The Role of Microvesicles in EMT and Tumour Microenvironment

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Abbreviations

A V	Annexin AV
BAPTA-AM	1,2-*Bis*(2-aminophenoxy)ethane-*N*,*N*,*N*',*N*'-tetraacetic acid tetrakis.
BSA	Bovine serum albumin
Ca2+	Intracellular calcium
Crtl	Control
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Doc	Docetoxel (Taxotere®)
ECM	Extracellular matrix
ECS	Extracellular space
ELISA	Enzyme immunosorbent assay
ER	Endoplasmic reticulum
Exos	Exosomes
FACS	Fluorescent activated cell sorter
FGF-1	Fibroblast growth factor -1
FSC	Forward scatter
g	Standard gravity
H_2O	Water
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-12	Interleukin 12
K	Potassium
Li	Lithium
Mg	Magnesium
MPs	Microparticles
mRNA	messenger Ribonucleic acid
MVs	Microvesicles
Na	Sodium
PBB	Permeabilisation buffer
PBS	Phosphate buffer saline

PE	Phosphatidylethanolamine
PFA	Paraformaldehyde
Pi	Propidium iodide
PI3K	Phosphatidyl inositol-3-kinase
PL	Phospholipid
PS	Phosphatidylserine
RNA	Ribonucleic acid
RT	Room temperature
SD	Standard deviation
SSC	Side scatter
TAMs	Tumour Associated Macrophages
TFG-β1	Transforming growth factor β 1
v/v	Volume per volume
w/v	Weight per volume

ABSTRACT

Microvesicles are heterogeneous population of micro-particles released constitutively and upon induction from healthy and unhealthy cells. The role of cancer cell derived-MV in intercellular communication gains an intensive aria of research. The influence of leukaemia cell derived-MVs in this study was determined on normal prostate epithelial cell lines. PNT2 cells were treated with Jurkat cell derived-MVs lost epithelial characteristic (decreased epithelial marker E-cadherin) and gained mesenchymal phenotype (increased expression of mesenchymal marker Vimentin).

TGF-β and intracellular Ca^{2+} concentration were partially involved in Epithelial Mesenchymal Transition (EMT) process. PNT2 cells acquire mesenchymal characteristic produced high level of resistances against apoptotic signals after exposed to serum starvation and anti-cancer drug docetaxel, produce excessive level of MMP-9 and 2/3 of total TPNT2 cell population were arrested in the G2/M phase of the cell cycle, and halts cell proliferation.

The influence of carcinoma cell derived-MVs on tumour microenvironment was examined through use of Non-small lung cancer cells (A549) derived-MVs on primary lung fibroblasts (MRC5). MRC5 cells were treated with A549 cell derived-MV produced significantly high level of myofibroblasts marker alpha-smooth muscle actin (α-SMA) cytoskeleton protein and FGF. MVs were isolated from the myofibroblasts were enriched with α-SMA protein. Primary fibroblasts were treated with MVs released myofibroblasts expressed high level of α-MSA protein. Elements present in the CGM cause

aggregation of cancer cell MVs and significantly reduced the effects of MVs on the target cells.

Original publications

Conference abstracts

2nd International meeting of the International Society for Extracellular Vesicles, Boston

- **Haidery, A. and Inal, J.M. (2013)** Microvesicles and epithelial mesenchymal transition in the development of cancer. *Journal of Extracellular Vesicles* Vol 2 Abstract 141

Microvesiculation and Disease 13th-14th September, 2012, London

- **Haidery, A. and Inal, J.M. (2012)** Microvesicles (MVs) and epithelial mesenchymal transition in the development of cancer (http://www.biochemistry.org/tabid/379/MeetingNo/SA133/view/Conference/default.aspx)

Publications

- **Kosgodage, U. Lourenco, C., Callum, S., Haidery, A., Inal, J.M. (2015)** Exosomes and Microvesicles in cancer immunotherapy. Review in preparation for *Journal of Extracellular Vesicles*.
- **Haidery, A. and Inal, J.M. (2015)** Microvesicles promote Epithelial Mesenchymal Transition of prostate, PNT2 cells. In preparation for imminent submission to *Biochem. Biophys. Res. Commun.*
- **Haidery, A. and Inal, J.M. (2015)** Microvesicular calcium promotes EMT in prostate bringing about a transformed phenotype. In preparation.
- **Haidery, A. and Inal, J.M. (2015)** A549Non-Small-Cell Lung Cancer cell-derived microvesicles induce MRC5, lung fibroblast-to-myofibroblast differentiation and myofibroblast-derived microvesicles induce further myofibroblastic differentiation. In preparation.

1. Introduction

1. Introduction

1.1 How MVs are formed

The mammalian body is made up of a complex collection of cells, whose interactions are important in term of homeostasis (1). Our understanding of the mechanism of cellular communication supports the discovery of microvesicles (MVs) as a pathway for the exchange of information between cells (1,2). MVs are cell membrane fragments released constitutively and upon activation from almost all mammalian cell types into their extracellular environment and into body fluids (1-3). MVs were first described by Chargaff and West in 1946 as a perceptible factor in plasma platelets with the capacity to produce thrombin (4). In 1967 they were described by Peter Wolf as platelet dust. These vesicles are composed of phospholipid bilayer, and are formed by regulated release through budding of the plasma membrane (5). Cells in their normal physiological states generally shed lower numbers of MVs than cancer cells. Cells under stressed condition, such as in malignancies and during hypoxia, have been shown to secret not only considerably higher numbers of MVs, but also MVs that are more heterogeneous in size compared to their normal counterparts (6). The release of MVs is induced by cell surface receptor activation, increased intracellular Ca^{2+} and apoptosis. Based on electron microscopy their sizes vary from 0.1 to 1 µm in diameter. Their contents reflect the phenotype and physiological state of the parent cells and allow the identification of the origin of the MV. A biochemical understanding in terms of the specific lipid composition is essential in order to understanding the mechanism of MV release from the parent cells. Aminophospholipids (Phosphatidylserine and

Phosphatidylethanolamine) are specifically localised in the inner leaflet of the lipid bilayer whereas Phosphatidylcoline and Sphingomyeline are present in the outer leaflet. It is generally believed that, there are three major proteins which play as important role in the regulation of lipid distribution in the plasma membrane namelyflippase, floppase and scramblase.

1.1.1 Flippase

Flippase is responsible for the translocation of aminophospholipid (PS and PE) from the outer layer of the plasma membrane to the inner monolayer of all mammalian cells. This lipid transporter protein can be ATP dependent or ATP independent and it can also be selective or non-selective. The newly synthesized PS and PE are however, moved to the cytoplasmic face of the membrane by an ATP dependent flippase protein (7).

1.1.2 Floppase

Floppase is another lipid transporter protein responsible for the mobilisation of substrates such as lipid (Phosphatidylcoline and Sphingomyeline) from the cytoplasmic to extracellular face of the plasma **(Fig.1)**. This class of transporter also consumes ATP to pump lipid outwards against a concentration gradient (8). Cytoplasmic Ca^{2}+ concentration is partially involved in floppase activity and its activation concomitantly inhibits the function of flippase.

This lipid transporter is responsible for the degradation of transbilayer phospholipid gradients. In the lipid bilayer, scramblase transports lipids bidirectionally (7). There are three types of scramblase activity in this lipid transporter and two of them participate in a lipid dissipation gradient in membrane bilayer. The third scramblase activity depends on Ca^{2+} stimulation, and once activated results in PS being scrambled from the inner to outer leaflet of the lipid bilayer in eukaryotic cells (8).

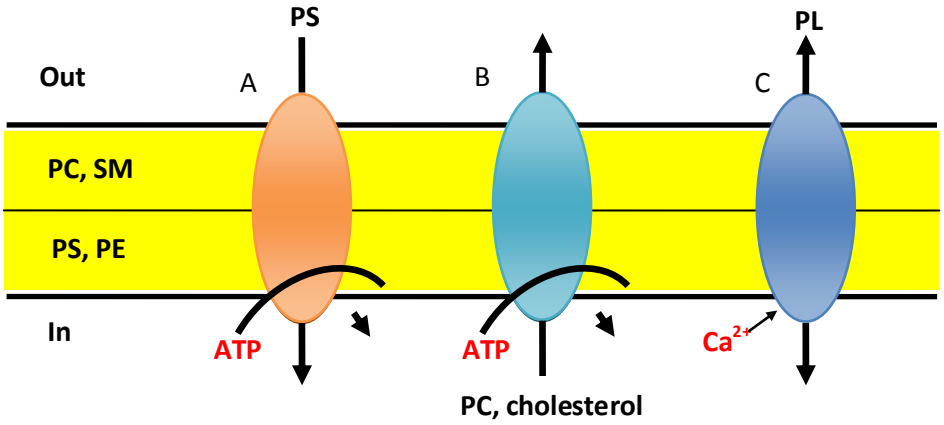

Figure 1 Flippase, Floppase and sramblase maintain lipid asymmetry in plasma membrane.

Fig.1 shows the action of lipid transporter protein in mammalian plasma cell membrane. PC and SM are restricted to the outer monolayer. PS and PE are localised on the cytoplasmic face of the plasma membrane. (A) The distribution of PS is maintained to the inner membrane by an inward directing action of the flippase protein. (B) PC is localised to the outer membrane which organised by an outward actin floppase. (C) Non-specific scramblase action in activated cells is caused by Ca^{2+} induction that leads to randomised PL distribution across the cell membrane.

Increased cytosolic Ca^{2+} concentration causes the collapse of membrane asymmetry by the action of floppase and scramblase which consequently down regulates the action of flippase. Translocation of PS from inner to outer biogenic membrane is one of the prominent changes that take place in the distribution of lipid in the plasma membrane of activated cells which is followed by the release of MVs. Therefore, the budding of these submicron particles from their parent cell membrane is due to the remodelling of the lipid bilayer and loss of phospholipid asymmetry **(Fig.2)** (9). After secretion, MVs can move within the extracellular environment and either be taken up by neighbouring cells or travel through body fluids for cargo delivery into recipient cells at distant sites **(Fig.2)** (10). MVs can be detected in blood (serum and plasma), urine, milk, sweat, saliva, ascites fluid, and cerebral spinal fluid (CSF) (11).

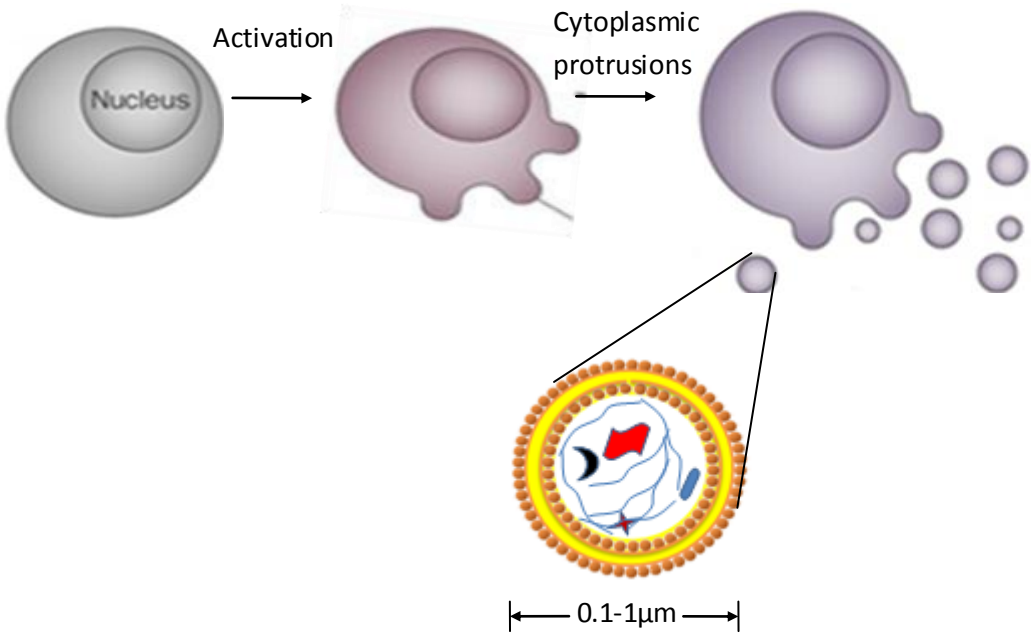

Figure 2 MVs bud off the plasma membrane into the extracellular environment.

