washed twice with PBS. The same procedure was followed as with the non-adherent cell line.

2.2.1.4 Thawing cells

Cryovials were removed from liquid nitrogen and thawed for one minute in a 37°C water bath. After cleaning the cryovial thoroughly with Bioguard the cells were transferred into a 15ml tube containing 9ml of pre-warmed CGM. Cells were mixed and centrifuged for 5 minutes to remove the DMSO. The centrifugation speed (~160*g*) was determined depending on the cell line. After centrifugation the supernatant with the DMSO was discarded and the pellet was resuspended in pre-warmed complete growth medium. The cells were transferred into a T75 culture flask and incubated at 37°C for 24 hours. After 24 hours cells were detached with Trypsin/EDTA if adherent and were washed with PBS twice to further remove the DMSO. This time the cells were seeded into a new flask according to the dilution and density desired.

2.2.2 Extracellular vesicles

2.2.2.1 Isolation of MVs from conditioned cell medium

Non-adherent cell culture supernatant was collected after centrifuging cells and the supernatant at 160*g* for 5 minutes. The adherent cell supernatant was removed from the T75 flask by serological pipette while the cells were still attached to the flask. The collected supernatant from any cell type was centrifuged at 4000*g* for 60 minutes

at 4°C to remove cell debris. The resultant supernatant was ultracentrifuged at 15,000*g* for 2 hours at 4°C. The MV pellet was resuspended in sterile MV-free PBS. The sample was quantified by Guava EasyCyte flow cytometer using ExpressPlus software (Guava Technologies). Isolated MVs were always kept until they were used for an experiment on the day or they were stored at -80°C for later experiments.

2.2.2.2 Detecting PS-positive MVs

MVs isolated from PC3 cells were quantified by flow cytometry using the GuavaEasyCyte flow cytometer and were resuspended in 50µl Annexin binding buffer and 5µl FITC-Alexa Fluor 488 was added or not (control) in to the sample(s). The sample was incubated at room temperature for 30 minutes with shaking. Precisely, 150µl of the binding buffer was added to the sample and was centrifuged at 25,000*g* for 2 hours to pellet MVs and wash off any unbound Annexin-V and the buffer. Samples were analysed immediately as directed by the manufacturer using the Guava EasyCyte flow cytometer.

2.2.3 Sample collection and analysis

2.2.3.1 Protein quantification

The protein concentration in MVs samples was determined using the BCA Protein Assay Kit. It uses a combination of the biuret reaction (in which the chelation of copper with protein forms a light blue complex in an alkaline environment) and the colour development reaction (bicinchoninic acid (BCA) which reacts with the reduced (cuprous) cation formed earlier). Samples were diluted with PBS (PBS;

dilution 1:5). A series of dilution standards was prepared using 2000 µg/ml BSA stock solution. Applied concentrations were, 0, 125, 250, 500, 750, 1000, 1500, 2000 µg/ml BSA. Following the manufacturer's instructions working reagent was prepared by mixing reagent A with reagent B in a ratio of 50:1 respectively. Accurately, 10 µl of standards and samples were added each on to a well on a 96 well plate. Next, 200 µl of working reagent was added to each standard and sample. The 96 well plate was incubated at 37°C for 30 minutes. After incubation, the plate was left at room temperature for 5 minutes before measuring the absorbance on a FLUOstar Omega micro plate reader at 562 nm.

2.2.3.2 Flow cytometry

The Guava flow cytometer GuavaEasyCyte 8HT is able to perform several complex immunological studies such as cell counting and viability, cytokine analysis, cell surface marker analysis. The ViaCcount assay was used for cell counting and viability. Express plus was used for cell markers and protein analysis.

2.2.3.3 Cell treatment prior to EMT

PNT2 cells were seeded into 24 well plates and incubated overnight prior to performing the EMT experiment. The cells were washed twice with MV-free filtered PBS to remove dead cells and any supernatant left in the wells. Exactly, 5µg of PC3 MVs was added to each well with semiconfluent PNT2 cells, except for the control. Each well had 1ml of MV- free and 0.2µm filtered CGM.

2.2.3.4 Staining PNT2 cells for EMT markers

After five days of incubation, supernatant was removed, cells were washed with PBS and then treated with trypsin. The cells were centrifuged at 200*g* for 5 minutes and were washed three times with PBS. Cell count and viability was determined using ViaCount reagent. Cells were fixed with 4% paraformaldehyde for 15 minutes and were washed three times before they are being transferred to 1.5ml Eppendorf tubes and being clearly labelled.

When labelling with E-cadherin, PNT2 cells (1 $x10^6$) cells were resuspended in ice cold antibody buffer (PBS containing 10% FBS and 1% NaN_3) and 2µg/million cells of primary antibody was added to the cells with thorough mixing. Cells were incubated at 4°C for 1 hour with shaking. After incubation, cells were washed three times with ice cold PBS at 400*g* for 5 minutes for each wash. When labelling cells with Vimentin, PNT2 cells were permeabilised first with 200 µl of 0.1% Triton X-100 in PBS for 20 minutes.

Cells were resuspended in 200 µl of blocking buffer for 1 hour. PNT2 cells were then resuspended in 200 µl of PBS to which a dilution of 1:200 of the secondary antibody (IgG-FITC) was added. The cells were incubated in the dark at 4°C with shaking for 1 hour. Cells were then washed after 1 hour with PBS three times before being resuspended in 200 µl antibody buffer ready for analysis on ExpressPlus.

Cells were stained with the isotype-matched controls (anti-mouse or anti-rabbit IgG-FITC, Abcam).

2.2.3.5 Preparing cells for fluorescent microscopy

Coverslips were inserted inside 24 well plates and poly -L- Lysine was applied to the cover slips. They were left to dry completely at room temperature. After five days of the experiment, supernatant was removed, and the cells were washed with PBS three times. The cells were then fixed with 4% paraformaldehyde at 37°C for 15 minutes. Coverslips were gently washed with ice cold PBS, for internal proteins, and cells on coverslips were permeabilised with 0.1% Triton-X-100 for 20 mins at room temperature. The coverslips were washed three times with ice-cold PBS before labelling with primary and secondary antibodies. Finally, the coverslips were removed and mounted on microscope slides with DAPI-VECTASHIELD medium. Images were taken with an Olympus IX81 inverted fluorescent microscope using an attached monochromatic camera. After acquisition, images were then coloured using imaging software Cell^M (from the Olympus Corporation).

2.2.3.6 Preparation of cell lysates

As much as (1 X 10^6) PNT2 cells were grown in culture flasks. Supernatant was removed and cells in the flask were treated with Trypsin/EDTA. After 5 minutes incubation at 37°C, cells were collected in a 15ml centrifuge tube and the cells pelleted by centrifugation at 9200*g*, 5 min at 21°C). Cells were washed by careful resuspension in PBS and re-centrifugation. The cell count was determined using a haemocytometer and cell lysis was then carried out. Depending on the protein of interest and its cellular location, the cells were lysed accordingly. Briefly, cell lysis was performed by resuspending the cell pellet obtained after the wash in 0.2% (w/v) Trion X-100 containing Protease inhibitor cocktail. For membrane protein

solubilisation, samples were thoroughly pipetted, and insoluble materials were pelleted by centrifugation at (10,000 rpm, 5min at 4°C, using 5810R centrifuge, with A-4- 62 swing out rotor, Eppendorf). Total protein concentration was determined using BCA kit according to procedure described under section 2.2.3.1.

2.2.3.7 Sample preparation for SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS sample buffer (4X) was added to samples in a ratio of 1:4 followed by 4 minutes incubation at 95°C. Prior to loading the samples on to the gel the samples were centrifuged to collect all the liquid at the bottom of the reaction tube.

2.2.3.8 SDS-PAGE protein molecular weight standards

Prestained protein molecular weight marker (BioRad) was used as a standard. 10µl of 2 to 212 kD or 10 to 250 kD prestained protein markers were loaded on to the gel as a standard marker. ECL detection system was used to visualise the bands on the gel.

2.2.3.9 SDS- Polyacrylamide Gel Electrophoresis

SDS- Polyacrylamide Gel Electrophoresis was used to separate the denatured proteins, according to their molecular masses, and was performed as described (Laemmli, 1970) using the Mini PROTEAN III Electrophoresis System (Bio-Rad). Gels with dimensions of 102 x 73 mm and a thickness of 0.75 mm were cast between two glass plates by pouring freshly prepared 12% separating gel solution containing acrylamide/bisacrylamide into the gel cassette fixed in a casting frame. Un-

polymerized separating gel solution was overlaid with H_2O-saturated butanol to achieve an even surface. After polymerization, H_2O-saturated butanol was poured off, washed twice with deionized water, and the excess water was blotted using a filter paper (Whatman 3 MM, Whatman AG). Freshly prepared stacking gel was poured into the gel cassette and a plastic comb was inserted from the top, to form the loading wells in the stacking gel. After polymerization, the gels were used immediately. For electrophoresis, the gel was placed into the electrode assembly device inside a clamping frame in the tank of the Mini PROTEAN III system.

Electrophoresis running buffer was added to the inner and outer chambers of the tank and the plastic comb was carefully removed. Wells were washed with the running buffer to remove any free unpolymerised acrylamide/bisacrylamide. Samples were loaded into the wells created in the stacking gel using extra-long loading pipette tips. Electrophoretic separation was performed at 150 V (constant voltage) until the bromophenol blue front of the SDS sample buffer reached the end of the resolving gel. Gels were either stained with Coomassie Brilliant Blue R250 or transferred onto nitrocellulose membrane for Western blot analysis.

2.2.3.10 Western blot analysis

Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane for further protein analysis. The semi-dry transfer method was always used (Bio-Rad Sartoblot system). A Hybond C nitrocellulose membrane, two pieces of thin blotting paper and two pieces of thick blotting paper (Whatman, BioRad) were cut to the size of the separating gel. The blotting papers and nitrocellulose papers were immersed in Sartoblot buffer before assembling the sandwich. To transfer proteins from the gel

on to the nitrocellulose membrane, a sandwich was made by placing the two blotting papers on either side, nitrocellulose membrane close to the cathode plate on the semi-transfer devise and the gel on top. The air bubbles were removed by simply rolling an empty 15 ml Eppendorf tube sideways. The anode plate was placed on top of the sandwich and the current was applied for 1hr 10mins at 15V (constant voltage). When the current was applied the negatively charged proteins were transferred on to the membrane.

2.2.3.11 Reversible Ponceau staining of protein

To ensure successful protein transfer on to the hybond C nitrocellulose membrane, the membranes were incubated with Ponceau S (Serva Electrophoresis GmbH) solution for 1 min. Excessive Ponceau solution was removed by washing the membranes with deionized water until the protein bands were visible. Membranes were labelled and cut when necessary for labelling with different primary antibody.

2.2.3.12 Immunochemical protein detection using the ECL System

In order to visualize the protein bands on the nitrocellulose membrane, the enhanced chemiluminescence reagent system (ECL, Amersham Pharmacia) was used. The membrane was first incubated in blocking buffer for 1 hr at room temperature on a shaker. Following blocking, membrane was rinsed with PBS-T and was incubated in 1:1000 dilution of primary antibody over night at 4^0 C shaking. Unbound primary antibody was washed three times for 5 mins each with PBS-T and the membrane was incubated with Goat IgG secondary antibody HRP conjugated at 1:5000 dilution for 1 hr. The unbound secondary antibody was removed by washing the membrane with

TBS-T six times for 5 mins each. The Last wash was performed using PBS without the Tween to avoid background noise. The ECL solutions A and B were mixed in a 1:1 ratio and the membrane was incubated with the solution for 1 min at room temperature. The chemiluminescence was detected using UVP ChemiDoc-It system (UVP systems, UK).

2.2.3.13 Cell count and viability

Cells number and viability were determined using ViaCount assay, which differentiates between viable and non-viable cells based on the differential permeability of two DNA binding dyes in the Guava ViaCount reagent. The nuclear dye binds to DNA in all cells, while the viability dye only binds to the DNA in dead cells. The intensity of the viability dye binding distinguishes viable and dead cells. Cell debris is excluded from the results based on negative staining with the nuclear dye.

2.2.3.14 Staining of MVs with PKH26 dye

PKH26 is a fluorescent membrane dye, which has been used as a cell tracer to locate the transplanted cell in host cells for a long time (Li *et al*, 2013). PC3 cells were cultured in T75cm^2 flasks; after 3 or 5 days of incubation supernatant was collected from the flasks for MV isolation. Supernatant was first centrifuged at 4000*g* for 60 mins, 4^0 C, to remove cell debris, the supernatant was removed and was further centrifuged at 25000*g* for 2 hours. The supernatant was discarded and the MVs pellet was re-suspended in sterile filtered PBS. The MVs pellet was washed by further centrifugation at 25000*g* for 2 hours 4^0 C, after resuspending the pellet in 200µl of

sterile PBS. After washing the MVs, the pellet was mixed in 250µl of diluent C and was left on ice. Meanwhile, 2µl of the PKH26 dye was mixed thoroughly with 250µl of diluent C, the dye mixture was added to the MVs pellet and was left to incubate for 5 minutes on ice. 500µl of serum (1% BSA) was added to the mixture to stop the reaction and was left for 1 minute on ice. The MV pellet was centrifuged at 25000*g* for 2 hours at 4°C to remove excess dye. After centrifugation, the MV pellet was resuspended in sterile PBS for washing. Again, MV were washed at 25000*g* for 2hours at 4°C to remove unbound dye. MVs were resuspended in 100µl of sterile, MV- free PBS prior to analysing by flow cytometry.

The number of PKH26 labelled MVs was determined by flow cytometry. 20 MVs per cell was added to resting PC3 cells for MV uptake analysis.

2.2.4 Proteomics

2.2.4.1 Sample preparation

After determining the concentration of the protein in cells and MV lysates, the protein samples were denatured and heated at 95°C for 5 minutes. The samples were mixed with Laemmli sample buffer prior to loading on the gel, samples were loaded on Any-KD Mini-PROTEAN TGX gels (bio-Rad) and were run for approximately 10 minutes just enough for the samples to reach the resolving gel. The bands on the gels were visualised with Coomassie blue. The gels were placed on a light box and the sample bands on the gels were cut carefully with a knife each at approximately 1mm^2. Each gel band was placed in a 96 well PCR plate and was destained, reduced (DTT mention concentration used/how long treatment) and alkylated (iodoacetamide

concentration/treatment length). The samples were digested enzymatically with chymotrypsin overnight at 37^0 C and the supernatant was then pipetted into a sample vial prior to loading onto an autosampler for automated LC-MS/MS analysis.

2.2.4.2 LC-MS/MS analysis

Protein samples were prepared and sent to Cambridge Centre for Proteomics for LC MS/MS analysis. Dionex Ultimate 3000 RSLC nanoUPLC and Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific Inc, Waltham, MA, USA) systems were used in all the LC-MS/MS experiments. The peptides in the samples were separated by reverse-phase nano Easy-spray column (Thermo Scientific PepMap C18, 2µm particle size, 100 Å pore size, 75µm i.d. x 50cm length). The isolated peptides were loaded onto a pre-column (Thermo Scientific PepMap 100 C18, 5µm particle size, 100 Å pore size, 300 µm i.d. x 5mm length) from the Ultimate 3000 autosampler with 0.1% formic acid for 3 minutes at a flow rate of 10µL/min. After this period, the column valve was switched to allow elution of peptides from the pre-column onto the analytical column. Solvent A was water + 0.1% formic acid and solvent B was 80% acetonitrile, 20% water + 0.1% formic acid. The linear gradient employed was 2-40% B in 30 minutes.

The LC eluant was sprayed into the mass spectrometer using the Easy-Spray source (Thermo Fisher Scientific Inc.). All m/z values of eluting ions were measured in an Orbitrap mass analyser, set at a resolution of 70000 and was scanned between m/z 380 – 1500. Data dependant scans were employed to automatically isolate and generate fragment ions by higher energy collisional dissociation (HCD, NCE: 25%) in the HCD collision cell and measurement of the resulting fragment ions was

performed in the Orbitrap analyser, set at a resolution of 17500. Ions that were singly charged and ions with unassigned charge states were excluded from being selected for MS/MS and a dynamic exclusion window of 20 seconds was employed.

2.2.4.3 Database searching

All the MS/MS data was converted to mgf files prior to submitting to the Mascot search algorithm (Matrix Science, London UK). The files were searched against the UniProt human database (71898 sequences, 38274 residues). Variable modification of oxidation (M), deamidation (NQ) and carbamidomethyl were applied. The peptide and fragment mass tolerances were set to 5ppm and 0.1 Da, respectively. A significance threshold value of $p < 0.05$ and a peptide cut-off score of 20 were also applied. Peak areas corresponding to the intact peptides were calculated from the chromatograms within PD. At the end all data was imported into the Scaffold program (Version_4.5.4, Proteome Software Inc, Portland, OR).

2.2.5 Statistical analysis

Statistical analysis was performed by the unpaired *t*-test or one-way ANOVA using GraphPad Prism software, version 5.0 (San Diego, CA, USA). Differences giving a value of $P < 0.05$ with confidence interval of 95% will be considered statistically significant.

2.2.6 Bioinformatics analysis

Gene lists of expressed proteins were generated using publically available datasets such as Uniprot and EVpedia.

2.2.7 Pathway analysis

For analysis of the data in chapter 3 quantitatively and qualitatively, three pathway analysis tools were used.

2.2.7.1 DAVID analysis

DAVID (Database for Annotation Visualisation and Integrated Discovery) (https://david.ncifcrf.gov/home.jsp) is a computational tool where several publicly available gene databases are combined allowing functional and biological patterns for a list of submitted proteins (Huang da *et al*, 2009a and Huang da *et al*, 2009b). Proteins involvement in cellular component, molecular function and biological process are some of the categories that are used in analysing proteins in this thesis.

2.2.7.2 PANTHER analysis

PANTHER (Protein Analysis Through Evolutionary Relationships) (http://pantherdb.org) classifies function related to evolutionary family trees through statistical analysis.

2.2.7.3 KEGG analysis

KEGG (Kyoto Encyclopaedia of Genes and Genomes) (http://www.kegg.jp/) is a database resource for understanding high level roles and functions of the biological system such as cells, organisms and the ecosystem at the molecular level. It has 72,728 genes in the database and it is involved in 472 pathways, these are used to search against submitted list of identifier genes (Kanehisa *et al*, 2016).

2.2.7.4 STRING analysis

STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) (https://string-db.org) is a database that provides protein – protein interactions which facilitates comparative protein analysis. This database also provides mapping and transferring interactions of proteins onto many organisms, facilitating evolutionary studies. STRING database is an online computerised system which allows information to be quickly accessed and it is best used for a quick initial overview of the functional partners of a query protein due to the database's varying confidence scores (von Mering *et al*, 2005).

In preparation of the data for pathway analysis the proteins identified from PC3, PNT2 and tPNT2 cells and its respective MVs were changed to corresponding human gene symbols

Each identified protein had its peptide sequences through UniProt (htttp://www.uniprot.org) to confirm its correct proteins name and obtain accession number. DAVID pathway analysis tools were used to paste a list of protein into the submission window, "OFFICIAL GENE SYMBOL" was selected as identifier, "Gene List" was selected for list type and finally the list of proteins was submitted for analysis. "Homo sapiens" species background was selected. The drop-down menu for "Gene- Ontology" was selected, three different categories of enrichment groupings were provided such as; molecular function (GOTERM- MF), biological process (GOTERM-BP) and cellular component (GOTERM- CC). Bonferroni test was applied to the data and the resulting data was copied into Excel and sorted according to the Bonferroni significance value of $p<0.05$. DAVID also gave the option for KEGG pathway analysis by selecting Pathways and then KEGG-

PATHWAY. Bonferroni significance test was also applied to this pathway. A graphical representation of the protein pathway for each term in the data table was provided. In Chapter 5, Pathways in cancer, integrin interaction and prostate cancer was selected and graphic representation of the proteins on our list was shown.

In PANTHER analysis (pantherdb.org) the list of gene for the proteins was submitted in the window box and Homo sapiens was selected as an organism and statistical overrepresentation test was chosen with default settings. Three categories of annotation were chosen again such as; molecular function, biological process and cellular components, Bonferroni significance of $p<0.05$ was used. The data was transferred to and further sorted in Excel.

In STRING (Search Tool for the Retrieval of Interacting Genes/proteins) protein – protein interaction networks are integrated over the tree of life (http://string-db.org). Analysis of information is gathered based on both known and predicted protein-protein interactions. The interaction between the proteins is predicted computationally based on direct (physical) and indirect (functional) associations. The STRING repository has information on 9 million proteins physical and functional associations across 2000 organisms.

2.2.8 Cell fluorescence intensity and densitometry using Image J

The corrected total cell fluorescence (CTCF) was calculated using image J software from the formula CTCF = Integrated Density – (area of selected cell x mean fluorescence of background readings). For analysis of Western blotting, the relative density of the bands, normalised using the loading control, β-actin, was calculated using image J software.

Chapter 3: Prostate cancer cells derived MVs and their uptake by recipient cells

3.1 Introduction

After skin cancer, Prostate cancer (PCa) is the second most common form of cancer amongst men in Western countries and there has been a significant amount of research carried out on PCa for many years (Hsing and Chokkalingam 2006). Different research groups have been studying different PCa models, which is manifest by the plethora of; established cell lines derived from metastatic human Prostate Cancers, xenograft models and genetically engineered mouse models. However, cell lines have had the longest history and have been most widely used in publications, in particular the PC3 cell line, which is commonly used and was originally derived from bone metastases, it is the most aggressive form of PCa prostate cancer cells (Jones *et al*, 1979).

In relation to MVs and cancer, and their roles as intercellular communicative vectors, very little is known about the mechanism(s) by which MVs are taken up by recipient cells. Different research groups have reported a host of possible routes of entry including such as; MVs direct contact of MVs with the recipient cells via their receptors, or through cleavage of surface proteins, as well as fusion with the recipient cell membrane (fusion of hemi-fusion) and endocytosis (Camussi *et al*, 2010). Due to the heterogenicity of MV populations it is likely that MVs utilise more than one route of entry, in a similar manner to viruses (Marsh and Helenius, 2006).

3.2 Methods:

3.2.1 MVs isolation from PC3 cells

The PC3 cell line was used extensively in this study. Essentially, MVs were isolated from PC3 cells, labelled with a membrane dye (PKH26) and were added to resting PC3 cells to study MV interaction with recipient cells and the different biological conditions that may affect the interaction in normal physiological or the diseased state were studied.

MVs were isolated from the supernatant of PC3 cells by collecting the supernatant and centrifuging at 4000 g for 1 hour. The pellet was discarded, and the supernatant was further centrifuged at 25,000 g for 2 hours. The resulting MV pellet was washed by resuspending in 200 µl of (MV free) PBS and was subjected to further centrifugation for 2 hours at 25,000 g. After centrifugation, the supernatant was discarded and the MV pellet was ready for labelling with the PKH26 dye. 250 µl of Diluent C (from the PKH26 dye kit) was added to the pellet and mixed thoroughly. Meanwhile, 250µl of the diluent C was mixed with 2µl of PKH26 dye, and this dye mixture added to the pellet and then topped up with an extra 500 µl of diluent C. The reaction was left for 5 minutes on ice, with occasional mixing. 500µl of 1% BSA was added for 1 minute, to stop the reaction. The MVs were centrifuged to remove the unbound dye and then further centrifuged to wash off excess buffers and dye. The MV pellet (labelled with PKH26 dye) was finally resuspended in MV free PBS ready for analysis by flow cytometry.

The number of labelled MVs was then determined using the flow cytometer. On a 24- well plate for each cell, 20 labelled MVs were added or not for control. The plate was incubated at 37^0 C, 5% CO_2 for 1 hour.

After incubation the supernatant was removed from the wells and the wells were washed 3 times with PBS. For fluorescent microscopy 200µl of PBS was added to each well ready to be examined under the microscope. However, for flow cytometry, supernatant was removed from each well and cells were treated with trypsin to dissociate them from the wells. Cells were washed 3 times with trypsin and were then resuspended in a 96 well plate with some PBS ready for flow cytometry analysis.

The objective of this study was to explore PC3 MVs interaction with the recipient cells and the effect of different physiological conditions (such as; temperature and change in pH) on the uptake of MVs by the recipient cells. It was deemed that an understanding of the mechanisms and/or routes of MV uptake will help aid our understanding of the role of MVs in EMT which was to be investigated in Chapter 4.

3.3 Results

3.3.1 MV characterisation and detection of phosphatidylserine exposed on the outer leaflet of MV

To explore MV interaction with recipient cells, MVs were isolated from PC3 cells according to the methods described in the Materials and Methods (section 2.2.2.1). PC3 cells were cultured in MV free CGM, and supernatant collected after 48 hours of incubation. Cells were washed with filtered DPBS three times. The PBS was centrifuged at 25,000 g for 4 hours and then filtered through a 0.1 μm pore size filter to remove MVs. This MV free PBS was also used to re-suspend the MVs pellet after isolation. The presence of MVs in every medium used in cell culture may increase the chance of interference/contamination during MV analysis using the EasyCyte Guava Flow Cytometer 8HT (Millipore) and Guava express plus software. Therefore, in this study MV free media was used exclusively. Previous researchers in Cellular and Molecular Immunology Research Centre (CMIRC) have 'quantified' MVs by determining the protein concentration of MVs when adding them to recipient cells, due to the miscalculation observed using the earlier model of the Guava EasyCyte. Using the new Guava 8HT, the counting of MVs is more accurate than that obtained with the previous version which gave too much background noise. In this study, I have therefore exclusively counted the number of MVs using the new Guava 8HT. Approximately, 20 MVs per recipient cell were added in these experiments.

3.3.2 MVs quantification and characterisation using the Guava-8HT

As the MVs field is still in its infancy, lacking standardisation of isolation and characterisation protocols, it is important to confirm that the MVs samples used in this study are MVs according to recent data in the literature and work carried out in

the lab of Prof. Inal and by numerous previous researchers. The electron microscopic image of EVs is shown below in figure 3 where the medium-sized EVs ('MVs' as termed throughout this thesis) are indicated by white arrows and small-sized EVs (likely 'exosomes') are indicated by black arrowheads.

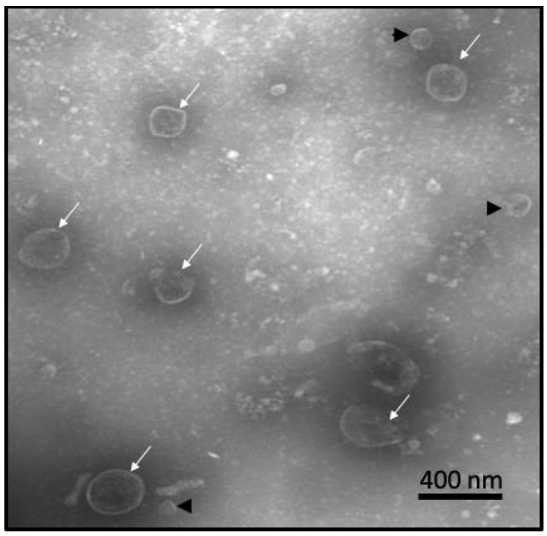


Figure 3: Characterisation of Microvesicles using electron microscopy. Transmission electron microscope image shows PC3 MVs. Medium-sized EVs 'MVs' are indicated by white arrows and small-sized 'exosomes' are indicated by black arrowheads.

The MVs were isolated from the metastatic prostate cancer cell line, PC3 cell line by differential centrifugation of cell culture supernatant (initial 4,000 *g* spin for 1h to remove cells and cell debris followed by a 15,000 *g* spin of the resultant supernatant for 2h at 4°C to collect a pellet of MVs (medium-sized EVS)). They were then negatively stained with potassium phosphotungstate (PTA) (which is non-disruptive to membrane systems). For this, the MVs were stained with phosphotungstic acid (pH 2.8) and aqueous bacitracin. Once stained, the samples were put on a 400-mesh copper grid with a pioloform support. Samples were pre-treated with Alcian blue (aq.) 8GX for 15 min. The samples were viewed using a JEOL JEM-1200 EX II

electron microscope and digital photographs taken using an AMT digital camera. The magnification is 75,000.

MVs show a typical distribution on the forward and side scatter graph as seen in figure 2 (A) below. To further confirm that isolated vesicles are indeed MVs, PC3-derived MVs were labelled with Annexin-V Alexa flour 488 antibody detecting phosphatidyl serine molecules on the MV membrane. In figure 4 (B) it is shown that 65% of the MVs isolated were Annexin-V positive, (C) shows the fluorescence peak. This confirms that the vesicles derived from PC3 cells are pure and biologically active MVs. The Guava EasyCyte 8HT also provides the estimation of MV number harvested from PC3 cells and it also gives a good account of the size and granularity of MVs from the FSC and SSC respectively

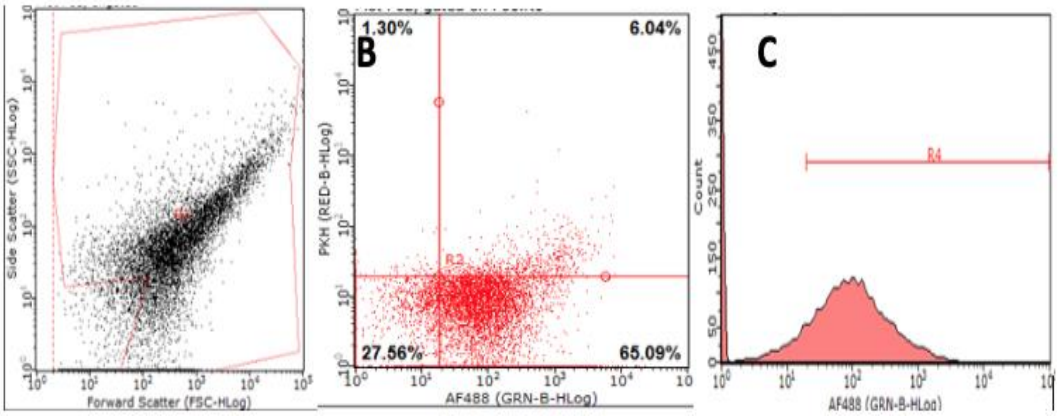


Figure 4: Characterisation of Microvesicles using Guava EasyCyte 8HT

The scatter plot in (A) shows the number of PC3 MVs isolated from 10ml of conditioned PC3 cell culture medium. The size and granularity of MVs are assessed from the logarithmic amplification of forward scatter (FCS) and side scatter (SSC) signals. The Guava easyCyte is a technique of choice, as it is well known and widely used due to measuring different parameters such as particle count and granularity. In (B) and (C) guava express software is used to analyse the population of PS positive microvesicles by gating on the population.

3.3.3 Flow cytometry analysis shows uptake of PC3 derived MVs by recipient cells

To determine PC3 derived MV uptake by the recipient cells, ($1x10^5$/well) PC3 cells were seeded in a 12- well plate for 14 hours in MV- free medium supplemented with 10% FBS. Cells were washed with MV- free DPBS twice to remove attached MVs. Fresh MV- free medium was added in each well together with 20 PKH26 labelled MVs per cell to each well except the control. After 2 hours of incubation the cells were detached from the wells by adding trypsin/EDTA. After 5 minutes of incubation at 37°C, 5% CO_2 with trypsin, the cells were removed from the wells and transferred to 1.5ml of Eppendorf tubes for washing. The cells were centrifuged at 400g for 5 minutes to remove the debris and dead cells. The pellet was washed twice with DPBS before it was re-suspended in MV- free DPBS ready for analysis by Flow cytometry.

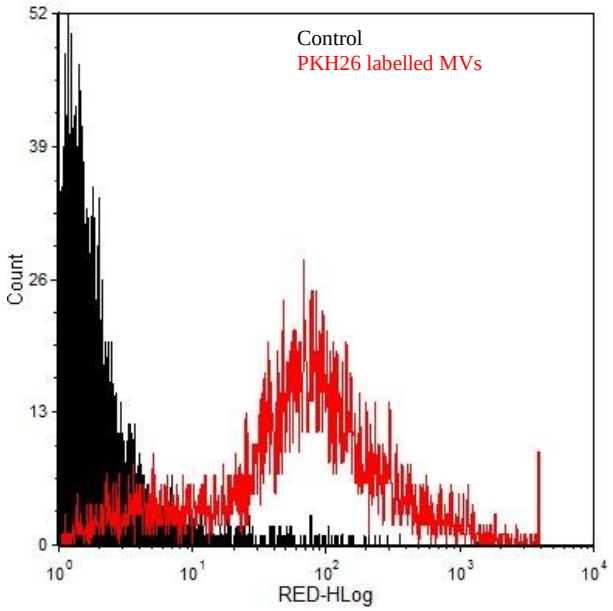


Figure 5: PC3 Microvesicles labelled with PKH26 dye prior to the experiment

This is the flow cytometric analysis of PC3 MVs labelled with PKH26 dye. PC3 MVs were isolated by centrifugation and were labelled with PKH26 dye according to the protocol mentioned in materials and methods. This histogram shows that 80% of the isolated MVs expressed PKH26 label out of the MVs sample analysed. The fluorescence was checked routinely before adding the MVs to the recipient cells.

3.3.4 Fluorescent microscopy analysis shows uptake of PC3 derived MVs by recipient cells

To determine PC3- derived MV uptake by the recipient cells, ($1x10^5$/well) PC3 cells were seeded in 12- well plate for 14 hours in MV- free medium supplemented with 10% FBS. Cells were washed with MV- free DPBS twice to remove attached MVs. Fresh MV- free medium was added in each well together with 20 PKH26 labelled MVs per cell to each well except the control. After 2 hours of incubation at 37^{o}C, 5% CO_2 the cells were washed with DPBS twice to remove the unbound MVs. 0.5ml of DPBS was added to each well to prevent the cells from drying while the fluorescent analysis was taking place.

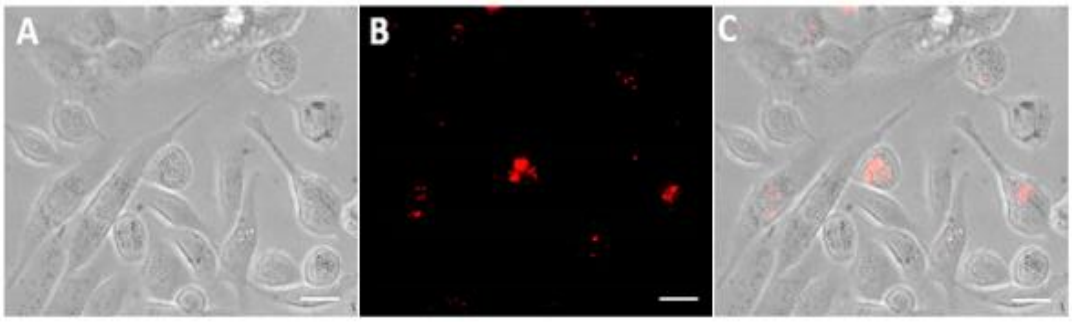


Figure 6: Prostate cancer MVs labelled with PKH26 dye treated cells show the diffusion of the dye inside the cells. (A) control PC3 cells, **(B)** PC3 cells treated with PC3 cells derived MVs and **(C)** combination of **(A)** and **(B)**. Scale bar = 50μm

The fluorescence microscopic images in figure 6 (B) and (C) shows the internalised PKH26 labelled MVs after 2 hours of incubating PC3 cells treated with PC3 derived MVs labelled with PKH26 dye. The internalisation of the MVs shows that the MVs are taken up by the recipient cells, the next step was to remove the surface proteins by treating the recipient cells with trypsin before adding PC3 MVs. This would ascertain the importance of protein – protein interaction between MVs and recipient cells as a likely precursor to attachment of some fusion/hemi-fusion event.

3.3.5 Flow cytometry analysis shows the effect of trypsin on PC3 MVs uptake by recipient cells.

Removing cell surface protein by treating the recipient cells with trypsin prior to adding MVs derived from PC3 cells was investigated in this study. PC3 cells ($1x10^5$/well) were seeded in a 12- well plate for 14 hours in MV- free medium supplemented with 10% FBS. Cells were washed with MV- free DPBS twice to remove attached MVs. Recipient cells were treated with 0.5ml of trypsin for 2 minutes except the positive control, to remove the cell surface proteins. Fresh MV-free medium was added in each well together with 20 PKH26- labelled MVs per cell to each well except the control. After 2 hours of incubation the cells were detached

from the wells by adding trypsin/EDTA. After 5 minutes of incubation at 37^{o}C, 5% CO_2 with trypsin the cells were removed from the wells and transferred to 1.5ml Eppendorf tubes for washing. The cells were centrifuged at 400g for 5 minutes to remove the debris and dead cells. The pellet was washed twice with DPBS before it was re-suspended in MV- free DPBS ready for analysis by Flow cytometry.