This process captures the cytosolic contents of the parent cells in the newly formed lumen and plasma membrane receptors in the surrounding membrane. The membrane bilayer of newly formed MVs has the same specific lipid composition as their parent cells. This well structured membrane is characterised by the transverse distribution of lipids and proteins between the inner and outer leaflets. Apart from this transverse distribution, a lateral organisation of so-called rafts also participates in the characterisation of the MV membrane. In the case of MV release, a general redistribution of lipid organisation takes place which leads to the raft structuration, PS externalization and MV release.

1.2 Heterogeneity of MVs

Human cells produce a heterogeneous population of MVs which differs from apoptotic bodies and exosomes in terms of size and morphology. MVs are larger than exosomes (50 to100 nm in diameter), their sizes ranging from 0.1 to1µm in diameter (12). There is a great deal of evidence in the literature suggesting that they are ubiquitously released mainly by cancer cells and other cells upon activation. The amounts of MVs are released by tumour cells increase considerably in aggressive types of cancer cells (13). While the number and composition of MVs released from normal cells are significantly different from cancer cells, it is still unclear whether cancer cell-derived MVs are similar in terms of structure, composition and function. In terms of size profile of MVs, researchers have used size bead-based FACS analysis as the primary means of MV characterization (14). However, isolation of MVs may easily result in an overlap at the upper end of the size range with apoptotic bodies and at the lower end with exosomes. Recent advances in capture and sorting capabilities may overcome these challenges (15). Another way to design more precise capture strategies is to establish a molecular profile of shed MV populations (16).

1.3 Size determination method of MVs

1.3.1 Pre-analytical argument

There are concerns about the pre-analytical changes of MVs in the diagnostic field. These changes are in terms of numbers which increase in response to shear stress and storage (17). Freeze-thaw cycles of MVs cause a substantial increase in the count of Annexin V labelled MVs by standard FACs analysis (18). It has also been shown that MV counts are altered significantly with storage time and by temperature. The type of buffer used to dilute the MVs can also change the MV count which shows the particles to be highly sensitive to environmental factors (19). MV analysis in terms of numbers, using standard FACS, therefore needs be considered in the routine diagnostic approach.

1.3.2 Determination of MVs by Transmission Electron Microscopy

The gold standard for size determination of MVs is transmission electron microscopy (20). However in this method, prior to analysis, the MVs need to be concentrated using high speed centrifugation, dehydration and fixation, leading to the possibility that these processes may in fact change the size and morphology of MVs (21). Advanced techniques such as cryo-EM demonstrated that the cup-shaped feature of exosomes is an artefact, due to the fixation and dehydration of MVs for EM. Transmission electron microscopy however, is the only technique so far that can simultaneously examine the size and structure of MVs (20).

1.3.3 Flow Cytometric detection of MVs

FACS is a standard method for MV detection and many researchers report that MV counts closely correlate with varieties of physiological and pathological conditions (22). A standard FACS can detect MVs above the size of 0.2 µm. This could be a major concern in terms of MV counts because smaller MVs (<0.2µm) cannot be analyzed and characterized directly by this method. Based on FACS analysis, Annexin V is used as a common marker for MVs, but there is evidence for example suggesting that more than 80 % of platelet-derived MVs are negative for Annexin V MVs. In fact, Annexin V requires around 1.5 mM Ca^{2+} to bind the PS contents of MVs. Therefore If PBS is used as a diluent for MVs, it is not suitable for Annexin V staining of MVs, because it can interfere with the binding capacity of Annexin V, Ca^{2+} precipitating with phosphate (7).

1.4 Structure of MVs

For a long period It has been known that MVs are released from a wide range of mammalian cell types such as epithelial cells, endothelial, neuronal and blood cells (23). These vesicles have the same phospholipid bilayer leaflet structures as the donor cells but harbour a variety of glycol phospholipids, miRNA and mRNA and their composition depends on the cell types from which they are derived. The major challenge is to establish a specific method to isolate different MVs according to their composition and origin (24). The content of MVs originates from the cytoplasm, cytoskeleton, proteasome, plasma membrane, mitochondria and endoplasmic reticulum

(25). Furthermore, the possibility of MVs carrying transcription factors could enable them to serve as genotypic biomarkers to the target cells (26).

1. 5 MVs and horizontal transfer of bioactive molecules

Importantly, MV release is not a random process, such as degradation of plasma membranes undergoing necrosis. In recent years, it has been widely accepted that miRNAs act as a major player in information transfer between cells (27). These RNA molecules in fact are very unstable inside the blood circulation. Therefore, a secure systemic delivery is required to prevent their degradation before reaching target cells. All bioactive molecules are systematically transferred from donor to recipient cells through MVs and thereby protected from degradation (28) **(Fig.3)** and a key function of MVs is the transportation of RNA in cancer invasion and metastasis which takes place in almost all types of cancer including colorectal adenocarcinoma, pancreatic adenocarcinoma, lung carcinoma, and glioblastoma (29).

1.6 Proteases in MVs

Cancer cells of epithelial origin specifically breast carcinoma and ovarian cancer secrete MVs into the microenvironment carrying MMP9 and MMP2 (30).These MVs interact with extracellular matrix causing matrix degradation. There are some interacting factors in fact playing a role between released MVs and recipient matrix such as β1 integrins. Thus, cancer cells release MVs as a mechanism to facilitate proteolysis at distal

sites creating paths for local invasion and metastasis (31). It could be suggested therefore that MV shedding selectively occurs at specific sites on the plasma membrane and is designed to release selected cellular components and matrix degradation.

1.7 MV Interaction with target cells

The role of MVs as intercellular communicative vectors in maintaining normal body homeostasis is principally due to their ability to release and to deliver their cargo to recipient cells. The cellular and molecular basis of MV targeting cells is still not fully understood but mechanisms are beginning to be elucidated (31). The specificity of these target cells for binding of MVs is likely to be determined by adhesion molecules. Therefore, interactions of MVs with the target cells are established through specific molecules such as surface receptors and adhesion molecules, for example integrins which are present at the surface of MVs (32,33). After binding to the recipient cells, MVs either fuse with the plasma membrane or are internalized through specific endocytic pathways. In some cases MVs are stably associated with the plasma membrane or remain dissociated from the target cells (17). There are some factors which can probably interfere or even block this interaction of MVs with the target cells and such interference can be due to the translocation of PS to the outer leaflet of MVs during their biogenesis.

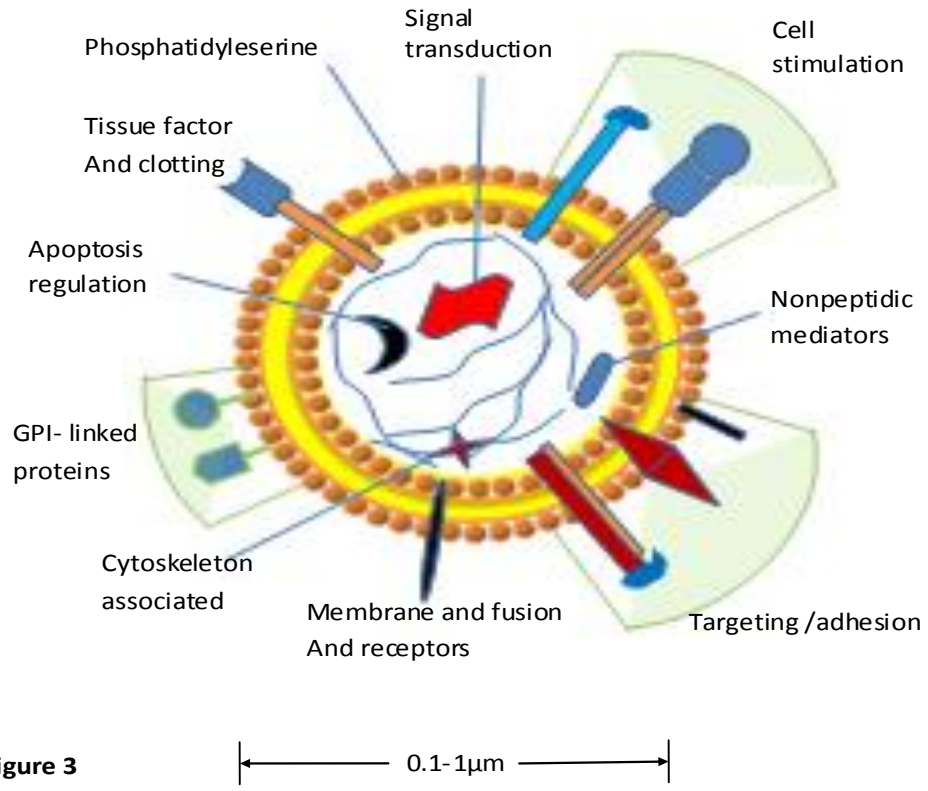

Figure 3

Figure 3 Types of bioactive molecules carried by MVs

Mammalian cells are exposed to a variety of stimulations that lead to the release of MVs. These MVs highjack phospholipid membrane constituents and functional proteins from the cytoplasm of the parent cells and these bioactive molecules have significant roles in intercellular communication. Furthermore, under normal conditions they participate in homeostasis or in the case of pathology by carrying pathogenic constituents, these MVs therefore cause disease progression. The nature of MV cargo depends on the cell type, and also the state of cells.

1.8 MVs as diagnostic tools

MVs influence the behaviour of target cells in a variety of ways and this is due to the variety of cargoes they deliver to the target cells (34). This ubiquitous formation of MVs enables the clinician to establish their diagnostic value in various pathological conditions. Much research is being invested in developing MVs as diagnostic tools. This is due to the accessibility of MVs through a variety of biological fluids such as blood, urine, ascites and saliva (35). MV levels are elevated in the plasma of individuals suffering from acute and chronic vascular diseases such as acute coronary syndrome, hypertension renal failure and pulmonary diseases (36), and many studies have implicated MVs as biomarkers for cancer diagnosis, demonstrating that the level of MVs in blood positively correlates with cancer prognosis. Patients suffering from glioblastoma for example, have high numbers of MVs in their blood which carry significant levels of mRNA, thus making them useful markers for the diagnosis of cancer (37).

1.8.1 MVs as therapeutic delivery vehicles

MVs have the capacity to modulate the phenotype of target cells. This effect is due to the diverse RNA species they carry and transfer into target cells (21). Recent studies have shown that stem cell-derived MVs can mimic stem cell function, therefore playing a relevant role as mediators of stem cell induced regeneration of injured cells and tissues (38). MVs can securely transfer miRNA into the target cells which cause epigenetic

changes in the recipient cells. Furthermore, the mRNA content of MVs may be delivered to recipient cells and translate to a functional protein in the target cells. Endothelial progenitor cells derived MVs for example are able to establish new vascularisation and muscle regeneration in hind limb ischaemia (38,39). However, the biological effects of MVs on the target cells can be altered by the functional state of the recipient cells. In fact, the effect of miRNA delivery into the target cells will also be dependent on which pathways are activated in the case of normal or inflammatory states and malignancy (38-40).

1.9 Normal function of MVs

Overall, there is not enough information about the functions of MVs in normal development. For example, the number of MVs detected in the peripheral circulation of women during pregnancy is higher than non-pregnant woman but the actual function of MVs is still not fully understood during pregnancy. However, some functional proteins such as β-Catenin are detected in the isolated MVs. It is generally believed that this protein plays central roles in tissue and organ development (41). Other functional proteins that can be detected in MVs during pregnancy are sonic hedgehog. This protein has critical functions in embryogenesis including nerve system development and the gastrointestinal system (42). MVs which are present in bronchoalveolar fluid may increase the secretion of proinflammatory cytokines from the epithelial cells of airways. The presence of MVs in the upper respiratory tract can function as tolerized molecules when exposed to

some kind of allergens (43). Within the neuronal system, MVs are secreted from neurons, oligodendroglial and microglial cells which can participate in myelin formation, neurite outgrowth and neuronal survival (44).

1.10 Pathological functions of MV

Levels of MVs in body fluid are raised in a range of pathological conditions (45). Patients suffering from thrombotic diseases such as cardiovascular and cerebrovascular diseases, for example, have high numbers of platelet derived MVs in their peripheral blood (46). It is not entirely clear how MVs can contribute to the pathogenesis of these diseases, but some previous work supports the idea of MV participation in the activation of the coagulation cascade through the presence of phosphatidyl serine. Patients suffering from chronic inflammation such as rheumatoid arthritis show high numbers of MVs in their body fluids. In this case the presence of MVs in body fluids confirms the function of MVs as chemoattractant molecules and inflammatory mediators (47). Cancer cells secrete an increased number of MVs which can be detected in a variety of human cancers including prostate and ovarian cancers. The role of MVs in human malignancies is thought to involve the progression of the diseases and metastasis of cancer cells to distal sites (48).

1.10.1 MV participation in inflammation

Inflammation is collectively established as a result of the interaction between various cell types. This communication takes place either directly between cells or through the mediation of cytokines and other soluble factors. MVs are released by those cells that actively participate in the inflammatory process (49). The role of such inflammatory MVs is generally dependent on the stages of inflammation. In the early stage, cells in the immune system especially neutrophils shed high numbers of MVs which cause activation of macrophages. Activation of macrophages in this stage of inflammation is accompanied by the release of anti-inflammatory mediators such as TGF β1 and IL 10 (50). At the later stage of inflammation, MVs released from the immune cells strengthen the inflammatory response. These functions of MVs are fulfilled by the transfer of chemokine receptors such as chemokine receptor4 and chemokine receptor 5 which stimulate the release of IL6 and monocyte chemotactic protein (51).

1.10.2 Immunomodulatory functions of MVs

Cancer cells release MVs to promote cancer progression, as shedding MVs suppress anti-tumour responses to significant levels. This function of MVs is caused by modulation in signalling pathways which are responsible for T-cell proliferation and activation (52). These suppressive effects of MVs in the tumour microenvironment are due to the presence of membrane-bound transforming growth factor beta, CD 73, Fas ligand and galectins. MVs derived from mammary tumours carrying interleukin inhibit the

differentiation of bone marrow precursors in dendritic cells (53). Cancer cell derived MVs are suggested to suppress the response of immune cells by preventing leukocyte adhesion to tumour necrosis factor alpha-(TNF-α) activated endothelial cells. This immune suppressive function of MVs is due the membrane bound intra cellular adhesion molecules -1 (ICAM-1) (54).

1.10.3 MVs and tumour growth

The number of MVs derived from cancer patients is significantly higher than that of healthy individuals (55); this elevation correlates with poor prognosis. However, not all MVs found in body fluids are secreted by cancer cells, the vast majority of these MVs being shed by activated platelets, lymphocytes, macrophages and erythrocytes (56). Recently, a large scale proteomic analysis was carried out to determine the differences between protein content of MVs from healthy and neoplastic patients (57). The majority of proteins in tumour derived MVs are known to be linked with cancer development and cancer progression, and these proteins include oncoproteins, oncogenes and chemokine receptors as well as soluble factors (58). Tumour-derived MVs participate in cancer growth by transferring oncogenic molecules to other cells in the tumour microenvironment at specific distal sites. Some caution is required when interpreting the results, however, because MV purification techniques and contamination of samples will change the consistency and reproducibility of data (59).

1.10.4 MVs and angiogenesis

MVs shedding from malignant cancer cells especially from glioblastomas carry high concentrations of angiogenic factors such as vascular endothelial growth factor, Interleukin8, interleukin 6 and tissue inhibitor of matrix metalloproteinase-1 (60). Another angiogenic cascade protein found recently in cancer derived MVs is delta like 4 Zigland (D 114) (61). This protein can block the Notch signalling pathway thereby increasing vessel density in the tumour microenvironment. Other components of MVs participate in angiogenesis and include sphingomyeline and matrix metalloprotease inducers (62).

1.10.5 MVs and phenotypic changes of target cells

The capacity of individual cells to acquire phenotypical changes has been clearly demonstrated by exchanging genetic material between donor and recipient cells (63). Mammalian cells use MVs as a mechanism to deliver the genetic composition safely and securely to the recipient cells. When MVs are taken up by recipient cells, they can profoundly change their biology. These changes can be either transitional or permanent. These phenotypic changes are due to the effect of the contents of MVs on the target cells (64). Microvesicle cargo includes membrane proteins, cytosolic proteins, nucleic acids and membrane lipids that are delivered into the recipient cells (57), which interfere with the physiological function of the recipient cells.

1.10.5.1 Epithelial to Mesenchymal Transition

There is evidence in the literature suggesting the role of cancer cell derived MVs in bringing about phenotypic changes of recipient cells (65). The epithelial to mesenchymal transition (EMT) is an embryonic cell transformation that occurs in almost all normal mammalian tissues (66). In this process epithelial cells lose their polarities by down regulating the expression of cell adhesion molecules such as E-cadherin (67). The decrease in the epithelial gene expression is accompanied by increase in mesenchymal genes such as Vimentin and fibronectin (68). EMT is a reversible biological process that allows epithelial cells which normally interact with their basement membrane by their basal surface, to undergo multiple biochemical changes that enable them to acquire mesenchymal characteristics (69). Several rounds of EMT and mesenchymal to epithelial transition (MET) are required for primary cells in order to become fully differentiated, specialized cell types. It is one of the most important changes that happens during development and disease progression (70). Epithelial to mesenchymal transition is triggered by a variety of extracellular signals. Generally, EMT is involved in major physiological and pathological changes including morphogenesis, fibrosis and tumour progression and metastasis (71).

1.10.6 Types of Epithelial to Mesenchymal Transition

1.10.6.1 Morphogenetic Epithelial to Mesenchymal Transition (Type 1)

Morphogenetic EMT is linked to embryogenesis when the primary mesenchymal tissues are formed from the upper epiblast epithelium which results in a three layered blastocyst, (ectoderm, mesoderm, and endoderm) (72). Epithelial cells undergoing mensenchymal changes result in the formation of the ectoderm, mesoderm and endoderm from the invaginating primary streak (73). Cells in the primary streak are transformed into a mesenchymal phenotype under the influence of the Wnt signalling pathway (74). Developmental epithelial to mesenchymal changes also take place during neurulation, started in the neural plate. This phenotypic change facilitates the neural tube formation and finally forms the spine and brain (75). Neural crest cells also acquire a mesenchymal characteristic that facilitates their migration and dissemination throughout the embryo. This transformation finally participates in the generation of tissues including the adrenal medulla and the peripheral nervous system (76).

1.10.6.2 Fibrotic Epithelial to Mesenchymal Transition (Type 2)

Fibrotic EMT occurs to maintain tissue homeostasis (77,78). This phenotypic change takes place during tissue injury and results in wound healing and tissue remodelling (77). The difference between morphogenetic and fibrotic epithelial to mesenchymal transition at the molecular level is that, the fibrotic type is triggered and governed by inflammatory reactions (79). There is much evidence to suggest that EMT takes place in

progressive kidney and lung disease, as a result of which functional epithelial cells in the kidneys are replaced by the deposition of dysfunctional collagens and other matrix components (80).

1.10.6.3 Epithelial to Mesenchymal Transition in cancer (Type 3)

This type of epithelial to mesenchymal transition is distinguished from its type 1 and type 2 counterparts in the cellular context, because in almost all cases it occurs in oncogenically transformed cells (81). More than 80% of tumours are carcinomas of epithelial origin and one of the hallmarks of these malignant tumours is loss of epithelial morphology and gain of mesenchymal characteristics (82). Cancer cells acquire this phenotypic change in order to infiltrate into surrounding tissue and ultimately metastasize to distant sites (83). It is very difficult to observe EMT in malignant tumours because tumours are made up of a complex collection of tumour cells and stroma. In cell culture models, however, this phenotypic change can be accurately studied. ‘’IF’’ as part of EMT tumour cells increase the production of proteases, these enzymes are able to degrade the surrounding basement membrane (84). However, the most important question in terms of genetic alteration and EMT, whether the genotypic changes in cancer cells are due to the acquisition of phenotypic change of cancer or as a result of an independent phenomenon has not yet been answered. The migration of cancer cells takes place through the basement membranes of mesenchymal cells into the adjacent tissue which normally functions as a barrier between epithelial cell tissue and stroma (85). Metastatic cells gain the migratory capacity at the expense of their

proliferative potential (86). Therefore, a reverse conversion of mesenchymal epithelial transition is required for cancer cells in order for them to become fully functional. Mesenchymal to epithelial transition (MET) therefore, enables the cancer cells to produce macro-metastasic cells similar to the primary tumour at a distal site (87). This phenotypic change in cancer cells in fact, defines a key feature of malignant cells with successful metastatic behaviour (88).

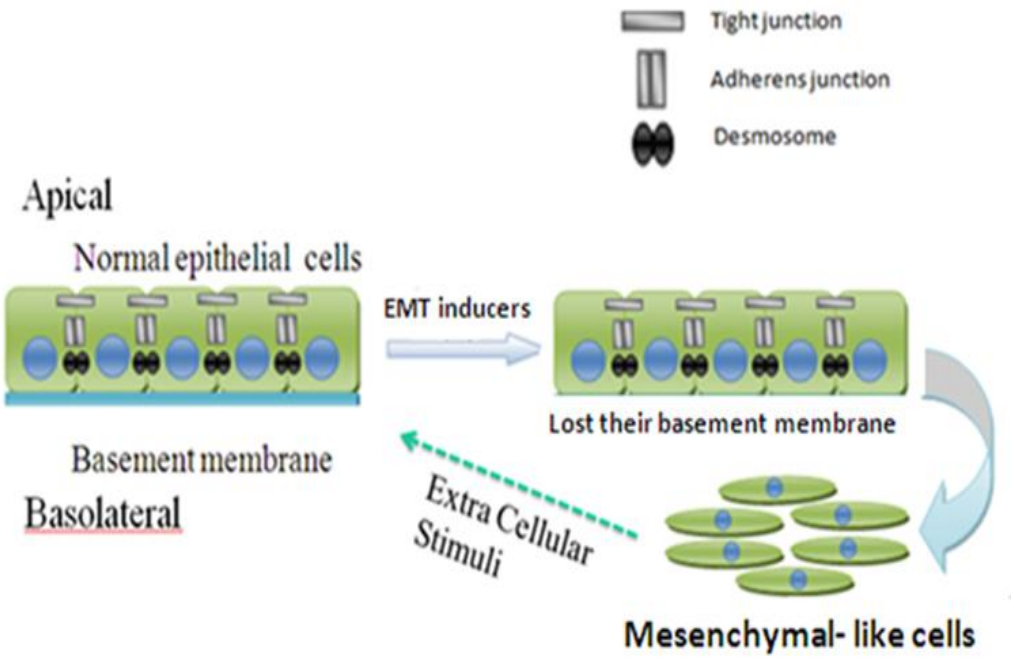

Figure 4 Epithelial Mesenchymal Transition is reversible process.

EMT is a biological process that allows epithelial cells that are normally attached to their basement membrane via their basal surface, to undergo multiple biochemical changes that enable them to acquire mesenchymal characteristics. This phenotypic change takes place in epithelial tissue during wound healing and malignancies. EMT takes place in carcinomas and leads to the progression of disease. Cancer cells use this physiological process for colonisation of local and distant organs. This phenotypic change is believed to be reversible, therefore, cancer cells using EMT to survive in blood circulation and lymphatic system. Malignant cells that have undergone EMT acquire a reversible process, so-called MET in the secondary organ and establish secondary tumours. However, it is still not clear whether transformation of a benign tumour into a malignant form can actually represent EMT, and if so how important it is in the process of cancer progression.

1.10.7 Molecular mechanism of EMT

The molecular processes involved in EMT include activation of transcription factors, expression of specific cell surface proteins, expression and reorganization of cytoskeletal proteins, degrading enzymes and changes in the expression of specific microRNAs. Assays such as loss of cell adhesion and cytoskeletal rearrangement are in fact used to assess the progression of EMT in highly metastatic cancer patients.

1.10.7.1 Adherens Junction and EMT

The major concept of EMT is the dissolution of adherens junctions that are derived from the interaction of E-cadherin between the epithelial cells (89). In principle, epithelial cells do not have the ability to dissociate from epithelial entities and escape to other organs or the circulatory system (90). This is due to the expression of junctional protein complexes including tight junctions, adherens junctions, desmosomes and gap junctions in epithelial cell lines (91). At the initial stage of EMT the reorganisation of the adherent junction and down regulation of E-cadherin lead to the loss of epithelial polarity and destroy the epithelial cell sheet which functions as a barrier between the epithelial and the extracellular environment (92).

1.10.7.2 Integrin signalling and EMT

Integrins partially participate in cell communication within the microenvironment (93). The role of integrin in cell-to-cell and cell-to-matrix interactions is defined in term of cell adhesions, cell migration, cell proliferation, invasion and cell survival (94): the level of integrin increases significantly during EMT (95) and integrins participate in EMT by creating traction force that activates transforming growth factor beta (TGF-β) (96).