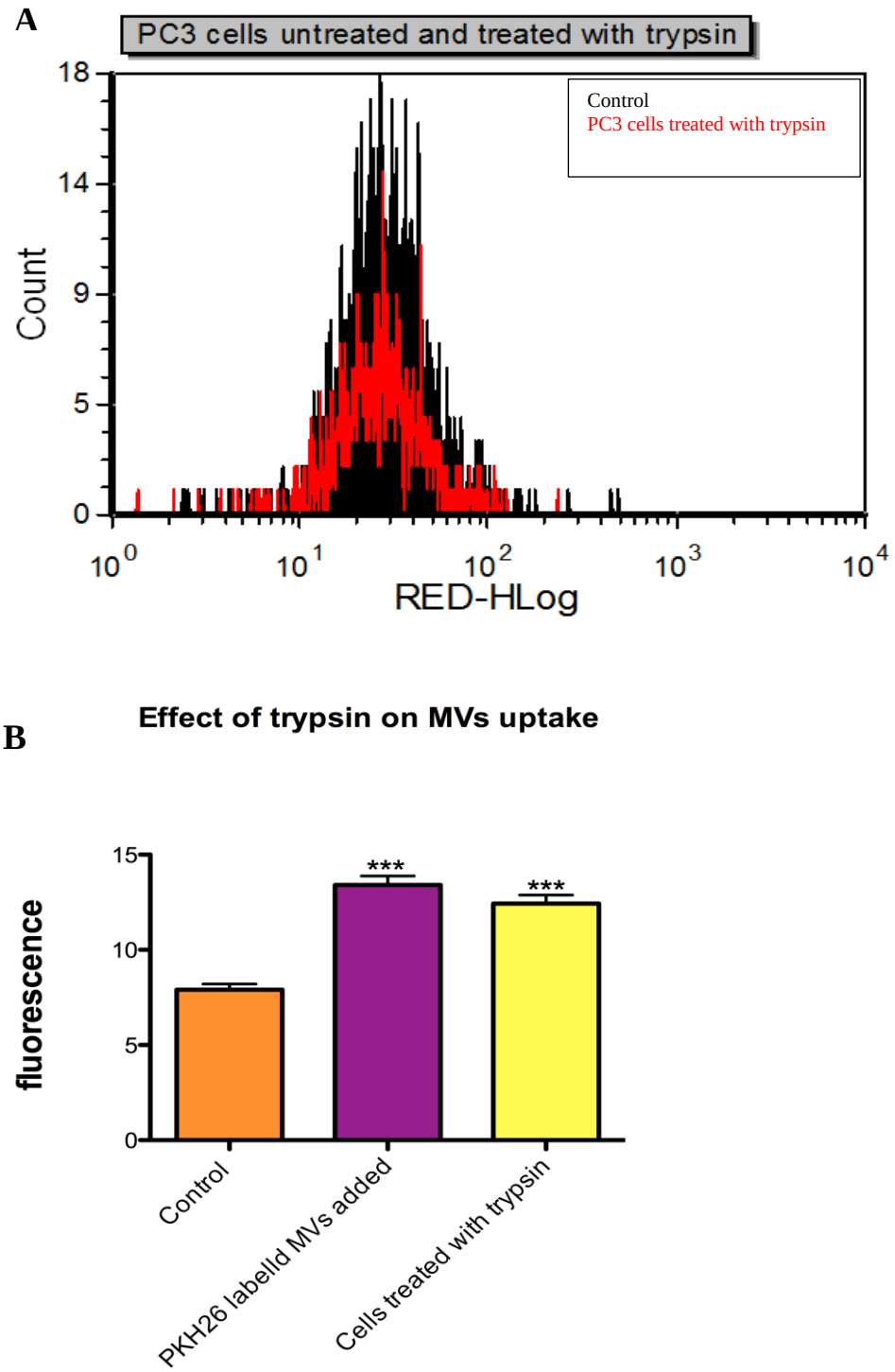
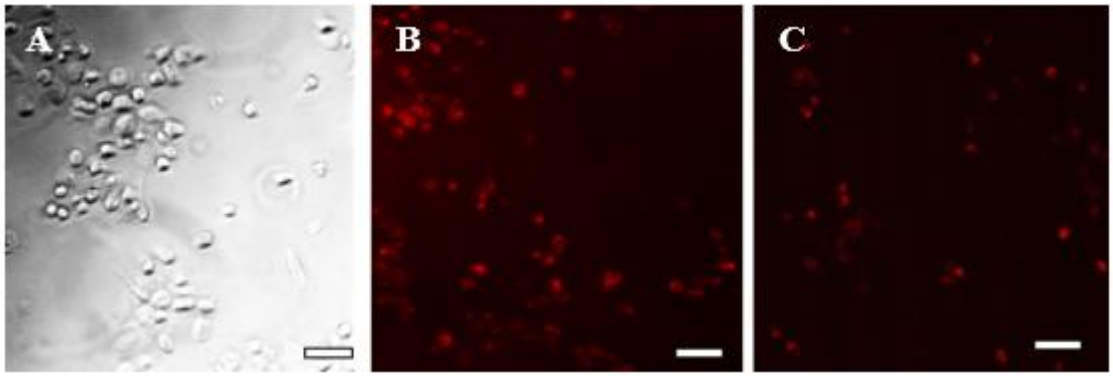


Figure 7: Flow cytometer analysis shows loss in fluorescence between the positive control and the test (recipient cells treated with trypsin before adding PC3 MVs labelled with PKH26). (A) Histogram shows an overly of control cells (not treated with trypsin) and cells treated with trypsin before adding PKH26 labelled MVs. Bar chart in **(B)** shows marked reduction in PC3 fluorescence after treated with trypsin. The experiment was repeated three times and the data presented are the mean ±SEM of the results (***P<0.001)

3.3.6 Fluorescent microscopy analysis shows uptake of PC3 derived MVs by recipient cells after treating the recipient cells with trypsin prior to adding the MVs.

PC3 cells were treated with trypsin prior to adding PC3 cell- derived MVs to remove cells surface proteins and observe its effect on MV uptake. Again ($1x10^5$/PC3 cells/well) were seeded in 12- well plates for 14 hours in MV- free medium supplemented with 10% MV-free FBS. Cells were washed with MV- free DPBS twice to remove attached MVs. For the test, cells were treated with 0.5ml of trypsin for 2minutes. Fresh MV- free medium was added to each well together with 20 PKH26 labelled MVs per cell except the control. After 2 hours of incubation at 37ºC, 5% CO_2 the cells were washed with DPBS twice to remove all the unbound MVs. 0.5ml of DPBS was added to each well to keep the cells alive while the fluorescent analysis was taking place.

A



B

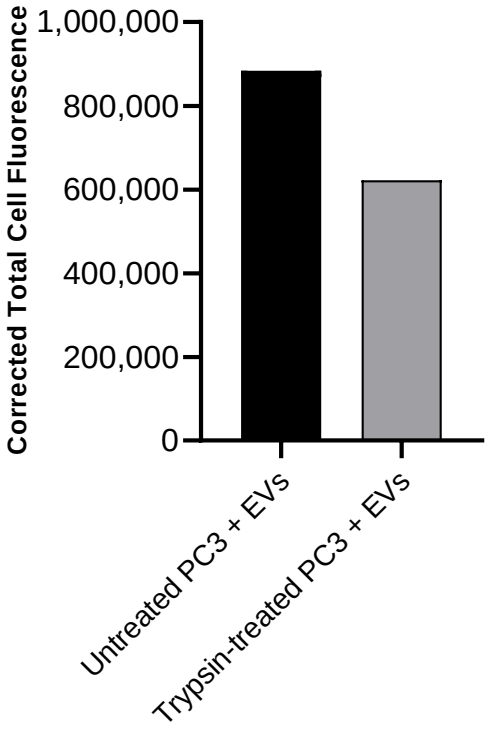


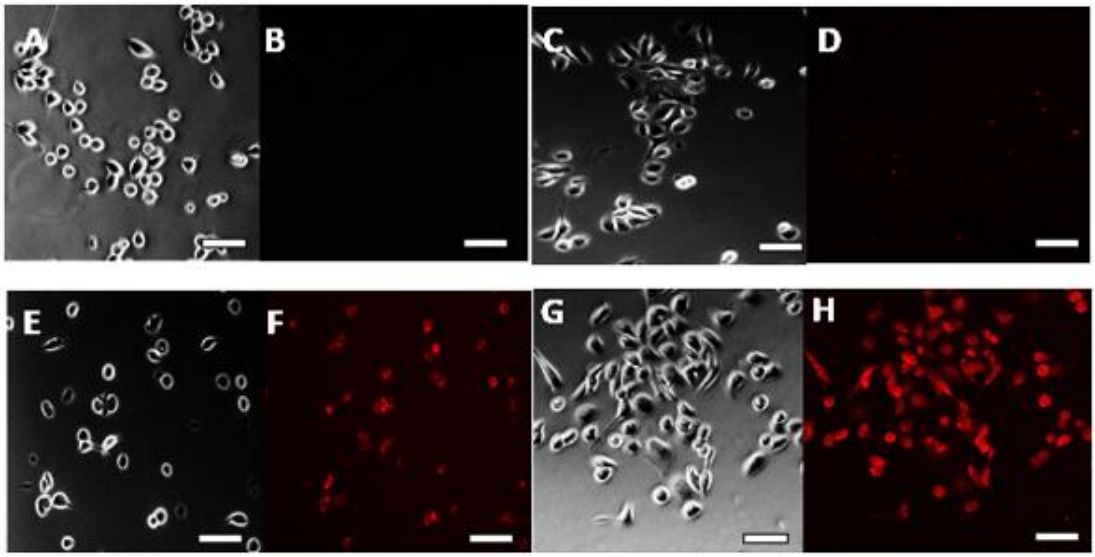
Figure 8: Prostate cancer (PC3) cells treated with trypsin before adding PC3 cell derived MVs labelled with PKH26 dye, show marked loss of PKH26 dye expression as determined by fluorescent microscopy. In panel A, **(A)** control untreated PC3 cells. **(B)**, positive control PC3 cells treated with PC3 cell derived MVs (20 MVs/cell) labelled with PKH26 dye **(C)**, PC3 cells treated with trypsin prior to adding 20MVs/cell PC3 derived MVs labelled with PKH26 dye. Scale bar = 200µm. In Panel B, the corrected total cell fluorescence (CTCF) of images in panel A was calculated, cells treated with Trypsin shows lower CTFC. This is representative of a triplicate experiment.

There is considerably less fluorescence observed in (C) where cells were treated with trypsin prior to adding PC3 MVs, compared to (B) where cells were not treated with trypsin prior to adding PC3 MVs. This finding is consistent with the hypothesis that removing cell surface proteins will decrease the uptake of MVs. However, the difference in fluorescence is not highly significant. This also suggests that MVs might well be taking a different uptake route upon interaction with the recipient cell as mentioned in the introduction.

3.3.7 Fluorescent microscopy analysis shows the effect of different pH levels on the uptake of PC3 derived MVs by recipient cells.

PC3- derived MV uptake by recipient cells was investigated at different pH levels. PC3 cells ($1x10^5$/well) were seeded in 12- well plates for 14 hours in MV- free medium supplemented with 10% FBS. Cells were washed with MV- free DPBS twice to remove attached MVs. Fresh MV- free medium was added to the control. DPBS of pH 7.4, 6.4 and 5.4 was prepared by calibrating with pH meter using HCl and NaOH. The DPBS was filtered through a 0.2µm pore size filter to obtain sterile medium for cells to be incubated in for the duration of the experiment. Each well was treated with 20 PKH26- labelled MVs per cell except for control. After 2 hours of incubation at 37^{o}C, 5% CO2 the cells were washed with DPBS twice to remove all the unbound MVs. 0.5ml of DPBS was added to each well to prevent the cells from drying while the fluorescent analysis was taking place. The following findings were observed.

A



B

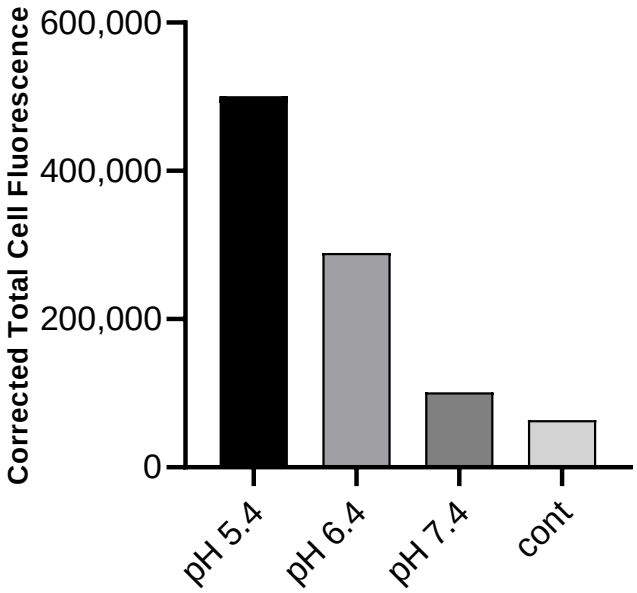


Figure 9: The effect of different pH levels on the MVs uptake by the recipient cells observed by fluorescent microscope. In panel **A**, **(A)** and **(B)** is the control. **(C)** and **(D)** shows PKH26 MVs internalised at pH 7.4. **(E)** and **(F)** shows uptake of PKH26 derived MVs by the recipient cells at pH 6.4. **(G)** and **(H)** shows uptake of PKH26 derived MVs by the recipient cells at pH 5.4. Scale bar = 200μm. In panel **B**, the corrected total cell fluorescence (CTCF) of images in panel A was calculated, pH5.4 (acidic) shows high CTFC compared to control. This is representative of a triplicate experiment.

In the above figure 9, MV- uptake by the recipient cells is greater in (H) where pH of the recipient cells was pH5.4, compared to (F) where pH of the cells was pH6.4. Overall both (F) and (H) show significantly higher MV uptake by recipient cells compared to (D) where cells pH was pH7.4. This shows that MV uptake was greater at lower pH. To further confirm that the MV uptake is favoured under acidic conditions, flow cytometry analysis of the experiment was also obtained. This can be observed in figure 10.

3.3.8 Flow cytometry analysis shows the effect of different pH levels on the uptake of PC3 derived MVs by recipient cells.

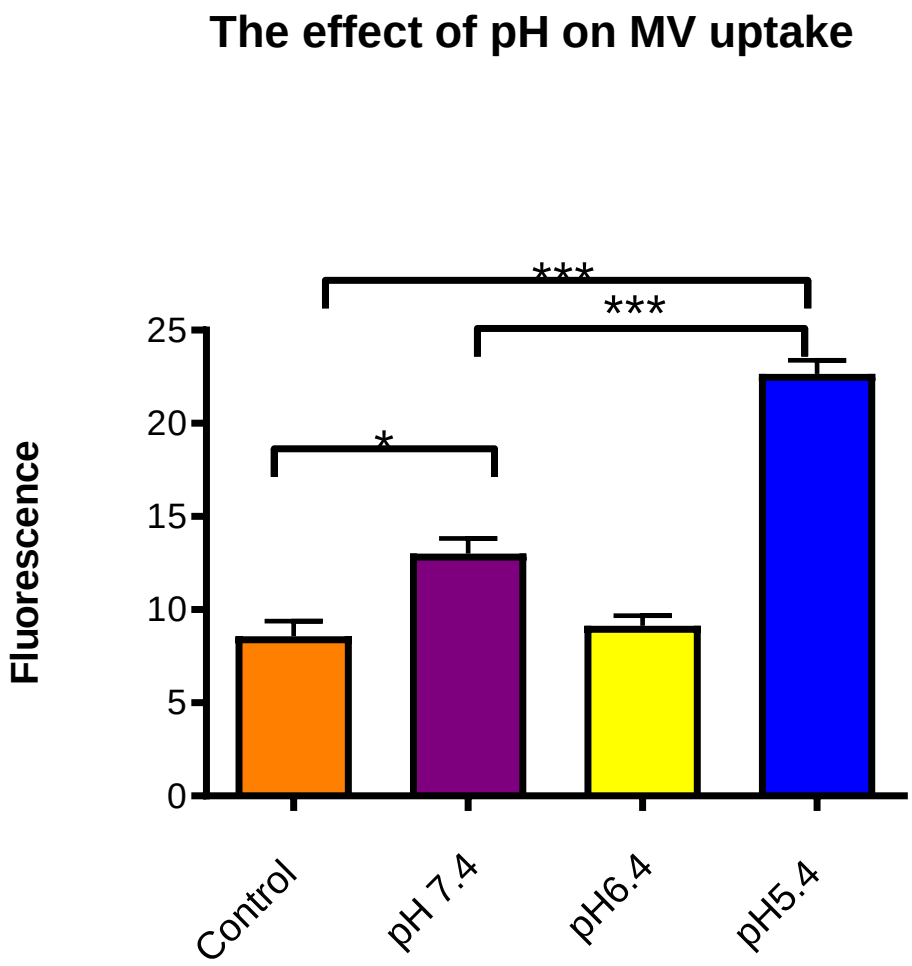


Figure 10: Flow cytometer analysis shows increased uptake of PKH26 labelled MVs by recipient cells at pH5.4. The lowest uptake is shown at pH 6.4 in the more alkaline environment. The experiment was repeated three times and the data presented are the mean ±SEM of the results (*<0.05 and ***P<0.001)

3.3.9 Flow cytometry analysis of temperature change shows difference in PC3 MVs uptake by control and cells treated with PKH26 labelled PC3 MVs.

Temperature is another parameter that was tested here in this chapter. Three different temperatures; 4ºC, 21ºC and 37ºC were tested to determine the optimum temperature for MVs interaction with the recipient cells. Once again ($1x10^5$ PC3 cells/well) were seeded in 12- well plates for 14 hours in MV- free medium supplemented with 10% FBS. Cells were washed with MV- free DPBS twice to remove attached MVs. PKH26 labelled PC3 derived MVs were added to PC3 cells and incubated at three different temperatures; 4ºC, 21ºC and 37ºC for 2 hours. After two hours of incubation at the above three different temperatures, cells were washed with DPBS twice to remove all the unbound MVs. Cells were detached from the wells with trypsin and were then transferred to tubes for washing. Cells were resuspended in PBS after three washes with DPBS and were analysed with flow cytometer.

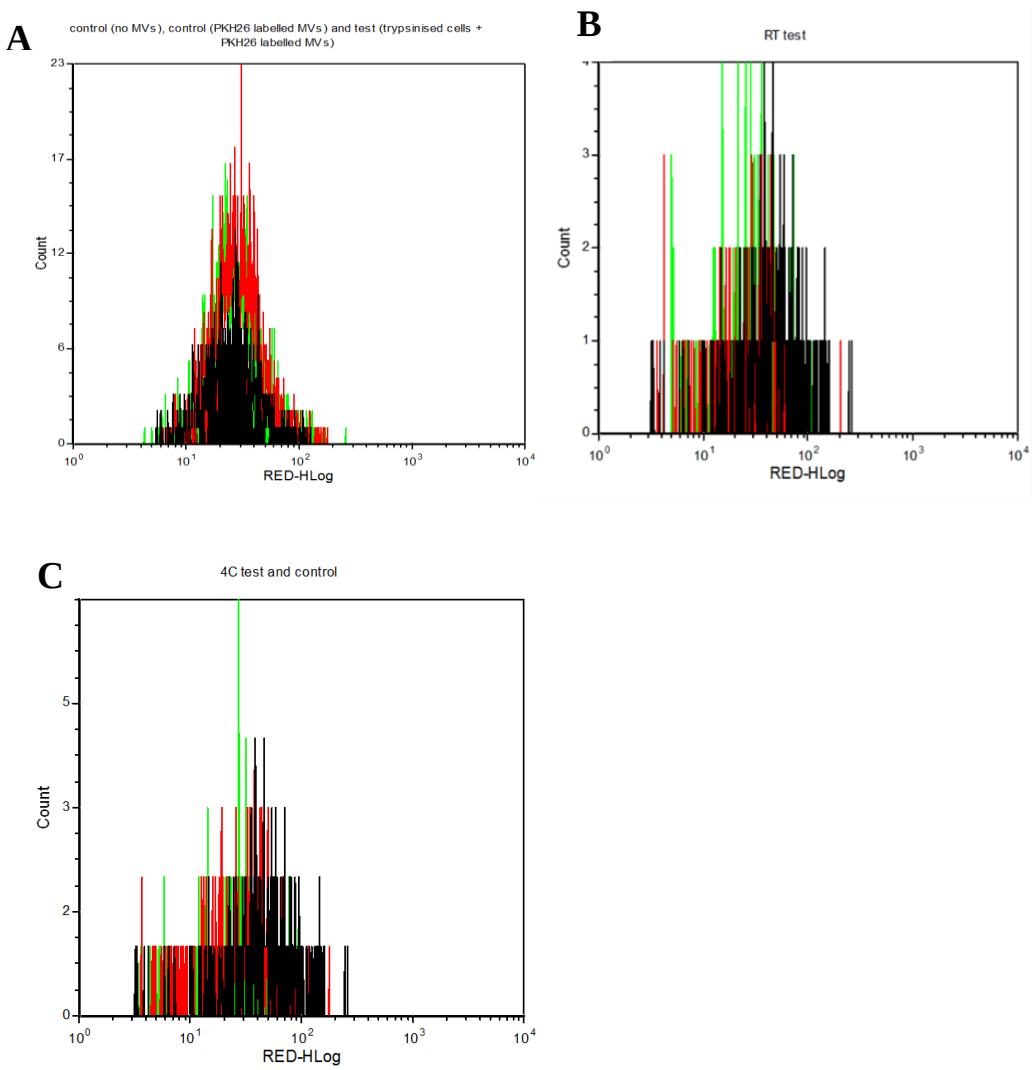


Figure 11: flow cytometer analysis shows the effect of temperature on MVs uptake by the recipient cells. (A) control, MVs uptake by recipient cells at 37°C shows highest fluorescence. **(B)**, shows level of fluorescence at Room Temperature, considerably lower than in **(A)** however, significantly higher than in **(C)**. **(C)**, shows very low fluorescence emitted by MVs taken up by recipient cells at 4°C.

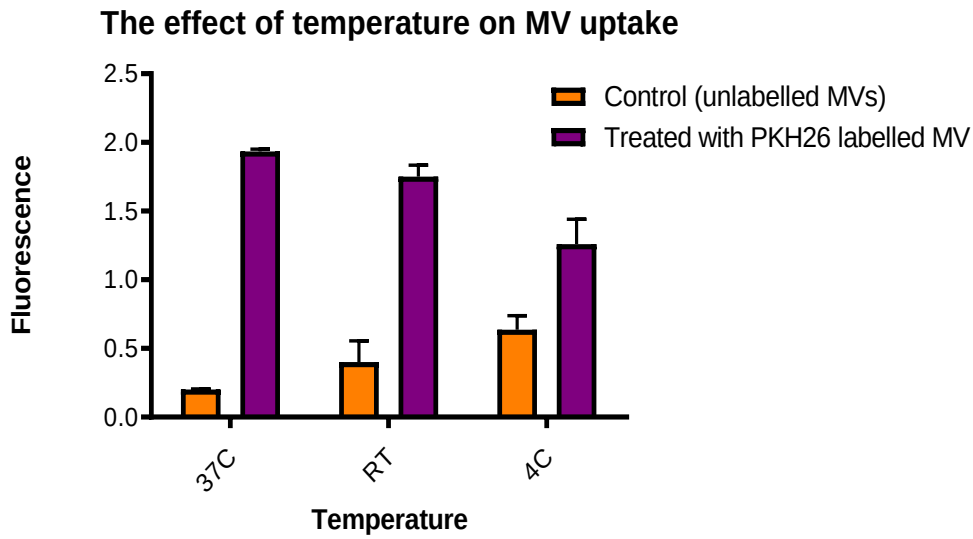


Figure 12: Flow cytometer analysis shows effect of temperature on the uptake of PKH26 labelled MVs by recipient cells. Uptake of MVs was shown significantly high at 37°C compared to 4°C. Nevertheless, there was some fluorescence at Room Temperature. The experiment was repeated three times.

3.3.10 Flow cytometry analysis shows the effect of blocking the PS site on MVs uptake on the recipient cells.

PC3 MVs were then treated with Annexin V prior to labelling with PKH26 dye, to block the PS site on the MV membrane. This is to test the hypothesis that PS on the outer leaflet of MVs is responsible for the uptake by recipient cells. Once again ($1x10^5$PC3 cells/well) were seeded in 12- well plates for 14 hours in MV- free medium supplemented with 10% FBS. Cells were washed with MV- free DPBS twice to remove attached MVs. For this experiment, PC3- derived MVs were labelled with Annexin V, washed with DPBS and the sample was tested for fluorescence prior to labelling it with PKH26 dye.

MV- free medium was added in each well together with 20 An-V and PKH26 labelled MVs per cell to each well except the control.

As a positive control, 20 labelled MVs per cell for three wells were lysed with RIPA buffer to ensure membrane disruption. This control was used to test the importance of an intact MV membrane. After 2 hours of incubation at 37^{o}C, 5% CO_2 the cells were washed with DPBS twice to remove all the unbound MVs. Cells were detached from the wells with trypsin and were then transferred to tubes for washing. After three washes with DPBS, cells were resuspended in PBS prior to analysing on flow cytometer.

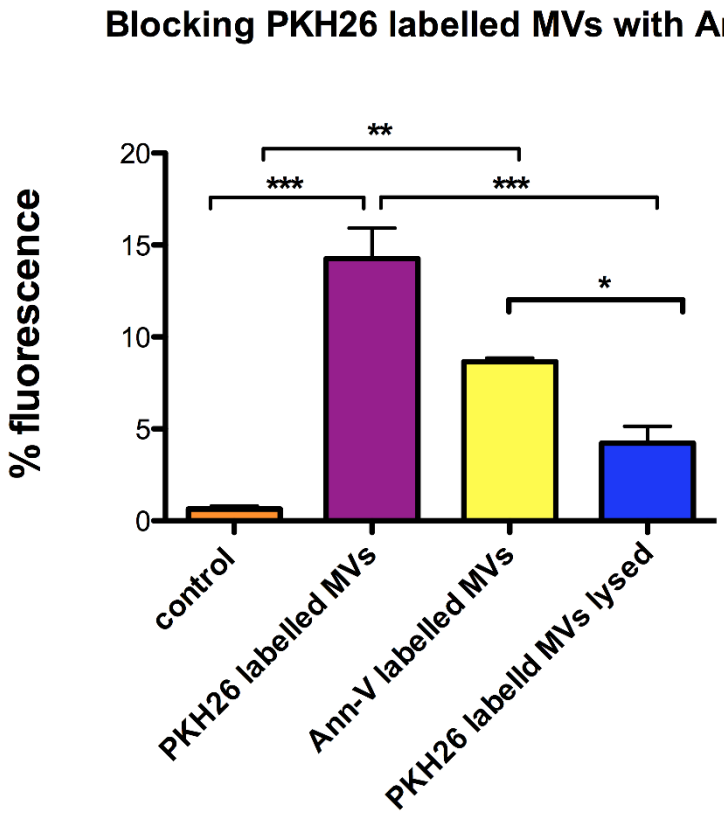


Figure 13: The effect of blocking PS with Annexin V on capacity for MV uptake by recipient cells analysed by flow cytometer. Annexin V labelled MVs showed reduced uptake by the recipient cells compared to untreated control (MVs labelled with PKH26 dye). PKH26 labelled MVs lysed showed some fluorescence compared to the control. The experiment was repeated three times and the data presented are the mean ±SEM of the results (*P<0.05, **P<0.01 and ***P<0.001)

3.3.11 Fluorescence microscopy analysis of PC3 recipient cells after treating with MVs labelled with Annexin-V and PKH26 Dye.

PC3 MVs were treated with Annexin V prior to labelling with PKH26 dye, to block the exposed PS on the MV membrane. This is to test the hypothesis that PS on the outer leaflet of MVs is responsible for the uptake by the recipient cells. Once again ($1x10^5$ PC3 cells/well) were seeded in 12- well plates for 14 hours in MV- free medium supplemented with 10% FBS. Cells were washed with MV- free DPBS twice to remove attached MVs. For this experiment PC3 derived MVs were labelled with Annexin V, washed with DPBS and the sample was tested for fluorescence prior to then labelling it with PKH26 dye.

MV- free medium was added in each well together with 20 An-V and PKH26 labelled MVs per cell to each well except the control.

As a positive control, 20 labelled MVs per cell for three wells were lysed with RIPA buffer to ensure membrane disruption. This control was used to test the importance of intact membranes in MVs for uptake. After 2 hours of incubation at 37ºC, 5% CO_2 the cells were washed with DPBS twice to remove all the unbound MVs. 0.5ml of DPBS was added to each well to keep the cells alive while the fluorescent analysis was taking place.

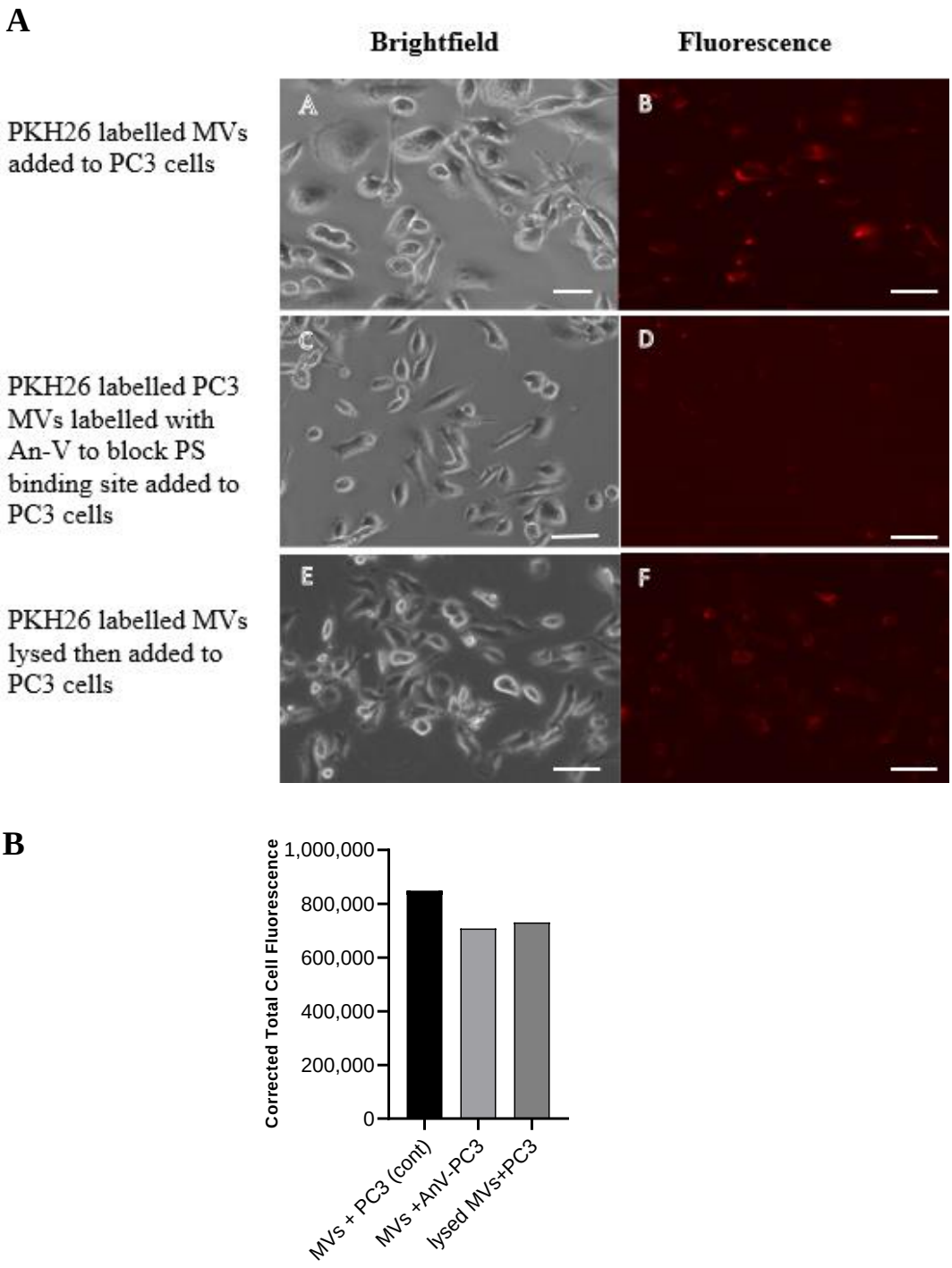


Figure 14: The effect of blocking PS with Annexin V on the MVs uptake by the recipient cells was analysed by flow cytometer. In panel A, **(A)** and **(B)** is control cells labelled with PKH26 dye only. **(C)** and **(D)** is cells treated with MVs labelled with An-V prior to labelling with PKH26 dye. **(E)** and **(F)** is cells treated with lysed labelled PKH26 labelled MVs. Annexin V labelled MVs in **(D)** showed reduced uptake by the recipient cells compared to control (MVs labelled with PKH26 dye) **(B)** not treated. PKH26 labelled MVs lysed showed low fluorescence **(F)** compared to the control. Scale bar = 200µm. In panel B, the corrected total cell fluorescence (CTCF) of images in panel A was calculated, cells treated with MVs (treated with An-V) shows lower CTFC. This is representative of a triplicate experiment.

3.3.12 Proteomic analysis of PC3 cells and PC3 MVs

PC3 cell lysates were obtained by growing PC3 cells in 24- well plates for 5 days until the cells were confluent. The supernatant of the cells was collected to use for MV isolation. Cells and MV lysates were obtained using RIPA buffer and protein concentration was determined. Samples of PC3 cells and MVs were loaded onto an SDSPAGE gel for 10 minutes. The protein bands were excised and placed in 1.5 ml centrifuge tubes ready for Mass spectrometry analysis. Lists of proteins for both PC3 cells and MVs were then generated.

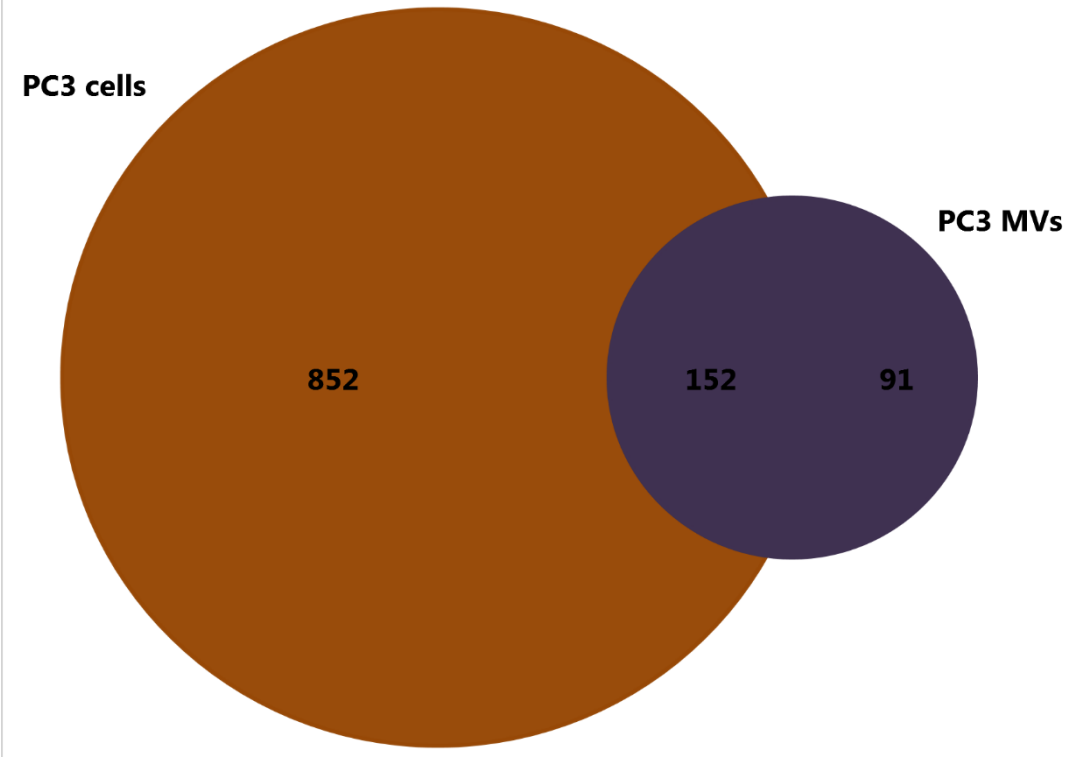
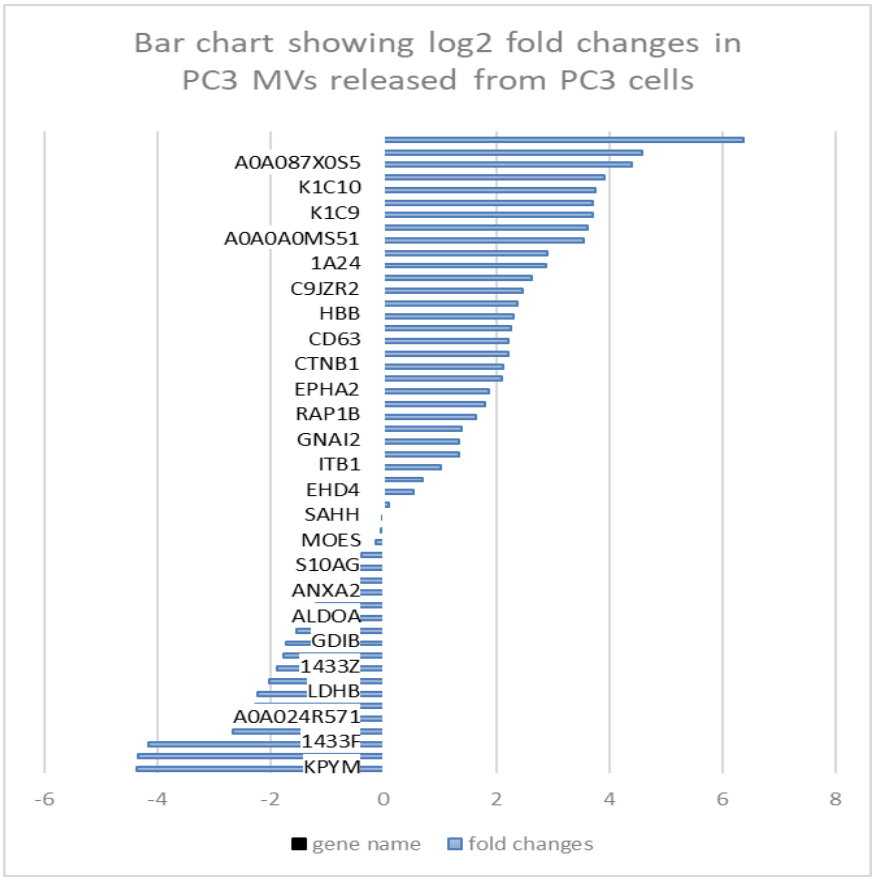


Figure 15: Venn diagram shows proteins present in PC3 cells and PC3 MVs after proteomic analysis. Venn diagram shows proteins present in PC3 cells and PC3 MVs after proteomic analysis. There are 152 proteins present in both parent cell and MVs derived from parent cells. Also, there are 91 proteins only present in PC3 derived MV.