1.10.7.3 Intermediate filament and EMT

Intermediate filaments are important components of the cytoskeletal compartment of almost all mammalian cells (97). They are found in the cell cytoplasm and nucleus of cells and their functions include giving mechanical support to cells, tissues and organs. During EMT the intermediate filament network of epithelial cells is reorganised (98). This reorganisation causes disruption to the desmosomal structure which consequently leads to the release of cells from well organised epithelial sheets. Another strong function of intermediate filament subunits observed in almost all EMT processes is the constant suppression of cytokeratin expression (99). This is the major desmosome linked intermediate filament protein in cells within the epithelial cells. In the last ten years Vimentin has been considered as an epithelial mesenchymal marker (100). Vimentin expression in EMT is characteristically unregulated, and it has been demonstrated that knocking down this gene results in down regulation of EMT events in mammary epithelial cells (101).

1.10.7.3.1 Vimentin and prostate cancer

The expression pattern of Vimentin in normal and malignant cells is of great value for cancer diagnosis and cancer progression. Vimentin expression is mainly detectable in the metastatic form of prostate cancer (102). Down regulation of this intermediate filament in the metastatic form of prostate cancer significantly decreases motility and invasiveness (103). Another independent study demonstrated that this protein is over expressed in highly aggressive types of prostate cancer (PC3-M; PC3-1E8). Over expression of Vimentin by and large is accompanied with the down regulation of the E-cadherin/ β-catenin complex (104).

1.10.7.3.2 E-cadherin and prostate cancer

Epithelial tissues are most abundant in mammalian organs and more than 90% of malignancies arise from carcinomas of epithelial origin. Malignancy is defined by the ability of cancer cells to overcome cell-cell adhesion and invade surrounding tissues, and E-cadherin plays a significant role in the maintenance of intercellular adhesion and cell polarity. In almost all types of epithelial cancer, the expression of this junctional protein is down regulated (105). This is a clear indication of the tumour suppressor effect of E-cadherin in carcinomas of epithelial tissue. The mechanism of this inhibitory effect is believed to come from blockage of the EMT process. However, a great deal of study is required in this field to identify the molecular mechanism by which E-cadherin can down regulate tumorigenesis and cancer invasion and metastasis.

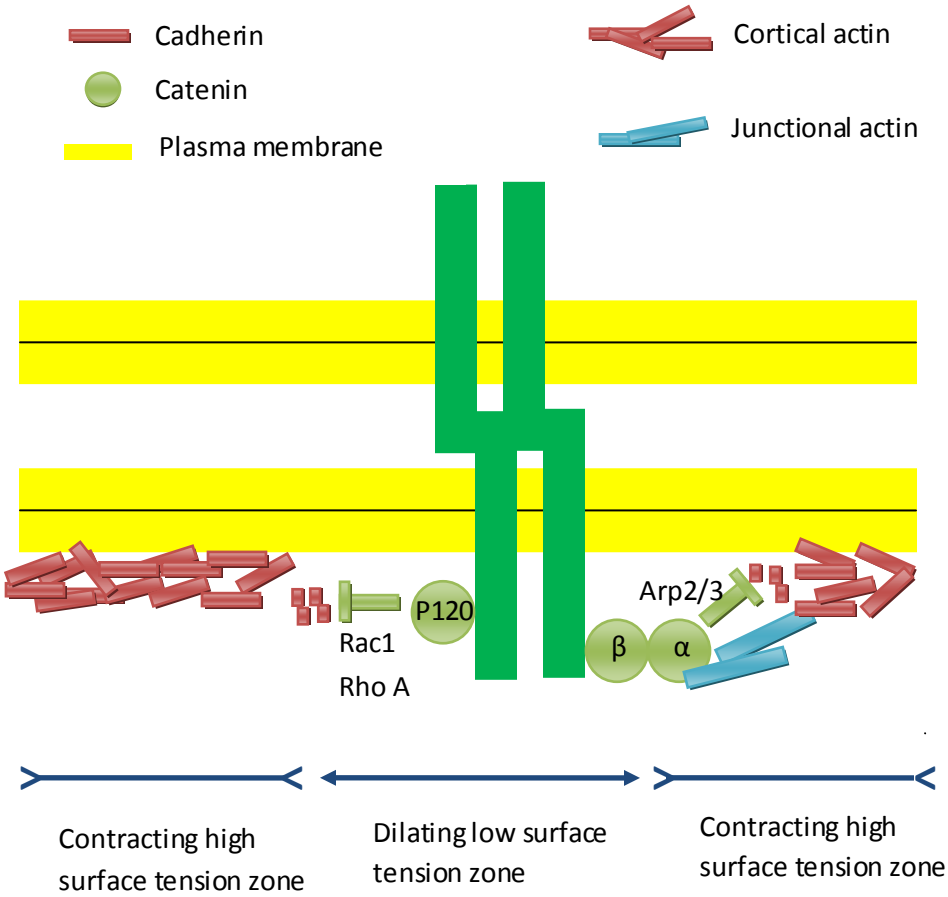

Figure 5 Adhesion signalling in epithelial cells.

The signalling pathway is formed by the action of P120 and catenin activating Rac 1. The activation of Rac 1 is accompanied by the inhibition of Rho A. Alpha catenin blocks the function of Arp2/3 in actin polymerization. When the cadherin signalling pathway becomes activated, it disrupts the acto-myosin protein arrangement and decreases the cell-cell interfacial tension thus widening the surface contact. The mechanical tension results in the adhesion complex breaking away from the cytoplasmic side. However, it is not entirely clear yet, whether dissociation takes place inside the cells between α-actin and β-actin. In the case of dissociation of these cytoskeleton proteins, maintaining the β-actin and α-actin interaction can be achieved by the establishment of chemical signalling from P120-catenin.

1.10.7.4 Microenvironment signals an EMT

EMT can be induced by a variety of extracellular signals both in an autocrine or paracrine way including extracellular matrix (collagen and hyaluronic acid) and secreted soluble factors such as transforming growth factor beta (TGF-β), Wnt, Hedgehog, epidermal growth factor (EGF) and hepatocyte growth factor (HGF) (106). The most critical signalling pathways participating in EMT are Wnt, TGF-β, EGF, hedgehog and Notch (107). These signalling pathways in fact, reorganise a set of genes and protein networks that are needed for the establishment of the mesenchymal cell type (108), their activation causing disassembly of epithelial architecture such as cell polarity and cellular junctions (109).

1.10.7.4.1 Cell polarity and EMT

Epithelial cell polarity is governed by a group of proteins known as partition defective proteins which include PAR3, PAR6 aPKC and PAR1 (110). Partition protein 3, 6 and protein kinase C, are localized predominantly in the apical junction and plasma membrane of epithelial cells (111). Partition defective protein 1 presents at the basolateral membrane of epithelial cells (111). These proteins establish and maintain the apical junction of epithelial cells. Epithelial cells undergoing EMT lose their top–bottom polarity leading to a more fibroblastic phenotype (112). The interaction of these two complexes in the apical and basolateral junctions leads to the formation of cellular asymmetry in epithelial cells (113).

Down regulation of partition defective protein 3 can cause TGF-β to disrupt the apico-basal polarity and consequently induce EMT in the target cells (114).

1.10.7.4.2 Transforming growth factor-β and EMT

Transforming growth factor beta is the major inducer of EMT in epithelial cells (115). This cytokine utilizes two signalling pathways, gene expression-dependent and gene expression-independent to induce EMT on target epithelial cells (116). TGF-β causes EMT not only during organ development but also induces EMT during inflammation and tumour progression (117). Knocking down of Smad 3 expression blocks TGF-β-induced EMT in primary epithelial cells (118). In the case of malignancy, it has a biphasic function in different stages of tumour development. At the early stage of cancer progression it inhibits tumorigenesis by induction of apoptosis and cell cycle arrest of target cells (119). At the late stage of disease, it acts as a positive modulator of tumour progression (120), the stimulatory function of TGF-β on cancer progression and metastasis being due to the induction of EMT in recipient cells (121).

1.10.7.5 Gene expression- dependent mechanism

Amongst the typical features of EMT are the loss of epithelial markers such as E-cadherin, Cytokeratin, Ocludin and desmoplakin and gain of mesenchymal markers such Vimentin, N-cadherin and fibronectin (122). Among these markers, loss of E-cadherin molecules is considered the

hallmark of EMT (123). A number of transcriptional factors are involved in this process. Transcription factors participating in E-cadherin repression during EMT include *Snail, ZEB*, and basic helix-loop-helix factors such *Twist, KLF8* and *Fox C2* (124). These transcriptions factor down regulate the expression of E-cadherin proteins by binding to the E box at the *CDH1* promoter region (125). Slug is another transcription factor that is involved in the down regulation of desmoplakin and plakoglobin expression. This down regulation in turns disassembles the junctional complex which is a specialized protein structure for cell-to-cell adhesion, the so-called desmosomes component of epithelial cells. This series of events causes defects in the adjoining epithelial cells and encourages the cancer cells to detach from primary tumour and make their way to the circulatory system.

1.10.7.6 Gene expression-independent mechanism

In the gene expression-independent mechanism, the type II TGF-β receptor becomes activated as a result of ligand receptor interaction (126). After it's activation, it interacts with a component of the tight junction, occluding, which leads to the phosporylation of the Par6 protein (127). This type of interaction and phosphorylation recruits Smurf1 that leads to degradation of RhoA in a ubiquitin-dependent manner (128). RhoA is a member of the GTPase family responsible for the maintenance of the apico-basal polarity and junctional stability. Therefore, the degradation of RhoA by TGF-β in fact, causes loss of epithelial cell polarity which is an important factor in the EMT process (104).

1.10.7.7 Wnt/β-catinin signalling and EMT

The β-catenin pathway is heavily involved in EMT cell remodelling (129). In the presence of Wnt ligands, Wnt binds to the receptor Frizzled and transmembrane low density lipoprotein receptors to inactive glycogen synthesis kinase-3 in the ‘’destruction complex’’ (130). This causes stabilization of β-catenin in the cytoplasm and then it’s translocation into the nucleus. Accumulation of β-catenin in the nucleus follows and it’s binding to the enhancer binding factor leads to the transcription of the target genes (131). Genes participating in EMT signalling pathway include *c- myc, cyclin D, surviving* and *slug.* The genetic alteration that is caused by the activation of the Wnt signalling pathway in particular leads to the reorganization of the cytoskeleton of epithelial cells (132,133).

1.10.8 EMT generates cells with stem cell-like properties

There is a group of transcription factors that become activated at the very early stages of life by coordinating EMT for new organ formation and development (134). These embryonic transcription factors are believed to become activated in the later stage of human life by participating in the formation of malignant traits such as, resistance to apoptosis, motility, and invasiveness of the cancerous cells (135). During EMT, epithelial cells gain a self-renewal capacity, which is the unique feature of stem cells (136). This process in fact, is similar to the stage that takes place during tissue repair and regeneration. At this stage the adult stem cells move out from the bone marrow and enter the circulation and reach their final destination

where they start a new phase of proliferation and differentiation to participate in tissue restructuring (135).

1.10.9 EMT and drug resistance

Drug resistance is a major concern in the medical field that results when diseases no longer respond to pharmaceutical treatment. The method of drug resistance can be specific or evolutionarily conserved. Many types of disease including cancer are susceptible to treatment to begin with but over time develop resistance. Overall, there are two specific mechanisms by which diseases can resist pharmaceutical treatment and these include DNA mutations and metabolic changes which cause drug inhibition and degradation. The role of neoplastic EMT in drug resistance is a new phenomenon in oncology (137). One reason cancer cells acquire mesenchymal characteristics is to survive intravasation and adopt a new environment during metastasis. Malignant cells use this phenotypic change as a mechanism against chemotherapy. There are many factors during EMT that can participate in the development of drug resistance (138), for example to chemotherapy. For example, TGF-β can also generate survival signals for neoplastic cells against chemotherapy (139). However, the level of drug resistance in cancer cells is determined by the stage of the tumour, and the grade of tumour is measured by the degree of EMT within the neoplastic tissue.

1.10.10 EMT and apoptotic regulation

Apoptosis or programmed cell death in multicellular animals is governed by the balanced expression of pro-apoptotic and anti-apoptotic proteins. Normal cell regulation is maintained by a balance between cell growth and cell death. Carcinomas of epithelial origin acquire phenotypic changes to develop resistance to chemotherapy, one of the mechanisms being due to the expression of anti-apoptotic proteins such as those of the Bcl-2 family. In a model of EMT breast epithelial cell lines were found to display stem cell-like characteristics with enhanced metastasis and secondary tumour formation. When these mesenchymal-like cells were exposed to apoptotic inducers, they produced a very poor response against the cytotoxic drug (135). This research group later found that, the expression of anti-apoptotic protein (Bcl-xL) was high in the breast epithelial cells lines that had undergone EMT compared to the epithelial cells under basal conditions. Importantly, by knocking down Bcl-xL expression in transformed cells, apoptotic resistance was eliminated.