Gene name	Protein name
A0A087X0S5	Collagen alpha-1 (vi) chain
K1C10	Keratin type1, cytoskeletal 10
K1C9	Keratin type1, cytoskeletal 9
A0A0A0MS51	Gelsolin
1A24	HLA class 1 histocompatibilityantigen
C9JZR2	Catenin delta-1
HBB	Haemoglobin subunit beta
CD63	CD63 antigen
CTNB1	Catenin beta-1
EPHA2	Ephrin type A receptor 2
RAP1B	Ras- related protein Rap-1b
GNAI2	Guanine nucleotide bindin protein
ITB1	Integrin beta-1
EHD4	EH domain containing protein 4
SAHH	Adenosyl homocysteinase
MOES	Moesin
S10AG	Protein S100
ANXA2	Annexin A2
ALDOA	Fructose - bisphosphate aldose A
GDIB	Rab GDP dissociation inhibitor beta
1433Z	14-3-3 protein zeta/delta
LDHB	L-lactate dehydrogenase B chain
A0A024R571	EH domain containing protein 1
1433F	HLA class 1 histocompatibilityantigen
KPYM	Pyruvate kinase PKM

Figure 16: Bar chart showing protein fold changes in PC3 MVs released from PC3 cells. Bar chart showing protein fold changes in PC3 MVs released from PC3 cells. Gene names are in black and fold changes are represented in blue. There are positive and negative fold changes in the genes.

The Proteomics data above shows that there are proteins present in PC3 cells and/or MVs. It is interesting to observe the proteins present in MVs, as this determines the potential activity of the MVs. Here the Venn diagram shows 152 proteins out of 852 to be present in MVs. However, there are 91 proteins that were only present in MVs. This is tightly linked to the process of microvesiculation. The list of some proteins that up regulated and down regulated is also shown.

3.4 Summary

In this chapter, PC3 cells derived MVs' uptake by the recipient cell was investigated. Different parameters were tested to study the uptake of MVs by the recipient cells. As mentioned in the introduction there are three different pathways via which the recipient cells may take up the MVs; membrane fusion, a variety of endocytic pathways, including phagocytosis/macropinocytosis and a lipid raft- mediated pathway (Carter *et al*, 2014). Carter *et al* (2014) also suggested that due to the heterogeneous nature of MVs and other extracellular vesicles, they may take more than one route to enter the recipient cell.

PC3 MV attached/fusion/hemi- fusion was observed by both fluorescent microscopy and flow cytometry analysis. Different time points were tested and PC3 MV uptake was observed at all the time points tested. The MVs were labelled with PKH26 dye, which stains the membrane of the vesicles red. Labelled MVs were added to recipient PC3 cells and recipient cells were then subjected to cell surface protein removal by treating the cells with trypsin to remove the cell surface proteins. Despite the removal of cell surface protein, the uptake was similar to the control in the experiment of cells not treated with trypsin. This observation is consistent with the suggestion from Carter *et al* (2014) that MV uptake by recipient cells may take different routes. To

further study this, the PS sites were blocked by Annexin-V antibody to stop it from binding to the membrane of the recipient cells, the results showed that there was significantly less uptake by the cells that were subjected to PS blocking.

Furthermore, MV uptake was tested in an acidic environment; low pH is a hallmark of tumour malignancy and potentially increasing the uptake of MVs by the recipient cells according to Parolini *et al*, (2009). Therefore, an acidic environment with pH 5.4 was compared to the physiological condition with pH 7.4 in this study. I observed that there was a higher uptake of MVs in acidic environment pH5.4 compared to other pH. This observation is consistent with the findings by Parolini *et al* and it showed that MVs were internalised by fusion in tumour microenvironment (Parolini *et al*, 2009). The proteomics analysis of PC3 cells and MVs show the proteins that are present on both the parent cell and the MVs isolated from the parent cell. However, there are also some proteins that are not present in the parent cell which suggests that these proteins are unique to the MVs, this finding aids our understanding of the potential of MVs in therapeutics and lays the foundation for the further study of the role of MVs in EMT, which are described in the following chapters.

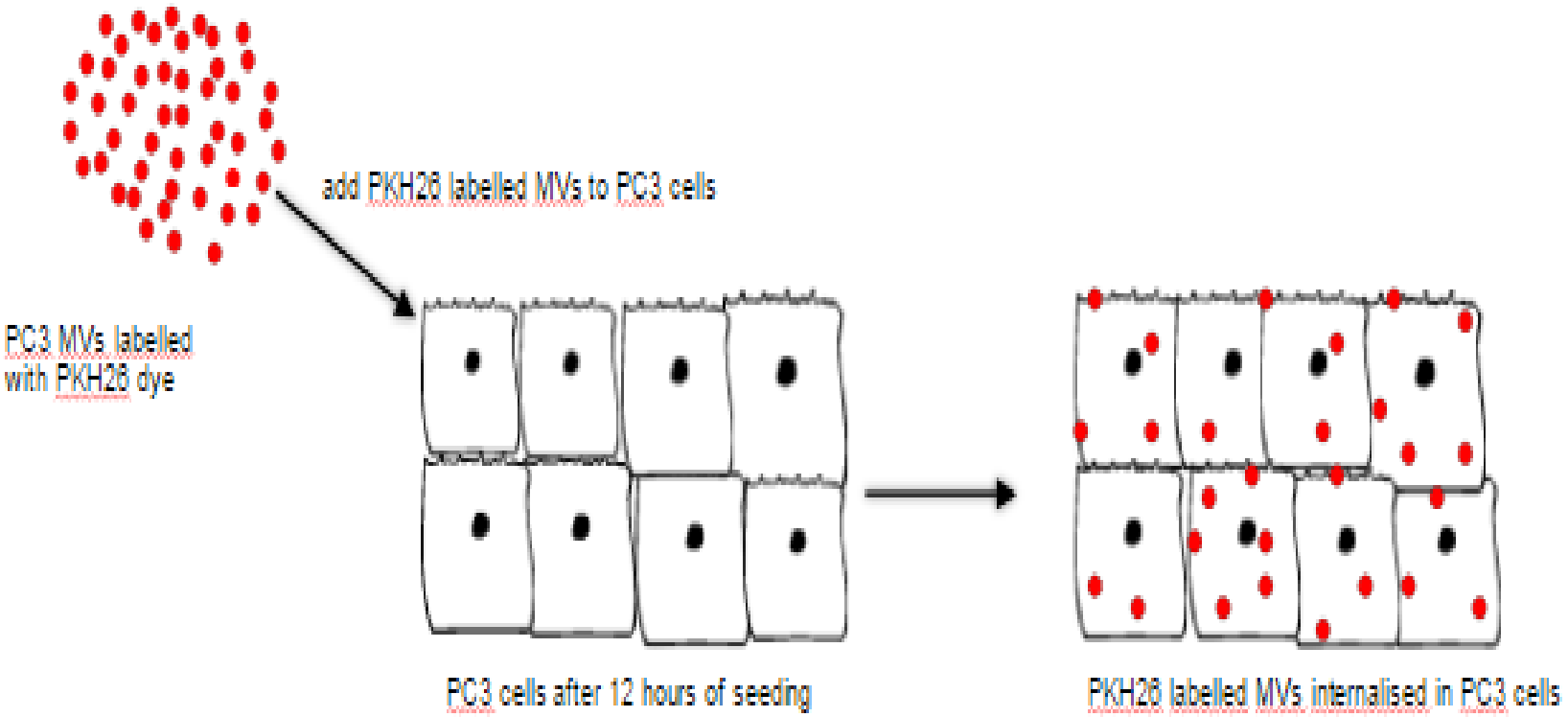


Figure 17: Schematic diagram representing the attachment/fusion of PKH26 labelled MVs (derived from PC3 cells) by recipient PC3 cells after two hours of incubation at 37°C and 5% CO_2.

Chapter 4: The effect of prostate cancer cell (PC3) derived MVs on epithelial mesenchymal transition in normal prostate cancer cell line (PNT2)

4.1 Introduction

Epithelial mesenchymal transition is a highly specialised physiological process that plays a vital role in cancer metastasis. It is defined by loss of epithelial characteristics and acquisition of mesenchymal phenotype and it is regulated by various transcription factors such as Snail, Slug, Zeb and Twist families. There are three different types of EMT as mentioned in the introduction. The functional consequences of EMT is generally dependant on the EMT regulators and the phenotypic changes are not always permanent. For example, during morphogenesis primary epithelial cells undergo EMT to induce morphogenesis such as neural crest formation and another organ formation in the embryo. Therefore, the functional consequences of this type of EMT is only restricted to its developmental processes.

The type2 EMT involved in wound healing is termed fibrotic EMT. This is also a physiological function of cells in the epithelial tissue during injury and inflammation. The process involves keratinocyte cells at the edge of the wound transforming to mesenchymal like cells. The EMT involved during the process of wound healing is termed fibrotic EMT. This fibrotic EMT also prevents infection and the entry of pathogen to the wound.

The cancerous type of EMT taking place during cancer metastasis in the epithelial cells is the type 3 EMT. This is a pathological process taking place in epithelial cells. Firstly, disseminating from the primary tumour and invading tumour microenvironment. The motile cells are then transported via blood into distant sites. In 2010 Quessenberry and Aliotta suggested that cancer cell derived MVs can bring phenotypical changes in the recipient cells. Therefore, MVs shed from tumour cells could aid in the process of metastasis considering its carrying the bioactive molecules

from the tumour cells it is released from. The objective of this study was to understand the role of PC3 derived MVs in the phenotypic change of normal prostate cells PNT2. This chapter is looking at the role of MVs in cancer metastasis through EMT.

4.2 Methods

Briefly, prostate epithelial cells (PNT2) were seeded at ($5x10^4$/well) in a 24 well plate in triplicates and were left over night to adhere to the bottom of the plate. Cells were then washed and 5mg/ml PC3 derived MVs were added. Cells were then incubated for 5 days at 37°C, 5% CO_2. Supernatant was collected for MVs isolation and the cells were washed and were subjected to either immunohistochemical analysis, immunofluorescence analysis or western blot analysis. In this results chapter results obtained from each analysis is explained.

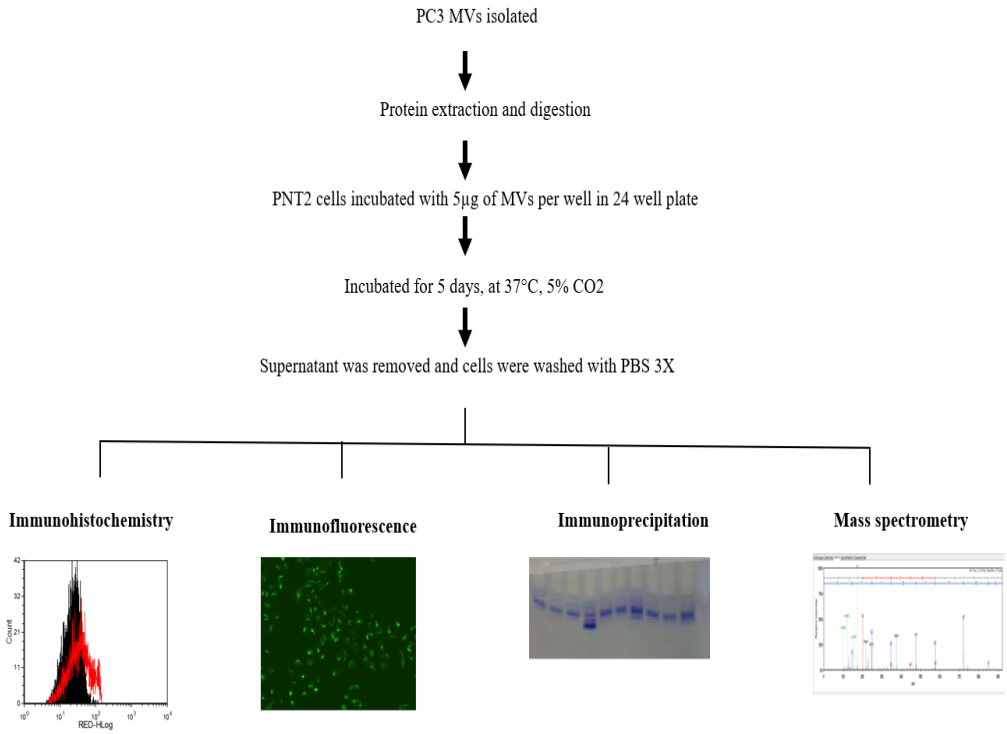


Figure 18: Flow chart showing the methods followed in this chapter.

4.3 Results

4.3.1 Morphological changes in PNT2 cells after treating with PC3 MVs.

Prostate epithelial cells (PNT2) were seeded at ($5x10^4$/well) in a 24 well plate in triplicates. The cells were grown in complete growth medium (RPMI supplemented with 10% FBS). Cells were incubated for 24 hours in 37°C, 5% CO_2 to ensure adherence to the culture plate. After 24 hours of incubation the media was removed, the adherent cells were washed with PBS and resuspended in MVs free complete growth medium (refer to methods in chapter 2). Cells were either treated with 5mg/ml of PC3 MVs or not for control. Treated and untreated cells were all incubated for 5 days at 37°C, 5% CO_2. On day 3, 5 and 7 of incubation the supernatant was removed, and the cells were washed three times with PBS to observe the morphological changes in the cells.

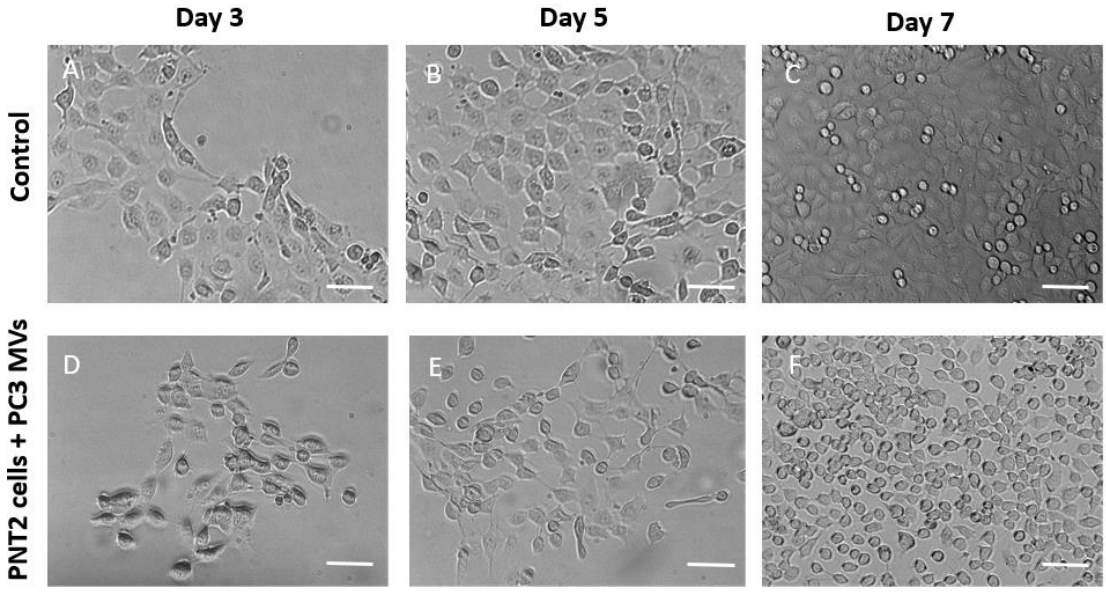


Figure 19: Morphological changes observed in PNT2 cells after treating with 5mg/ml of PC3 derived MVs for 5 days at 37C, 5%CO2. Images obtained using Olympus fluorescent microscope after 3 days of incubation (A and D) the treated cells started to lose adherence with the neighbouring cells, PNT2 cells at day 5 (B and E) show marked loss of polarity between cells and PNT2 cells after incubation at day 7 (C and F) show complete loss of polarity. Scale bar = 200μm

The morphology of the treated PNT2 cells was significantly changed to that of the control PNT2 cells due to treatment with PC3 MVs. Upon testing the viability of the PNT2 cells at day 3, 5 and 7, it was observed that cells had high viability on day 5 compared to day 7, therefore cells were incubated until day 5 for studying EMT. Cell proliferation was significantly reduced on day 7 of the incubation for both the control and treated, however more reduction observed in the treated cells on day 7.

4.3.2 Analysis of E-cadherin expression in PNT2 cells treated with PC3 derived MVs by fluorescent microscopy.

For fluorescent microscopy analysis, cover slips were sterilised with ethanol prior to placing them in the 24 well plate. The cover slips in the wells were coated with poly L-Lysine and were left at room temperature for 15mins to dry. Prostate epithelial cells (PNT2) were seeded at ($5x10^4$/well) in the 24 well plate in triplicates. The cells were grown in complete growth medium (RPMI supplemented with 10% FBS). Cells were incubated for 24 hours in 37°C, 5% CO_2 to ensure adherence to the culture plate. After 24 hours of incubation the media was removed, the adherent cells were washed with PBS and resuspended in MVs free complete growth medium (refer to methods in chapter 2). Cells were either treated with 5mg/ml of PC3 MVs or not for control. Treated and untreated cells were all incubated for 5 days at 37°C, 5% CO_2. At day 5 of incubation the supernatant was removed and stored for MVs isolation. The cells were washed with ice cold PBS and the fixed with 4% PFA and labelled with monoclonal anti E-cadherin antibody. The cover slips were removed from the wells and were placed on a microscope slide which was coated with DAPI VECTASHIELD medium which helped locate the nucleus within the cells.

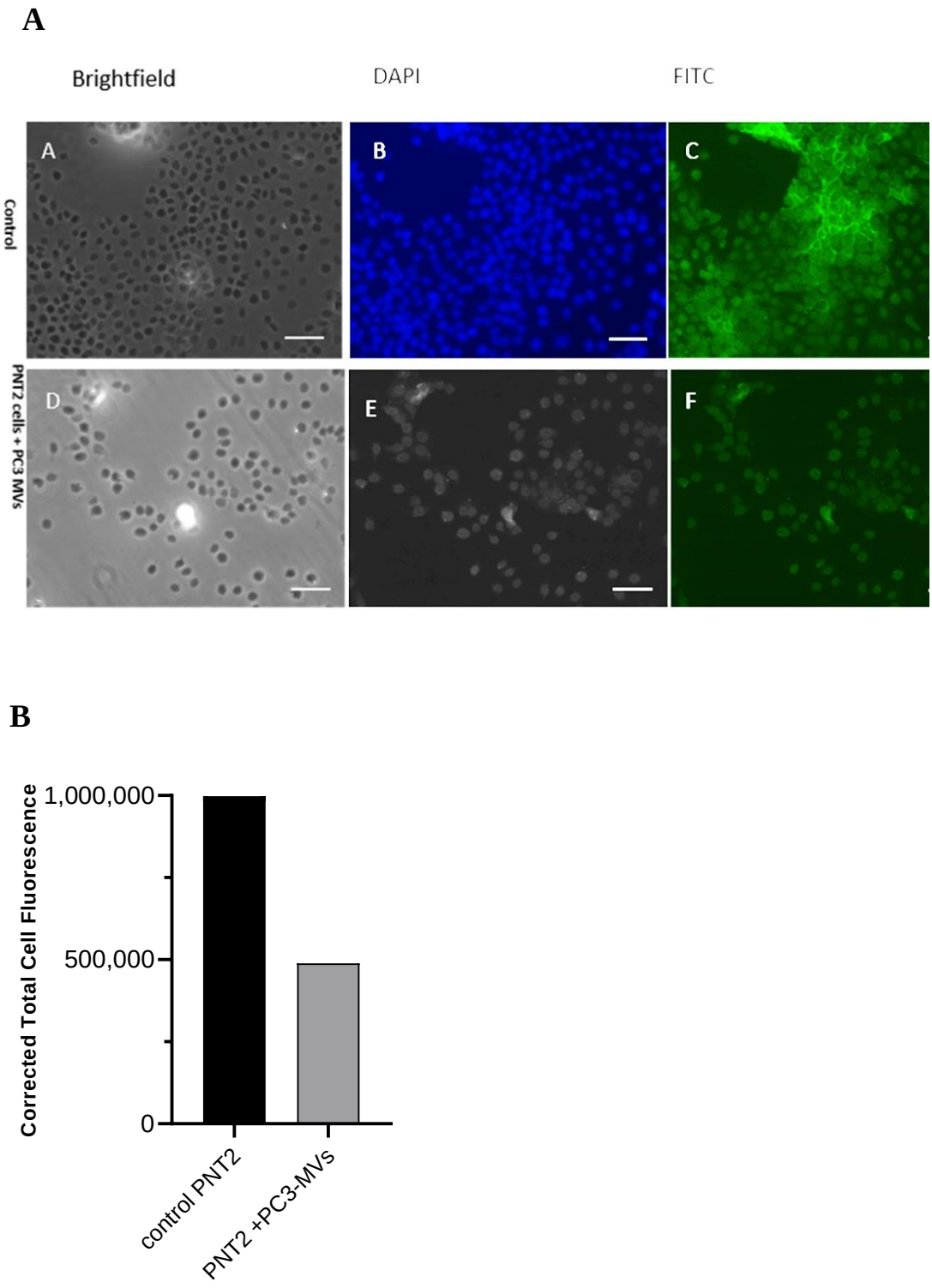


Figure 21: Fluorescent microscopy analysis show the expression of E-cadherin in PNT2 cells treated/not treated with PC3 derived MVs for 5 days. In panel A, bright field images show PNT2 cells after 5 days **(A)** control and **(D)** after treatment with 5mg/ml of PC3 MVs. **(B)** and **(E)** show the nuclei in the cells stained with DAPI. **(C)** shows expression of E-cadherin in control PNT2 cells and **(F)** shows loss of E-cadherin expression after treatment. In panel B, the corrected total cell fluorescence (CTCF) of images in panel A was calculated, cells treated with PC3 MVs show marked decrease in fluorescence compared to control cells. Scale bar = 200μm.

The images below were obtained using higher magnification on the fluorescent microscope. Here treated PNT2 cells were labelled with E-cadherin and the expression was detected. It is clear in the figure below that the E-cadherin expression is concentrated on one side of the cell, showing polarity. This could be due to the cytoskeletal muscles concentrated on one end of the cell to aid motility of the cell.

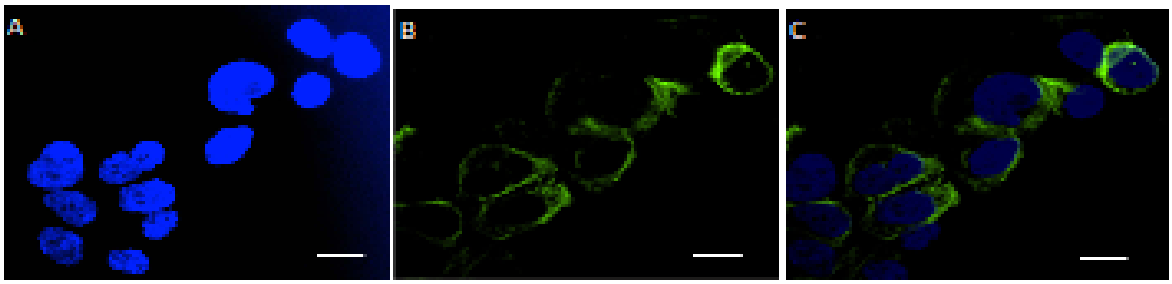


Figure 22: Expression level of E-cadherin on PNT2 cells treated with 5mg/ml PC3 MVs for 5 days. (A) shows DAPI staining of the nucleus **(C)**, shows nuclear staining combined with E-cadherin expression in **(B)**. Scale bar = 250μm

4.3.3 Flow cytometric analysis of E-cadherin expression following treatment of PNT2 cells with PC3 derived MVs.

For flow cytometry analysis, prostate epithelial cells (PNT2) were seeded at ($5x10^4$/well) in the 24 well plate in triplicates. The cells were grown in complete growth medium (RPMI supplemented with 10% FBS). Cells were incubated for 24 hours in 37C, 5% CO_2 to ensure adherence to the culture plate. After 24 hours of incubation the media was removed, the adherent cells were washed with PBS and resuspended in MVs free complete growth medium (refer to methods in chapter 2). Cells were either treated with 5mg/ml of PC3 MVs or not for control. Treated and untreated cells were all incubated for 5 days at 37°C, 5% CO_2. At day 5 of incubation the supernatant was removed and stored for MVs isolation. The cells were washed with ice cold PBS and were dissociated from the wells by Trypsin/EDTA. Cells were fixed with 4% PFA prior to labelling with monoclonal anti E-cadherin antibody. The labelled cells were resuspended in 100µl of filtered MVs free PBS ready for analysis on Guava easyCyte.

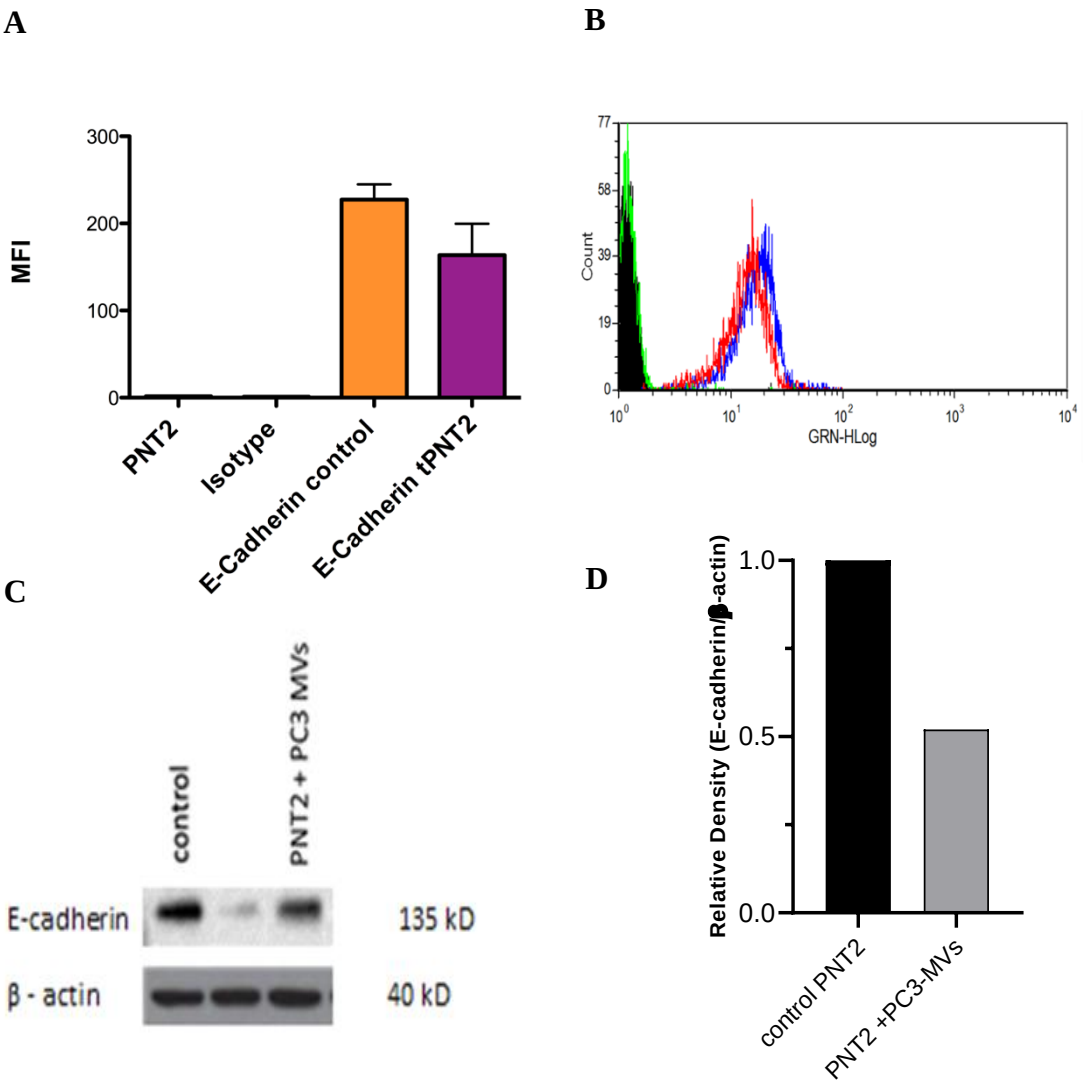


Figure 23: Analysis of E-cadherin expression in PNT2 cells treated with PC3 derived MVs by fluorescent microscopy. Flow cytometry and western blot analysis show the expression of E-cadherin in PNT2 cells treated/not treated with 5mg/ml for 5 days. **(A)** shows the expression of E-cadherin in PNT2 cells measured by Mean Fluorescence Intensity, PNT2 cells treated with PC3 MVs show marked loss in E-cadherin expression. The experiment was repeated three times and the data presented are the mean ±SEM of the results. The histogram in **(B)** shows loss of E-cadherin (red) in PNT2 cells treated with PC3 MVs compared to the control PNT2 cells (blue). Similarly, in **(C and D)** Western blot analysis show that there is lower expression of E-cadherin in the treated PNT2 cells compared to the control.

The results in figure 23 indicate that PNT2 cells treated with PC3 MVs show mesenchymal like morphology. Also, at the molecular level control PNT2 cells in the experiment expressed more E-cadherin compared to cells treated with PC3 MVs. This is due to loss of cell to cell adherence in the epithelial cells.

4.3.4 Analysis of vimentin expression in PNT2 cells treated with PC3 derived MVs by fluorescent microscopy.

For fluorescent microscopy analysis, cover slips were sterilised with ethanol prior to placing them in the 24 well plate. The cover slips in the wells were coated with poly L-Lysine and were left at room temperature for 15mins to dry. Prostate epithelial cells (PNT2) were seeded at ($5x10^4$/well) in the 24 well plate in triplicates. The cells were grown in complete growth medium (RPMI supplemented with 10% FBS). Cells were incubated for 24 hours in 37°C, 5% CO_2 to ensure adherence to the culture plate. After 24 hours of incubation the media was removed, the adherent cells were washed with PBS and resuspended in MVs free complete growth medium (refer to methods in chapter 2). Cells were either treated with 5mg/ml of PC3 MVs or not for control. Treated and untreated cells were all incubated for 5 days at 37°C, 5% CO_2. After the incubation the supernatant was removed and stored for MVs isolation. The cells were washed with ice cold PBS prior to fixing with 4% PFA. As Vimentin is a cytoskeletal protein expressed in the cytoplasm of the cells the cells were permeabilised with Triton X-100 (0.1%) prior to labelling with monoclonal anti-Vimentin antibody. The cover slips were removed from the wells and were placed on a microscope slide which was coated with DAPI VECTASHIELD medium which helped locate the nucleus within the cells.

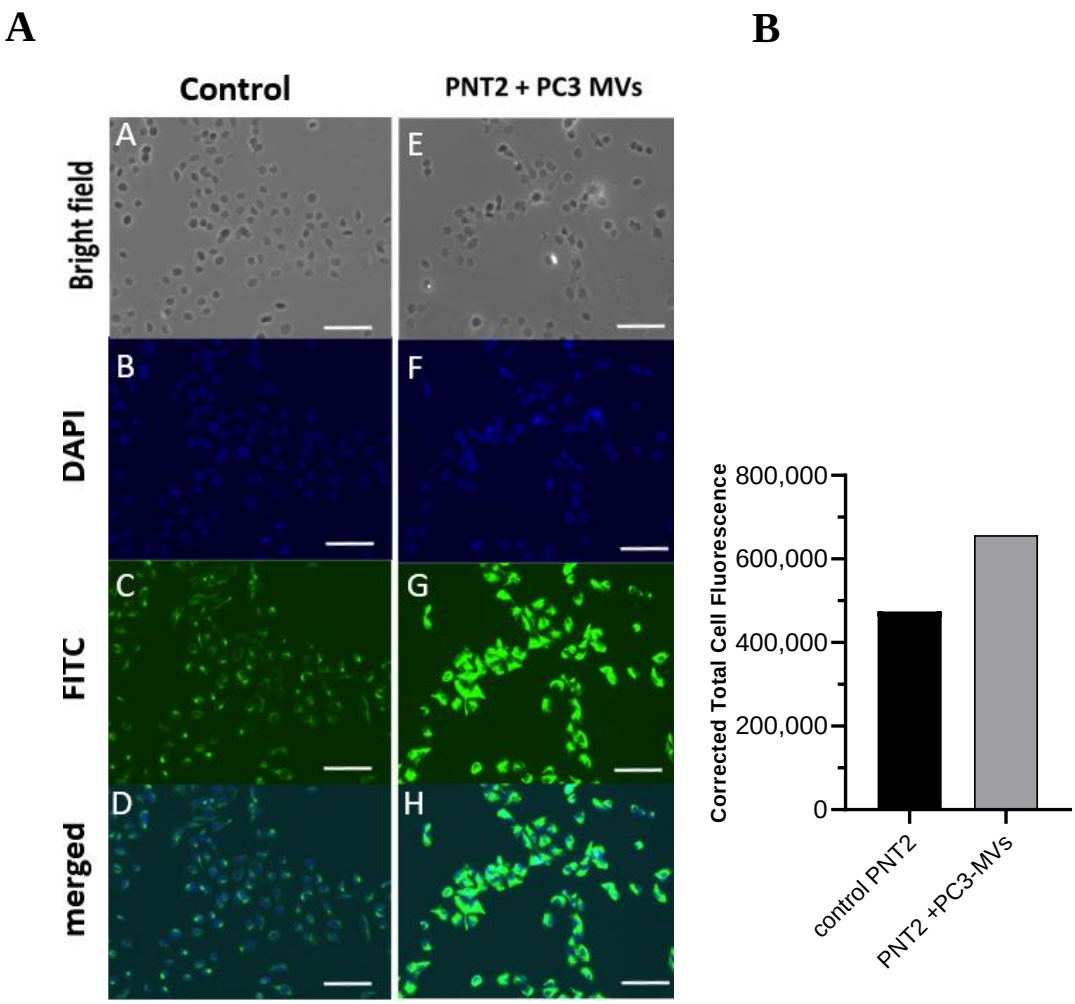


Figure 24: PNT2 cells treated with Prostate cancer (PC3) cell derived MVs show marked increase in Vitamin expression as determined by fluorescent microscopy. In Panel A, (A) and (E) show bright field images of the cells. (B) and (F) show the nuclei in the cells using DAPI. (C) and (G) show Vimentin expression in control and treated PNT2. (D) and (H) show merged images of DAPI and Vimentin to show the localisation of the protein. In panel B, the corrected total cell fluorescence (CTCF) of images in panel A was calculated, cells treated with PC3 MVs show marked increase in fluorescence compared to control cells. Scale bar= 200μm.

4.3.5 Flow cytometry analysis of vimentin expression following treatment of PNT2 cells with PC3 derived MVs.

For flow cytometry analysis, prostate epithelial cells (PNT2) were seeded at ($5x10^4$/well) in the 24 well plate in triplicates. The cells were grown in complete growth medium (RPMI supplemented with 10% FBS). Cells were incubated for 24 hours in 37°C, 5% CO_2 to ensure adherence to the culture plate. After 24 hours of incubation the media was removed, the adherent cells were washed with PBS and resuspended in MVs free complete growth medium (refer to methods in chapter 2). Cells were either treated with 5mg/ml of PC3 MVs or not for control. Treated and untreated cells were all incubated for 5days at 37°C, 5% CO_2. At day 5 of incubation the supernatant was removed and stored for MVs isolation. The cells were washed with ice cold PBS and were dissociated from the wells by Trypsin/EDTA. Cells were fixed with 4% PFA prior to permeabilisation with Triton X-100 (0.1%) due to Vimentin being a cytoskeletal protein expressed mainly in the cytoplasm of the cells. After permeabilisation cells were labelled with monoclonal anti Vimentin antibody. The labelled cells were resuspended in 100µl of filtered MVs free PBS ready for analysis on Guava easyCyte.

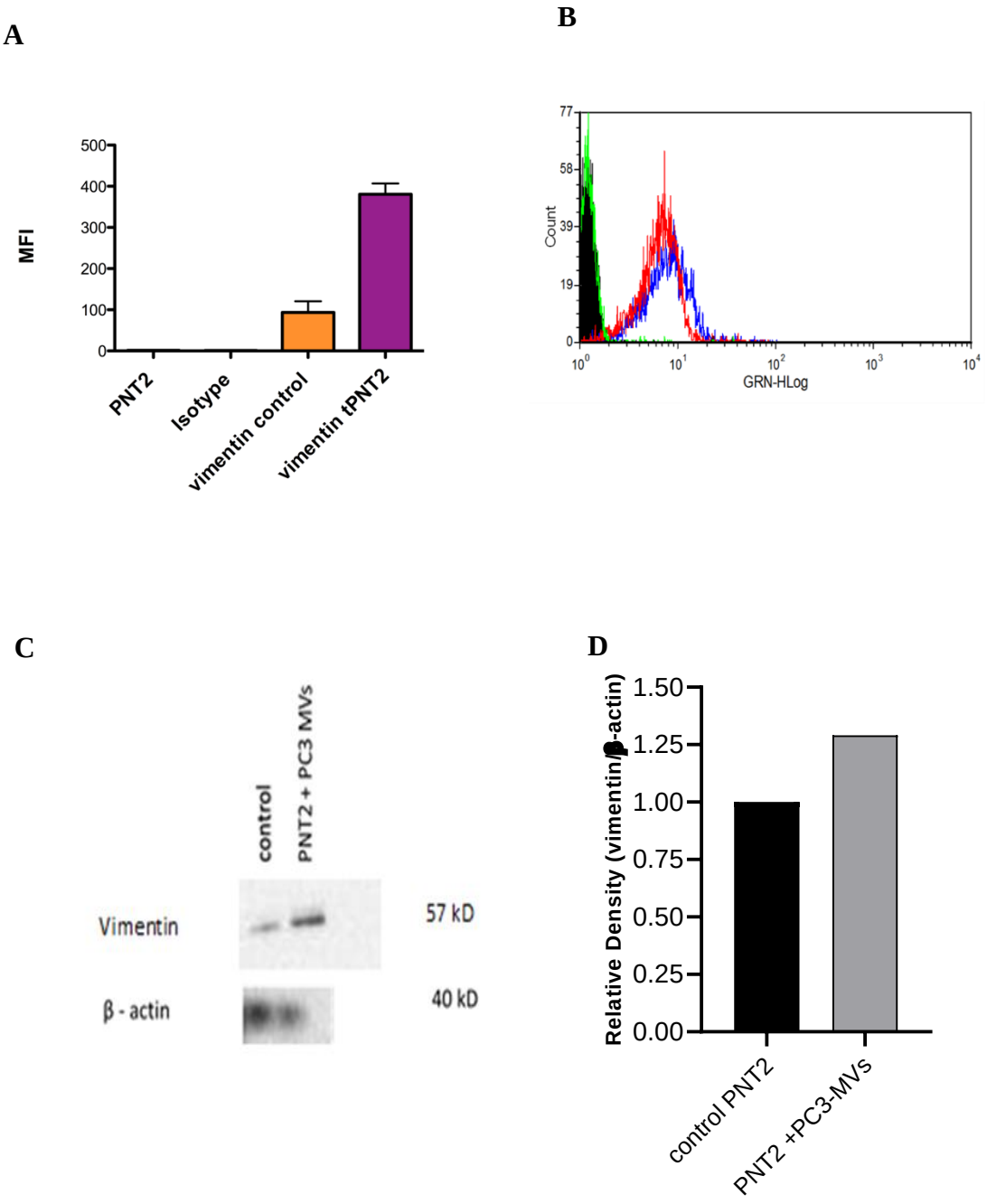


Figure 25: Analysis of Vimentin expression in PNT2 cells treated with PC3 derived MVs by fluorescent microscopy. Flow cytometry and western blot analysis show the expression of Vimentin in PNT2 cells treated/not treated with 5mg/ml for 5 days. **(A)** shows the expression of Vimentin in PNT2 cells measured by Mean Fluorescence Intensity, PNT2 cells treated with PC3 MVs show marked increase in Vimentin expression. The histogram in **(B)** shows marked increase in the expression of Vimentin (red) in PNT2 cells treated with PC3 MVs compared to the control PNT2 cells (blue). Similarly, in **(C and D)** Western blot analysis show that there is higher expression of Vimentin in the treated PNT2 cells.

The above figure shows the expression of EMT marker vimentin in control PNT2 cells and treated PNT2 cells. PNT2 cells showed high expression of Vimentin in PNT2 cells treated with 5mg/ml PC3 MVs and expression of this intermediate filament protein is observed only in cells undergoing EMT in cancer or fibrosis. The above results show that PC3 derived MVs play an active role in the process of EMT due to MVs carrying bioactive material. Studies carried out in CMIRC previously showed that MVs can induce apoptosis in monocytic cells by carrying TGF-β on the surface of the MVs.

Similarly, in figure 25 the same EMT marker, Vimentin is shown to have increased in medium fluorescence intensity (MFI) compared to the control cells. Also, the bar chart shows a slight increase in the percentage expression compared to the control. The western blot analysis shows intense band on treated cells compared to the control.

4.3.6 Analysis of β-catenin expression in PNT2 cells treated with PC3 derived MVs by fluorescent microscopy.

Transcription factors were also studied in this study to further confirm the epithelial mesenchymal transition observed with the above to biomarkers. PNT2 cells were treated with PC3 MVs as in above and were incubated for 5 days at 37°C, 5% CO_2. Cells were labelled with primary and secondary antibody and were analysed with fluorescent microscope.

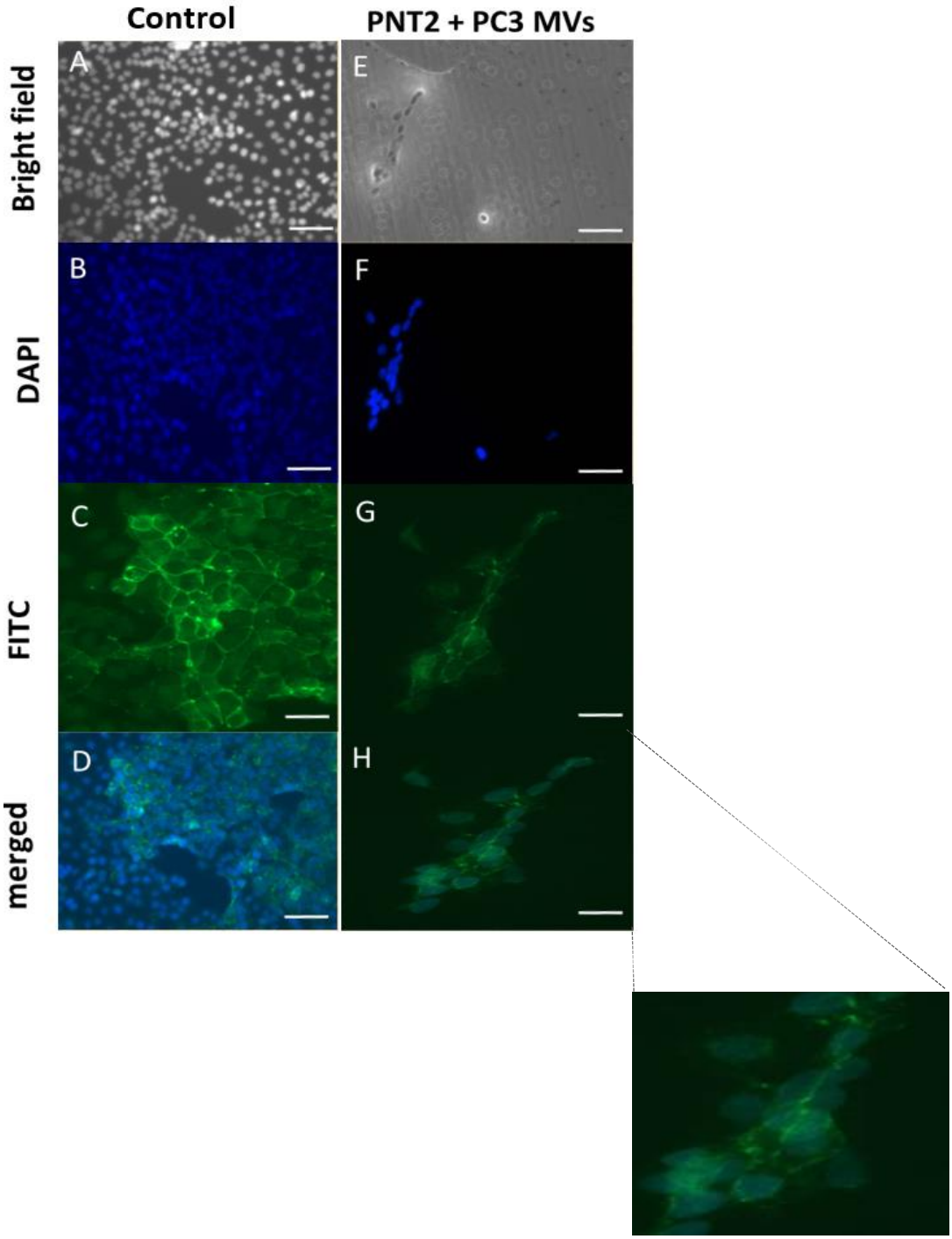


Figure 26: Fluorescent microscopy analysis show the expression of β-catenin in PNT2 cells treated/not treated with PC3 derived MVs for 5 days.
Bright field images show PNT2 cells after 5 days **(A)** control and **(E)** after treatment with 5mg/ml of PC3 MVs. **(B)** and **(F)** show the nuclei in the cells stained with DAPI. **(C)** and **(G)** shows expression of β-catenin in control PNT2 cells and treated PNT2 cells. **(D)** shows merging of DAPI and Fluorescence in control **(H)** shows phosphorylation of β-catenin after treating PNT2 cells with PC3 MVs. Scale bar = 200μm

4.3.7 Flow Cytometry analysis of β-catenin expression following treatment of PNT2 cells with PC3 derived MVs.

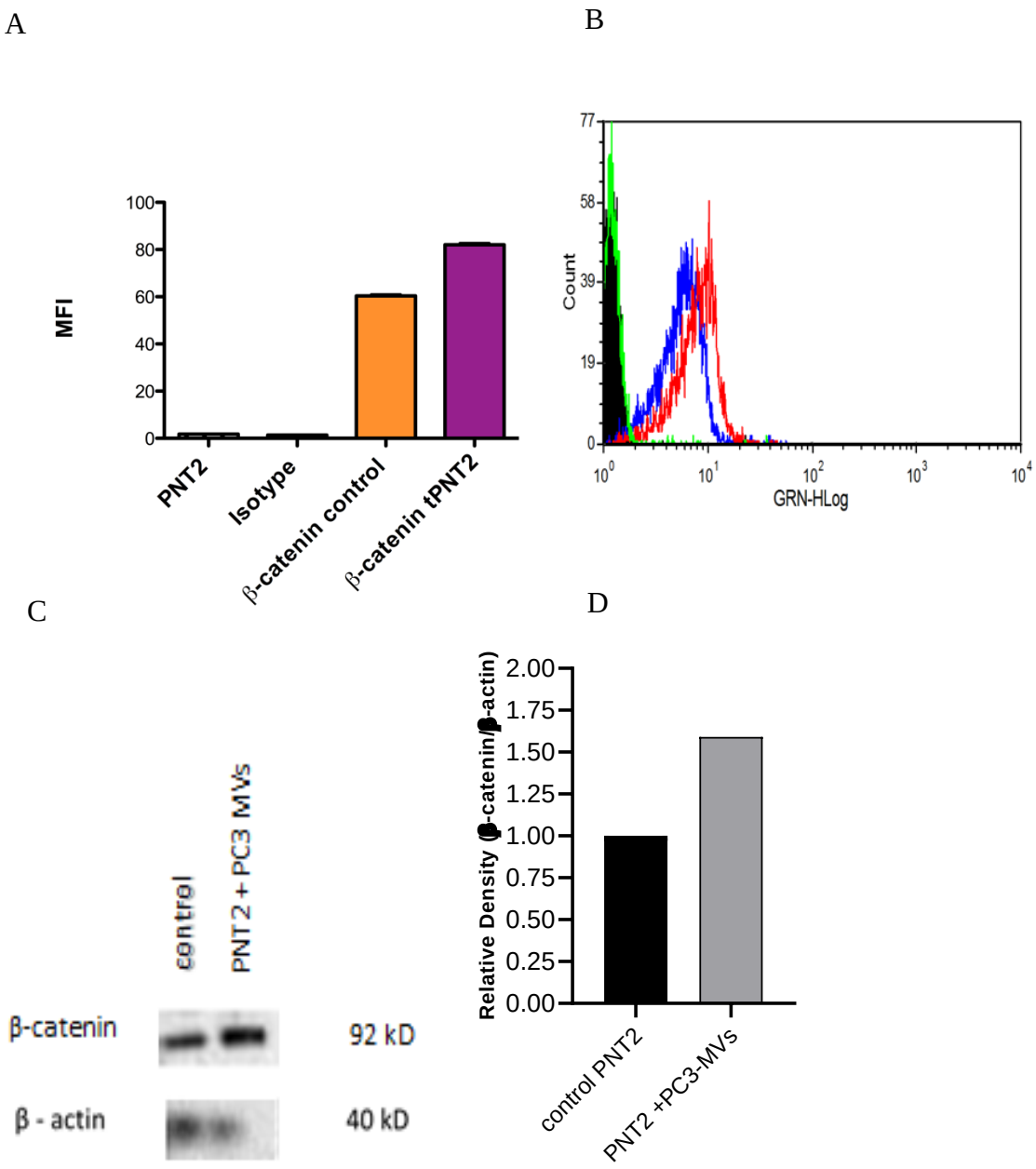


Figure 27: Analysis of β-catenin expression in PNT2 cells treated with PC3 derived MVs by flow cytometry and western blot. Flow cytometry and western blot analysis show the expression of β-catenin in PNT2 cells treated/not treated with 5mg/ml for 5 days. **(A)** shows the expression of β-catenin in PNT2 cells measured by Mean Fluorescence Intensity. The experiment was repeated three times and the data presented are the mean ±SEM of the results. The histogram in **(B)** shows marked increase in β-catenin (red) in PNT2 cells treated with PC3 MVs compared to the control PNT2 cells (blue). Similarly, in **(C and D)** Western blot analysis show that there is higher expression of β-catenin in the treated PNT2 cells.

The above figure shows the expression levels of β-catenin in PNT2 cells treated and untreated. β-catenin is a transcription factor and has shown to be translocated in the nucleus taking control of the cell cycle. This suggests that indeed the prostate cancer cell derived MVs are capable of inducing EMT in normal epithelial cell line.

4.3.8 Analysis of Slug expression in PNT2 cells treated with PC3 derived MVs by fluorescent microscopy.

The EMT experiment was set up as in the above, however in this experiment prior to labelling the cells with the monoclonal anti-Slug, the cells were permeabilised with Triton- X100 (0.1%) to get to the cytoplasm and nucleus of the cells.

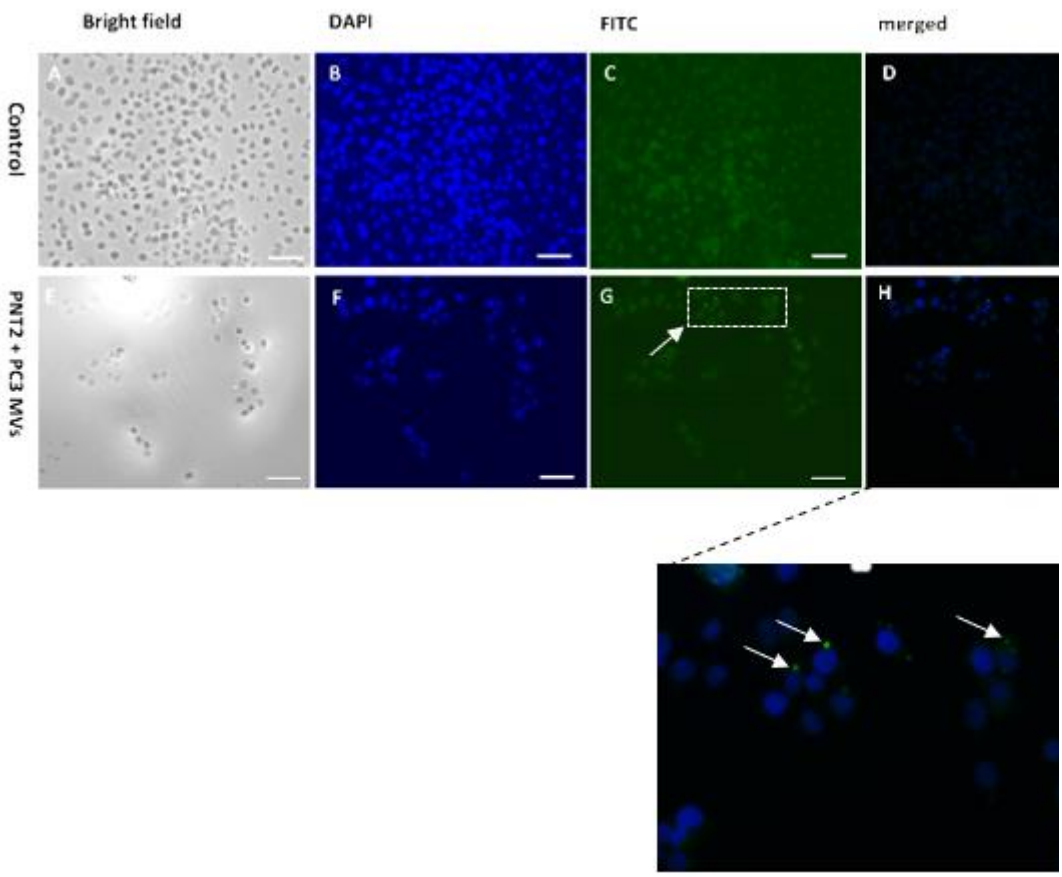


Figure 28: Fluorescent microscopy analysis show the expression of Slug in PNT2 cells treated/not treated with PC3 derived MVs for 5 days. Bright field images show PNT2 cells after 5 days **(A)** control and **(E)** after treatment with 5mg/ml of PC3 MVs. **(B)** and **(F)** show the nuclei in the cells stained with DAPI. **(C)** and **(G)** shows expression of Slug in control PNT2 cells and treated PNT2 cells. **(D)** shows merging of DAPI and Fluorescence in control **(H)** shows phosphorylation of Slug after treating PNT2 cells with PC3 MVs and in enlarged form in **(I)**. Scale bar = 200µm

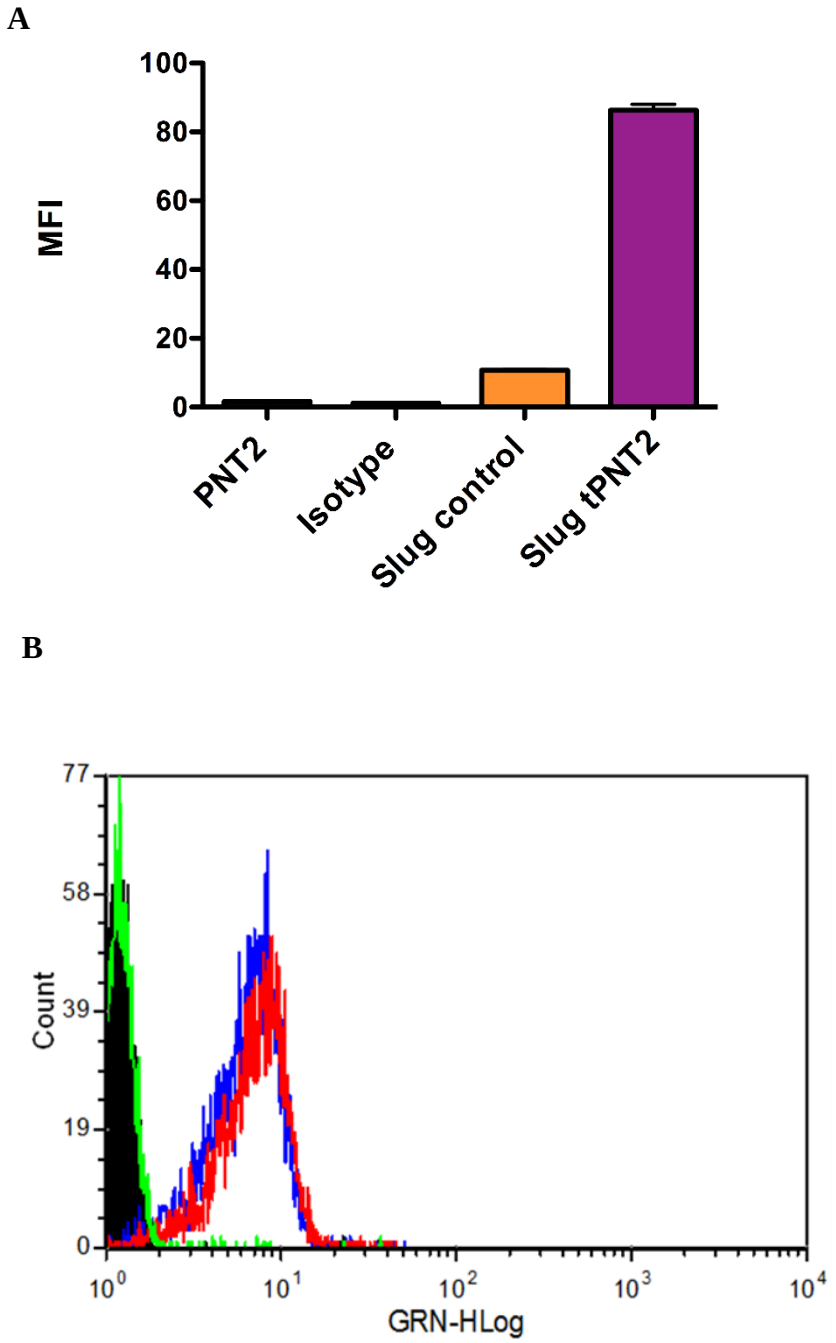


Figure 29: Analysis of Slug expression in PNT2 cells treated with PC3 derived MVs by flow cytometry. (A) shows the expression of Slug in PNT2 cells measured by Mean Fluorescence Intensity, PNT2 cells treated with PC3 MVs show marked increase in Slug expression. The experiment was repeated three times and the data presented are the mean ±SEM of the results. The histogram in **(B)** shows slight shift to the right, which shows an increase in the expression of Slug (red) in PNT2 cells treated with PC3 MVs compared to the control PNT2 cells (blue).

4.4 Summary

EMT is a physiological process in which epithelial cells are disseminated from the surrounding epithelial tissue and acquire mesenchymal like properties. There are three different types of EMT identified so far and out of the three types of EMT two are physiological and one is cancerous, and it only occurs when and where it is needed. The physiological types of EMT are the morphogenesis and fibrotic. These two occur in the body naturally and stops once the new tissue is made. However, the cancerous EMT spreads through the blood and the cancer cells end up in a distant site for metastasis, destroying the tissue eventually.

In this chapter, the hypothesis was tested to observe the potential of PC3 derived MVs in initiating EMT in PNT2 cells. This was PC3 MVs were added to PNT2 cells to observe the EMT in PNT2 (non-cancer cell line). PNT2 cells were sub cultured and were incubated with PC3 MVs for 5 days to observe the effect of EMT. Morphological and biochemical changes were observed and analysed using flow cytometer, fluorescent microscope and western blot.

E-cadherin and Vimentin expression were measured against the control with no PC3 MVs. When analysed the E-cadherin was observed to have reduced its expression after the addition of PC3 MVs and Vimentin's expression was increased with the addition of PC3 MVs. These results indeed exhibited the behaviour of EMT cells. To further confirm the transitional changes in the cells, transcription factors of EMT were also investigated.

In total, three different transcription factors were tested namely; β-catenin, Slug and N-cadherin. There was marked increase in the expression of the transcription factors in all three cases. This suggests that the cells have undergone EMT.

The fluorescent microscope images indicate that the epithelial cells (PNT2) generally lost adherence, gaps were observed in between cells. Cells that were stained with E-cadherin showed marked decrease in expression in cells after adding PC3 MVs compared with control. Also, Vimentin was shown to have increased in expression in cells with PC3 MVs added compared to control. This can also be seen in the western blot analysis. The Vimentin band appears darker (more concentrated) in the cells treated with PC3 MVs.

Transcription factors were also shown to have increased slightly in expression in cells treated with PC3 MVs. The fluorescence was observed around the nucleus which is significant of phosphorylation.

Based on the above results, it can be concluded that PC3 derived MVs are capable of inducing EMT in non-cancer PNT2 epithelial cells.

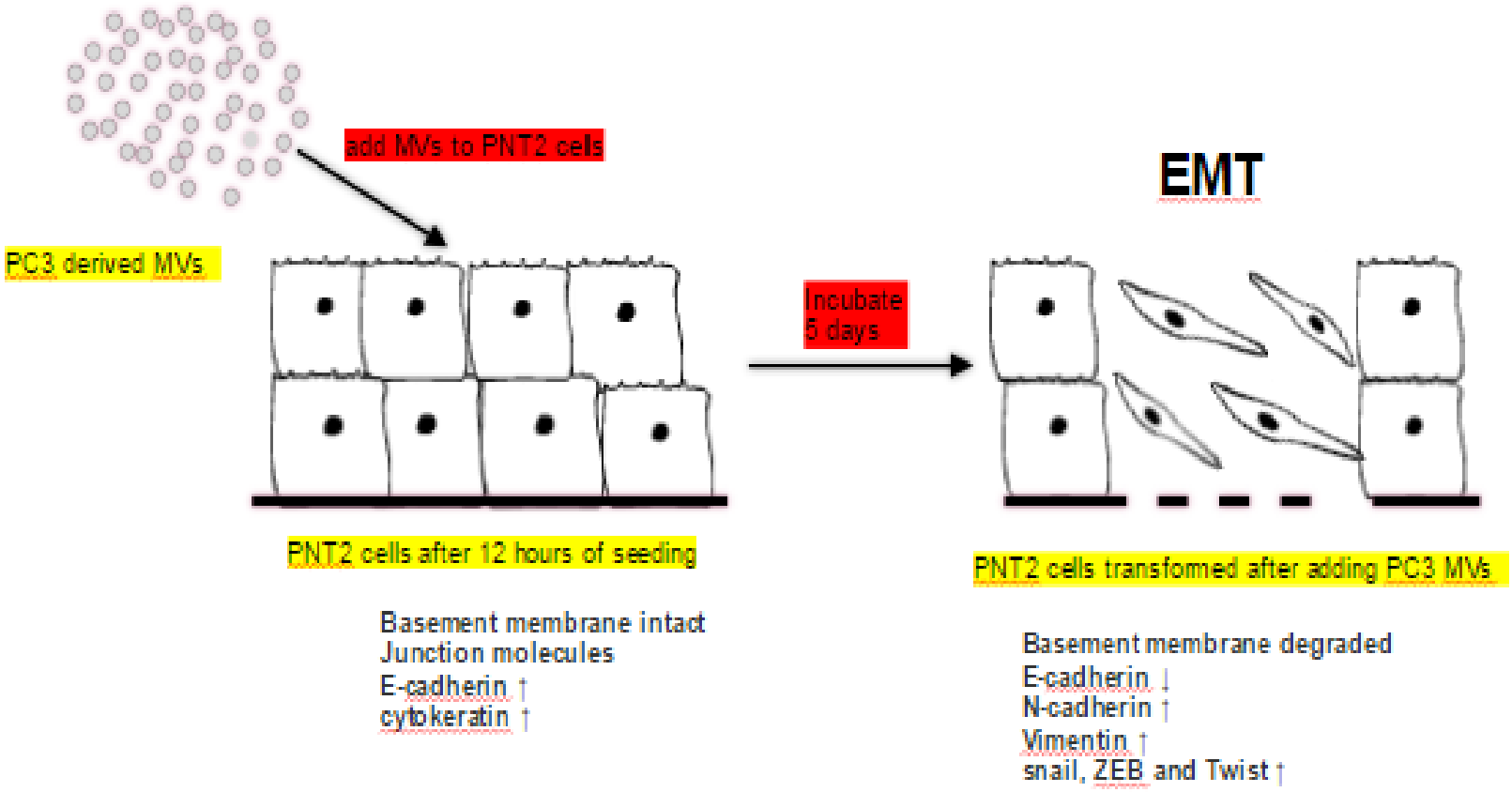


Figure 30: **Schematic diagram shows the effect of PC3 cells derived MVs on EMT in PNT2 cells. The loss of epithelial markers and gain of mesenchymal markers is also shown.**

Chapter 5: Proteomics analysis of PC3 Microvesicles

5.1 Introduction

The experiment presented in chapter 4 clearly showed that prostate cancer derived MVs added to normal (immortal) prostate epithelial cells (PNT2 cell line), induced the process of epithelial mesenchymal transition in the PNT2 cells. This was shown using different molecular biology techniques. This also suggests that PC3 derived MVs have active biomaterials that make the MVs potent and capable of inducing EMT. To further investigate, the protein cargo carried by these vesicles was considered.

Proteomic techniques and analysis were used to study the up regulation and/or down regulation of different proteins involved in inducing the process of EMT, presence in PNT2 MVs. Furthermore, to delineate which proteins specifically taken up in MVs could be playing a role in EMT, the cells from which the MVs were isolated was also studied for their protein content. In this study a quantitative label-free LC-MS/MS proteomics procedure was used. This technique allows identification and quantification of hundreds and thousands of proteins simultaneously. Unlabelled peptide fragments were submitted directly to the LC-MS/MS, the data generated the being mapped to online protein databases allowing for protein identification and quantification. The relative abundance of species in the sample was quantified by the spectral count, which is the number of MS/MS spectra identified as arising from the species. The average total number of spectra in each sample was calculated, also the average number of spectra across all the samples was also calculated. The number of spectral count for each triplicate of each sample was multiplied by (the average of spectral count across the samples / average number of spectral counts in each

sample). This calculation of normalising the spectrum count was done using the Scaffold software.

The significantly enriched protein IDs obtained from this study were then used for further bioinformatics analysis such as predicting involvement in specific cellular pathways and involvement in other cellular processes. Two widely cited online software tools were used to analyse the data in this chapter; DAVID (to determine the involvement of proteins in cellular processes), KEGG (to understand high level functions of the biological system) and STRING (to look at protein pathways). Both software required UniProt accession numbers which were found for each protein from the LC-MS/MS database IDs. The UniProt accession IDs were then used to perform pathway analysis and protein interaction analyses found in this chapter.

5.2 Methods

Throughout this study isolation of enough MVs was a major hurdle and proved to be time consuming. Therefore, providing enough samples for proteomics (each sample in biological and experimental triplicate) was a very crucial step and samples had to be prepared carefully. Therefore, cells and MVs protein samples were prepared as in the steps below.

MVs derived from PC3 cells were isolated and their protein concentration determined using the BCA protein assay. As many as (5×10^4) PNT2 cells per well were grown in 24-well plates overnight. Cells were washed and 5 mg/ml of PC3 MVs were added to each well of PNT2 cells. After an incubation period of 5 days at 37^0 C and 5% CO_2, the cells were collected and treated with RIPA buffer to analyse the proteins.

PC3, PNT2 and tPNT2 cells ('transformed' PNT2 also called 'EMT cells') and MV lysates were obtained and heated at 95^0 C for 5 minutes to denature the proteins in the sample. All samples were mixed with Laemmli sample buffer and loaded on precast Any-KD Mini-PROTEAN TGX gels (Bio-Rad). Samples were run for approximately 10 minutes to concentrate the proteins through the stacking gel and obtain distinct bands in the resolving gel. The protein bands were excised and placed in an Eppendorf tube with ddH_2O for storage. Samples were sent to the Cambridge Proteomics centre for LC-MS/MS analysis. Initially, the gel bands were cut into $1mm^2$ pieces, de-stained, reduced (DTT) and alkylated (iodoacetamide) and subjected to enzymatic digestion with chymotrypsin overnight at 37^0 C. After digestion the supernatant was pipetted into a sample vial and loaded onto an autosampler for automated LC-MS/MS analysis. LC-MS/MS experiments was

performed on the samples using a Dionex Ultimate 3000 RSLC nanoUPLC system and a Q Exactive Orbitrap mass spectrometer. The peptides were separated by reverse-phase chromatography and were loaded onto a pre-column from the Ultimate 3000 autosampler. The elution of peptide was performed by switching the column valve from the pre-column onto the analytical column. The eluent was then sprayed into the mass spectrometry. All m/z values of eluting ions were measured in an Orbitrap mass analyser set to a specific resolution range.

The data obtained from the MS/MS analysis was converted to mgf files and the files were submitted to the Mascot search algorithm and searched against UniProt human database (71898 sequences; 24121858 residues (and a common contaminant sequences (115 sequences, 38274 residues). Variable modification of oxidation, deamidation and carbamidomethyl were applied. For significance of the data, threshold value of $p<0.05$ and peptide cut off score of 20 were also applied. The peak area of the intact peptides on the chromatograms were calculated and all data was imported into the Scaffold program (Version _4.5.4, Proteomes Software Inc, Portland, OR)

Protein IDs from the analysis were then subjected to statistical tests by exporting to excel and sorting protein identifications according to number of peptides present. Proteins expressed on MVs were then mapped on the vesicleopedia database to obtain their accession numbers. Also, proteins were grouped according to presence or absence in all the cells lines and MVs samples. This helped in identifying proteins that were present in PC3 and not PNT2 however otherwise present in tPNT2. The identified list of proteins was further analysed using three pathway analysis tools; DAVID, KEGG and STRING. This software required each proteins UniProt

accession numbers or its gene names, so that each protein could be matched and identified. In figure 31, a flow chart of the process used to harvest the cells and MVs for proteomic analysis are shown.

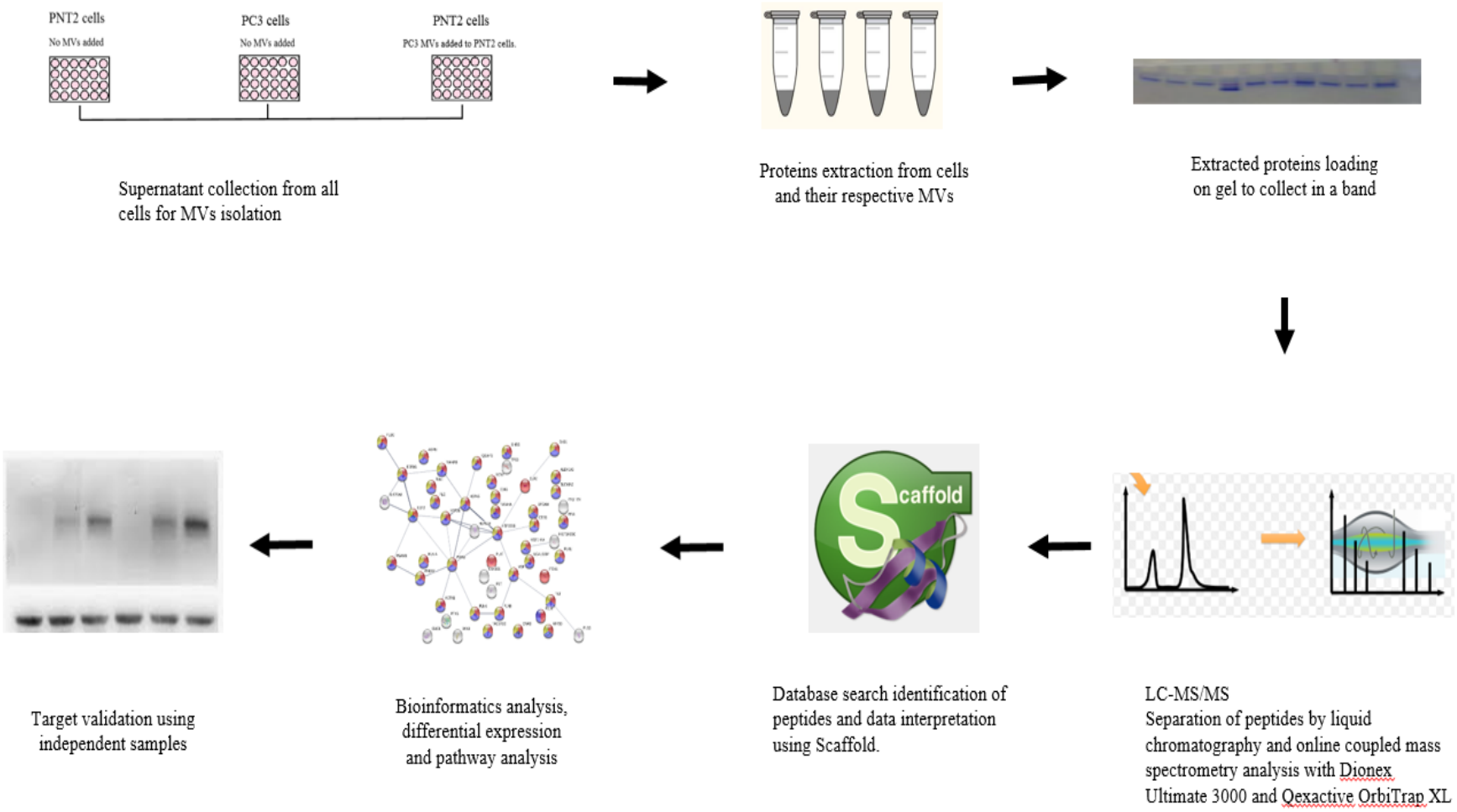


Figure 31: Flow chart shows methods and techniques used in this chapter.

5.3 Results

Briefly, LC - MS/MS was used to analyse the protein cargo of MVs and cells. The results were uploaded on to a proteomics software 'Scaffold.' accession numbers for each protein were obtained from the software. The proteins were mapped onto human gene database and were identified.

Data analysis was performed using Excel. Further data analysis was carried out using DAVID, KEGG and STRING analysis pathway analysis tools. DAVID (the Database for Annotation, Visualization and Integrated Discovery) was used for gene enrichment analysis of differentially expressed proteins.

EVpedia is an online database of proteins that are expressed in Extracellular Vehicles (EVs). This database was used to review the proteins differentially expressed in all the different MVs samples.

To ascertain putative protein- protein interactions, STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) analysis was used to draw associated links between the different proteins involved in different biological processes.

BioCarta was used to determine gene expression coherence in biological pathways. Co-expression of genes was observed using pathways annotated by KEGG (Kyoto Encyclopedia of Genes and Genomes).

Since the proteomics study in this chapter is observing protein expression in three different cell lines (PC3, PNT2 and tPNT2) and their respective MVs, comparing

different cell lines, MVs derived from different cell types and cells versus MVs were considered to be the most significant comparative conditions.

5.3.1 Proteins detected in cells and MVs

All cells samples and MVs samples that were tested for proteins by LC-MS/MS were sorted and grouped together into two categories. Venn diagrams were produced for the proteins present on all three cells and another Venn diagram for the respective MVs samples.

In total 1290 different proteins were detected collectively on cells and MVs that were analysed. The data of all the proteins was sorted on Excel, and the proteins were grouped into two categories; cellular proteins and MVs proteins. List of proteins in each sample were sorted and Venn diagrams were generated for both different cells and respective MVs.

The Venn diagram below (Figure 32) illustrates the number of proteins in each cell line and MVs analysed. In (A) there are 678 common proteins present in all three cells. There are 175 proteins commonly were present in both PC3 and tPNT2, whereas only 28 common proteins present in PNT2 and tPNT2.

This suggests that 38% the proteins in of PC3 cells' proteins are also present in the transformed tPNT2 cells compared to only 6% of the PC3 proteins present in PNT2 cells. This is representative of the transition to a mesenchymal phenotype (tPNT2) cells. In (B), there are more proteins in PC3 MVs compared to PNT2 and tPNT2 MVs. Also, there are 110 proteins that are present in both PC3 MVs and tPNT2 MVs,

which accounts for 47% of the total number of proteins detected for PC3 MVs. There are approximately 4% of PNT2 MVs proteins present commonly in both PNT2 and tPNT2 MVs. This is also representative of the transformation of PNT2 cells by PC3 MVs.