Therefore, cancer cells acquire EMT as a mechanism for cell survival and motility. One of the mechanisms by which in fact cancer cells acquire resistance is thought to be through the EMT process by disrupting programmed cell death and by over expression of anti-apoptotic proteins (140).

1.10.11 EMT and ECM regulation

Extracellular matrix (ECM) is the major component in the tissue microenvironment and in the case of epithelial tumours, around 60 % of the solid tumour is composed of stroma. ECM remodelling by cancer cells can in fact facilitate tumour progression, local invasion and metastasis. Epithelial cells are separated from mesenchymal counterparts by a specialized connective tissue in the ECM called the basement membrane, and the phenotypic characteristic of epithelial cells is governed by this layer of connective tissue. In order for a carcinoma *in situ* to establish a relationship with ECM, tumour cells need to cross this barrier of basement membrane.

Therefore, cancer cells acquire EMT in order to firstly remove the partition which is located between epithelial and mesenchym and secondly, to orchestrate the remodelling of the ECM. After the dissolution of the basement membrane, cancer cells exposed to the ECM component present in the mesenchymal compartment. This exposure causes further reduction of epithelial function. Therefore, malignant cells undergoing EMT are capable of expressing high amount of MMPs (141), which are the major modulators of the tumour microenvironment. The major MMP subtypes include, collagenases (MMP-1, MMP-8, MMP-13), Gelatinases (MMP-2, MMP-9), Matrylisins (MMP-8, MMP-26), membrane type MMPs (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, MMP-25) and stromelysins (MMP-3, MMP-10, MMP 11), MMP-1, MMP-7 and MMP-9 being important in the development of epithelial carcinomas (142).

1.10.12 EMT and cell cycle regulation

Normal tissue morphology and function in the human body is maintained by a strict number of cells. This strict limitation of cells can only be achieved through a well governed regulation of the cell cycle. In mammals the cell cycle is believed to be regulated by a complex network which is not yet fully defined. There are a number of cell cycle related genes which are thought to participate in cell cycle progression. These complex networks include cyclins, cyclin-dependent kinases and histone modification enzymes such as histone deacetylases (HDACs) (143). Although cell cycle state is considered one of the most significant factors for cellular response to extracellular stimuli, very little attention is paid to the cellular responses generated during EMT. It is generally accepted that growth arrest is a step that can be defined by cell cycle regulation and that it is required for cells undergoing differentiation. For example, TGF-β induces EMT in epithelial tissues by arresting target cells in the G1/S phases of the cell cycle. This cell cycle arrest in fact, could explain one of the mechanisms by which cancer cells develop resistance to chemotherapy and bypass apoptosis. Indeed, cells undergoing EMT acquire resistance against apoptosis in fatal rat hepatocytes (144).

Although there is an obvious relationship between the cell cycle regulation and cellular transformation, there is little knowledge about the cellular changes which influence the cell cycle dependent EMT.

1.11.1 Carcinoma cell derived MVs and tumour microenvironment

It is generally believed that almost all cells in normal tissues communicate with each other through complex interactions such as direct contact or indirectly by soluble or insoluble biochemical molecules (145). Over the last decade, scientists have proved that one of the most important roles of MVs is to establish communication between cells in different organs. In pathological conditions 'however' the normal interaction between cells in the microenvironment becomes disrupted. In the case of inflammatory conditions or malignancy for example, up-regulation of degrading enzymes such as MMPs by stromal cells and expression of cytokines and chemokines by immune cells cause major changes in ECM. In the case of carcinoma, a complex proliferative signalling network is established between the cancer cells, stromal cells and the immune cells, such that the tumour microenvironment is made of a collection of cancer cells, stroma cells, fibroblasts, endothelial cells, adipocytes and immune cells (146).

1.11.2 Cell components of ECM

1.11.2.1 Tumour Associated Macrophages (TAMs)

Macrophages encompass the major portion of the leukocyte population and their role in innate and adaptive immunity have been known for over century, but their involvement in the biology of cancer are recognised recently (147). TAMs are heterogeneous population of stromal cells in the tumour microenvironment and the role of TAMs in tumour progression is complex. Although these activated immune cells are supposed to have anti-tumour activities, according to the literature, their anti-tumour activity is evaded by cancer cells (148). Macrophage infiltration in the tumour site in fact is accompanied with tumour vascularity and invasiveness. The recruitment of TAM is mediated by a group of chemoattractants, such as monocytechemoattractant protein 1(MCP- 1/ CCL2), macrophage inflammatory protein-1α (MIP-1α/ CCL3), and the infiltration of macrophages to the tumour site is accompanied by increases in tumour vascularity and invasiveness (149). However, accurate identification of TAMs are not an easy task when the plasticity of these cells population are considered and the lack of unique surface markers between the lineages has made it difficult to pinpoint which myeloid cell population have been studied (150). Making the matter more complicated is the fact that most of our the knowledge about myeloid cell phenotypes in tumour is derived from animal studies; trans-species differences in the expression of certain marker have made the direct translation of human cancer difficult (151).

1.11.2.1.1 TAMs and tumour growth

TAMs are the main regulators of carcinogenesis in epithelial tumours. The origin of TAMs is either from resident tissue or they are attracted from the bone marrow or spleen (152). Their role is demonstrated at the leading edge of the tumour which drives the invasive cellular phenotypes (153), and their participation in cancer invasion is due to tumour derived colony stimulating factor-1 (CSF-1) and macrophage derived EGF. Furthermore, TAMs are demonstrated to be the major source of proteases such as cysteine cathepsins, enzymes that have strong pro-tumorigenic activities and that also result in resistance against chemotherapy (153).

1.11.2.2 Fibroblast cells

Fibroblasts are a heterogeneous population of mesenchymal cells. From a morphological point of view, these stromal cells are characterized by their spindle shape and oval nuclei. The main function of these mesenchymal cells is the synthesis and deposition of collagen and in maintaining homeostasis of adjacent cells (154). This function of fibroblasts is mainly fulfilled by their proliferation, differentiation and production of collagen and MMPs. They can also respond to growth factors, cytokines and chemokines and consequently orchestrate the remodelling of ECM and they are main contributors of angiogenesis in almost all tissues (154). Tumour associated fibroblasts are stromal cells in the tumour microenvironment but differ morphologically from the fibroblasts in normal adjacent tissue.

1.11.2.3 Fibroblast differentiation

Fibroblast differentiation is considered to have an important role in tissue remodelling and cancer progression and metastasis. The activation of fibroblasts is mostly regulated by certain mediators such as growth factors and cytokines that are released by cancer cells. TGF-β is recognised to be one of the central inducers of fibroblast differentiation causing the expression of cytoplasmic alpha Smooth Muscle Actin (α-SMA) cytoskeleton protein (155). Alpha-SMA is widely used as a molecular marker for the determination of fibroblast differentiation.

The physiological function of fibroblasts is the synthesis of collagen, and once this mesenchymal cell has transformed to a myofibroblast, it can participate in the production of proteases, pro-angiogenic factors and pro-metastatic molecules. Excessive production of α-SMA interferes with the normal physiological functions of target organs and remodelling of ECM (156). There are some other novel markers and modulators such as endosialin in cancer associated fibroblasts and P311 in hypertrophic myofibroblasts during wound healing which are used to assess myofibroblast differentiation (157).

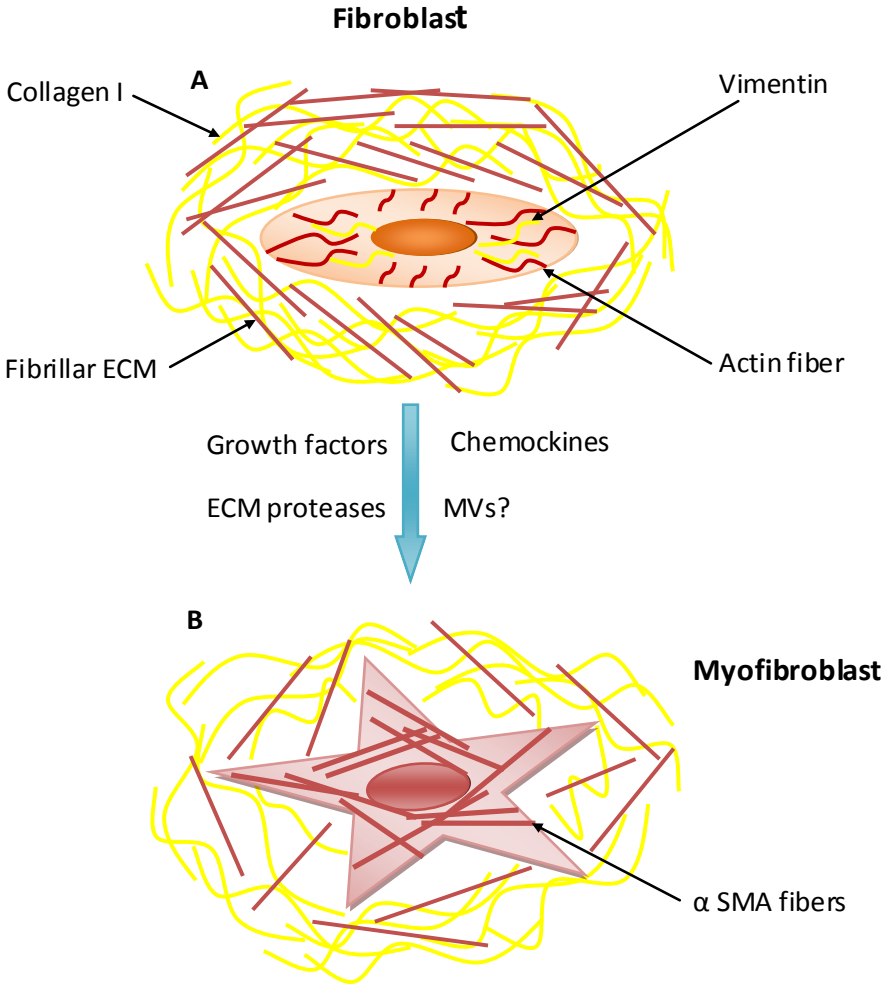

Figure 6 Schematic diagram of fibroblast differentiation.

(A) Normal fibroblast cells surrounded with ECM. Fibroblast cells exhibit mesenchymal characteristics with front–back polarity and an oval shaped nucleus. At the molecular level, a fibroblast cell is characterized by the cytoplasmic expression of a mesenchymal marker, the intermediate filament protein Vimentin. (B) Shows the activated form of a fibroblast cell, the so-called myofibroblast. Myofibroblast cells differ from fibroblasts in terms of morphology, myofibroblast cells looking star shaped compared to fibroblasts that look elongated. At the molecular level, myofibroblast cells are recognised by the expression of cytoplasmic α-SMA cytoskeleton protein. There are a range of growth factor proteins and cytokines which cause activation of fibroblasts to myofibroblasts.

1.11.2.4 Tumour associated fibroblasts or Myofibroblasts

Myofibroblasts are intermediate cells between fibroblasts and smooth muscle cells that can generally be found in fibrotic tissues and solid tumours. Myofibroblasts are characterised by the expression of α-SMA cytoskeleton protein which is used as a reliable marker for these activated stromal myofibroblast cells (158). However, there is some evidence suggesting that myofibroblasts are presents in normal tissues such as alveolar septa and bone marrow stroma (159).

1.11.2.4.1 Biology of myofibriblasts

Myofibroblasts are the most prominent cell type in the tumour micro-environment, and in the context of epithelial carcinoma, myofibroblasts are referred to as tumour associated fibroblasts. The biological characteristics of myofibroblasts have been well defined in the last decade and significant improvements have been made regarding their participation in physiological and pathological environments. In breast, prostate, and pancreatic carcinoma for example, these specialised cells correlate with malignancy grade and poor prognosis.

However, despite much research on the biological features of myofibroblasts, there are many questions yet to be answered (160); what are the progenitor cells of myofibroblasts?; how is the contractile activity of myofibroblasts regulated?; is the α-SMA cytoskeleton protein a specific marker for the recognition of myofibroblasts?