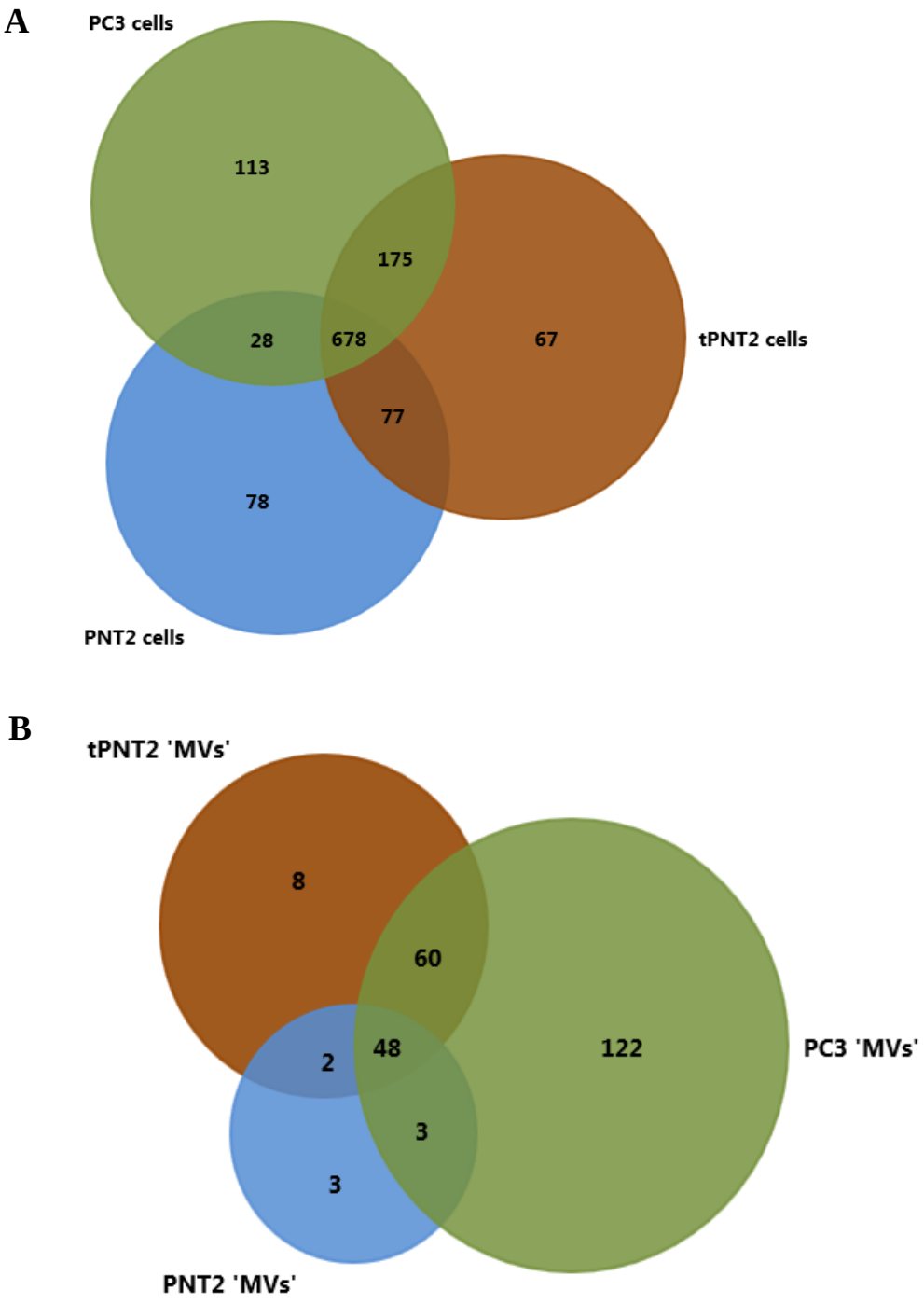


Figure 32: Number of proteins present in three different cell lines and the respective MVs samples in this study. (A) Venn diagram showing proteins expressed in PC3, PNT2 and tPNT2 cells. **(B)** Venn diagram showing proteins expressed in MVs isolated from those same cell lines.

Proteins identified as present in at least two of the replicates were considered for submission to DAVID. One hit identification across three replicates were not included in this analysis and the resulted proteins that were included in the search were as follows; 135, 60 and 148 proteins in PC3, PNT2 and tPNT2 cells respectively. Similarly, number of proteins in MVs were also reduced to 236, 59 and 120 proteins in PC3, PNT2 and tPNT2 MVs respectively.

5.3.2 Protein comparison between MVs and parent cell

To understand how many proteins are carried by the host cells and its respective MVs, proteins expressed in parent cells and proteins expressed in MVs were compared and overlapped.

The differences noted in protein up-regulation or down-regulation between the parent cell and its respective released MVs could be useful in understanding the nature of microvesiculation and using MVs as vectors in cellular communication.

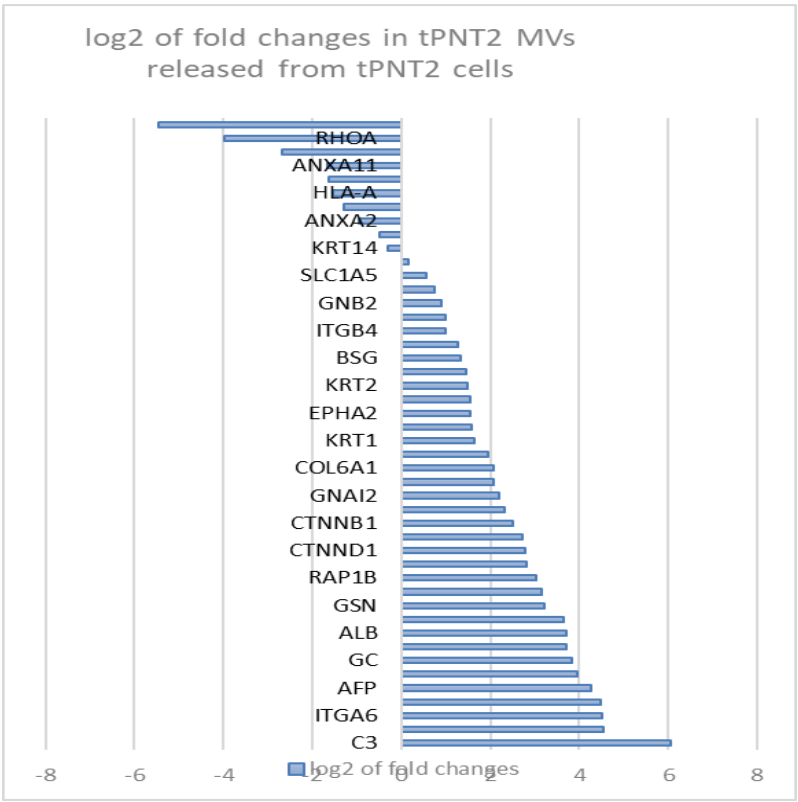
Here in Figure 33, protein expressed in the transformed cells; tPNT2 cells and MVs derived from tPNT2 cells were compared. There are 921 proteins present in tPNT2 cells and 40 proteins in tPNT2 MVs. In addition, there are 77 proteins that are expressed in both tPNT2 cells and respective tPNT2 MVs. Due to the relatively small size of MVs compared to their respective parent cells, MVs are expected to express far less protein compared to the cells.

Fold change in some proteins expressed in tPNT2 cells and MVs was determined by log2 of fold changes. Protein RHOA, ANXA5, ANXA11, HLA-A, ANXA2 and KRT14 were down regulated in MVs compared to their respective parent cells. The rest of the proteins on the list were up regulated as on the fold change.

Annexins

There are three proteins from the annexin family that were down regulated in the fold changes in MVs compared to parent cells. Annexins play a significant role in two apoptosis related pathways; caspase and p53, particularly Annexin A11. Annexins also act as receptors for calcium in the process of exocytosis (Mirsaeidi *et al*, 2016).

A



Gene name	Protein name
RHOA	Transforming protein RhoA
ANXA11	Annexin A11
HLA-A	HLA class 1 histocompatability
ANXA2	Annexin A2
KRT14	Keratin type1 cytoskeletal 14
SLC1A5	Neutral amino acid transporter
GNB2	Guanine nucleotide binding protein
ITGB4	Integrin beta 4
BSG	Basigin
KRT2	Keratin, type11 cytoskeletal 2
EPHA2	Ephrin type- A receptor 2
KRT1	Keratin, type1 cytoskeletal 1
COL6A1	Collagen alpha-1
GNA12	Guanine nucleotide binding protein
CTNNB1	Catenin beta 1
CTNND1	Catenin delta 1
RAP1B	Ras related protein Rap-1b
GSN	Gelsolin
ALB	Albumin
GC	Vitamin D binding protein
AFP	Alpha fetoprotein
ITGA6	Integrin alpha 6
C3	Complement C3

B

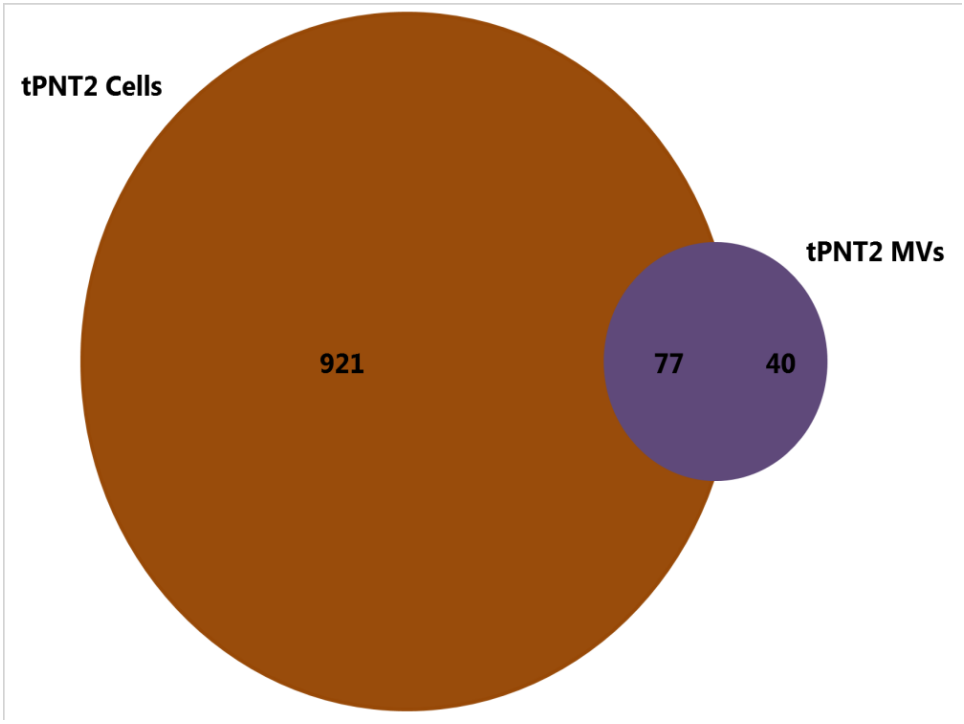


Figure 33: Part (A) shows log2-fold changes of proteins in tPNT2 cells compared to tPNT2 MVs. The protein names for genes are displayed in the table. Part **(B)** is a Venn diagram that shows the number of proteins present in tPNT2 cells and tPNT2 MVs.

Similarly, in figure 34 proteins present in PC3 cells and respective PC3 MVs show fold changes and the Venn diagram shows the number of proteins that were present on the MVs compared to the parent cell.

As many as 852 proteins were expressed in PC3 parent cells and 91 proteins were expressed in MVs isolated from the same PC3 cells. There were 152 proteins that were expressed in both PC3 cells and PC3 derived MVs. Also, fold changes in the two samples are shown below.

Fold change in some proteins expressed in PC3 cells and MVs was determined by log2 of fold changes. Protein SAHH, S1OAG, ALDOA, 1433Z, MOES, ANXA2, GD1B, LDHB, AOAO24R571, 1433B and KPYM were down regulated in MVs compared to cells. The rest of the proteins on the list were up regulated as on the fold change.

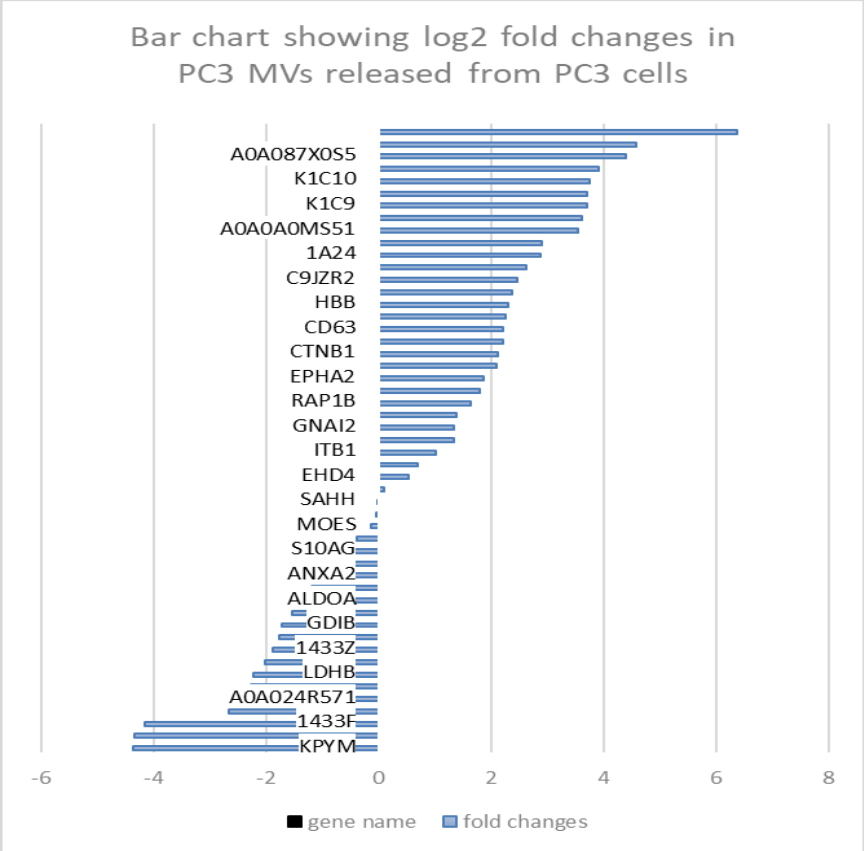
Pyruvate kinase (KPYM)

Pyruvate kinase was shown to be one of the most down-regulated proteins in PC3 MVs compared to PC3 cells. Pyruvate kinase is an enzyme that plays a part in converting ADP to ATP in glycolysis and it also has a role in regulating cell metabolism. Its role has also been implicated in cancer pathologies for the last 40 years (Israelson and Vander Heiden, 2015). Specifically, pyruvate kinase helps the cell to be metabolically flexible and helps with cellular adaptation to changing conditions (Israelson and Vander Heiden, 2015).

Gelsolin (AOAO24MS51)

Gelsolin is the protein that has up regulated the most in the "PC3 MVs" vs "PC3 cell" comparison. Gelsolin is an actin binding protein that is regulated by calcium and it is involved in filament assembly, stabilisation and disassembly. It is found in both cytoplasm and mitochondria and it plays a role in regulating apoptosis (Burtnick *et al*, 2004). Having both pro and anti-apoptotic activities (Desouza *et al*, 2012)

A



Gene name	Protein name
A0A087X0S5	Collagen alpha-1 (vi) chain
K1C10	Keratin type1, cytoskeletal 10
K1C9	Keratin type1, cytoskeletal 9
A0A0A0MS51	Gelsolin
1A24	HLA class 1 histocompatibility antigen
C9JZR2	Catenin delta-1
HBB	Haemogloin subunit beta
CD63	CD63 antigen
CTNB1	Catenin beta-1
EPHA2	Ephrin type A receptor 2
RAP1B	Ras- related protein Rap-1b
GNAI2	Guanine nucleotide bindin protein
ITB1	Integrin beta-1
EHD4	EH domain containing protein 4
SAHH	Adenosyl homocysteinase
MOES	Moesin
S10AG	Protein S100
ANXA2	Annexin A2
ALDOA	Fructose - bisphosphate aldose A
GDIB	Rab GDP dissociation inhibitor beta
1433Z	14-3-3 protein zeta/delta
LDHB	L-lactate dehydrogenase B chain
A0A024R571	EH domain containing protein 1
1433F	HLA class 1 histocompatibility antigen
KPYM	Pyruvate kinase PKM

B

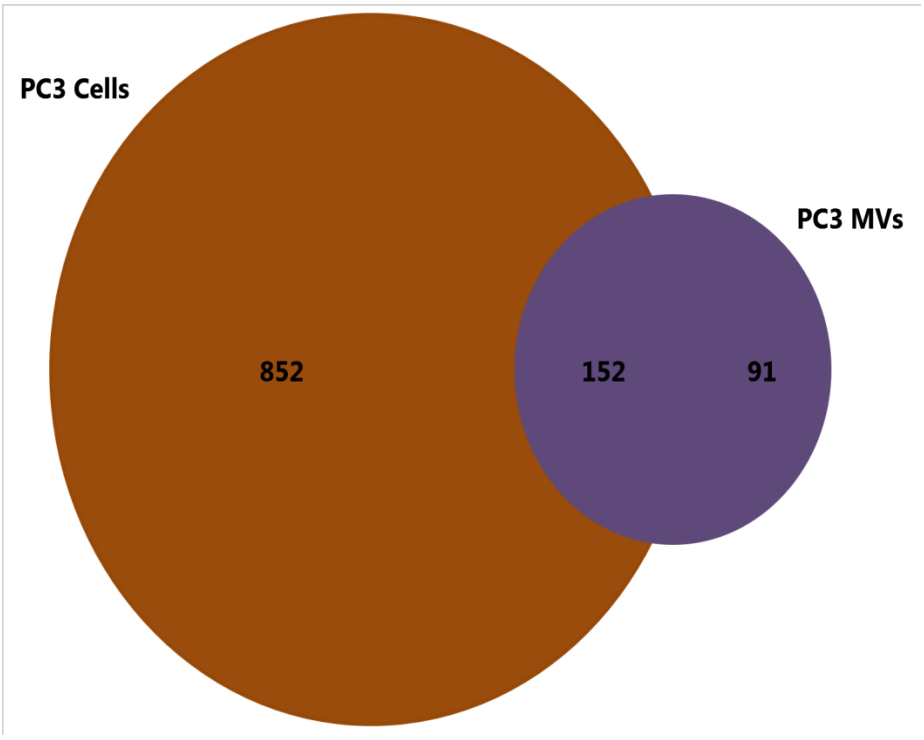


Figure 34: Part (A) shows log2 fold changes in PC3 cells compared to PC3 MVs. The protein names for genes are displayed in the table. Part **(B)** is a Venn diagram that shows the number of proteins present on PC3 cells and PC3 MVs.

5.3.3 Proteins present or absent in PC3, PNT2, tPNT2 and their respective MVs

Mass spectrometry allows for a large number of proteins to be identified in a small sample based on the behaviour of their peptide fragments in a magnetic field. Therefore, MS analysis produces information on peptide fragmentation and does not identify intact proteins directly. This is known as a bottom up approach and has statistical limitations when identifying proteins. The peptide information is matched, scored and assigned a significance value to a potential protein it belongs to on a programmed database using algorithms. Identifiers from these databases are then assigned to proteins which in turn are assigned significance values based on the

confidence of their constituent peptides as a result peptide can be of low enough significance as to not result in a protein identification being assigned. “Absent” therefore in relation to the following comparisons relates at the very most to proteins that are not present in one condition but present in its comparator or at the very least it represents proteins that didn’t have enough peptide information to result in a protein identification in one condition compared to another.

Here, proteins present or absent on all three cell lines and their respective MVs were compared. The samples analysed were in triplicates, proteins present in two samples or more being regarded as ‘present’. Similarly, protein present on one sample and not in the other two being regarded as ‘absent’.

As the role of PC3 MVs in epithelial mesenchymal transmission was being explored in this chapter, proteins up regulated or down regulated in PC3 MVs is clearly regarded as of high importance. However, proteins present or absent on all the cells studied in this work is used as a reference for our study and all samples tested were analysed in experimental and biological triplicates. The following table of proteins was used as a control for the proteins present or absent for cells compared with the MVs.

Table 1: Table of proteins showing proteins present and/or absent in PC3 cells, PNT2 cells and tPNT2 cells.

		CELLS					CELLS		
No.	Protein Name	PC3	PNT2	tPNT2	No.	Protein Name	PC3	PNT2	tPNT2
1	26S proteasome non-ATPase regulatory subunit 7	+	-	+	76	Glutaminase kidney isoform, mitochondrial	-	+	+
2	26S proteasome non-ATPase regulatory subunit 8	+	+	+	77	Glutathione reductase, mitochondrial	-	+	+
3	26S proteasome non-ATPase regulatory subunit 9	+	-	+	78	Glutathione S-transferase	+	-	+
4	60S ribosomal protein L31	+	+	+	79	Glycylpeptide N-tetradecanoyltransferase 1	+	+	+
5	6-phosphogluconolactonase	+	-	+	80	Growth/differentiation factor 2	-	+	+
6	Actin-related protein 2/3 complex subunit 2	+	-	+	81	GTP-binding protein SAR1a	+	-	+
7	Actin-related protein 2/3 complex subunit 3	+	-	+	82	Hemoglobin subunit alpha	-	+	+
8	Acylamino-acid-releasing enzyme	+	-	+	83	Heterochromatin protein 1-binding protein 3	+	-	+
9	Acyl-coenzyme A thioesterase 9, mitochondrial	+	-	+	84	Hippocalcin-like protein 1	+	-	+
10	Adenylosuccinate synthetase isozyme 2	+	+	+	85	Histidine--tRNA ligase, cytoplasmic	+	+	+
11	ADP-sugar pyrophosphatase	-	+	+	86	Histone H1.0	+	-	+
12	AFG3-like protein 2	-	+	+	87	Histone H2A	+	+	+
13	Alcohol dehydrogenase [NADP(+)]	+	-	+	88	HLA class I histocompatibility antigen	-	+	+
14	Aldose reductase	-	+	+	89	Hornerin	-	+	+
15	alpha 2	-	+	+	90	Hsp70-binding protein 1	+	-	+
16	Alpha-2-macroglobulin	-	+	+	91	Hydroxymethylglutaryl-CoA synthase	-	+	+
17	Aminopeptidase B	-	+	+	92	Importin subunit alpha-3	+	-	+
18	Anterior gradient protein 2 homolog	+	-	+	93	Importin-4	+	-	+
19	Antithrombin-III OS=Homo sapiens	-	+	+	94	Importin-5	+	+	+
20	Ataxin-10	+	-	+	95	Integrin alpha-6	+	-	+
21	ATP synthase subunit d, mitochondrial	+	-	+	96	Integrin beta-4	+	-	+
22	ATP synthase subunit g, mitochondrial	+	-	+	97	Inter-alpha-trypsin inhibitor heavy chain H2	-	+	+
23	ATPase ASNA1	+	-	+	98	ITIH4 protein	-	+	+
24	ATPase family AAA domain-containing protein 3A	+	-	+	99	Keratin, type I cytoskeletal 14	-	+	+
25	BAG family molecular chaperone regulator 2	-	+	+	100	Keratin, type I cytoskeletal 16	-	+	+
26	BAG6	+	-	+	101	Keratin, type II cuticular Hb1	+	-	+
27	Beta-2-glycoprotein 1	-	+	+	102	Keratin, type II cytoskeletal 5	-	+	+
28	Calcium load-activated calcium channel	+	-	-	103	Lactoylglutathione lyase	-	+	+
29	Calcium/calmodulin-dependent protein kinase	+	-	+	104	Long-chain-fatty-acid--CoA ligase 3	+	-	-
30	Calpain small subunit 1 (Fragment)	+	-	+	105	Macrophage-capping protein	+	-	+
31	Catenin delta-1	+	-	+	106	Malignant T-cell-amplified sequence 1	+	-	+
32	Caveolae-associated protein 1	-	+	+	107	MICOS complex subunit	+	+	+
33	CD59 glycoprotein	+	-	+	108	Mitochondrial 2-oxoglutarate/malate carrier protein	-	+	+
34	Cellular nucleic acid-binding protein	+	+	+	109	Mitochondrial carrier homolog 2	+	-	-
35	Charged multivesicular body protein 1b	-	+	+	110	Mitochondrial import receptor subunit TOM70	+	-	+
36	Chloride intracellular channel protein 4	+	-	+	111	Mitotic checkpoint protein BUB3	-	+	+

37	C-Jun-amino-terminal kinase-interacting protein 4	+	-	+	112	Myosin-10	-	+	+
38	Clathrin interactor 1	+	-	+	113	N(G)-dimethylarginine dimethylaminohydrolase 1	+	-	+
39	Coactosin-like protein	+	_	+	114	N(G)-dimethylarginine dimethylaminohydrolase 2	+	-	+
40	Coatomer subunit gamma-1	+	_	+	115	Nascent polypeptide	+	-	+
41	Coiled-coil domain-containing protein 47	+	-	+	116	Nestin	+	-	+
42	Complement C3	-	+	+	117	NSFL1 cofactor p47	+	-	+
43	Copine-3	+	+	+	118	Nucleoprotein TPR	+	+	+
44	Creatine kinase B-type	-	+	+	119	OCIA domain-containing protein 2	+	-	+
45	Cysteine-rich protein 2 (Fragment)	+	-	+	120	Omega-amidase NIT2	+	-	
46	Cytochrome b-c1 complex subunit Rieske	+	-	+	121	Paxillin	+	-	+
47	Cytochrome c1, heme protein, mitochondrial	+	+	+	122	PDZ and LIM domain protein 1	+	-	+
48	Cytoplasmic dynein 1 intermediate chain 2	+	-	+	123	Peptidyl-prolyl cis-trans isomerase F	+	-	+
49	Cytosolic acyl coenzyme A thioester hydrolase	+	-	+	124	Phosphoribosyl pyrophosphate synthase-associated protein 1	+	+	+
50	Delta-1-pyrroline-5-carboxylate synthase	+	+	+	125	Plastin-2	+	-	+
51	DNA polymerase-transactivated protein 6	+	-	-	126	Platelet-activating factor acetylhydrolase IB	+	+	+
52	DNA replication licensing factor MCM4	-	+	+	127	Prolyl 4-hydroxylase subunit alpha-1	+	+	+
53	DNA replication licensing factor MCM5	-	+	+	128	Prostaglandin reductase 1	-	+	+
54	DNA topoisomerase 1	+	-	+	129	Proteasome activator complex subunit 1	+	-	+
55	Dynamin-like 120 kDa protein, mitochondrial	+	+	+	130	Protein ERGIC-53	+	+	+
56	Dynein light chain 1, cytoplasmic	+	-	+	131	Protein NipSnap homolog 2	+	-	+
57	EH domain-containing protein 1	+	-	+	132	Protein TFG	+	-	+
58	Endoplasmic reticulum	+	-	+	133	Protein transport protein Sec24C	+	-	+
59	Endoribonuclease LACTB2	+	-	+	134	Protein transport protein Sec31A	+	-	+
60	Ephrin type-A receptor 2	+	-	+	135	Quinone oxidoreductase	+	+	+
61	Eukaryotic peptide chain release factor GTP	+	-	+	136	Ras GTPase-activating protein-binding protein 2	-	+	+
62	Eukaryotic translation initiation factor 3 subunit H	+	+	+	137	Regulator of nonsense transcripts 1	+	-	+
63	FACT complex subunit SSRP1	+	+	+	138	Retinol dehydrogenase 11	-	+	-
64	Far upstream element-binding protein 1	-	+	+	139	Rho GTPase-activating protein 1	+	-	+
65	Fatty aldehyde dehydrogenase	+	-	+	140	Ribosome maturation protein SBDS	+	-	+
66	Four and a half LIM domains protein 1 (Fragment)	+	-	+	141	RNA transcription	+	-	+
67	Fragile X mental retardation syndrome	+	-	+	142	Saccharopine dehydrogenase-like oxidoreductase	+	+	+
68	Galectin-3	+	-	+	143	Sec1 family domain-containing protein 1	-	+	+
69	Gamma-enolase	+	-	+	144	Selenium-binding protein 1	+	-	+
70	Gasdermin domain containing 1, isoform CRA_d	+	-	+	145	Sequestosome-1	-	+	+
71	General vesicular transport factor p115	+	-	+	146	Serine/threonine-protein kinase OSR1	+	-	+
72	Glia maturation factor beta (Fragment)	+	-	+	147	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	-	+	+
73	GluC	-	+	-	148	Serpin B5	+	-	+
74	Glucosamine 6-phosphate N-acetyltransferase	+	-	+	149	Serrate RNA effector molecule homolog	+	+	+
75	Glutamate--cysteine ligase catalytic subunit	-	+	+					

Table 2: Table of proteins showing proteins present and/or absent in PC3, PNT2 and tPNT2 cell derived MVs.

		MVs					MVs		
No.	Protein name	PC3	PNT2	tPNT2	No.	Protein name	PC3	PNT2	tPNT2
1	14-3-3 protein beta/alpha	+	-	-	125	Immunoglobulin heavy constant mu	-	-	+
2	14-3-3 protein epsilon	+	-	+	126	Immunoglobulin kappa constant	-	-	+
3	14-3-3 protein eta	+	-	-	127	Immunoglobulin lambda constant 2	-	-	+
4	14-3-3 protein gamma	+	-	-	128	Importin subunit beta-1	+	-	-
5	14-3-3 protein sigma	+	-	-	129	Importin-5	+	-	-
6	14-3-3 protein theta	+	-	+	130	Integrin alpha-1	-	-	+
7	14-3-3 protein zeta/delta	+	+	+	131	Integrin alpha-2	+	-	+
8	4F2 cell-surface antigen heavy chain	+	-	+	132	Integrin alpha-3	+	-	+
9	60 kDa heat shock protein, mitochondrial	+	-	-	133	Integrin alpha-6	+	-	+
10	Actin, cytoplasmic 1	+	+	+	134	Integrin beta-1	+	-	+
11	Adenosylhomocysteinase	+	-	+	135	Integrin beta-3	-	-	+
12	ADP/ATP translocase 3	+	-	-	136	Integrin beta-4	+	-	+
13	ADP-ribosylation factor 1	+	-	+	137	Inter-alpha-trypsin inhibitor heavy chain H1	+	+	+
14	ADP-ribosylation factor 4	+	-	-	138	Inter-alpha-trypsin inhibitor heavy chain H2	+	+	+
15	Agrin	+	-	-	139	Inter-alpha-trypsin inhibitor heavy chain H3	+	+	+
16	Aldehyde dehydrogenase family 1-member A3	+	-	-	140	ITIH4 protein	+	+	+
17	Alpha-2-HS-glycoprotein	+	+	+	141	Junction plakoglobin	+	-	-
18	Alpha-2-macroglobulin	+	+	+	142	Junctional adhesion molecule A	+	-	-
19	Alpha-actinin-1	+	-	-	143	Keratin, type I cytoskeletal 10	+	+	+
20	Alpha-actinin-2	+	-	-	144	Keratin, type I cytoskeletal 14	+	+	+
21	Alpha-actinin-4	+	+	+	145	Keratin, type I cytoskeletal 16	+	+	+
22	Alpha-enolase	+	+	+	146	Keratin, type I cytoskeletal 18	+	-	-
23	Alpha-fetoprotein	+	+	+	147	Keratin, type I cytoskeletal 19	+	+	-
24	Alpha-S1-casein	+	+	-	148	Keratin, type I cytoskeletal 9	+	+	+
25	Aminopeptidase N	+	-	+	149	Keratin, type II cytoskeletal 1	+	+	+
26	Aminopeptidase	+	-	-	150	Keratin, type II cytoskeletal 2 epidermal	+	+	+
27	Amyloid-beta A4 protein (Fragment)	+	-	-	151	Keratin, type II cytoskeletal 5	+	+	+
28	Amyloid-beta A4 protein (Fragment)	+	-	+	152	Keratin, type II cytoskeletal 6C	+	+	+
29	Annexin A2	+	+	+	153	Keratin, type II cytoskeletal 7	+	-	-
30	Annexin A5	+	-	+	154	Lactadherin	+	-	+

31	Annexin A6	+	-	+	155	Lactotransferrin (Fragment)	+	+	+
32	Annexin A7	+	-	+	156	Large neutral amino acids transporter small subunit 1	+	-	-
33	Antithrombin-III	+	+	+	157	Leukocyte surface antigen CD47	+	-	+
34	Apolipoprotein A-IV	-	-	+	158	L-lactate dehydrogenase A chain	+	-	+
35	Apolipoprotein B-100	+	+	+	159	L-lactate dehydrogenase B chain	+	-	+
36	Apolipoprotein E	+	-	-	160	Malate dehydrogenase, cytoplasmic	+	-	-
37	Basement membrane-specific heparan sulfate proteoglycan core	+	-	-	161	Malate dehydrogenase, mitochondrial	+	-	-
38	Basigin	+	-	+	162	Moesin	+	+	+
39	Beta-2-glycoprotein 1	+	+	+	163	Monocarboxylate transporter 1	+	-	+
40	Beta-2-microglobulin	+	-	-	164	Monocarboxylate transporter 4	+	-	+
41	Calcium-binding protein 39	+	-	-	165	Myosin-9	+	-	-
42	Calreticulin	+	-	-	166	Neutral alpha-glucosidase AB	+	-	-
43	Carboxypeptidase N catalytic chain	+	-	-	167	Neutral amino acid transporter B(0)	+	-	+
44	Catenin beta-1	+	-	+	168	Neutrophil gelatinase-associated lipocalin	+	-	-
45	Catenin delta-1	+	-	+	169	Nucleophosmin	+	-	-
46	Cathepsin D	+	-	-	170	Nucleoside diphosphate kinase	+	-	+
47	CD151 antigen	+	-	+	171	Pentraxin-related protein PTX3	+	-	-
48	CD166 antigen	+	-	-	172	Peptidyl-prolyl cis-trans isomerase A	+	-	-
49	CD44 antigen	+	-	-	173	Peptidyl-prolyl cis-trans isomerase B	+	-	-
50	CD63 antigen	+	-	-	174	Periostin	-	+	-
51	Cell division control protein 42 homolog	+	-	+	175	Peroxidasin homolog	+	-	-
52	Ceruloplasmin	-	+	+	176	Peroxiredoxin-1	+	-	+
53	Chloride intracellular channel protein 1	+	-	-	177	Peroxiredoxin-2	+	-	-
54	Choline transporter-like protein 2	+	-	-	178	Peroxiredoxin-6	+	-	+
55	Clathrin heavy chain	+	-	-	179	Phosphatidylethanolamine-binding protein 1	+	-	-
56	Claudin-4	-	-	+	180	Phosphoglucomutase-1	+	-	-
57	Coactosin-like protein	+	-	-	181	Phosphoglycerate kinase 1	+	-	+
58	Coagulation factor V	+	-	+	182	Phosphoglycerate mutase 1	+	-	-
59	Coagulation factor X	+	-	+	183	Phospholipid scramblase	+	-	-
60	Cofilin-1	+	-	+	184	Pigment epithelium-derived factor	+	+	+
61	Cofilin-2	+	-	-	185	Plasminogen	+	+	-
62	Collagen alpha-1(I) chain	+	+	-	186	Plastin-2	+	-	
63	Collagen alpha-1(III) chain	+	-	-	187	Plastin-3	+	-	-
64	Collagen alpha-1(VI) chain	+	+	+	188	Plectin	+	-	-

65	Collagen alpha-2(I) chain	-	+	-	189	Profilin-1	+		+
66	Complement C3	+	+	+	190	Programmed cell death 6-interacting protein	+	-	-
67	Complement C4-A	+	+	+	191	Prostaglandin F2 receptor negative regulator	+	-	-
68	Complement C5	+	+	+	192	Proteasome endopeptidase complex	+	-	-
69	Complement component C7	-	+	-	193	Proteasome subunit alpha type	+	-	-
70	Coronin-1C	+	-	-	194	Protein CYR61	+	-	-
71	CUB domain-containing protein 1	+	-	+	195	Protein disulfide-isomerase A3	+	-	-
72	Dickkopf-related protein 1	+	-	-	196	Protein disulfide-isomerase A6	+	-	-
73	Disintegrin and metalloproteinase domain-containing protein 10	+	-	-	197	Protein disulfide-isomerase	+	-	-
74	EGF-like repeat and discoidin I-like domain-containing protein 3	+	-	+	198	Protein FAM49B	+	-	-
75	EH domain-containing protein 1	+	-	-	199	Protein NDRG1	+	-	-
76	EH domain-containing protein 4	+	-	-	200	Protein S100-A16	+	-	-
77	Elongation factor 1-alpha 1	+	-	+	201	Protein-glutamine gamma-glutamyltransferase 2	+	-	-
78	Elongation factor 1-gamma	+	-	-	202	Prothrombin	+	+	+
79	Elongation factor 2	+	-	-	203	Pyruvate kinase PKM	+	+	+
80	Endoplasmic reticulum chaperone BiP	+	-	-	204	Rab GDP dissociation inhibitor beta	+	-	+
81	Endoplasmin	+	-	-	205	Ras GTPase-activating-like protein IQGAP1	+	-	-
82	Ephrin type-A receptor 2	+	-	+	206	Ras-related C3 botulinum toxin substrate 1	+	-	-
83	Epithelial cell adhesion molecule	+	-	-	207	Ras-related protein Rab-11B	+	-	-
84	Equilibrative nucleoside transporter 1	+	-	+	208	Ras-related protein Rab-1A	+	-	-
85	Eukaryotic translation initiation factor 2 subunit 3	+	-	-	209	Ras-related protein Rab-7a	+	-	-
86	Ezrin	+	-	-	210	Ras-related protein Ral-A	+	-	-
87	Farnesyl pyrophosphate synthase	+	-	-	211	Ras-related protein Ral-B	+	-	-
88	Fatty acid synthase	+	-	-	212	Ras-related protein Rap-1b	+	-	+
89	Fibroblast growth factor-binding protein 1	+	-	-	213	Serotransferrin		+	+
90	Fibronectin	+	+	+	214	Serum albumin	+	+	+
91	Fibulin-1	+	+	+	215	Serum albumin	+	+	+
92	Filaggrin-2	+	-	-	216	Sex hormone-binding globulin (Fragment)	+	-	-
93	Filamin-A	+	-	-	217	Sodium/potassium-transporting ATPase subunit α-1	+	-	+
94	Filamin-B	+	-	-	218	Sodium/potassium-transporting ATPase subunit β-1	+	-	-
95	Follistatin	+	-	-	219	Sodium/potassium-transporting ATPase subunit β-3	+	-	-

96	Fructose-bisphosphate aldolase A	+	-	+	220	Sodium-coupled neutral amino acid transporter 2	+	-	-
97	Galectin-3-binding protein	+	-	-	221	Solute carrier family 2	+	-	+
98	Gelsolin	+	+	+	222	Sulfhydryl oxidase 1	+	-	-
99	Glucose-6-phosphate isomerase (Fragment)	-	-	-	223	Syntenin-1	+	-	-
100	Glucosidase 2 subunit beta	+	-	-	224	Talin-1	+	-	-
101	Glutathione S-transferase P	+	-	-	225	Tetranectin	+	-	+
102	Glyceraldehyde-3-phosphate dehydrogenase	+	+	+	226	Tetraspanin	+	-	+
103	Granulins	+	-	-	227	Tetraspanin	+	-	+
104	GTP-binding nuclear protein Ran (Fragment)	+	-	-	228	Thioredoxin reductase 1, cytoplasmic	+	-	-
105	Guanine nucleotide-binding protein G(i) subunit alpha-2	+	-	+	229	Thrombospondin-1	+	+	+
106	Guanine nucleotide-binding protein G(I)	+	-	+	230	Thrombospondin-4	+	+	+
107	Guanine nucleotide-binding protein subunit beta-2	+	-	+	231	Thyroxine-binding globulin	+	-	-
108	Guanine nucleotide-binding protein G(s) subunit alpha	+	-	-	232	Tissue-type plasminogen activator	+	-	-
109	Heat shock 70 kDa protein 1B	+	-	-	233	Transferrin receptor protein 1	+	-	+
110	Heat shock cognate 71 kDa protein	+	+	+	234	Transforming protein RhoA	+	-	+
111	Heat shock protein HSP 90-alpha	+	-	+	235	Transgelin-2	+	-	-
112	Heat shock protein HSP 90-beta	+	+	+	236	Transitional endoplasmic reticulum ATPase	+	-	-
113	Hemoglobin subunit alpha	+	+	+	237	Transketolase	+	-	-
114	Hemoglobin subunit beta	+	-	+	238	Triosephosphate isomerase	+	-	+
115	Heterogeneous nuclear ribonucleoprotein A1	+	-	-	239	Trypsin	+	+	+
116	Heterogeneous nuclear ribonucleoproteins A2/B1	+	-	-	240	Tubulin alpha-1B chain	+	+	+
117	Histone H1.2	+	-	-	241	Tubulin alpha-4A chain	-	-	+
118	Histone H2B type 1-C/E/F/G/I	+	-	-	242	Tubulin beta chain	+	+	+
119	Histone H4	+	-	-	243	Tumor-associated calcium signal transducer 2	+	-	-
120	HLA class I histocompatibility antigen, A-24 alpha chain	+	-	-	244	Ubiquitin-40S ribosomal protein S27a	+	-	+
121	HLA class I histocompatibility antigen, A-34 alpha chain	+	-	+	245	Urokinase-type plasminogen activator	+	-	-
122	HLA class I histocompatibility antigen, B-56 alpha chain	-	-	-	246	Vimentin	+	+	+
123	Hornerin	+	+	+	247	Vinculin	+	-	+
124	Immunoglobulin heavy constant gamma 1 (Fragment)	-	-	+	248	Vitamin D-binding protein	+	+	+
					249	Vitronectin	+	+	+

5.3.4 Integrins detected on MVs samples

David Lyden and colleagues showed that tumour derived extracellular vesicles play an important role in preparing the site of metastasis in secondary organs (the so-called 'pre-metastatic niche', due to tumour derived extracellular vesicles' ability to interact with specific resident cells on target organs such as lung, liver and brain (Hashino et al, 2015). One specific secondary organ is targeted, due to the distinct combinations of integrin proteins expressed on the surface of the tumour derived extracellular vesicles.