1.11.2.5 Myofibroblast inducers

1.11.2.5.1 TGF-β induces fibroblast activation

Mammals have three different forms of TGF-β (TGF-β 1, β2 and β3). Each of these cytokines is expressed widely throughout the body (161). When tissue homeostasis breaks down, TGF-β is believed to act as a pro-fibrotic cytokine which causes proliferation and differentiation of fibroblasts into myofibroblasts, fibroblast activation leading to the over expression of ECM and α-SMA cytoskeleton protein. However, endogenous expression of TGF-β under normal conditions is sufficient to increase the function of this cytokine (162).

1.11.2.5.2 Connective Tissue Growth Factor (CTGF)

CTGF is a member of the extracellular signalling protein family which is characterised by a high content of cysteine amino acid residues (163). CTGF induces fibroblast differentiation either by directly binding to the target fibroblast cells or indirectly acting on other mediators to interact with their receptors on the fibroblasts (164). CTGF is shown to stimulate lung fibroblast cells which lead to proliferation and a high level of expression of proteins, including, type 1 collagen, cytoskeleton protein vinculin, moesin, ezrin and IQ motif containing GTPase activating protein 1(IQGTAP1), a scaffolding protein; the cytoskeleton protein that is elevated in stimulated lung fibroblasts participates in cell migration (165).

1.11.2.5.3 FGF as inducer of fibroblast differentiation

The FGFs are a polypeptide growth factor family composed of 22 structurally similar ligands and found in all mammalian cells (166). FGF activities are mediated through the activation receptor tyrosine kinases. The signalling stimulated by these regulates cell proliferation, differentiation, and migration of almost all mammalian cells from embryogenesis to adult life (166). Many scientists believe that apart from its role in myofibroblast differentiation, it is largely expressed by cancer cells as an autocrine growth factor which provides self-sufficiency in growth signals (167).

1.11.2.6 Myofibroblast response to tumour cells

There is much evidence that myofibroblats participate in the multistep processing of tumorigenesis. The pro-tumorigenic activities of myofibroblasts are due to their direct influence on cancer proliferation, differentiation, invasion and metastasis. Myofibroblasts are believed to pave the way for cancer cells to invade neighbouring tissues (168). Cancer cells can use the myofibroblast as a vehicle to invade the tissue microenvironment. Furthermore, myofibroblasts can be used as a prognostic tool to assess tumour grade and provide information about response to anti-cancer therapy. However, there is still no concrete evidence to prove whether myofibroblasts are capable by themselves of inducing malignant cell transformation (169). One important question yet to be answered is: since neither the cancer nor the myfibroblasts are normal, how and where does the pathology begin?

1.11.2.7 Tumour angiogenesis

Malignant cells utilise more energy than normal tissues, this being due to the high level of activity of cancer cells in the tumour microenvironment. Biopsies of the solid tumour always emphasise the abundance of vascular structure. However, these blood vessels are poorly perfused compared to blood vessels in normal tissues (170). The main reasons for the poor vascularity are the constant activity of growth factors such as VEGF-A, PDGFs and FGFs and cytokines which are released by cancer cells and stromal cells in the tumour microenvironment causing proliferation of endothelial cells and thus leading to formation of new blood vessels. These continuous vascular activities in fact prevent vascular maturation (171). Furthermore, continuous vascular formation causes nascent blood vessels which are prone to rupture. Therefore, intravascular coagulation and fibrin deposition which can be seen in the tumour microenvironment is mainly due the rupturing of immature blood vessels and therefore anti-angiogenic therapies are used to prevent vascularisation and diminish carcinoma growth (172).

1.11.2.8 Pro-tumorigenic activities of Myofibroblasts

It is generally believed that cancer cells cannot survive on their own unless they build a relationship with local and distant host cells, and indeed a morphological analysis of stromal cells clearly demonstrates the participation of host cells in the formation of primary tumours, as well as premetastatic and metastatic cancer cells (173). Myofibroblasts can be used in cancer prognosis and in ascertaining response to chemotherapies of malignant carcinomas. Immunohistochemical analysis of epithelial carcinomas revealed that, many functional proteins that are expressed in the tumour microenvironment are believed to be encoded by genes that are expressed by myofibroblasts (174). However, we need to consider that the tumour microenvironment is not only composed of cancer cells and myofibroblasts. Inflammatory cells are also attracted to the cancer microenvironment by excessive expression of pro-inflammatory cytokines produced by myofibroblasts. Although the response of myofibroblasts to cancer cells is emphasised towards cancer progression, however, transgenic mouse experiments suggest that resident tissue fibroblast cells initiate signals that down regulate cancer cell proliferation and tumour progression (175).

1.11.2.9 ECM of normal organs

Stromal ECM is made up of a collection of different tissues including collagens, elastin, fibronectin, hyaluronic acid, proteoglycans and glycoproteins. The main function of ECM in healthy organ is to maintain tissue architecture. ECM is also capable of modifying the function of growth factors and cytokines (176). Furthermore, when growth factors and cytokines are released from the activated cells, the ECM provides them with biological latency and protects them from degradation. In the case of TGF-β for example, its activity and latency is governed by binding to a stromal proteins such as decorin or thrombospondin-1(177). The activity of growth factors such as VEGF is controlled by binding to heparin or Proteogycans before triggering endothelial cell activation and proliferation. Breaking down of the ECM homeostasis is caused by imbalanced production and degradation of ECM which consequently leads to the disruption of tissue architectures.

1.11.2.10 ECM of solid tumour

The collagen component of solid tumours is morphologically different to the collagen in normal connective tissue. The fibril portions of carcinoma tumours are multi-lobular and coarse, displaying a heterogeneous distribution of diameters. In normal connective tissue these collagen fibrils appear smooth and circular (178). Furthermore, the expression of collagen fibrils in solid tumours is considerably high compared to that in normal tissue. The synthesis of collagen in epithelial carcinomas is believed to be regulated by myofibroblasts (179). Cancer cells proliferate more rapidly than normal tissues and therefore require more energy, and these cells use collagen as a source of fuel for their growth and proliferation. Proline which is released as a result of collagen degradation by tumour cells is considered as a source of energy and also as a precursor for other amino acids (180). The activity of cross-linking enzymes such as Lysyloxidase is changed in solid tumours. The increased activity of these cross-linking collagen enzymes causes excessive collagen scaffolding stiffness which consequently increases the invasiveness of tumours (181).

1.11.2.11 Fluid balance in the tumour microenvironment

Fluid balance is disturbed in the tumour microenvironment, and this is due to the immature vascular system of the tumour which is caused by excessive expression of angiogenic factors such as VEGF. The blood vessels become leaky and lose plasma protein in the tumour stroma that leads to a breakdown of osmotic pressure between the interstitium and the blood (182). The lymphatic system is also not functional at the standard level compared to normal tissue leading to a lack of drainage of excess interstitial water. The blood pressure in tumour stroma ranges between 7-31 mm Hg, thus leading to severe oedema. This in fact leads in to inefficient transport of water and other molecules in the tumour microenvironment. Furthermore, the transport of larger protein molecules is far less efficient compared to normal tissues. This is generally due to the excessive composition of the ECM which is orchestrated by myofibroblasts, carcinoma cells and TAM (183).

1.11.2.12 How injured epithelial cells respond to the microenvironment

Epithelial cells are exposed to physical, chemical and pathological damage and can be repaired through the formation of granulation tissue, connective tissue produced by ECM deposition, fibroblast activation, endothelial proliferation and immune cell mobilization (184). Physiological repair mechanisms are carried out by the degradation of excessive ECM, and apoptosis of myofibroblasts. Inflammation is sustained when myofibroblasts remain active and continues with the production of ECM, MMPs, angiogenesis and immune cell influx. Therefore, the presence of continued inflammation, fibrotic lesions and excessive production of MMPs leads to the dissolution of the basement membrane which normally functions as a barrier between the epithelial tissue and stroma. Dissolution of this membrane disrupts the epithelial cell polarity and also the epithelial tissue becomes physically exposed to the inflamed microenvironment. It is unsurprising then that the risk of cancer is significantly increased in the target tissue (185).

1.12. AIMS OF THE PRESENT STUDY

The objective of this thesis was to study the influence of cancer cell derived microvesicles on normal epithelial cell lines and primary fibroblast cells.

In chapter III, the aim was to study the role Jurkat cell (T cell leukaemia cell line) derived microvesicles in generating phenotypic change of normal prostate epithelial cell lines (PNT2).

In chapter IV, aimed to investigate causes and functional consequences of Epithelial Mesenchymal Transition in prostate epithelial cells induced by Jurkat cell derived MVs.

In chapter V, the aim was to examine the influence of small lung cancer cell- (A549) derived microvesicles on primary fibroblast cell lines (MRC5), in the tumour microenvironment.

2. Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

Annexin V Alexa Fluor 488	Invitrogen
Annexin V reagent	R&D Systems
BAPTA-AM	Sigma-Aldrich
BCA protein assay kit	Pierce Biosciences
BSA (Bovine serum albumin)	Sigma-Aldrich
Calcium chloride	Sigma-Aldrich
DAPI-VECTASHIELD	Vector Laboratories Inc, CA, USA
DMSO (Dimethyl sulfoxide)	Sigma-Aldrich
Ethanol	Fisher Scientific
Fura-2-AM	Sigma-Aldrich
FBS (Foetal Bovine Serum)	Fisher Scientific
Guava ViaCount reagent	Guava Technologies, UK
Guava Nexin reagent	Guava Technologies, UK
Halt Protease Cocktail	Pierce, Thermo-Scientific
HCl (Hydrochloric acid)	Fisher Scientific
Human FGF-1 ELISA kit	R&D Systems
Human MMP9 ELISA kit	R&D Systems
Human TGF-β1 ELISA kit	R&D Systems
Kanamycin	Sigma-Aldrich
Magnisium chloride	Sigma-Aldrich
Methanol	Fisher Scientific
NHS (Normal Human Serum)	Sigma-Aldrich
Paraformaldehyde	Agar Scientific
PBS (Phosphate Buffer Saline)	Sigma-Aldrich
Penicillin / Streptomycin	Fisher Scientific
Potassium chloride	Sigma-Aldrich
Propidium iodide	Sigma-Aldrich
RPMI	Sigma-Aldrich
SB-431542	Fisher Scientific
Sodium azide	Sigma-Aldrich
Sodium chloride	Sigma-Aldrich
Sodium hydroxide	Sigma-Aldrich
Tris base	Sigma-Aldrich
Trichloroacetic acid	Fisher Scientific
Triton X-100	Sigma-Aldrich
Trypsin/EDTA solution	Sigma-Aldrich

2.1.2 Technical devices

AMT Digital camera	Advanced Microscopy Tech.
Cell culture flasks (75 cm^2)	Fisher Scientific
Centrifuge 5804 R	Eppendorf
Centrifuge 5810 R	Eppendorf
Gel loading tips	Corning
Guava EasyCyte flow cytometer	Guava Technologies, UK
Fluorescence microscope (1X81)	Olympus Corporation, Germany
FLUOstar Omega plate reader	BMG Labtech, UK
Incubator Heraeus CO_2-Auto-Zero	Thermo Electron Corporation
Microcentrifuge 5417R	Eppendorf
Microplate (12-well)	Sigma-Aldrich
Microplate (24-well)	Sigma-Aldrich
Microplate (96-well)	Sigma-Aldrich
pH-Meter 766 Calimatic	Jenway
Roto-Shake Genie	Denley
Semi-dry transfer system	BioRad
Small volume tips	Sigma-Aldrich
Sorvall ultracentrifuge RC6	Thermo Electron Corporation
Sorvall T-865 rotor	Sorvall
F-20 micron rotor	Sorvall
SE-12 rotor	Sorvall

2.1.3 Antibodies

Anti-Annexin V AlexaFlour 488	Biosciences
Anti-CD 25 FITC	BioLegend
Anti- E-cadherin	Abcam
Mouse anti-IgG AlexaFluor 488	Biosciences
Mouse anti-E-cadherin	Abcam
Mouse anti-FGF-1	Abcam
Anti- Smad3	R&D Systems
Anti- Smad3 (Phospho S423+ S425)	Abcam
Anti- Vimentin	Abcam
Mouse anti-TGF-β1	R&D Systems
Mouse anti-IgG FITC	AbD Serotec
Mouse anti Vimentin	Abcam
Rabit IgG anti- Human FITC	Abcam

2.1.4 Eukaryotic Cell lines

Jurkat cells (Human T- cancer cells) were used in most experiments as the source of cancer cells derived vesicles (MVs). However, in some experiments, MVs were isolated from A549 cells (Non small lung cancer cells), MOLM 13 cells (human leukaemia cells) were used in some experiments. Primary monocytes were also obtained from healthy donors.