Integrins are transmembrane receptors that play a critical role in the regulation of adhesion, migration, proliferation and differentiation of cells. Integrins are capable of rapid and flexible responses to events at the cell surface (Rippa *et al*, 2013).

In this proteomic study integrins expressed on MVs derived from PC3, PNT2 and tPNT2 cells were gathered in Table 3 below.

Table 3: List of integrin expressed in MVs isolated from all three different cells; PC3, PNT2 and the transformed tPNT2.

Protein name	Accession number	MW (kDa)	PC3 cells	PNT2 cells	tPNT2 cells
Integrin beta-1	ITB1_HUMAN	88	+	-	+
Integrin alpha-3	ITA3_HUMAN	117	+	-	+
Integrin alpha-6	ITA6_HUMAN	127	+	-	+
Integrin alpha-2	ITA2_HUMAN	129	+	-	+
Integrin beta 4	ITB3_HUMAN	202	+	-	+
Integrin alpha-1	ITA1_HUMAN	131	-	-	+
Integrin beta-3	ITB3_HUMAN	87	-	-	+

The top 5 integrins on the table were detected on PC3 and tPNT2 MVs, while the last two integrins on the list were only detected on tPNT2. It is also possible that integrin alpha-1 and beta-3 could have been present on PC3 MVs however at a very low level. As in the rest of this study proteins with 2 or more unique peptides were recorded as expressed.

Integrins such as β-1 are activated on metastatic prostate cancer cells, causing further metastasis to lymph nodes and bones (Jin *et al,* 2014). Also, over expression of integrin α6β4 has been reported to correlate with malignant progression and poor survival in different types of cancers. Integrin β4 is involved in the process of EMT (Kariya *et al*, 2017).

For validation analysis, the same protein samples that were used to do LC-MS/MS analysis in this results chapter were also used to validate the findings here in Table 3. Protein samples of PC3, PNT2 and tPNT2 cells and PC3 cell derived MVs were used (in triplicate where possible) to confirm the presence of some of the above integrins. Due to the cost of the monoclonal

antibodies, four of the above integrins were purchased for this validation analysis namely; Integrin alpha 1 and 6, beta 1 and 3.

Western blot analysis was carried according to the protocols in (section 2.2.3.10, 2.2.3.11 and 2.2.3.12) to confirm the presence of the above integrins in the samples tested. The following findings were observed.

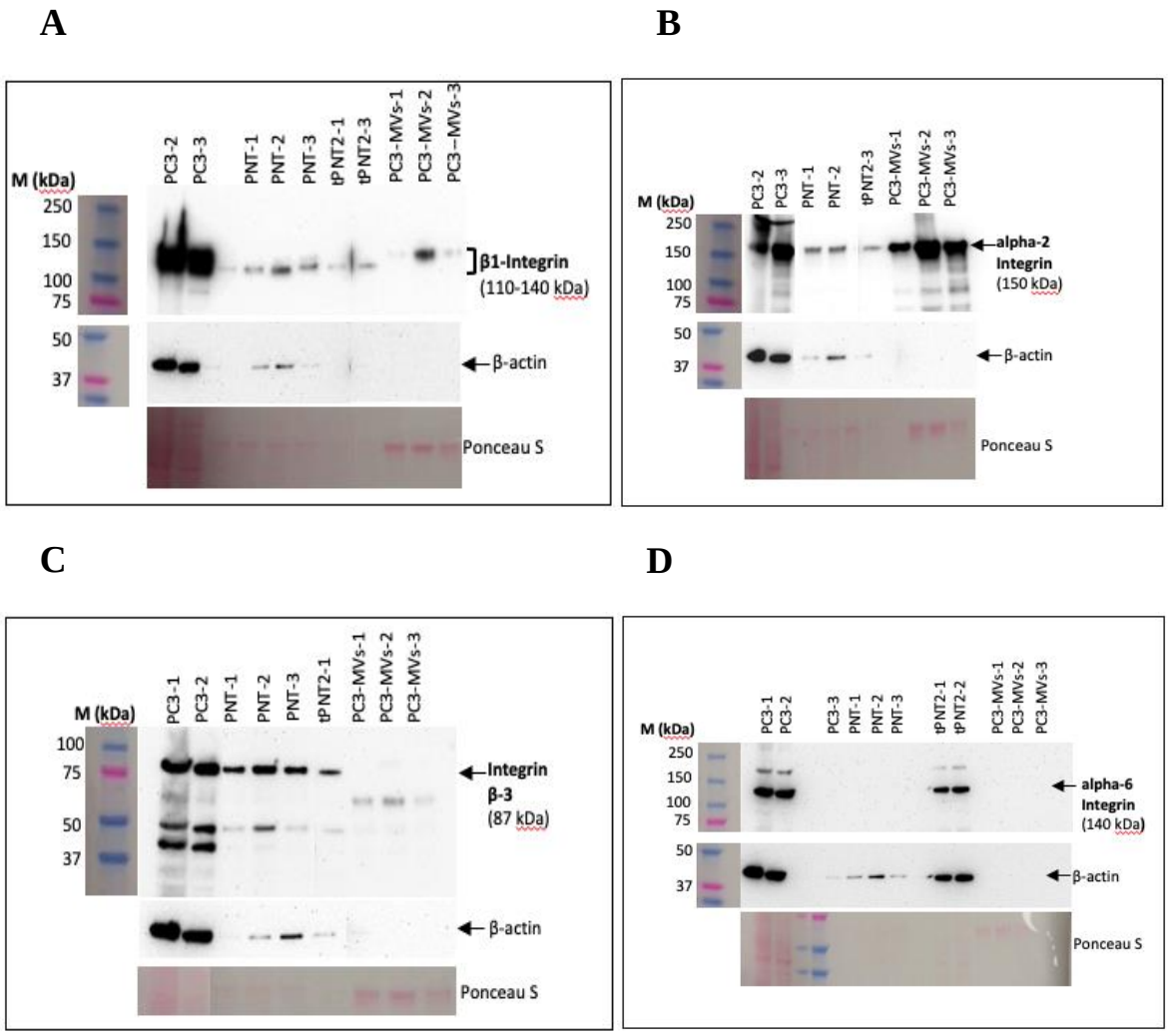


Figure 35: Western blot analysis used to validate the presence of Integrin beta 1, alpha 2, beta 3 and alpha 6 in PC3, PNT2, tPNT2 cells and PC3 MVs. β-actin is shown as a control and Ponceau S shows even loading of the protein lysates.

Panel A shows Integrin beta 1 protein bands are shown to be down regulated in tPNT2 cells compared to the other samples. Similarly, integrin alpha 2 in panel B also shows down regulation in tPNT2 cells. These findings correlate with the findings in literature where all primary and metastatic carcinomas express α2β1 integrins (Koistinen and Heino, 2000; Bianconi et al, 2016). Moreover, a research group in 1993 found the level of expression in α2β1 was down regulated in grade I and II tumours (Bonkhoff et al, 1993). Therefore, perhaps the low expression can be due to the early stages of metastasis in our experimental model. In addition, many experimental models show that integrins α2β1 is essential for cancer cell migration, invasion and metastasis (Vinhenin et al, 1996).

In panel C integrin β3 shows down regulation in tPNT2, however its expression is high in the immortal PNT2 cells. This does not correlate with the findings of other researchers which shows that integrin β3 is highly expressed in endometriosis and has been studied extensively for its role as cancer therapeutic (May et al, 2011). integrin β3 coupled with alphav has been shown to play a part in cancer angiogenesis in prostate cancer and many other tumours (Suyin et al, 2013).

In panel D integrin α6 has shown to be over expressed in the transformed tPNT2 cells. This corroborates with the findings in several literature, where integrin α6 has been found to be involved in tumour metastasis and overall tumour progression (Feitsma and Cuppen, 2008; Shull et al, 2017). It is said to play an important part in tumour angiogenesis, in that high levels of integrin α6 is needed to make the blood vessels that stem from the tumour (Emanueli et al, 2001).

As a control for the above experiment an anti tetraspanin antibody (CD63) tubulin proteins were used as a positive control. These proteins are present on both microvesicles and exosomes therefore they are a good control used for EVs.

Briefly, PC3 MVs were isolated by differential centrifugation as described earlier. They were then immunoblotted, to detect the presence of two EV markers, CD-63 and tubulin. The primary antibodies used were mouse anti-CD63 and mouse anti-tubulin (both at 1/1,000; 1h at room temperature). The secondary antibody was goat anti-mouse IgG (HRP conjugated) (1/10,000; 16 h at 4 °C). Both CD63 (a broad band in the 30-60 kDa range) and tubulin (50-51 kDa) were detected. The Ponceau S shows even loading of the MV protein lysates.

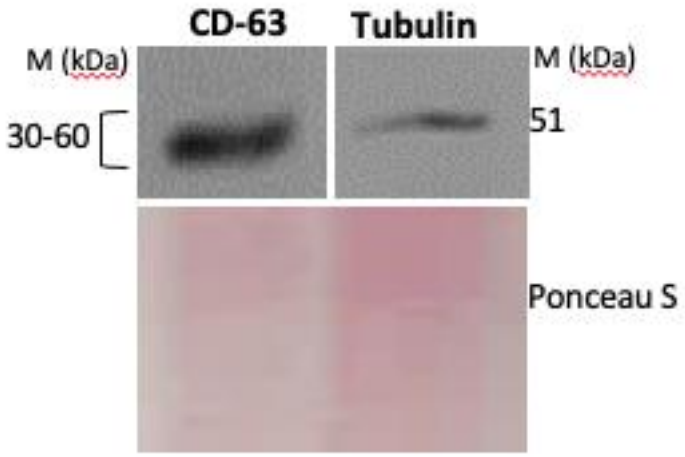
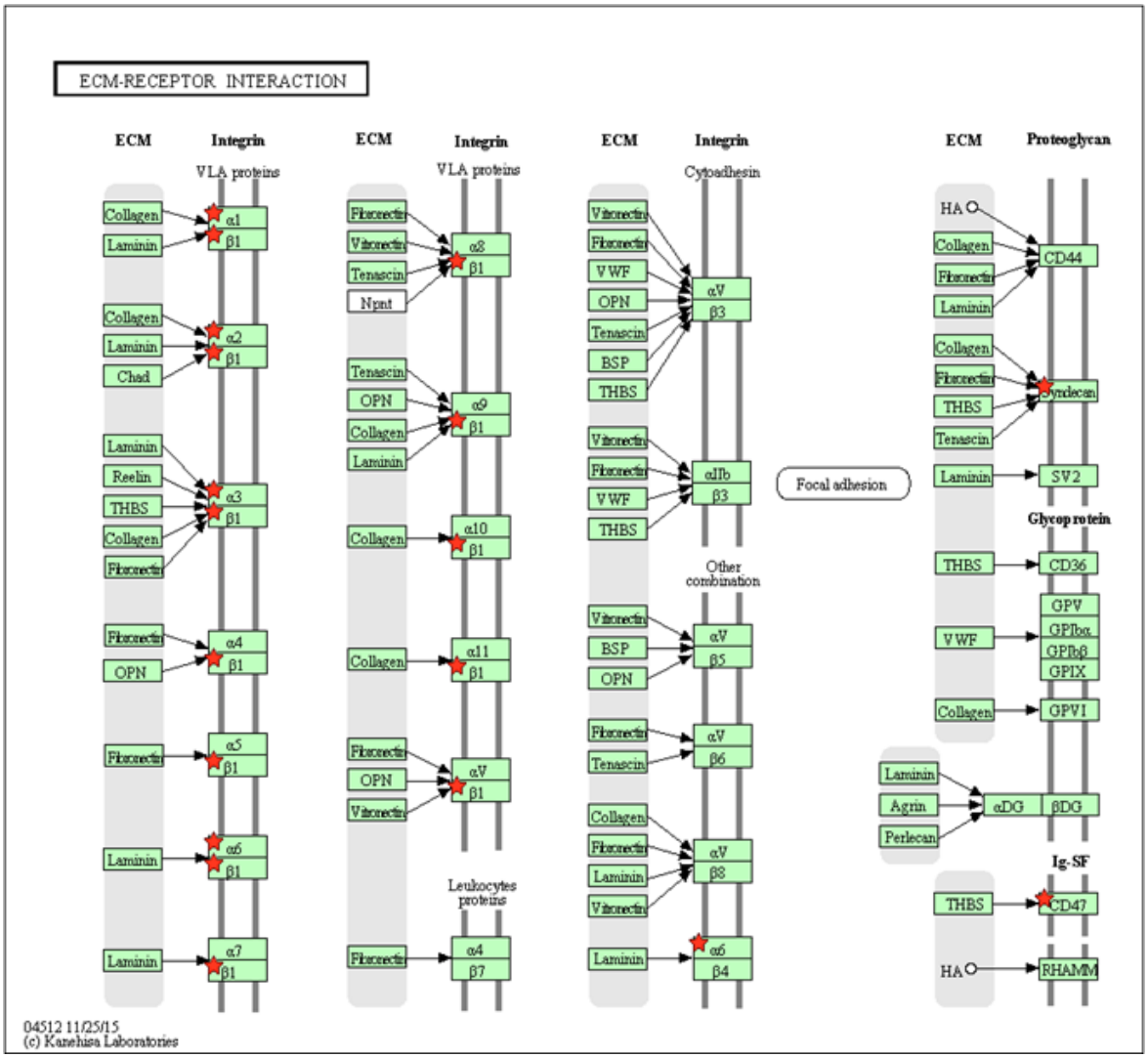


Figure 36: Immunoblot of two EV markers CD63 and tubulin, used as positive control.

On KEGG analysis about 5 integrins from the list above were shown to be involved in extracellular membrane interaction.



Term	Count	Genes	Adjusted p-value
ECM- receptor interaction	5	ITGA6, ITGA1, ITGA2, ITGA3, ITGB1	3.13 E 05

Figure 37: KEGG analysis showing Integrin involved in ECM (Extracellular Membrane) receptor interaction

5.3.5 Comparison of proteins expression between cell lines and cells derived MVs

Comparative studies on the list of proteins provided for each cell line and MVs could help understand and identify proteins that were upregulated or down regulated in the process of epithelial mesenchymal transition.

The following comparisons were made:

1. Comparing proteins expressed on PC3 MVs and not tPNT2 MVs
2. Comparing proteins expressed in tPNT2 MVs and not in PNT2 MVs

Tables of the expressed proteins below show that there are around 45 proteins on both tables that were present on one set of MVs and not the other. In Table 5.4: below shows proteins present in PC3 cell-derived MVs and not tPNT2. Since PC3 is a metastatic prostate cancer cell line, proteins present in this cell line and not in tPNT2 would suggest that they are strongly involved in metastasis and that the tPNT2 cells might not have become fully transformed to the EMT state.

Proteins expressed in tPNT2 and not PNT2 are shown in Table 5.5, which present 42 reviewed proteins expressed in tPNT2 MVs that are not expressed in PNT2, this suggests that some of the proteins expressed are matched to the proteins present in PC3 cells.

No.	UniProt accession	UniProt name	Status	Protein name
1	Q00610	CLH1	reviewed	Clathrin heavy chain 1
2	O00299	CLIC1	reviewed	Chloride intracellular channel protein 1
3	P55072	TERA	reviewed	Transitional endoplasmic reticulum ATPase
4	Q9Y490	TLN1	reviewed	Talin-1
5	P32119	PRDX2	reviewed	Peroxiredoxin-2
6	P21333	FLNA	reviewed	Filamin-A
7	P14625	ENPL	reviewed	Endoplasmin
8	P12814	ACTN1	reviewed	Alpha-actinin-1
9	P46940	IQGA1	reviewed	Ras GTPase
10	Q9H4M9	EHD1	reviewed	EH domain-containing protein 1
11	P18669	PGAM1	reviewed	Phosphoglycerate mutase 1
12	P26641	EF1G	reviewed	Elongation factor 1-gamma
13	P30101	PDIA3	reviewed	Protein disulfide-isomerase A3
14	P68366	TBA4A	reviewed	Tubulin alpha-4A chain
15	P29401	TKT	reviewed	Transketolase
16	P07339	CATD	reviewed	Cathepsin D
17	O75369	FLNB	reviewed	Filamin-B
18	P10809	CH60	reviewed	60 kDa heat shock protein, mitochondrial
19	Q9P2B2	FPRP	reviewed	Prostaglandin F2 receptor negative regulator
20	O00468	AGRIN	reviewed	Agrin
21	Q15149	PLEC	reviewed	Plectin
22	Q8IWA5	CTL2	reviewed	Choline transporter-like protein 2
23	P08962	CD63	reviewed	CD63 antigen
24	P13797	PLST	reviewed	Plastin-3
25	Q9Y624	JAM1	reviewed	Junctional adhesion molecule A
26	P00747	PLMN	reviewed	Plasminogen
27	P08727	K1C19	reviewed	Keratin, type I cytoskeletal 19
28	P16422	EPCAM	reviewed	Epithelial cell adhesion molecule
29	Q5JWF2	GNAS1	reviewed	Guanine nucleotide-binding protein G(s)
30	P01871	IGHM	reviewed	Immunoglobulin heavy constant mu
31	P12236	ADT3	reviewed	ADP/ATP translocase 3
32	P05534	1A24	reviewed	HLA class I histocompatibility antigen
33	P09758	TACD2	reviewed	Tumor-associated calcium signal transducer 2
34	P0DMV9	HS71B	reviewed	Heat shock 70 kDa protein 1B
35	P00749	UROK	reviewed	Urokinase-type plasminogen activator
36	P56199	ITA1	reviewed	Integrin alpha-1
37	P26022	PTX3	reviewed	Pentraxin-related protein PTX3
38	P47895	AL1A3	reviewed	Aldehyde dehydrogenase family 1member A3
39	P00750	TPA	reviewed	Tissue-type plasminogenactivator
40	P80188	NGAL	reviewed	Neutrophil gelatinase-associated lipocalin
41	Q12841	FSTL1	reviewed	Follistatin-related protein 1
42	O94907	DKK1	reviewed	Dickkopf-related protein 1
43	Q24JP5	T132A	reviewed	Transmembrane protein 132A
44	Q9Y536	PAL4A	reviewed	Peptidyl-prolyl cis-trans isomerase A-like 4A
45	P47710	CASA1	reviewed	Alpha-S1-casein

Table 4: Table showing proteins present in PC3 MVs and not in tPNT2 MVs. The list of proteins has been matched with the EVpedia database. The proteins were all reviewed by the database.

No.	UniProt accession	UniProt name	Status	Protein name
1	P08758	ANXA5	reviewed	Annexin A5
2	P04075	ALDOA	reviewed	Fructose-bisphosphate aldolase A
3	P68104	EF1A1	reviewed	Elongation factor 1-alpha 1
4	P61224	RAP1B	reviewed	Ras-related protein Rap-1b
5	P23526	SAHH	reviewed	Adenosylhomocysteinase
6	P04899	GNAI2	reviewed	Guanine nucleotide-binding protein alpha-2
7	P35613	BASI	reviewed	Basigin (5F7)
8	P02786	TFR1	reviewed	Transferrin receptor protein 1
9	P50395	GDIB	reviewed	Rab GDP dissociation inhibitor beta
10	P50995	ANX11	reviewed	Annexin A11
11	P08133	ANXA6	reviewed	Annexin A6
12	P62879	GBB2	reviewed	Guanine nucleotide-binding protein β-2
13	P84077	ARF1	reviewed	ADP-ribosylation factor 1
14	P20073	ANXA7	reviewed	Annexin A7
15	P68366	TBA4A	reviewed	Tubulin alpha-4A chain
16	Q08431	MFGM	reviewed	Lactadherin
17	P60033	CD81	reviewed	CD81 antigen
18	P11166	GTR1	reviewed	Solute carrier family 2
19	P23229	ITA6	reviewed	Integrin alpha-6
20	P68871	HBB	reviewed	Hemoglobin subunit beta
21	P62979	RS27A	reviewed	Ubiquitin-40S ribosomal protein S27a
22	P26006	ITA3	reviewed	Integrin alpha-3
23	P53985	MOT1	reviewed	Monocarboxylate transporter 1
24	P50502	F10A1	reviewed	Hsc70-interacting protein
25	P15144	AMPN	reviewed	Aminopeptidase N
26	P17301	ITA2	reviewed	Integrin alpha-2
27	P35222	CTNB1	reviewed	Catenin beta-1
28	O60716	CTND1	reviewed	Catenin delta-1
29	P48509	CD151	reviewed	CD151 antigen
30	Q08722	CD47	reviewed	Leukocyte surface antigen CD47
31	P12259	FA5	reviewed	Coagulation factor V
32	P29317	EPHA2	reviewed	Ephrin type-A receptor 2
33	O43854	EDIL3	reviewed	EGF-like repeat
34	P01857	IGHG1	reviewed	Immunoglobulin heavy constant gamma 1
35	P01871	IGHM	reviewed	Immunoglobulin heavy constant mu
36	Q9H5V8	CDCP1	reviewed	CUB domain-containing protein 1
37	P56199	ITA1	reviewed	Integrin alpha-1
38	O14713	ITBP1	reviewed	Integrin beta-1-binding protein 1
39	Q9BYZ2	LDH6B	reviewed	L-lactate dehydrogenase A-like 6B
40	B7Z2T5	AT1A1	reviewed	Sodium/potassium-transporting ATPase subunit α
41	P47710	CASA1	reviewed	Alpha-S1-casein
42	Q5SWX2	VINC	reviewed	Vinculin

Table 5: Table showing proteins expressed in tPNT2 MVs and not PNT2 MVs.

When comparing the expressed proteins on both tables above, there are four common proteins that are expressed in PC3 and tPNT2 MVs and not in PNT2. These four proteins are; heat shock protein 70 kDa, Alpha-S1 Casein, Integrin alpha-1 and Immunoglobulin heavy constant mu.

Heat shock protein 70 (HSP70)

Heat shock proteins 70 are found in nearly all subcellular fractions of the cells, where they support polypeptides folding, prevent proteins from aggregation and aid protein transport across the plasma membrane (Schmitt *et al*, 2007). These proteins are said to be present in high levels in tumour cells exposed to environmental stress hence, high cytosolic levels of the proteins is known to protect cancer cells from apoptotic cell death while promoting tumour proliferation and migration (Calderwood *et al*, 2006).

HSP70 appears amongst the many cytosolic chaperone proteins in plasma membrane bound protein global proteomic profiling suggesting its role in receptor mediated cell to cell communication (Shin *et al*, 2003).

Alpha-S1 Casein (α-s1)

Casein is the most abundant form of casein in bovine milk, it is present in milk in the form of micellar particles. It is relatively heat stable and capable of surviving pasteurisation. It is also expressed outside the mammary gland in primary human monocytes and $CD4^+$ and $CD8^+$ T cells (Vordenbaumen *et al*, 2011) as well as in benign prostate hyperplasia of humans (Xu *et al*, 2006). α-s1 protein has been reported to have antioxidant properties and is involved in transport of casein from Endoplasmic reticulum to Golgi apparatus (Le Parc *et al*, 2010).

Integrin alpha-1

Integrins are cell surface glycoproteins that act as key mediators of both cell to matrix and cell to cell interaction. They are expressed on the cell as heterodimers containing α- and/or β-subunit or a combination of both (Alberts *et al*, 2002). Integrins are involved in adhesion, hence they have been shown to function in signalling pathways to mediate migration and differentiation of epithelial cells (Alberts *et al*, 2002). Integrin α-1 is a receptor for collagen, and it supports cell migration through collagen rich extracellular matrices. Studies have shown increased integrin α-1expression in response to injury (Wehrle-Hallerand and Imhof, 2001) and high TGF-β levels. Also, integrin α-1 has been shown to favour interstitial collagen binding in mesenchymal tissues (Popova *et al*, 2004).

5.3.6 String analysis

STRING analysis of the above two comparative list of proteins was performed. Proteins are shown with a different colour and each colour corresponds to the different pathways the proteins are involved in, based on the database.

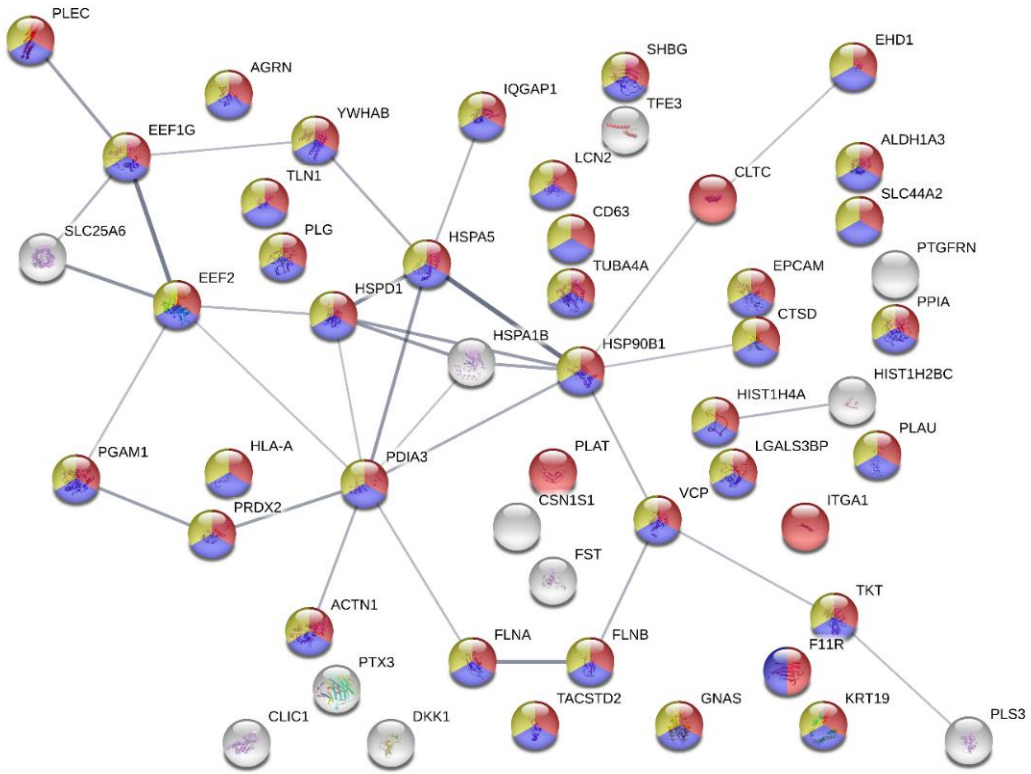


Figure 38: STRING analysis revealing the interaction partners of the significantly altered proteins present in PC3 MVs and not tPNT2 MVs. Proteins appearing in Red are proteins involved in extracellular exosomes, proteins in Blue are involved in membrane bound vesicles and proteins in Yellow are involved in vesicles.

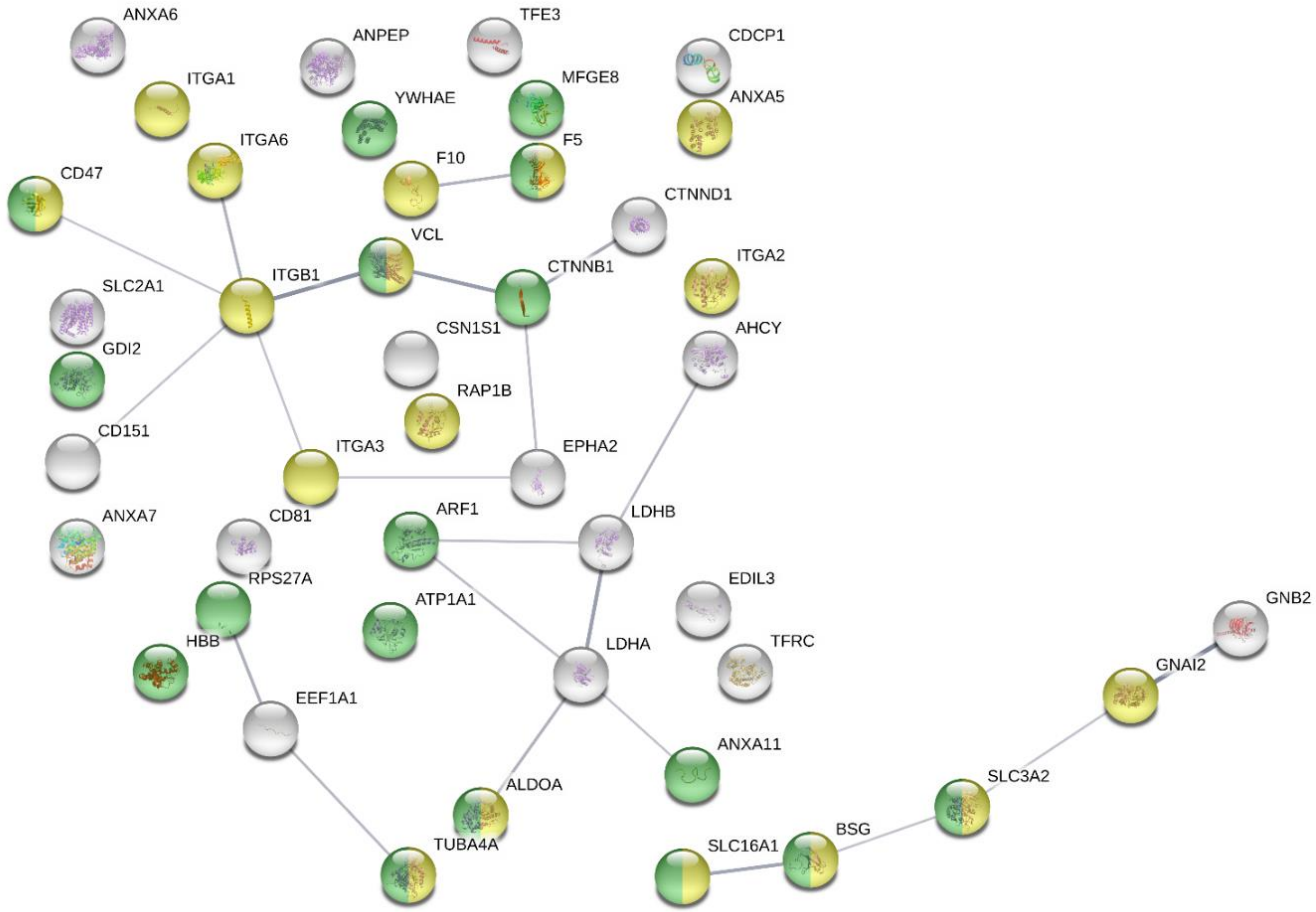


Figure 39: STRING analysis revealing the interaction partners of the significantly altered proteins present in tPNT2 MVs and not PNT2 MVs. Proteins appearing in Yellow are involved in wound healing, proteins in Green are involved in transport.

Proteins expressed on transformed (tPNT2) cells and not on PNT2 cells were used to draw this pathway in cancer, using KEGG analysis.

Term	Count	Genes	Adjusted p-value
Pathways in cancer	10	1974-S-AT, 33684-AT, 37983-AT, 1419-G-AT, 919-AT, 2090-I-AT, 35373-AT, 1855-AT, 1901-S-AT	0.01177

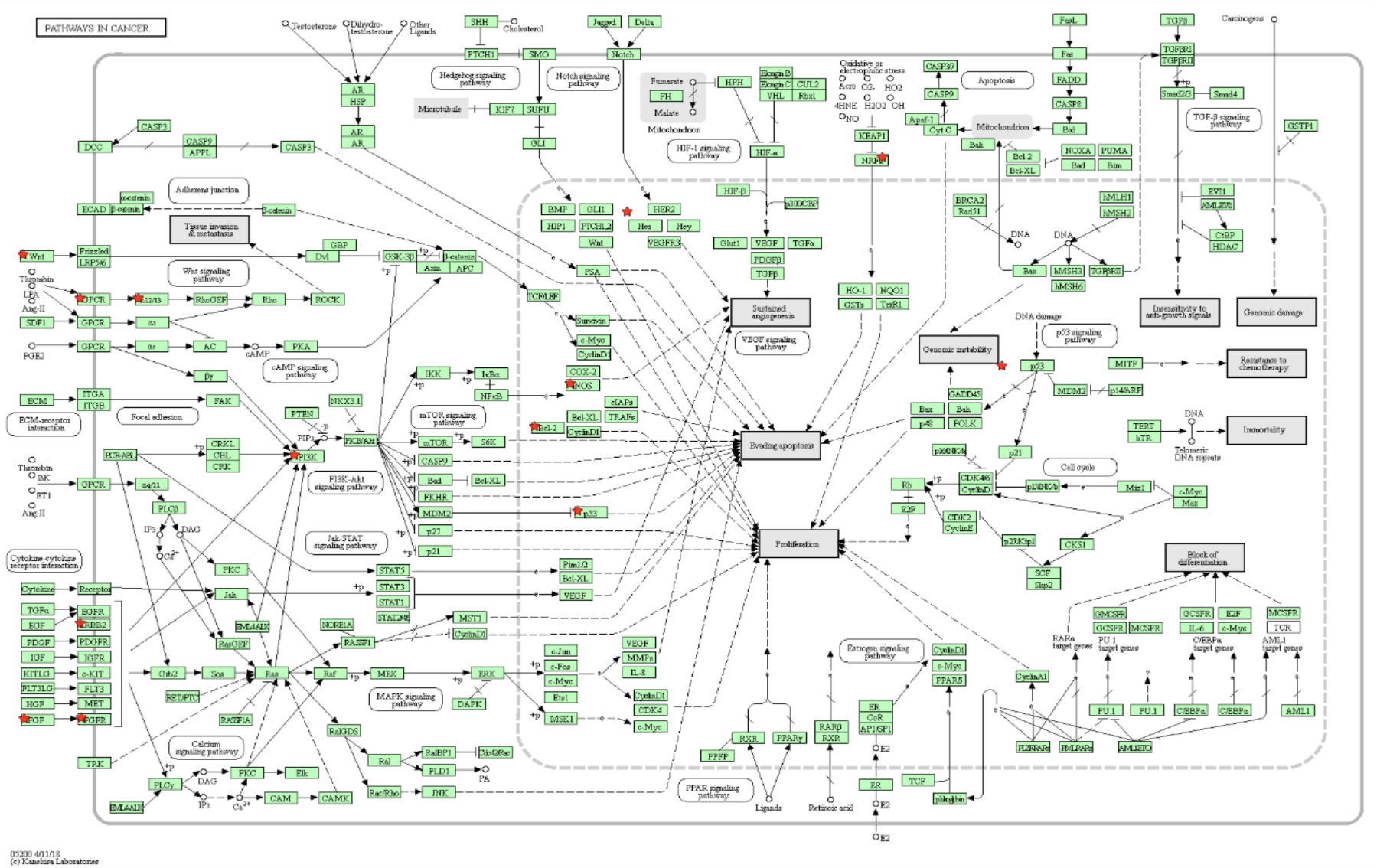


Figure 40: KEGG analysis showing proteins involved in cancer pathways, using proteins expressed in tPNT2 cells and not PNT2 cells.