Eukaryotic cell lines: MOLM 13 (Received as kind gift from Gareth Williams at UCL)

A549 cells (Received as kind gift from Gareth Williams at UCL)

Jurkat cells (ECACC; Ref No. 88042803)

MRC5 cell (Received as kind gift from Gareth Williams at UCL)

PNT2 (ECACC; Ref No. 86012803)

PC3 (ECACC; Ref No; 92127018)

2.1.5 Eukaryotic Cell Culture Media: Cell Growth Medium (GM)

The RPMI 1640 supplemented growth medium was used to cultivate PNT2 cells (immortalised prostate cells) and Jurkat cells. RPMI 1640 medium supplemented with 10% FBS (v/v) and 1% Penicillin / Streptomycin in 500 ml volumes and stored at 4°C.

2.2 Experimental Solutions

2.2.1 Mammalian cell freeze medium

20%	FBS (v/v)
10%	DMSO (v/v)
1%	Penicillin/Streptomycin (v/v)
69%	RPMI (v/v)

2.2.2 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

Made up to 50 ml and stored as 1 ml aliquots at -20°C

2.2.3 (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

2.2.4 Resolving gel solution (12%)

2 ml	ddH_2O
1.25 ml	1.5 M 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

2.2.5Antibody dilution buffer (WB)

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

2.2.6Permeabilisation Buffer (PB)

0.5%	Tween 20 (v/v)
	PBS

2.2.7 Immunofluorescence antibody dilution buffers

3.2.7.1Cell Dilution Medium

10%	FBS
1%	NaN_3
	PBS solution

2.2.7.2 Primary and secondary antibody dilution buffer

3%	BSA (w/v)
	PBS

2.2.8 Flow cytometry analysis buffer

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

All components dissolved in 500 ml deionised water

2.2.9 Annexin V binding buffer–pH 7.4

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

All components dissolved in 100 ml of deionised water

2.2 Methods

2.2.1 Maintaining cell lines

2.2.1.1 Non-adherent cell lines

Non-adherent Jurkat cells was maintained in growth medium containing RPMI 1640 supplemented with 10% foetal bovine serum (FBS), 1% penicillin-streptomycin. In addition, cells were occasionally maintained for a week in growth medium supplemented with 1% kanamycin at 37°C in 5% CO_2 atmospheric conditions. The cells were split, depending on confluency every 3 to 4 days by washing twice with serum-free RPMI 1640. The cells were transferred into 50 ml centrifuge tubes and spun at 160 *g* for 5 min. Resulting supernatant was discarded and cells were gently resuspended in the remaining medium. RPMI was added and cells were centrifuged as mentioned earlier. Resulting supernatant was discarded and cell pellets were resuspended in the appropriate volume of growth medium. Cells were then seeded in the desired dilution into new 75 cm^2 culture flasks. Cells were only cultured in medium supplemented with kanamycin for a week to avoid development of resistance to the antibiotic.

2.2.1.2 Adherent cell lines

Adherent cells (PNT2) were also maintained at 37°C with 5% CO_2, in RPMI growth medium. These cells were also split depending on confluency every 3 to 4 days. Cells were washed twice by changing GM with serum-free RPMI and addition of 0.25% (v/v) trypsin/EDTA in RPMI. After 6 min incubation at 37°C with 5% CO_2, the flask was tapped several times to

detach the cells and growth medium was added to inactivate the trypsin. The trypsin solution was removed by centrifugation at 200 *g* for 5 min followed by one wash in serum-free RPMI. Cell pellets were resuspended in the appropriate GM volume and seeded in the desired dilution into new culture flasks. Exponentially growing cells with viability of 95% or higher was used in every experiment. The number of cells and viability were determined before the start of every experiment using the flow cytometer (ViaCount assay, Guava Technologies).

2.2.1.3 Freezing of eukaryotic cells

To prepare frozen stocks for long term storage, non-adherent cells grown to almost 100% confluency were washed twice (160 *g*, 5 min) with serum-free RPMI and cell number determined using Guava viacount as mentioned in section 3.8.1.2. Cells were carefully resuspended in freeze mix, transferred into cryo-vials (Greiner) at $1x10^7$ cells/ml in 1 ml volumes and immediately placed on ice. The cryo-vials were frozen at –80°C in special cryo boxes, which ensure a temperature decrease of 1°C per minute. For long-term storage the deep frozen cryo-vials were transferred to liquid nitrogen cell storage tanks. Adherent cells were also frozen by a similar procedure except that cells were first trypsinised to bring them into suspension, as described earlier. Suspended cells were washed by centrifugation at 200 *g* for 5 min. Cells were resuspended in the freeze mix and transferred into cryo-vials.

2.2.1.4 Thawing of cells

To defrost cells, cryo-vials were removed from liquid nitrogen and immediately thawed in a waterbath at 37°C. After cleaning the lid with 70% ethanol, the content was transferred to a 15 ml centrifuge tube containing 9 ml of fresh growth medium, prewarmed to 37°C and cells were sedimented by centrifugation at the appropriate speed (200*g* or 160 *g*, 5 min for mammalian cells). To remove DMSO, the medium was discarded and the pellet was resuspended in fresh growth medium. The cells were then placed into culture flasks of the same size as had been used prior to freezing, and incubated at 37°C with 5% CO_2.

2.3 Biochemical Methods

2.3.1 Purification of MVs from medium

Cells cultured supernatant containing 10% FBS (MVs free) was centrifuged once at 160*g* for 5 min to remove the cells. The supernatant was then centrifuged at 4,000 *g* for 1 hrs to remove cell debris. Supernatant was centrifuged at 25,000 *g* for 2 hrs to pellet MVs. Pelleted MVs were washed once by resuspending in sterile 0.1 µm filter PBS and centrifuged again at 25,000 *g* for 2 hrs. The MV pellet was resuspended in sterile PBS and quantified, or analysed for PS exposure as described under section 3.9.1.

2.3.2 Annexin labelling of MVs

Isolated MVs were resuspended in Annexin binding buffer and Guava Nexin-FITC was added or not (control) in a 100 µl final volume. Sample was then incubated at room temperature for 45 min with shaking and centrifuged at 25,000 *g* for 2 hrs to pellet MVs. Samples were analysed immediately as directed by the manufacturer using the flow cytometer (Guava EasyCyte, Guava Technologies).

2.3.3 Measurement of intracellular calcium

2.3.3.1 Omiga Plate reader

PNT2 cells ($1x10^6$/ml) were washed twice with serum-free RPMI by centrifugation at 200 *g* for 5 min and incubated in 0.5 ml volumes with 2 mM Fura-2-AM at RT for 1 hrs in the dark with shaking. Labelled cells were washed a further three times with RPMI and resuspended in a physiological salt solution (PSS) containing 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 10 mM HEPES in the presence or absence of 1 mM $CaCl_2$ or 5 mM EGTA. Measurements were made before and after addition of Jurkate cells MVs after45 min with continual stirring, whilst monitoring fluorescence on a spectrofluorimeter at 505 nm upon excitation at 340 and emission at 380 n. Triton-X-100 (1%) was added after 150 seconds to measure the maximum fluorescence. Intracellular calcium was calculated using the equation: $[Ca^{2+}]_i$ = Kd[(R-Rmin)/(Rmax-R)x(Sf2/Sb2)] where Kd (dissociation constant) for Fura-2-AM is 224 nM. R is the ratio of the emission intensities measured at 505 nm on excitation at 340 and 380 nm. R-min is the ratio of intensities

obtained at zero intracellular Ca^{2+} (before the addition of 1% Triton X-100 to cells resuspended in buffer containing 5 mM EGTA) and

R-max is the ratio of emission intensities measured after lysing the cells with 1% Triton-X-100 thus allowing Fura-2 measurement in the maximum external calcium concentration of 1 mM.

Sf2 is the emission intensities of free calcium measured at 380 nm [ratio of intensities measured after addition of 1% Triton-X-100 to cells resuspended in buffer containing with 5 mM EGTA (here, the calcium is free as it is bound to EGTA and not to Fura-2-AM)]. Sb2 is the emission intensities of bound calcium measured at 380 nm (ratio of maximum intensities measured after addition of 1% Triton X-100). All measurements were made at 37°C.

2.3.3.2 Ion chromatography

PNT 2 cells were transferred into marked 1.5 ml appendrof tubes. Pellet cells at 450 g for 5 minutes. Cells are resuspend it in 1 ml double distilled water. Tubes are dropped in liquid nitrogen to freeze and thaw sample on ice (thawing takes a few minutes). Vortex briefly and Repeat freeze-thaw cycle four more times. Samples were centrifuged at 450 g 10 minutes and the supernatants were transferred into new marked 1.5 ml appendrof tubes. Ion chromatograph is able to measure concentrations of major cations (lithium, sodium, ammonium, potassium, calcium) and anions, (fluoride, chloride, nitrate, nitrite, and sulphate). Ion chromatography is form of liquid chromatography that measures concentrations of ionic species by

separating them based on their interaction with a resin. Ionic species separate differently depending on species type and size. Sample solutions pass through a pressurized chromatographic column where ions are absorbed by column constituents. As an ion extraction liquid, known as eluent, runs through the column, the absorbed ions begin separating from the column. The retention time of different species determines the ionic concentrations in the sample. Small quantity of the sample (no less than 100 um) are injected into the machine using a small sterile syringe. The syringe needle passes through a thick rubber disc (known as a septum) which reseals itself when the syringe is pulled

2.3.4 Growth Inhibition Assays

PNT2 cells were seeded into 12-well plates at $1x10^5$ cells/well in triplicate. 30 µg/ml jurkat cells derived MVs were added to each well (except controls) and plates were incubated at 37°C for 5 days. On the days indicated, PNT2 cells were removed using typsin/EDTA and counted by ViaCount assay on a Guava EasyCyte flow cytometer.

2.3.5 Differentiation Assay

PNT2 cells were determined following treatment with 30 µg/ ml of Jurkat cells MVs. To investigate EMT, PNT2 cells $1x10^4$ cells/well were seeded into 24 well plates in triplicate. Cells were left untreated (control) or treated either with 30 µg Jurkat cells MVs and incubated at 37°C in a humidified atmosphere of 5% CO_2 for five days. After 5 days the cells washed twice with ice cold double PBS and the attached cells were released with trypsin/EDTA. The trypsinised cells, in suspension were collected by centrifugation (200 *g*, 5 min) and immuno-labelled at 4°C for 1 h with the anti- E-cadherin antibody and anti- Vimentin antibody.

2.3.6 Determination of Protein Concentration

The concentration of a protein solution was determined using the BCA Protein Assay Kit (Pierce, Thermo Scientific, UK). It uses a combination of the biuret reaction (reduction of Cu^{2+} ions to Cu^{+} ions by proteins in an alkaline medium) and the colorimetric detection of the Cu^{+} - cations by a bicinchoninic acid-containing colour reagent. Following the manufacturer's instructions, 10 µl of protein samples were diluted in double distilled water (ddH_2O; dilution 1:5) and added to 200 µl working solution consisting of a mixture of kit reagent A and reagent B (ratio 50:1 respectively). In parallel a dilution series of a 0.5 µg/µl BSA stock solution in ddH_2O were prepared and used as standard in later evaluation. Applied concentrations were 0, 62.5, 125, 250, 500, 750, 1,000, 1,500 and 2, 000 µg/ml BSA in 500 µl ddH_2O. Volumes equal to samples were added to 200 µl of a mixture of kit reagent A and B (ratio 50:1) in a 96 well plate followed by incubation at 37°C for 30 min. After incubation at room temperature for 10 min to cool samples, Absorbance at 562 nm readings was taken on a FLUOstar Omega microplate reader. Protein concentrations of the unknown samples were determined by interpolation on a standard curve multiplied by a dilution of factor of 5.

2.3.7 Preparation of cell lysates

PNT2 cells ($1x10^6$) grown in culture flasks, after removing the medium, cells were trpsininsed and sedimented by centrifugation (200 *g*, 5 min at 15°C). The pellet was washed once by careful resuspension in RPMI followed by centrifugation. After that, cells were counted using a haemocytometer and lysis was performed to give a protein concentration. If defined amounts of protein were required, the pellet was subjected to detergent based lysis followed by determination of total protein concentration using procedure for the BCA kit according to the procedure described under section 3.9.11. Briefly, cell lysis was performed by resuspension of the pellet in 0.2% Triton X-100 (w/v) containing protease inhibitor cocktail. To solubilise membrane proteins, samples were repeatedly pipetted and insoluble materials were sedimented by centrifugation (10,000 rpm, 5 min at 4°C, A-4-62 swing-out rotor, using 5810R centrifuge, Eppendorf). The total protein concentration was determined of the resultant supernatant and then subjected to SDS-PAGE analysis.