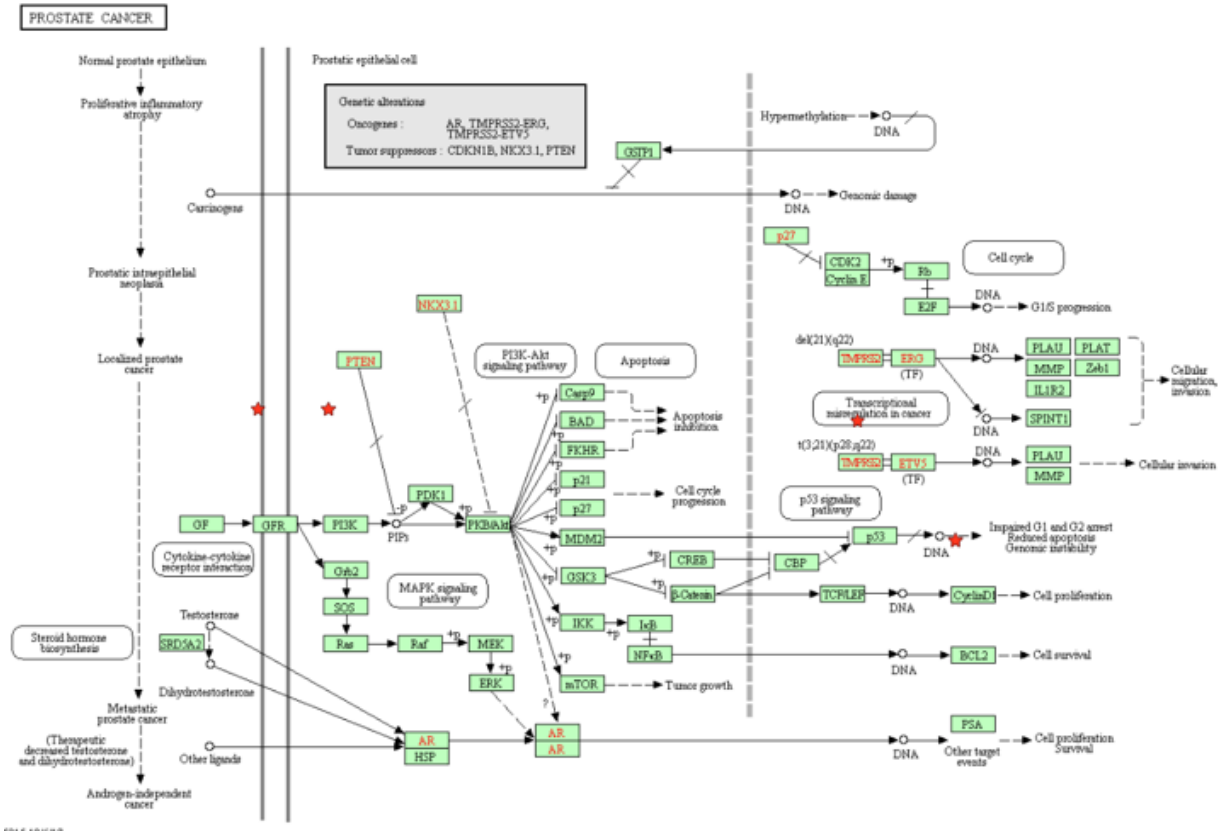


Figure 41: KEGG analysis showing proteins involved in Prostate Cancer, using proteins expressed in tPNT2 cells and not PNT2 cells.

Term	Count	Genes	Adjusted p-value
Prostate cancer	4	1974-S-AT, 35373-AT, 1901-S-AT, 1910-S-AT	0.0514

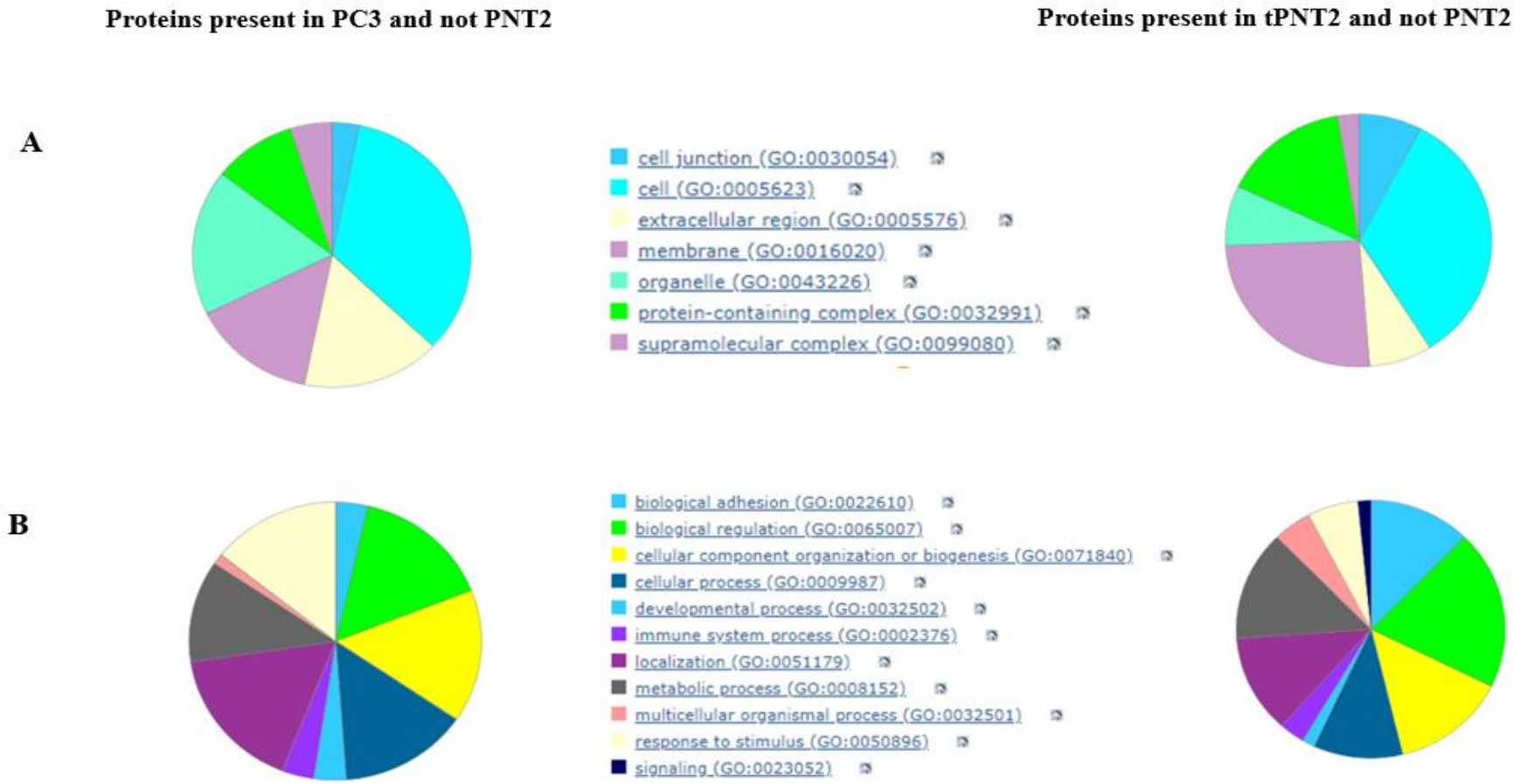


Figure 42: Shows the difference in proteins involved in cellular components of the cells and proteins involved in biological processes in the cells. (A) shows proteins involved in cellular components and **(B)** shows proteins involved in biological processes**.**

Identifying proteins that are present in PC3 MVs and transformed tPNT2 cells.

When proteins in PNT2 cells and proteins in tPNT2 (transformed) cells were compared, those proteins that were only present in the transformed cells were identified. This list of proteins was then compared to PC3 cells and proteins common in both cells were listed. Lastly, the list of identified proteins was compared to the proteins in PC3 MVs and 7 proteins were identified: (i) Plastin-2, (ii) Integrin beta-4, (iii) Ephrin type-A receptor 2, (iv) Integrin alpha-6, (v) EH domain-containing protein 1, (vi) Catenin delta-1 and (vii) Coactosin-like protein.

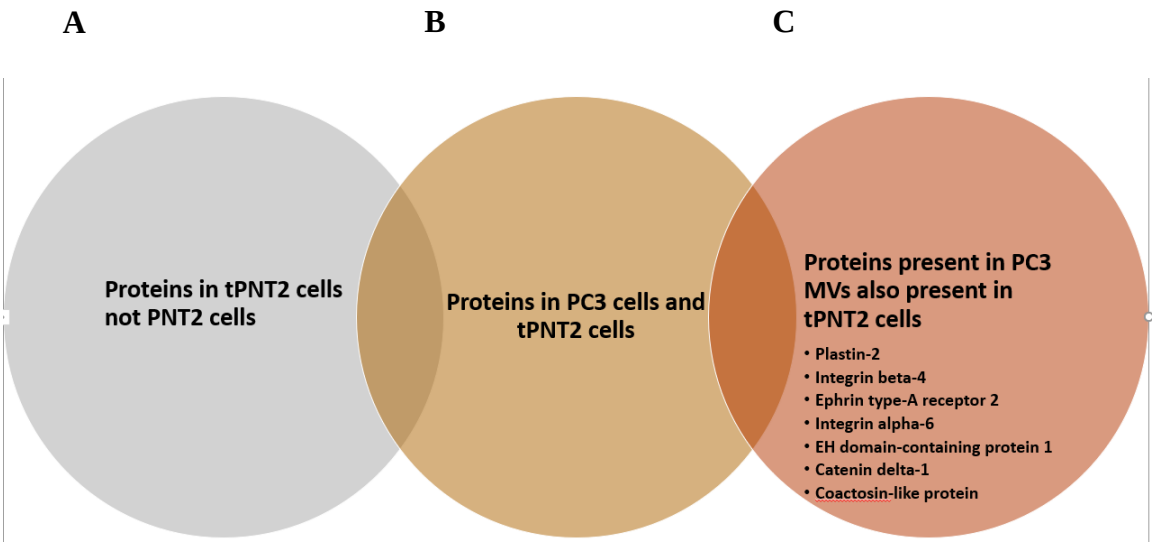


Figure 43: Comparing proteins in PNT2 cells with tPNT2 cells and cross comparing the resulting proteins with PC3 cells and PC3 MVs. The 7 proteins shown in panel C shows proteins that are present in PC3 MVs that are also present in the transformed tPNT2 cells.

i. **Plastin-2** is an actin binding protein which plays a role in the activation of T-cells in response to costimulation through TCR/CD2 or CD28.

ii. **Integrin beta-4** is a receptor for laminin. It plays a critical structural role in the hemidesmosome of epithelial cells. This integrin is required for the regulation of keratinocyte polarity and motility.

iii. **Ephrin type-A receptor 2** is a tyrosine kinase receptor which binds promiscuously membrane bound ephrin- A family ligands residing on adjacent cells leading to contact dependent bidirectional signalling into neighbouring cells.

iv. **Integrin alpha-6** is a receptor for laminin on platelets. Integrin alpha6/beta4 is a receptor for laminin in epithelial cell ad it plays a critical structural role in the hemidesmosome.

v. **EH domain-containing protein 1** is ATP and membrane binding protein that controls membrane reorganisation and tabulation upon ATP hydrolysis. This protein also causes vesiculation of endocytic membranes as it acts in early endocytic membrane fusion and membrane trafficking of recycling endosomes.

vi. **Catenin delta-1** binds to and inhibits the transcriptional repressor ZBTB33, which may lead to activation of target genes of the Wnt signalling pathways. This protein belongs to the beta catenin family.

vii. **Coactosin-like protein** binds to F-actin in a calcium independent manner. It has no direct effect on actin depolymerisation however it acts as a chaperone for ALOX5, influencing both its stability and activity in leukotrienes synthesis.

The above identified proteins were then analysed on STRING analysis for its proteins - proteins interaction.

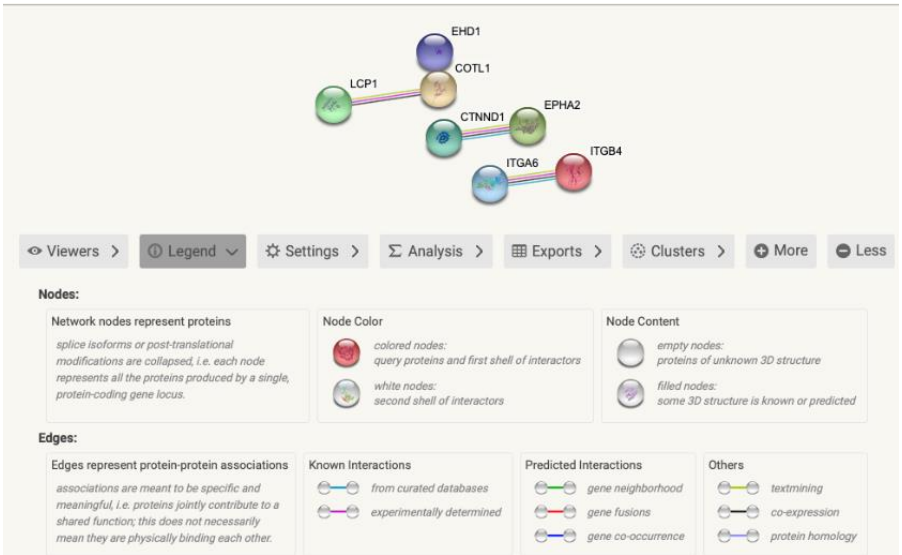


Figure 44: String analysis used for protein – protein interaction. Lines between nodes represent known interactions (from curated databases, blue; experimentally determined, pink), predicted interactions (gene neighbourhood, green; gene fusions, red; gene co-occurrence, blue) and other interactions (text-mining, lime green; co-expression, black; protein homology, grey).

5.4 Summary

Quantitative LC-MS/MS analysis was used to determine the proteins up regulated or down regulated in PC3 MVs that were used to induce EMT in PNT2 cells.

The results presented show that there are less proteins expressed in MVs compared to the host cells they are released from. This is complimentary with the size of the cell compare to the small size of MVs. The comparison between proteins expressed in PC3 and not PNT2 showed a list of proteins that were up regulated or down regulated. The same comparison of proteins expression was carried out between PNT2 MVs and tPNT2 cells.

Integrins play an important role in regulating cell adhesion, migration, proliferation and differentiation. Most of the Integrins in Table 3 were expressed in PC3 cells and all of them were present in tPNT2 cells and none of the integrins were present in PNT2 MVs. This suggest that PNT2 cells were transformed. This was further confirmed with functional validation of four integrins using Western blotting.

Of the proteins expressed on tPNT2 and not PNT2, the heat shock protein and integrin α-1 were found to have a role in cell adhesion, communication and migration. All these characteristics are needed for PNT2 (epithelial) cells to transform to tPNT2 by the process of EMT. The cells need to be able to communicate and allow migration to travel to a distant site for secondary metastasis. This is supported by studies carried out by David Lyden and colleagues that showed tumour derived extracellular vesicles to play an important role in preparing site of metastasis in secondary organs. In the case of prostate cancer MVs the metastatic site is in bone.

String analysis showed the proteins that are involved in different biological processes that are then connected to each other based on the database used. In Figure 40, about 10 proteins from the list of proteins that were expressed on tPNT2 MVs were involved in pathways in cancer. Also, four other proteins were involved in the prostate cancer pathway also as shown in Figure 41, using the KEGG analysis pathway.

Panther analysis was used to look at proteins involved in different cellular and biological processes in the cells. Proteins that were expressed in PC3 MVs and not PNT2 MVs were shown to be present in extracellular region of the cell more than compared to proteins that were present in tPNT2 and not PNT2 MVs.

Chapter 6: Discussion

6.1 Challenges of Extracellular Vesicles analysis

Extracellular vesicles (EVs) are small membrane bound vesicles released by most mammalian cells into the extracellular environment under both normal and pathological conditions as a form of intercellular communication (Cocucci *et al*, 2009). EVs mediate cell-to-cell communication by transmitting specific information from their host cell to their target cells and based on this key property it makes EVs novel tools for various treatment modalities, including: anti-tumour therapy, pathogen vaccination, regenerative therapies and drug delivery. The process of microvesiculation does not affect cell viability as observed *in vitro* and *in vivo* (Cocucci *et al*, 2009). There are two different types of EVs, exosomes and microvesicles (MVs). Although, they are both released by the cells, however they have different mechanisms of production. Exosomes are released by the inward budding of the cell membrane (endocytosis and subsequent intraluminal budding of endosomes), whereas MVs are released by an external budding of the cell membrane. Although according to MISEV 2018 the more appropriate terms are small EVs (sEVs) and medium EVs (mEVs) for exosomes and MVs, respectively, these terms have not been used throughout this thesis. They are both present in a variety of biological fluids in the body including blood, urine, cerebro-spinal fluids, saliva and ascites (Balaj *et al*, 2011; Lee *et al*, 2018; Becker *et al*, 2016)

There have been extraordinary advances in the use of EVs as therapeutics over the last 5 years, but fundamental techniques of isolation and characterisation have not kept apace. Thus a lack of efficient and standardised methods of EV isolation, purification, concentration, size measurement and molecular content, has limited the capacity to develop EVs in the clinical diagnostic and cancer therapeutic fields (Momen- Heravi F *et al*, 2012). In light of the recent explosion of EV studies, it has become increasing clear, and therefore a firm aim of ISEV (the International Society for Extracellular Vesicles) to generate standardised guidelines on

isolation and characterisation to avoid over interpreting exciting findings. In 2014 with a follow up in 2018 (Thery *et al*, 2018; Lotvall *et al*, 2004) the ISEV board members published Position Editorials detailing their recommendations for the most appropriate nomenclature, methods of isolation with a particular new focus on EV subtypes as well as the latest methodology for accurate characterisation.

6.2 Analysis of Extracellular Vesicles

After collecting exosomes (sEVs) by size exclusion chromatography or density gradient centrifugation or even differential centrifugation (as used in this thesis), an analysis by Nanosight Tracking Analysis (NTA) would reveal a typical histogram showing a modal peak size of 100 nm diameter. Considering that 100 nm is the size for large exosomes and small MVs, it is no longer expected that a pure sample of exosomes or MVs can be obtained. Nevertheless, upon labelling MV samples with Annexin-V Alexa Flour 488, which has a high affinity for PS, as shown earlier in this thesis, on average 65% of the samples were positive. Phosphatidylserine (PS) is a marker for MVs as during microvesiculation the PS present on the inner cytosolic face of the lipid membrane bilayer loses its asymmetric distribution and translocates to the extracellular leaflet of the plasma membrane where its presence is detected by Annexin-V Alexa Flour 488. Studies have however also shown that exosomes are also PS positive and detectable by Annexin-V, and that some EVs are PS negative (Miyanishi *et al*, 2007; Thery *et al*, 2018; Lea *et al*, 2017). Also, as using the Guava easyCyte 8HT for MV analysis means that the instrument cannot detect particles lower than 100nm (due to limits of resolution) therefore a decision was made to include both exosomes and MVs and refer to them as extracellular vesicles (EVs). MVs characterisation remains a big challenge as there are no reliable techniques to distinguish the size of MVs and exosomes. In this report MVs were analysed mainly by flow cytometry using the Guava easyCyte 8HT, which characterises

particles based on the spectral properties of the fluorescence signal that defines the morphology and granularity of the particles. The particle sample is analysed through hydrodynamic focusing where the suspended cells are moved through a compressed channel in front of a laser beam. As the laser hits the particle, the emitted scatter and fluorescence is detected. The intensity of the light is recorded as forward scatter (FSC) and side light scatter (SCC). Calibration beads of a known size and number are used to identify the heterogenous population of MVs.

6.3 Biological factors affecting EV interaction with recipient cells

Apart from the limitations in the characterisation of EVs, its interaction with the recipient cells is also an area that needs a lot of attention, as the fundamental cell biology needs to be elucidated to enable EVs to be utilised as therapeutic agents. In this study, EV interaction with recipient cells was observed by labelling EVs with a lipid dye (PKH26 dye). It is still however unclear which mechanism EVs use to interact with recipient cells; indeed, of the several candidate mechanisms, it may be that one of more may be involved, and that this may anyway differ between cells. As documented by Mulcahy *et al*, (2014) some of these routes for EV-recipient cell interaction include clatherin-mediated or caveolae-mediated endocytosis, micropinocytosis, phagocytosis and lipid raft-mediated uptake routes (Zech *et al*, 2012), as well as membrane fusion (or hemi-fusion). Due to the heterogeneous nature of EVs, it is possible that EVs utilise more than one uptake route, depending on cell type and EV constituents. This was demonstrated by several research groups, having observed that inhibition of any given pathway did not completely abrogate EV uptake by the recipient cell (Zech *et al* 2012, Rana *et al* 2012 and Tian *et al*, 2010).

Though researchers in the EV world are looking at the interaction of the vesicles with the recipient cells, this study however investigated the different physiological factors affecting EV uptake by the recipient cells. By varying the environmental conditions in which EVs were allowed to interact with the recipient cells it was possible to observe changes in the degree of these interactions and to thus better understand the nature of these interactions.

6.4 Effect of temperature and pH on MV interaction with recipient cells

When PC3 cells were treated with PC3-derived, labelled MVs at different temperatures, MV uptake was found to be at its lowest at low temperature (**figure 11)**. This finding was in line with the findings of Tian *et al*, which similarly showed almost no uptake of exosomes by the recipient cells. This demonstrated that EV uptake is energy dependent (Tian *et al* 2010). As EV interaction with recipient cells takes place in the body under either physiological or pathological conditions controlling the temperature only clarifies that the process of interaction is energy dependent, but there is insufficient research on the effect of temperature on the nature of EV: recipient cell interaction.

While low temperature reduced EV uptake by the recipient cells, low pH on the other hand increased the uptake of EVs as shown in (**Figure 9)**. When labelled PC3 MVs were added to PC3 cells, higher fluorescence was observed at low, more acidic pH. In terms of EV interactions in the tumour microenvironment, which is particularly relevant to the EV-recipient cell interactions involved in inducing EMT as studied in this thesis, the effect of low pH on these interactions is particularly pertinent. In cancer, extracellular acidity is due to the production of acidic metabolites (Sánchez-Tilló et al, 2011), such as, lactate caused by anaerobic glycolysis under the hypoxia (Warburg *et al*, 1924; Kallinowski and Vaupel 1986).

It is also known that the acidic environment acts as a trigger for pain in cancer patients (Helmlinger *et al*, 1997).

Temperature change and pH changes can also affect membrane fluidity, this can in turn affect the uptake of EVs by the recipient cell. Studies have shown the effect of pH on the conformation of membrane proteins (Yamaguchi T. *et al*, 1982). Membrane fluidity can be investigated further to help understand EV interaction with the recipient cell. Furthermore, a range of different pH (3.0 – pH 12) can also be investigated.

6.5 Role of surface proteins and EV interaction with recipient cells

Surface proteins on EVs and recipient cells plays a vital role in the uptake of EVs. Studies have shown EV uptake by endocytosis through protein - protein interaction (Raposo *et al*, 1996, Morelli *et al*, 2004 and Christianson *et al*, 2013). Proteins on EVs have been shown to interact with the membrane receptors on target cells (Nagae *et al*, 2007; Record *et al*, 2014). When cell surface proteins were removed in this study by treating the recipient cells with trypsin, a significantly reduced EVs uptake was observed as shown in figures 7 & 8. These observations are in line with observations of other research groups where EV interaction with the recipient cells was elucidated by the use of a specific antibody that recognises receptors or ligands leading, thus able to block a particular region of the membrane from interacting with EVs. (Escrevente *et al*, 2011). Furthermore, EV uptake was also reduced when PC3 EVs were treated with Annexin-V (blocking the PS sites on EVs), this observation was also on a par with the research groups, above.

In both experiments above, protein-mediated or facilitated EV-cell interaction or PS-mediated interaction, EV uptake was reduced but not fully stopped and this is because EV interact with recipient cells occurs via different mechanisms, such that when one pathway is blocked the

other can be adopted or perhaps due to the heterogeneity of EVs any interaction pathway could be used at one time (Mulcahy *et al*, 2014). The ability to pin point the uptake pathway utilised by specific sized EVs is very complex, nevertheless imperative in the EV research field. Understanding EVs uptake mechanisms and finding biochemical markers to characterise and isolate specific types of EVs will expand and develop our ability to use EVs as therapeutic agents.

In future, the use of Imaging Flow Cytometry (IFC) and Förster Resonance Energy Transfer (FRET) analysis to measure fusion and endocytosis for the study of EVs uptake could help our understanding (Ofir- Birin *et al*, 2018 and Rowland *et al*, 2015) of EVs uptake route/s to recipient cells. Indeed, where membrane fusion or hemi-fusion of EV and recipient cells occurs (which is measured by lipid mixing assays Ansa-Addo *et al*., 2010), it may be that this is dependent on an initial low affinity interaction between EVs and cells (PS-PS receptor or protein-protein mediated).

6.6 MVs from cancer cells capable of inducing EMT in non-cancer cells

With the aid of our understanding of EV uptake by recipient cells and EV interaction with recipient cells as demonstrated here, and by other researchers, the role of EVs in the process of EMT and cancer metastasis should be pertinent to explore. EVs have long been reported to be playing a role in EMT as described by Quesenberry and Alliota, in 2010. The mechanisms that drive carcinogenesis in normal cells is not yet fully understood. However, studies have shown exosomes released from tumour cells to contribute to cancer progression as they are involved in adhesion to the substratum, an important feature for metastatic cells (Hemler, 2005). Exosomes are also involved in angiogenesis induction (Koumangoye *et al*, 2011) and they can deliver miRNA to target cells (Nazaenko *et al*, 2010). Moreover, when exosomes from tumour

cells were added to adipose tissue derived mesenchymal stem cells, the mesenchymal stem cells adopted a myofibroblast phenotype, and myofibroblast cells are important in supporting tumours (Kogure *et al*, 2011). To understand the effect of Prostate cancer derived EVs on Prostate epithelial cells, it is important to look at the process of EMT at the cellular, molecular and genetic levels. EMT is a process in which epithelial cells that are tightly packed together by junction and adherent proteins, differentiate into mesenchymal like cells. This causes the cells to become mesenchymal stem cells by gaining migratory and invasive properties (Cho *et al*, 2012). Cell to cell contact at adherent junction is lost, and this is demonstrated by marked loss in E-cadherin (Hollier *et al*, 1992) and on the other hand mesenchymal proteins such as vimentin and fibronectin are expressed (Thomson *et al*, 2011).

When Prostate cancer (PC3) derived MVs were added to prostate epithelial cells (PNT2) to observe changes in the recipient cells, and a possible triggering of the metastatic process of EMT, the expression of two markers were studied, the epithelial marker (E-cadherin) and the mesenchymal marker (vimentin).

The morphological changes that occurred in PNT2 cells during transformation and subsequent EMT after adding PC3 MVs was presented in **Figure 20**. When comparing control PNT2 cells with transformed PNT2 (tPNT2) cells after 5 days, the transformed cells are no longer, packed together, and the cells are generally dispersed in **Figure 20** panel **E** and **F**.

EMT molecular markers were tested in this study. We found that when PNT2 cells were treated with PC3 MVs, E-cadherin levels were down-regulated while vimentin levels were up-regulated. **Figure 23** shows the molecular changes that had taken place in PNT2 cells post treatment with PC3 MVs, these changes being demonstrated by flow cytometry. When

comparing control, untreated and treated groups, all three experimental panels demonstrated down-regulation of E-cadherin protein. E-cadherin is the characteristic marker of epithelial cell lines, such as PNT2 cells. During EMT, the epithelial cells dissociate from one another because of alterations in intercellular adhesion molecules such as E-cadherin (Pain *et al*, 2014). The transmembrane glycoprotein E-cadherin is connected to β-catenin and the actin cytoskeleton. During destabilisation of adherens junctions, E-cadherin is cleaved at the plasma membrane, disturbing cell-to-cell adhesion, causing cell dissociation (Lamouille *et al*,2014; Fujii *et al*, 1996). The cleaved β-catenin is however, either degraded or protected from degradation so that it can participate in transcription (Shibamoto *et al*, 1994). The loss of cell-to-cell adhesion in PNT2 cells is demonstrated in (**Figure 21**, **F**) by fluorescence microscopy analysis, where a reduction in E-cadherin expression is observed. This molecular change also affects cytoskeletal rearrangement of epithelial cells that eventually leads to a mesenchymal phenotype. In EMT, cells lose top-to-bottom polarity and acquire front-to-back polarity, to allow the cells to stretch out and become motile, ready for invasion (Niehrs et al, 2012; Ridley, 2011). This can be seen in a close-up image in **Figure 22** when comparing the treated cells with the control cells in **Figure 21**, the treated cells show back-to-front polarity when labelled with E-cadherin, whereas the control cells exhibit a more classical round shape with apical-basal polarity.

Based on fluorescent microscopy, immunoprecipitation and flow cytometry analysis, PNT2 cells treated with PC3 MVs have been shown to undergo some significant molecular changes and this finding corroborates the findings of Panangopoulos *et al*, 2013) that induction of cell

malignancy was observed in non-malignant prostate cancer cell line upon EV exposure (Nelson, 2009).

In work leading up to focus on MV-cell interaction and the induction of EMT in prostate cancer cells, previous work carried out at CMIRC (Haidery, A., PhD thesis) the effect of T cell leukaemic cell (Jurkat) MVs had been studied on normal prostate epithelial cells (PNT2). This work showed a similar effect, to that which had been observed elsewhere (Antonyak *et al*, 2011; Kreger *et al*, 2016) of imparting characteristics typical of a 'transformed' cell. Essentially the PNT2 cells treated with Jurkat MVs became resistant to apoptotic signals induced by serum starvation and upon treating with the chemotherapeutic drug, docetaxel. Furthermore these 'transformed' cells ('tPNT2') produced excessive amounts of matrix metalloproteinase-9 (MMP-9) and 66% of the treated population underwent cell cycle arrest in G2/M. Corroborating the findings of Haidery in the current work the tPNT2 cells have been shown in detail to undergo an MV-induced EMT, the cells losing epithelial and acquiring mesenchymal characteristics.

E-cadherin has been shown to also play a role in intracellular signalling pathways and plays part in regulating the events (Panagopoulos *et al*, 2013).

The activation of transcription factors was also observed in the process of EMT induced by PC3 MVs, when PNT2 cells were treated with PC3 MVs. Transcription factors play an important role in coordinating the process of EMT (Lamouille *et al*, 2014). During embryogenesis, EMT is induced by the effect of TGFβ together with fibroblast growth factor (FGF) on transcription factors such as Snail (Barrallo-Gimeno and Nieto *et al*, 2005). Snail and Slug are key transcription factors in EMT that promote the breaching of the epithelial cells from the basal membrane just before the process of EMT by supressing E-cadherin expression

and activating metalloproteinases (Cano *et al*, 2000) and (Medici *et al*, 2008). After suppressing E-cadherin, the cell adhesion in the epithelial cells is lost and the extracellular matrix is degraded allowing for the process of cell migration to take place (Cano *et al*, 2000). The up regulation of Slug during EMT when PC3 MVs were added to PNT2 cells was shown by fluorescent microscopy and flow cytometry in chapter 4 of this thesis. These findings corroborate those of Ester *et al*, (2011) in also showing the role of β-catenin in EMT. In normal epithelial cells, the oncoprotein β-catenin is localised at the cellular membrane with the adhesion molecule E-cadherin away from the nucleus. However, in metastatic cells canonical Wnt pathway triggers the translocation of β-catenin to the nucleus where it activates ZEB-1 and other transcription factors such as Snail and Slug (Ester *et al*, 2011). This is in line with our findings in chapter 4, where the nuclear translocation of β-catenin is observed in the PNT2 treated cells with PC3 MVs.

As previously mentioned, the hall marks of EMT include the reduction in epithelial markers and increased expression in mesenchymal markers such as vimentin. After treating PNT2 cells with PC3 derived MVs an increase in the expression of vimentin was observed in treated cells compared to cells in the control group. This is demonstrated in **Figure 25** where FACS analysis showed increase in vimentin, as did Western blot analysis showing high relative density (densitometry readings) for vimentin compared with control β-actin. These results were further confirmed by fluorescence microscopy analysis in **Figure 24** where treated cells showed increased expression in vimentin compared to control cells in **Figure 24, D and H**. vimentin is a 57 KDa, type three intermediate filament that is found in mesenchymal cells of different types of tissue during their developmental stages and wound healing (McEwen *et al*, 2012). Vimentin is known to regulate cell migration in many cell types, whilst in contrast cells with no vimentin display reduced mechanical stability and motility *in vitro* (Coulombe and Wong,

2004). Vimentin null mice show impaired ability to heal wounds *in vivo* (Ivaska *et al*, 2007). Studies have shown that the changes in the expression of vimentin is associated with malignancy and the phenotypic changes that occur in malignant cells undergoing EMT are a result of changes at the genetic level (Eckes *et al*, 2000). These changes are facilitated by specific transcription factors to some extent and can modulate the expression of E-cadherin as mentioned above and other EMT associated genes *in vitro* (Vuoriluoto *et al*, 2010). Vimentin is a commonly recognised biomarker for EMT, although it is not yet known whether vimentin functionally contributes to the gene expression pattern responsible for the EMT phenotype or whether vimentin expression is simply a result of EMT (Satelli and Li, 2011). Nevertheless, the significant increase in the expression of vimentin in treated PNT2 cells corroborates well with the findings of Zhao *et al*, where vimentin expression was detected in poorly differentiated tumours and bone metastasis (Peinado *et al*, 2007; Zhao *et al*, 2008). Several other studies also showed that vimentin is over expressed in prostate cancer and contributes to cancer metastasis (Lang *et al*, 2002). Although, vimentin has been widely used as a biomarker for many different cancers, nevertheless the function of vimentin in the process of tumorigenesis remains to be elucidated (Sethi *et al*, 2011).

6.7 Proteomics analysis and identification of proteins in PC3 MVs that take part in the process of EMT

One of the most exciting outcomes of this PhD research was the identification of key proteins in PC3 MVs that may have contributed to the process of EMT that was observed in PNT2 cells when treated with PC3 MVs.

Mass spectrometry analysis was used to obtain protein profiles of the cell lines and their respective EVs samples. The analysis matches peptide fragment information given by mass

spectrometry to similar proteins using algorithms implemented by the program which perform alignments with proteins from known databases such as UniProt to predict what proteins are in the sample with a degree of certainty.

When proteins in PNT2 cells and proteins in tPNT2 (transformed) cells were compared, those proteins that were only present in the transformed cells were identified. This list of proteins was then compared to PC3 cells and proteins common in both cells were listed. Lastly, the list of identified proteins was compared to the proteins in PC3 MVs and 7 proteins were identified: (i) Plastin-2, (ii) Integrin beta-4, (iii) Ephrin type-A receptor 2, (iv) Integrin alpha-6, (v) EH domain-containing protein 1, (vi) Catenin delta-1 and (vii) Coactosin-like protein. Two of these proteins are integrins; namely Integrin alpha-6 and Integrin beta-4. Integrins are transmembrane glycoprotein receptors which are heterodimers comprised of alpha and beta subunits. The different alpha and beta combinations of polypeptides form complexes that gives variable ligand-binding specificities. Integrins also play a role in cell-to-cell adhesion and transduced signals that help regulate gene expression and cell growth. The integrin subunit beta-4 tends to associate with integrin alpha-6 subunit and is likely to play a vital role in the biology of invasive carcinoma. The results in this study are consistent with the studies that have found integrins to be involved in EMT in cancer progression (Desgrosellier & Cheresh *et al*, 2010). There is a correlation between integrin expression levels in human tumours and pathological outcomes, such as patient survival and metastasis. Integrin α6β4 is amongst the integrins that are associated with increased bone metastasis (Sloan *et al*, 2006). One of the principle problems clinicians are faced with is the management of pain and morbidity due to the growth of cancer within bones (McCabe *et al*, 2007). McCabe and team found that integrin receptors in prostate cancer cells are needed for progression within bone and that they determine tumour induced bone tissue transformation. David Lyden and colleagues have shown that exosomal integrins promote adhesion as well as triggering

signalling pathways and inflammatory responses in target cells. This results in a preparation of that organ rendering it permissive for the growth of metastatic cells. This finding is in line with the findings of this PhD thesis, where MVs derived from prostate cancer cells transported integrins from prostate cancer cells to the immortal prostate cell causing mesenchymal transformation. This suggests that integrins and/or other proteins carried by extracellular vesicles to the different organs, prepare the organ for future metastasis. The findings in this thesis have added to those of David Lyden and colleagues in demonstrating the important role of extracellular vesicles in dictating organ specific metastasis, providing information towards understanding the mechanism of organotropism.

The important role that integrins play in the biology of both tumour cells and tumour associated cells has made them very desirable targets for therapeutic studies. Integrin antagonists are being trialled in phase III clinical trials now. This will aid anti-integrin therapy in the clinical world (Desgrosellier & Cheresh 2010). In taking our research further, more extracellular vesicle integrins and proteins should be identified.

6.8 Conclusion and further directions

The mechanisms that drive metastasis in normal cells are yet to be fully understood. However, the data in this thesis demonstrates that cancer derived EVs can initiate the process of EMT and indeed trigger tumour metastasis. Due to the ability of EVs to spread to distant sites in the bloodstream and lymphatic vessels they are able to promote the development of metastasis. The new metastatic site becomes predisposed to the cancer cells by earlier arrival of EVs, prior to the recruitment of cancer cells, implying that EVs help set up the pre-metastatic niche. Therefore, inhibiting microvesiculation from the primary tumour could help stop the spread of integrins and other potent biomolecules and proteins being transported in the body fluid by

EVs. Moreover, using inhibitors to inhibit MVs from the primary tumour could not only help stop spreading the tumour but also allow the tumour cells to retain the chemotherapy drugs in the tumour site (Takahashi et al, 2017; Kosgodage *et al*, 2018; Kosgodage *et al*, 2017; Jorfi *et al*, 2015).

The role of small non-coding RNAs (miRNA) has been well established in cancer biology in that they regulate carcinogenesis, develop drug resistance and aid metastasis. miRNAs are also carried by EVs (Rocco *et al*, 2018). Therefore, understanding the molecular mechanisms of miRNA and profiling miRNAs in EVs of different tissues is vital for basic and clinical cancer research. This thesis can positively contribute to the current knowledge in the field of using EVs as therapeutics in cancer and other diseases.

References

Aga, M., Bentz, G. L., Raffa, S., Torrisi, M. R., Kondo, S., Wakisaka, N., Shackelford, J. (2014). Exosomal HIF1alpha supports invasive potential of nasopharyngeal carcinoma-associated LMP1-positive exosomes. *Oncogene, 33*(37), 4613-4622. doi:10.1038/onc.2014.66

Al-Nedawi, K., Meehan, B., Micallef, J., Lhotak, V., May, L., Guha, A., & Rak, J. (2008). Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat Cell Biol, 10*(5), 619-624. doi:10.1038/ncb1725

Ansa-Addo, E. A., Lange, S., Stratton, D., Antwi-Baffour, S., Cestari, I., Ramirez, M. I., Inal, J. M. (2010). Human plasma membrane-derived vesicles halt proliferation and induce differentiation of THP-1 acute monocytic leukemia cells. *J Immunol, 185*(9), 5236-5246. doi:10.4049/jimmunol.1001656

Antonyak, M. A., Li, B., Boroughs, L. K., Johnson, J. L., Druso, J. E., Bryant, K. L., Cerione, R. A. (2011). Cancer cell-derived microvesicles induce transformation by transferring tissue transglutaminase and fibronectin to recipient cells. *Proc Natl Acad Sci U S A, 108*(12), 4852-4857. doi:10.1073/pnas.1017667108

Balaj, L., Lessard, R., Dai, L., Cho, Y. J., Pomeroy, S. L., Breakefield, X. O., & Skog, J. (2011). Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. *Nat Commun, 2,* 180. doi:10.1038/ncomms1180

Barrallo-Gimeno, A., & Nieto, M. A. (2005). The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development, 132*(14), 3151-3161. doi:10.1242/dev.01907

Bianconi, D., Unseld, M., & Prager, G. W. (2016). Integrins in the Spotlight of Cancer. *Int J Mol Sci, 17*(12). doi:10.3390/ijms17122037

Bobrie, A., Colombo, M., Raposo, G., & Thery, C. (2011). Exosome secretion: molecular mechanisms and roles in immune responses. *Traffic, 12*(12), 1659-1668.

doi:10.1111/j.1600-0854.2011.01225.x

Bonkhoff, H., Stein, U., & Remberger, K. (1993). Differential expression of alpha 6 and alpha 2 very late antigen integrins in the normal, hyperplastic, and neoplastic prostate: simultaneous demonstration of cell surface receptors and their extracellular ligands. *Hum Pathol, 24*(3), 243-248.

Burch, T. C., Watson, M. T., & Nyalwidhe, J. O. (2013). Variable metastatic potentials correlate with differential plectin and vimentin expression in syngeneic androgen independent prostate cancer cells. *PLoS One, 8*(5), e65005. doi:10.1371/journal.pone.0065005

Burtnick, L. D., Urosev, D., Irobi, E., Narayan, K., & Robinson, R. C. (2004). Structure of the N-terminal half of gelsolin bound to actin: roles in severing, apoptosis and FAF. *Embo j, 23*(14), 2713-2722.

Calderwood, S. K., Khaleque, M. A., Sawyer, D. B., & Ciocca, D. R. (2006). Heat shock proteins in cancer: chaperones of tumorigenesis. *Trends Biochem Sci, 31*(3), 164-172. doi:10.1016/j.tibs.2006.01.006

Camussi, G., Deregibus, M. C., Bruno, S., Cantaluppi, V., & Biancone, L. (2010). Exosomes/microvesicles as a mechanism of cell-to-cell communication. *Kidney Int, 78*(9), 838-848. doi:10.1038/ki.2010.278

Cano, A., Perez-Moreno, M. A., Rodrigo, I., Locascio, A., Blanco, M. J., del Barrio, M. G., . . . Nieto, M. A. (2000). The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol, 2*(2), 76-83. doi:10.1038/35000025

Chargaff, E., & West, R. (1946). The biological significance of the thromboplastic protein of blood. *J Biol Chem, 166*(1), 189-197.

Cho, J. A., Park, H., Lim, E. H., & Lee, K. W. (2012). Exosomes from breast cancer cells can

convert adipose tissue-derived mesenchymal stem cells into myofibroblast-like cells. *Int J Oncol, 40*(1), 130-138. doi:10.3892/ijo.2011.1193

Christianson, H. C., Svensson, K. J., van Kuppevelt, T. H., Li, J. P., & Belting, M. (2013). Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity. *Proc Natl Acad Sci U S A, 110*(43), 17380-17385. doi:10.1073/pnas.1304266110

Clark, M. R. (2011). Flippin' lipids. *Nat Immunol, 12*(5), 373-375. doi:10.1038/ni.2024

Coulombe, P. A., & Wong, P. (2004). Cytoplasmic intermediate filaments revealed as dynamic and multipurpose scaffolds. *Nat Cell Biol, 6*(8), 699-706. doi:10.1038/ncb0804-699

Daleke, D. L. (2003). Regulation of transbilayer plasma membrane phospholipid asymmetry. *J Lipid Res, 44*(2), 233-242. doi:10.1194/jlr.R200019-JLR200

Desgrosellier, J. S., & Cheresh, D. A. (2010). Integrins in cancer: biological implications and therapeutic opportunities. *Nat Rev Cancer, 10*(1), 9-22. doi:10.1038/nrc2748

Desouza, M., Gunning, P. W., & Stehn, J. R. (2012). The actin cytoskeleton as a sensor and mediator of apoptosis. *Bioarchitecture, 2*(3), 75-87. doi:10.4161/bioa.20975

D'Souza-Schorey, C., & Clancy, J. W. (2012). Tumor-derived microvesicles: shedding light on novel microenvironment modulators and prospective cancer biomarkers. *Genes Dev, 26*(12), 1287-1299. doi:10.1101/gad.192351.112

Duband, J. L., & Thiery, J. P. (1982). Appearance and distribution of fibronectin during chick embryo gastrulation and neurulation. *Dev Biol, 94*(2), 337-350.

Eckes, B., Colucci-Guyon, E., Smola, H., Nodder, S., Babinet, C., Krieg, T., & Martin, P. (2000). Impaired wound healing in embryonic and adult mice lacking vimentin. *J Cell Sci, 113 (Pt 13)*, 2455-2462.

Emanueli, C., Minasi, A., Zacheo, A., Chao, J., Chao, L., Salis, M. B., Madeddu, P. (2001). Local delivery of human tissue kallikrein gene accelerates spontaneous angiogenesis in

mouse model of hindlimb ischemia. *Circulation, 103*(1), 125-132.

Escrevente, C., Keller, S., Altevogt, P., & Costa, J. (2011). Interaction and uptake of exosomes by ovarian cancer cells. *BMC Cancer, 11*, 108. doi:10.1186/1471-2407-11-108

Escrevente, C., Keller, S., Altevogt, P., & Costa, J. (2011). Interaction and uptake of exosomes by ovarian cancer cells. *BMC Cancer*, *11*, 108. doi:10.1186/1471-2407-11-108

Feitsma, H., and Cuppen, E. "Zebrafish as a Cancer Model." Molecular Cancer Research. 6(2008):685-694.

Feng, D., Zhao, W. L., Ye, Y. Y., Bai, X. C., Liu, R. Q., Chang, L. F., Sui, S. F. (2010). Cellular internalization of exosomes occurs through phagocytosis. *Traffic, 11*(5), 675-687. doi:10.1111/j.1600-0854.2010.01041.x

Fleissner, F., Goerzig, Y., Haverich, A., & Thum, T. (2012). Microvesicles as novel biomarkers and therapeutic targets in transplantation medicine. *Am J Transplant, 12*(2), 289-297. doi:10.1111/j.1600-6143.2011.03790.x

Fujii, K. (1996). Ligand activation of overexpressed epidermal growth factor receptor results in loss of epithelial phenotype and impaired RGD-sensitive integrin function in HSC-1 cells. *J Invest Dermatol, 107*(2), 195-202.

Gonzalez, D. M., & Medici, D. (2014). Signaling mechanisms of the epithelial-mesenchymal transition. *Sci Signal, 7*(344), re8. doi:10.1126/scisignal.2005189

Hagiwara, K., Ochiya, T., & Kosaka, N. (2014). A paradigm shift for extracellular vesicles as small RNA carriers: from cellular waste elimination to therapeutic applications. *Drug Deliv Transl Res, 4*(1), 31-37. doi:10.1007/s13346-013-0180-9

Harding, C., Heuser, J., & Stahl, P. (1984). Endocytosis and intracellular processing of transferrin and colloidal gold-transferrin in rat reticulocytes: demonstration of a pathway for receptor shedding. *Eur J Cell Biol, 35*(2), 256-263.

Hargett, L. A., & Bauer, N. N. (2013). On the origin of microparticles: From "platelet dust" to mediators of intercellular communication. *Pulm Circ, 3*(2), 329-340. doi:10.4103/2045-8932.114760

Hashimoto, S., Onodera, Y., Hashimoto, A., Tanaka, M., Hamaguchi, M., Yamada, A., & Sabe, H. (2004). Requirement for Arf6 in breast cancer invasive activities. *Proc Natl Acad Sci U S A, 101*(17), 6647-6652. doi:10.1073/pnas.0401753101

Helmlinger, G., Yuan, F., Dellian, M., & Jain, R. K. (1997). Interstitial pH and pO2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. *Nat Med, 3*(2), 177-182.

Hemler, M. E. (2005). Tetraspanin functions and associated microdomains. *Nat Rev Mol Cell Biol, 6*(10), 801-811. doi:10.1038/nrm1736

Hollier, B. G., Tinnirello, A. A., Werden, S. J., Evans, K. W., Taube, J. H., Sarkar, T. R., Mani, S. A. (2013). FOXC2 expression links epithelial-mesenchymal transition and stem cell properties in breast cancer. *Cancer Res, 73*(6), 1981-1992. doi:10.1158/0008-5472.Can-12-2962

Hoshino, A., Costa-Silva, B., Shen, T. L., Rodrigues, G., Hashimoto, A., Tesic Mark, M., Lyden, D. (2015). Tumour exosome integrins determine organotropic metastasis. *Nature, 527*(7578), 329-335. doi:10.1038/nature15756

Hsing, A. W., & Chokkalingam, A. P. (2006). Prostate cancer epidemiology. *Front Biosci, 11*, 1388-1413.

Huang da, W., Sherman, B. T., & Lempicki, R. A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc, 4*(1), 44-57. doi:10.1038/nprot.2008.211

Huang da, W., Sherman, B. T., Zheng, X., Yang, J., Imamichi, T., Stephens, R., & Lempicki, R. A. (2009). Extracting biological meaning from large gene lists with DAVID. *Curr*

Protoc Bioinformatics, Chapter 13, Unit 13.11. doi:10.1002/0471250953.bi1311s27

Inal, J. M., Ansa-Addo, E. A., Stratton, D., Kholia, S., Antwi-Baffour, S. S., Jorfi, S., & Lange, S. (2012). Microvesicles in health and disease. *Arch Immunol Ther Exp (Warsz), 60*(2), 107-121. doi:10.1007/s00005-012-0165-2

Israelsen, W. J., & Vander Heiden, M. G. (2015). Pyruvate kinase: Function, regulation and role in cancer. *Semin Cell Dev Biol, 43*, 43-51. doi:10.1016/j.semcdb.2015.08.004

Ivaska, J., Pallari, H. M., Nevo, J., & Eriksson, J. E. (2007). Novel functions of vimentin in cell adhesion, migration, and signaling. *Exp Cell Res, 313*(10), 2050-2062. doi:10.1016/j.yexcr.2007.03.040

Jin, J. K., Tien, P. C., Cheng, C. J., Song, J. H., Huang, C., Lin, S. H., & Gallick, G. E. (2015). Talin1 phosphorylation activates β1 integrins: a novel mechanism to promote prostate cancer bone metastasis. *Oncogene, 34*(14), 1811-1821. doi:10.1038/onc.2014.116

Johnstone, R. M., Adam, M., Hammond, J. R., Orr, L., & Turbide, C. (1987). Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J Biol Chem, 262*(19), 9412-9420.

Jones, C. U., Hunt, D., McGowan, D. G., Amin, M. B., Chetner, M. P., Bruner, D. W., Shipley, W. U. (2011). Radiotherapy and short-term androgen deprivation for localized prostate cancer. *N Engl J Med, 365*(2), 107-118. doi:10.1056/NEJMoa1012348

Jorfi, S., Ansa-Addo, E. A., Kholia, S., Stratton, D., Valley, S., Lange, S., & Inal, J. (2015). Inhibition of microvesiculation sensitizes prostate cancer cells to chemotherapy and reduces docetaxel dose required to limit tumor growth in vivo. *Sci Rep, 5*, 13006. doi:10.1038/srep13006

Kallinowski, F., & Vaupel, P. (1986). Concurrent measurements of O2 partial pressures and pH values in human mammary carcinoma xenotransplants. *Adv Exp Med Biol, 200*, 609-621.

Kalluri, R., & Weinberg, R. A. (2009). The basics of epithelial-mesenchymal transition. *J Clin Invest, 119*(6), 1420-1428. doi:10.1172/jci39104

Kalra, H., Simpson, R. J., Ji, H., Aikawa, E., Altevogt, P., Askenase, P., Mathivanan, S. (2012). Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. *PLoS Biol, 10*(12), e1001450. doi:10.1371/journal.pbio.1001450

Kariya, Y., Kariya, Y., & Gu, J. (2017). Roles of Integrin α6β4 Glycosylation in Cancer. *Cancers, 9*(7), 79. Retrieved from https://www.mdpi.com/2072-6694/9/7/79.

Kogure, T., Lin, W. L., Yan, I. K., Braconi, C., & Patel, T. (2011). Intercellular nanovesicle-mediated microRNA transfer: a mechanism of environmental modulation of hepatocellular cancer cell growth. *Hepatology, 54*(4), 1237-1248. doi:10.1002/hep.24504

Koistinen P, Heino J. Integrins in Cancer Cell Invasion. In: Madame Curie Bioscience Database [Internet]. Austin (TX): Landes Bioscience; 2000-2013.

Kosgodage, U. S., Mould, R., Henley, A. B., Nunn, A. V., Guy, G. W., Thomas, E. L., Lange, S. (2018). Cannabidiol (CBD) Is a Novel Inhibitor for Exosome and Microvesicle (EMV) Release in Cancer. *Front Pharmacol, 9*, 889. doi:10.3389/fphar.2018.00889

Koumangoye, R. B., Sakwe, A. M., Goodwin, J. S., Patel, T., & Ochieng, J. (2011). Detachment of breast tumor cells induces rapid secretion of exosomes which subsequently mediate cellular adhesion and spreading. *PLoS One, 6*(9), e24234. doi:10.1371/journal.pone.0024234

Kreger, B. T., Dougherty, A. L., Greene, K. S., Cerione, R. A., & Antonyak, M. A. (2016). Microvesicle Cargo and Function Changes upon Induction of Cellular Transformation. *J Biol Chem, 291*(38), 19774-19785. doi:10.1074/jbc.M116.725705

Lamouille, S., Xu, J., & Derynck, R. (2014). Molecular mechanisms of epithelial-mesenchymal

transition. *Nat Rev Mol Cell Biol, 15*(3), 178-196. doi:10.1038/nrm3758

Lamouille, S., Xu, J., & Derynck, R. (2014). Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol, 15*(3), 178-196. doi:10.1038/nrm3758

Lamouille, S., Xu, J., & Derynck, R. (2014). Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol, 15*(3), 178-196. doi:10.1038/nrm3758

Lang, S. H., Hyde, C., Reid, I. N., Hitchcock, I. S., Hart, C. A., Bryden, A. A., Maitland, N. J. (2002). Enhanced expression of vimentin in motile prostate cell lines and in poorly differentiated and metastatic prostate carcinoma. *Prostate, 52*(4), 253-263. doi:10.1002/pros.10088

Le Parc, A., Leonil, J., & Chanat, E. (2010). AlphaS1-casein, which is essential for efficient ER-to-Golgi casein transport, is also present in a tightly membrane-associated form. *BMC Cell Biol, 11*, 65. doi:10.1186/1471-2121-11-65

Lea, J., Sharma, R., Yang, F., Zhu, H., Ward, E. S., & Schroit, A. J. (2017). Detection of phosphatidylserine-positive exosomes as a diagnostic marker for ovarian malignancies: a proof of concept study. *Oncotarget, 8*(9), 14395-14407. doi:10.18632/oncotarget.14795

Lindoso, R. S., Collino, F., & Camussi, G. (2015). Extracellular vesicles derived from renal cancer stem cells induce a pro-tumorigenic phenotype in mesenchymal stromal cells. *Oncotarget, 6*(10), 7959-7969. doi:10.18632/oncotarget.3503

Lotvall, J., Hill, A. F., Hochberg, F., Buzas, E. I., Di Vizio, D., Gardiner, C., Thery, C. (2014). Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. *J Extracell Vesicles, 3*, 26913. doi:10.3402/jev.v3.26913

Marsh, M., & Helenius, A. (2006). Virus entry: open sesame. *Cell, 124*(4), 729-740. doi:10.1016/j.cell.2006.02.007

Mathivanan, S., Ji, H., & Simpson, R. J. (2010). Exosomes: extracellular organelles important in intercellular communication. *J Proteomics, 73*(10), 1907-1920. doi:10.1016/j.jprot.2010.06.006

May, K. E., Villar, J., Kirtley, S., Kennedy, S. H., & Becker, C. M. (2011). Endometrial alterations in endometriosis: a systematic review of putative biomarkers. *Hum Reprod Update, 17*(5), 637-653. doi:10.1093/humupd/dmr013

McCabe, N. P., De, S., Vasanji, A., Brainard, J., & Byzova, T. V. (2007). Prostate cancer specific integrin alphavbeta3 modulates bone metastatic growth and tissue remodeling. *Oncogene, 26*(42), 6238-6243. doi:10.1038/sj.onc.1210429

McEwen, A. E., Escobar, D. E., & Gottardi, C. J. (2012). Signaling from the adherens junction. *Subcell Biochem, 60*, 171-196. doi:10.1007/978-94-007-4186-7_8

Medici, D., Hay, E. D., & Olsen, B. R. (2008). Snail and Slug promote epithelial-mesenchymal transition through beta-catenin-T-cell factor-4-dependent expression of transforming growth factor-beta3. *Mol Biol Cell, 19*(11), 4875-4887. doi:10.1091/mbc.E08-05-0506

Mirsaeidi, M., Gidfar, S., Vu, A., & Schraufnagel, D. (2016). Annexins family: insights into their functions and potential role in pathogenesis of sarcoidosis. *J Transl Med, 14*, 89. doi:10.1186/s12967-016-0843-7

Miyanishi, M., Tada, K., Koike, M., Uchiyama, Y., Kitamura, T., & Nagata, S. (2007). Identification of Tim4 as a phosphatidylserine receptor. *Nature, 450*(7168), 435-439. doi:10.1038/nature06307

Miyanishi, M., Tada, K., Koike, M., Uchiyama, Y., Kitamura, T., & Nagata, S. (2007). Identification of Tim4 as a phosphatidylserine receptor. *Nature, 450*(7168), 435-439. doi:10.1038/nature06307

Momen-Heravi, F., Balaj, L., Alian, S., Tigges, J., Toxavidis, V., Ericsson, M., Kuo, W. P. (2012). Alternative methods for characterization of extracellular vesicles. *Front*

Physiol, 3, 354. doi:10.3389/fphys.2012.00354

Morelli, A. E., Larregina, A. T., Shufesky, W. J., Sullivan, M. L. G., Stolz, D. B., Papworth, G. D., Thomson, A. W. (2004). Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. *Blood, 104*(10), 3257-3266. Retrieved from http://www.bloodjournal.org/content/bloodjournal/104/10/3257.full.pdf. doi:10.1182/blood-2004-03-0824

Mulcahy, L. A., Pink, R. C., & Carter, D. R. (2014). Routes and mechanisms of extracellular vesicle uptake. *J Extracell Vesicles, 3*. doi:10.3402/jev.v3.24641

Mulcahy, L. A., Pink, R. C., & Carter, D. R. (2014). Routes and mechanisms of extracellular vesicle uptake. *J Extracell Vesicles, 3*. doi:10.3402/jev.v3.24641

Nagae, M., Hiraga, T., & Yoneda, T. (2007). Acidic microenvironment created by osteoclasts causes bone pain associated with tumor colonization. *J Bone Miner Metab, 25*(2), 99-104. doi:10.1007/s00774-006-0734-8

Nauseef, J. T., & Henry, M. D. (2011). Epithelial-to-mesenchymal transition in prostate cancer: paradigm or puzzle? *Nat Rev Urol, 8*(8), 428-439. doi:10.1038/nrurol.2011.85

Nazarenko, I., Rana, S., Baumann, A., McAlear, J., Hellwig, A., Trendelenburg, M., Zoller, M. (2010). Cell surface tetraspanin Tspan8 contributes to molecular pathways of exosome-induced endothelial cell activation. *Cancer Res, 70*(4), 1668-1678. doi:10.1158/0008-5472.Can-09-2470

Nelson, W. J. (2009). Remodeling epithelial cell organization: transitions between front-rear and apical-basal polarity. *Cold Spring Harb Perspect Biol, 1*(1), a000513. doi:10.1101/cshperspect.a000513

Nickel, W., & Rabouille, C. (2009). Mechanisms of regulated unconventional protein secretion. *Nat Rev Mol Cell Biol, 10*(2), 148-155. doi:10.1038/nrm2617

Niehrs, C. (2012). The complex world of WNT receptor signalling. *Nat Rev Mol Cell Biol,*

13(12), 767-779. doi:10.1038/nrm3470

Nolte-'t Hoen, E. N., Buschow, S. I., Anderton, S. M., Stoorvogel, W., & Wauben, M. H. (2009). Activated T cells recruit exosomes secreted by dendritic cells via LFA-1. *Blood, 113*(9), 1977-1981. doi:10.1182/blood-2008-08-174094

Ofir-Birin, Y., Abou Karam, P., Rudik, A., Giladi, T., Porat, Z., & Regev-Rudzki, N. (2018). Monitoring Extracellular Vesicle Cargo Active Uptake by Imaging Flow Cytometry. *Front Immunol, 9*, 1011. doi:10.3389/fimmu.2018.01011

Okada, H., Danoff, T. M., Kalluri, R., & Neilson, E. G. (1997). Early role of Fsp1 in epithelial-mesenchymal transformation. *Am J Physiol, 273*(4), F563-574. doi:10.1152/ajprenal.1997.273.4.F563

Owens, A. P., 3rd, & Mackman, N. (2011). Microparticles in hemostasis and thrombosis. *Circ Res, 108*(10), 1284-1297. doi:10.1161/circresaha.110.233056

Pain, M., Bermudez, O., Lacoste, P., Royer, P. J., Botturi, K., Tissot, A., . . . Magnan, A. (2014). Tissue remodelling in chronic bronchial diseases: from the epithelial to mesenchymal phenotype. *Eur Respir Rev, 23*(131), 118-130. doi:10.1183/09059180.00004413

Pan, B. T., Teng, K., Wu, C., Adam, M., & Johnstone, R. M. (1985). Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. *J Cell Biol, 101*(3), 942-948. doi:10.1083/jcb.101.3.942

Panagopoulos, K., Cross-Knorr, S., Dillard, C., Pantazatos, D., Del Tatto, M., Mills, D., Chatterjee, D. (2013). Reversal of chemosensitivity and induction of cell malignancy of a non-malignant prostate cancer cell line upon extracellular vesicle exposure. *Mol Cancer, 12*(1), 118. doi:10.1186/1476-4598-12-118

Parolini, I., Federici, C., Raggi, C., Lugini, L., Palleschi, S., De Milito, A., Fais, S. (2009). Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J Biol Chem,*

284(49), 34211-34222. doi:10.1074/jbc.M109.041152

Peinado, H., Olmeda, D., & Cano, A. (2007). Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer, 7*(6), 415-428. doi:10.1038/nrc2131

Perez-Hernandez, J., & Cortes, R. (2015). Extracellular Vesicles as Biomarkers of Systemic Lupus Erythematosus. *Dis Markers, 2015*, 613536. doi:10.1155/2015/613536

Perez-Hernandez, J., Redon, J., & Cortes, R. (2017). Extracellular Vesicles as Therapeutic Agents in Systemic Lupus Erythematosus. *Int J Mol Sci, 18*(4). doi:10.3390/ijms18040717

Pompa, A., De Marchis, F., Pallotta, M. T., Benitez-Alfonso, Y., Jones, A., Schipper, K., Bellucci, M. (2017). Unconventional Transport Routes of Soluble and Membrane Proteins and Their Role in Developmental Biology. *Int J Mol Sci, 18*(4). doi:10.3390/ijms18040703

Popova, S. N., Rodriguez-Sanchez, B., Liden, A., Betsholtz, C., Van Den Bos, T., & Gullberg, D. (2004). The mesenchymal alpha11beta1 integrin attenuates PDGF-BB-stimulated chemotaxis of embryonic fibroblasts on collagens. *Dev Biol, 270*(2), 427-442. doi:10.1016/j.ydbio.2004.03.006

Quesenberry, P. J., & Aliotta, J. M. (2010). Cellular phenotype switching and microvesicles. *Adv Drug Deliv Rev, 62*(12), 1141-1148. doi:10.1016/j.addr.2010.06.001

Rana, S., & Zoller, M. (2011). Exosome target cell selection and the importance of exosomal tetraspanins: a hypothesis. *Biochem Soc Trans, 39*(2), 559-562. doi:10.1042/bst0390559

Raposo, G., Nijman, H. W., Stoorvogel, W., Liejendekker, R., Harding, C. V., Melief, C. J., & Geuze, H. J. (1996). B lymphocytes secrete antigen-presenting vesicles. *J Exp Med, 183*(3), 1161-1172. doi:10.1084/jem.183.3.1161

Raposo, G., Nijman, H. W., Stoorvogel, W., Liejendekker, R., Harding, C. V., Melief, C. J., & Geuze, H. J. (1996). B lymphocytes secrete antigen-presenting vesicles. *J Exp Med, 183*(3), 1161-1172. doi:10.1084/jem.183.3.1161

Record, M., Carayon, K., Poirot, M., & Silvente-Poirot, S. (2014). Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiologies. *Biochim Biophys Acta, 1841*(1), 108-120. doi:10.1016/j.bbalip.2013.10.004

Rennebeck, G., Martelli, M., & Kyprianou, N. (2005). Anoikis and survival connections in the tumor microenvironment: is there a role in prostate cancer metastasis? *Cancer Res, 65*(24), 11230-11235. doi:10.1158/0008-5472.Can-05-2763

Ridley, A. J. (2011). Life at the leading edge. *Cell, 145*(7), 1012-1022. doi:10.1016/j.cell.2011.06.010

Rippa, A. L., Vorotelyak, E. A., Vasiliev, A. V., & Terskikh, V. V. (2013). The role of integrins in the development and homeostasis of the epidermis and skin appendages. *Acta Naturae, 5*(4), 22-33.

Rocco, G. D., Baldari, S., & Toietta, G. (2017). Exosomes and other extracellular vesicles-mediated microRNA delivery for cancer therapy. *Translational Cancer Research*, S1321-S1330. Retrieved from http://tcr.amegroups.com/article/view/16316.

Rozmyslowicz, T., Majka, M., Kijowski, J., Murphy, S. L., Conover, D. O., Poncz, M., Ratajczak, M. Z. (2003). Platelet- and megakaryocyte-derived microparticles transfer CXCR4 receptor to CXCR4-null cells and make them susceptible to infection by X4-HIV. *Aids, 17*(1), 33-42. doi:10.1097/01.aids.0000042948.95433.3d

S, E. L. A., Mager, I., Breakefield, X. O., & Wood, M. J. (2013). Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat Rev Drug Discov, 12*(5), 347-357. doi:10.1038/nrd3978

Sanchez-Tillo, E., de Barrios, O., Siles, L., Cuatrecasas, M., Castells, A., & Postigo, A. (2011).

beta-catenin/TCF4 complex induces the epithelial-to-mesenchymal transition (EMT)-activator ZEB1 to regulate tumor invasiveness. *Proc Natl Acad Sci U S A, 108*(48), 19204-19209. doi:10.1073/pnas.1108977108

Santiago-Dieppa, D. R., Steinberg, J., Gonda, D., Cheung, V. J., Carter, B. S., & Chen, C. C. (2014). Extracellular vesicles as a platform for 'liquid biopsy' in glioblastoma patients. *Expert Rev Mol Diagn, 14*(7), 819-825. doi:10.1586/14737159.2014.943193

Satelli, A., & Li, S. (2011). Vimentin in cancer and its potential as a molecular target for cancer therapy. *Cell Mol Life Sci, 68*(18), 3033-3046. doi:10.1007/s00018-011-0735-1

Schmitt, E., Gehrmann, M., Brunet, M., Multhoff, G., & Garrido, C. (2007). Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy. *J Leukoc Biol, 81*(1), 15-27. doi:10.1189/jlb.0306167

Segura, M. M., Garnier, A., Durocher, Y., Coelho, H., & Kamen, A. (2007). Production of lentiviral vectors by large-scale transient transfection of suspension cultures and affinity chromatography purification. *Biotechnol Bioeng, 98*(4), 789-799. doi:10.1002/bit.21467

Sethi, S., Macoska, J., Chen, W., & Sarkar, F. H. (2011). Molecular signature of epithelial-mesenchymal transition (EMT) in human prostate cancer bone metastasis. *Am J Transl Res, 3*(1), 90-99.

Shibamoto, S., Hayakawa, M., Takeuchi, K., Hori, T., Oku, N., Miyazawa, K., Ito, F. (1994). Tyrosine phosphorylation of beta-catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells. *Cell Adhes Commun, 1*(4), 295-305.

Shin, B. K., Wang, H., Yim, A. M., Le Naour, F., Brichory, F., Jang, J. H., Hanash, S. M. (2003). Global profiling of the cell surface proteome of cancer cells uncovers an abundance of proteins with chaperone function. *J Biol Chem, 278*(9), 7607-7616.

doi:10.1074/jbc.M210455200

Shull, A. Y., Hu, C. A., & Teng, Y. (2017). Zebrafish as a model to evaluate peptide-related cancer therapies. *Amino Acids, 49*(12), 1907-1913. doi:10.1007/s00726-017-2388-3

Simpson, R. J., Kalra, H., & Mathivanan, S. (2012). ExoCarta as a resource for exosomal research. *J Extracell Vesicles, 1*. doi:10.3402/jev.v1i0.18374

Skromne, I., & Stern, C. D. (2001). Interactions between Wnt and Vg1 signalling pathways initiate primitive streak formation in the chick embryo. *Development, 128*(15), 2915-2927.

Sloan, E. K., Pouliot, N., Stanley, K. L., Chia, J., Moseley, J. M., Hards, D. K., & Anderson, R. L. (2006). Tumor-specific expression of alphavbeta3 integrin promotes spontaneous metastasis of breast cancer to bone. *Breast Cancer Res, 8*(2), R20. doi:10.1186/bcr1398

Slomka, A., Urban, S. K., Lukacs-Kornek, V., Zekanowska, E., & Kornek, M. (2018). Large Extracellular Vesicles: Have We Found the Holy Grail of Inflammation? *Front Immunol, 9*, 2723. doi:10.3389/fimmu.2018.02723

Souza, A. G., B. Silva, I. B., Campos-Fernández, E., Marangoni, K., F. Bastos, V. A., Alves, P. T., . . . Alonso-Goulart, V. (2018). Extracellular vesicles as drivers of epithelial-mesenchymal transition and carcinogenic characteristics in normal prostate cells. *Molecular Carcinogenesis, 57*(4), 503-511.

Stratton, D., Moore, C., Antwi-Baffour, S., Lange, S., & Inal, J. (2015). Microvesicles released constitutively from prostate cancer cells differ biochemically and functionally to stimulated microvesicles released through sublytic C5b-9. *Biochem Biophys Res Commun, 460*(3), 589-595. doi:10.1016/j.bbrc.2015.03.074

Suyin Paulynn Chin, Dickinson Joanne Louise and Holloway Adele Frances “Integrins in Prostate Cancer Invasion and Metastasis”. 9(2013)

Thery, C., Duban, L., Segura, E., Veron, P., Lantz, O., & Amigorena, S. (2002). Indirect

activation of naive CD4+ T cells by dendritic cell-derived exosomes. *Nat Immunol, 3*(12), 1156-1162. doi:10.1038/ni854

Thery, C., Ostrowski, M., & Segura, E. (2009). Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol, 9*(8), 581-593. doi:10.1038/nri2567

Thery, C., Regnault, A., Garin, J., Wolfers, J., Zitvogel, L., Ricciardi-Castagnoli, P., Amigorena, S. (1999). Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73. *J Cell Biol, 147*(3), 599-610. doi:10.1083/jcb.147.3.599

Thery, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R., Zuba-Surma, E. K. (2018). Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles, 7*(1), 1535750. doi:10.1080/20013078.2018.1535750

Thery, C., Zitvogel, L., & Amigorena, S. (2002). Exosomes: composition, biogenesis and function. *Nat Rev Immunol, 2*(8), 569-579. doi:10.1038/nri855

Thery, C., Zitvogel, L., & Amigorena, S. (2002). Exosomes: composition, biogenesis and function. *Nat Rev Immunol, 2*(8), 569-579. doi:10.1038/nri855

Thomson, S., Petti, F., Sujka-Kwok, I., Mercado, P., Bean, J., Monaghan, M., Haley, J. D. (2011). A systems view of epithelial-mesenchymal transition signaling states. *Clin Exp Metastasis, 28*(2), 137-155. doi:10.1007/s10585-010-9367-3

Tian, T., Wang, Y., Wang, H., Zhu, Z., & Xiao, Z. (2010). Visualizing of the cellular uptake and intracellular trafficking of exosomes by live-cell microscopy. *J Cell Biochem, 111*(2), 488-496. doi:10.1002/jcb.22733

Tian, T., Zhu, Y. L., Hu, F. H., Wang, Y. Y., Huang, N. P., & Xiao, Z. D. (2013). Dynamics of exosome internalization and trafficking. *J Cell Physiol, 228*(7), 1487-1495.

doi:10.1002/jcp.24304

Vader, P., Mol, E. A., Pasterkamp, G., & Schiffelers, R. M. (2016). Extracellular vesicles for drug delivery. *Adv Drug Deliv Rev, 106*(Pt A), 148-156. doi:10.1016/j.addr.2016.02.006

Valadi, H., Ekstrom, K., Bossios, A., Sjostrand, M., Lee, J. J., & Lotvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol, 9*(6), 654-659. doi:10.1038/ncb1596

Vega, F. M., & Ridley, A. J. (2008). Rho GTPases in cancer cell biology. *FEBS Lett, 582*(14), 2093-2101. doi:10.1016/j.febslet.2008.04.039

Vicovac, L., & Aplin, J. D. (1996). Epithelial-mesenchymal transition during trophoblast differentiation. *Acta Anat (Basel), 156*(3), 202-216.

Vihinen, P., Riikonen, T., Laine, A., & Heino, J. (1996). Integrin alpha 2 beta 1 in tumorigenic human osteosarcoma cell lines regulates cell adhesion, migration, and invasion by interaction with type I collagen. *Cell Growth Differ, 7*(4), 439-447.

Vordenbäumen, S., Braukmann, A., Altendorfer, I., Bleck, E., Jose, J., & Schneider, M. (2013). Human casein alpha s1 (CSN1S1) skews in vitro differentiation of monocytes towards macrophages. *BMC Immunol, 14*, 46. doi:10.1186/1471-2172-14-46

Vuoriluoto, K., Haugen, H., Kiviluoto, S., Mpindi, J. P., Nevo, J., Gjerdrum, C., Ivaska, J. (2011). Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer. *Oncogene, 30*(12), 1436-1448. doi:10.1038/onc.2010.509

Wehrle-Haller, B., & Imhof, B. A. (2003). Integrin-dependent pathologies. *J Pathol, 200*(4), 481-487. doi:10.1002/path.1399

Wolf, P. (1967). The nature and significance of platelet products in human plasma. *Br J Haematol, 13*(3), 269-288.

Wu, G., Dawson, E., Duong, A., Haw, R., & Stein, L. (2014). ReactomeFIViz: a Cytoscape app for pathway and network-based data analysis. *F1000Res, 3,* 146. doi:10.12688/f1000research.4431.2

Yamaguchi T, Koga M, Fujita Y, Kimoto E. (1982). Effects of pH on membrane fluidity of human erythrocytes. J Biochem, 91(4):1299-304.

Yang, J. S., Gad, H., Lee, S. Y., Mironov, A., Zhang, L., Beznoussenko, G. V., Hsu, V. W. (2008). A role for phosphatidic acid in COPI vesicle fission yields insights into Golgi maintenance. *Nat Cell Biol, 10*(10), 1146-1153. doi:10.1038/ncb1774

Yang, M., Chen, J., Su, F., Yu, B., Su, F., Lin, L., Song, E. (2011). Microvesicles secreted by macrophages shuttle invasion-potentiating microRNAs into breast cancer cells. *Mol Cancer, 10,* 117. doi:10.1186/1476-4598-10-117

Yilmaz, M., & Christofori, G. (2009). EMT, the cytoskeleton, and cancer cell invasion. *Cancer Metastasis Rev, 28*(1-2), 15-33. doi:10.1007/s10555-008-9169-0

Yuana, Y., Bertina, R. M., & Osanto, S. (2011). Pre-analytical and analytical issues in the analysis of blood microparticles. *Thromb Haemost, 105*(3), 396-408. doi:10.1160/th10-09-0595

Yuana, Y., Sturk, A., & Nieuwland, R. (2013). Extracellular vesicles in physiological and pathological conditions. *Blood Rev, 27*(1), 31-39. doi:10.1016/j.blre.2012.12.002

Zech, D., Rana, S., Buchler, M. W., & Zoller, M. (2012). Tumor-exosomes and leukocyte activation: an ambivalent crosstalk. *Cell Commun Signal, 10*(1), 37. doi:10.1186/1478-811x-10-37

Zeisberg, M., & Neilson, E. G. (2009). Biomarkers for epithelial-mesenchymal transitions. *J Clin Invest, 119*(6), 1429-1437. doi:10.1172/jci36183

Zhao, Y., Yan, Q., Long, X., Chen, X., & Wang, Y. (2008). Vimentin affects the mobility and invasiveness of prostate cancer cells. *Cell Biochem Funct, 26*(5), 571-577.

doi:10.1002/cbf.1478

Zitvogel, L., Regnault, A., Lozier, A., Wolfers, J., Flament, C., Tenza, D., Amigorena, S. (1998). Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med*, *4*(5), 594-600.