2.3.8 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 incubation at 95°C for 4 min. Before loading the samples onto the gel a centrifugation step was performed (2,000 *g*, 1min) to collect all liquid at the bottom of the reaction microtube and loaded into the gel wells.

2.3.9 SDS-PAGE Protein Molecular Weight Standards

As a protein molecular weight standard, prestained Protein-Marker I (BioRad) was used. Prestained markers, ranging from 2 to 212 kD or 10 to 250 kD were used when analysing gels by Western blot using the ECL detection system. Markers were applied by loading 10 µl into wells.

2.3.10 SDS-Polyacrylamide Gel Electrophoresis

To separate proteins, which were denatured by sodium dodecyl sulphate (SDS) according to their molecular masses, SDS polyacrylamide gel electrophoresis (SDS-PAGE) 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. Unpolymerized 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 deionised water, and the excess water was blotted using a filter paper (Whatman 3 MM, Whatman AG). Then unpolymerized 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. To perform 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 unpolymerized acrylamide/bisacrylamide. Samples were loaded into the wells of 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 or transferred onto nitrocellulose membrane for Western blotting analysis.

2.3.11 Flow cytometry

The Guava flow cytometer allows complex biological studies such as cell counting and viability testing, cytokine detection, cell activation marker analysis and other complex molecular analyses to be performed simultaneously. This saves time, but also generates accurate results, which comes much closer to mimicking the complex biological responses within the body. The Guava flow cytometer can be used to perform ten different assays; however, for these studies only three assays were implemented. These were the ViaCount assay (for counting cells and determining viability), ExpressPlus assay (for cell activation marker analysis, cytokine expression, microvesicle analysis and studies on protein-protein interaction), Nexin assay (for reporting apoptosis) and cell cycle analysis.

2.3.12 Cell counting and viability assessment

Cell number and viability were determined using the ViaCount assay, which distinguishes between viable and non-viable cells based on the differential permeabilities of two DNA-binding dyes in the Guava ViaCount reagent. The nuclear dye only stains nucleated cells, while the viability dye brightly stains dying or dead cells. This proprietary combination of dyes enables the Guava ViaCount assay to distinguish viable, apoptotic and dead cells. Cell debris is excluded from results based on negative staining with the nuclear dye.

2.3.13 Cell labelling

PNT2 cells were washed three times with serum-free RPMI prior to experiments. Cells were labelled with MMP 9 and AlexaFluor 488, at RT for 1 h with shaking at 4^{o}C. MMP9 AlexaFluor 488 was diluted in a binding buffer provided with the kit and used at 1 µg/10^{6} cells, according to the manufacturer's instructions. Cells were washed three times with serum-free RPMI and then diluted in 200 µl of flow cytometer dilution buffer prior to examination by guava flow cytometer.

2.3.14 Immunofluorescence staining for EMT markers

In order to determine the expression of cell activation markers as well as surface and intracellular cytokines by specific antibody staining, cells were processed according to the following protocol and analysed using flow cytometry. Cells were harvested, washed by centrifugation at 200 *g* for 5 min and total cell number and viability (usually >95%) was determined using ViaCount reagent (refer to 3.10.1). Cells were usually stained in eppendorf tubes and later transferred into a 96-well microtiter plate for analysis on the flow cytometer. However, they can also be stained in polystyrene round-bottom, 12 x 75 mm Falcon tubes. Cell surface expression of E-cadherin molecules were analysed by flow cytometry (EasyCyte, Guava Technologies, UK). PNT2 cells (1×10^6) were washed twice (200 *g*, 5 min each) with PBS and resuspended in ice cold PBS containing 10% FBS and 1% NaN_3. Cells were incubated in the dark with primary antibodies (2 µg/million cells, Abcam) against tested molecules at 4°C for 1 hrs with shaking. After three washes (400 *g*, 5 min), cells were stained with the isotype-matched controls (anti-mouse or anti-rabbit IgG-FITC, Abcam) diluted 1: 200 in PBS with 3% BSA and incubated with shaking, in the dark, at 4°C for 1 hrs. Cells were again washed three times with cold PBS, resuspended in 200 µl of PBS containing 3% BSA, 1% NaN_3 and analysed immediately using flow cytometry and the ExpressPlus assay program. For staining of intracellular expression, PNT2 cells ($1x10^6$/ reaction) in triplicate were resuspended in PB (0.5% Tween 20 in PBS) at room temperature for 20 min.

Cells were permeabilised by washing three times with PB and incubated with 2 µg of primary antibody (anti-Vimentin antibody) at 4°C for 1 hrs in PBS with 10% FBS/ 1% NaN_3. Cells were washed three times and incubated with IgG-FITC-labelled secondary antibodies (4°C, 1 hrs), and again washed three times with ice cold PBS. Samples were resuspended in 200 µl each of PBS containing 3% BSA, 1% NaN_3 and analysed immediately by flow cytometry.

2.3.15 Fluorescence microscopy

For fluorescent microscopy analysis, PNT2 cells, cultured on coverslips were fixed with 4% paraformaldehyde at 37^{o}C for 15 min. Plates were gently washed three times with ice cold PBS and coverslips were mounted on microscope slides with DAPI-VECTASHIELD medium. Images were collected using fluorescence microscope (1X81 motorized inverted fluorescence microscope, Olympus Corporation).

2.3.16 Quantitation of proteases by ELISA

MVs were isolated from PNT2 and transform PNT2 cells and the concentration of MMP 9 measured in MMP9 ELISA kits (Abcam) according to the manufacturer's instructions. In some experiments, PNT2 and transform PNT2 cells (1x10^4/ well), in duplicate lysed before measuring the proteases level in target cells.

2.4 Statistical Analysis

Statistical analysis for all data presented was performed by using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, USA). Statistical correlations between data values were also determined using GraphPad Prism software. Differences giving a value of $P < 0.05$ with confidence interval of 95% were considered statistically significant.

3. Microvesicles from a T lymphocytic leukaemia cell lines (Jurkat) and induction of Epithelial Mesenchymal Transition of normal prostate epithelial cell lines

3.1 Introduction

The Jurkat cell line was established in 1970 from the peripheral blood ofa14 year old boy with T lymphocytic leukaemia. This immortalized blood cancer cell line is used primarily to determine the susceptibility of cancer cells to chemotherapy and radiotherapy. The precedent for my line of investigation into the role of T cell leukaemia MVs is two-fold based firstly on evidence from the literature that mammary epithelial tumour cells are stimulated by activated CD8 T cells to undergo EMT, acquire resistance against chemotherapeutic agents and increase their tumorigenic capability. Secondly, from previous work showing Jurkat MVs to express high levels of TGF-β1 in a form that can be functionally activated (Ansa-Addo, 2010) and because of the described role of TGF-β1 in EMT.

The objective of this study was to have an insight into the role Jurkat cell-derived MVs have in the phenotypic change of normal prostate epithelial cell lines. EMT is a fundamental physiological process by which epithelial cells lose their epithelial characteristic (limited intercellular space, free apical surface, vascular and mitotic capability) and acquire mesenchymal characteristics (spindle shaped, end-to-end polarity). There are three types of EMT, physiologic EMT (Type I), Fibrotic EMT (Type II), and cancerous EMT (Type III). Type I EMT is the best characterised during organogenesis, Type II EMT is activated during wound healing and type III is a pathologic process that is functional during cancer invasion and metastasis.

Microvesicles comprise a heterogeneous population of particles (0.1 to 1µm in diameter) derived from normal and cancer cells under physiological and stressed conditions, and act as communicative vectors between cells.

3.2 Results

3.2.1 MV enumeration, characterisation and detection of exposed phosphatidylserine

Jurkat cells MVs were used in this study to determine their biological effects on normal prostate epithelial cell lines. MVs were isolated according to the method described in the Materials and Methods, from Jurkat cell culture medium containing10% MV-free FBS without antibiotics. Calcium-free PBS was used during the washing steps and to dilute the MVs in the last stage of MVs isolation. PBS was also filtered through a 0.1 µm pore size filter to remove particles that otherwise might interfere with MV analysis using the EasyCyte Guava Flow Cytometry (Millipore) and Guava express plus software. The analysis of MVs using this program is quite challenging and is mainly due to the lower sensitivity of this technique for MV analysis. However, to minimise the miscalculation of MV numbers with background noise using the Guava EasyCyte technology, I decided to place less of an emphasis on MV number. Rather, protein quantification of MVs was utilised using the FLUOstar Omega plate reader (Bio-Rad). Therefore, PNT2 cells were treated according to the protein content of blood cancer MVs rather than their number.

3.2.2 MV quantitation and characterization

This study is generally performed in every microvesicle laboratory, firstly to confirm the MV purification method has been performed correctly, secondly to have an approximate estimation of MV numbers harvested from the parent cells and thirdly, to look at the size and granularity of MVs from the FSC and SSC respectively.

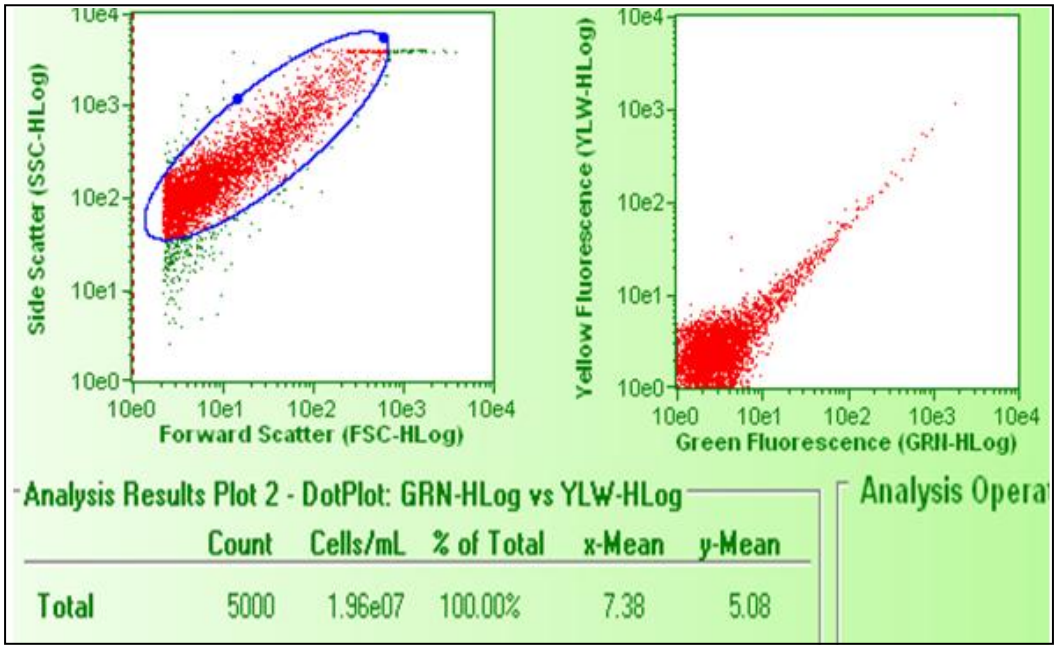

Figure 4.1 The direct analytical method of MVs performed using the Guava Easy Cyte. This scatter plot shows the number of MVs isolated from 70 ml conditioned (Jurkat) cell culture medium. The Guava Easy Cyte is a versatile technique for counting and characterizing MVs compared to the sophisticated electron microscope simply because it does not disturb the physiological property of MVs and unlike Nanosight Tracking Analysis enable other parameters such as the granularity of the sample to be determined and determination using fluorescence. The Guava Express software is used to gate the MVs and afterwards, analyze the relative proportion of MVs expressing phosphatidyleserine on their outer surface. Thus MVs were characterized by their size and granularity which was assessed from the logarithmic amplification of forward scatter (FSC) and side scatter (SSC) signals.

Essentially, this Guava EasyCyte technology uses a single screen dashboard of flow cytometer where simplified ease of use and the use of single colour further simplifies the data acquisition. However, this instrument has a throwback. As the particles below 0.2 µm cannot be detected by this machine, therefore MVs with range of 0.1 to 0.2 µm cannot be discriminated from the instrument noise. Furthermore, another major element that is absent from this technique which in fact undermines the accuracy of the assay is the ability to compensate for signal crossover when both fluorescent channels are used. This crossover signal effect that compromises the assay's accuracy is partially dependent on the fluorophores used and laser chosen for the system.

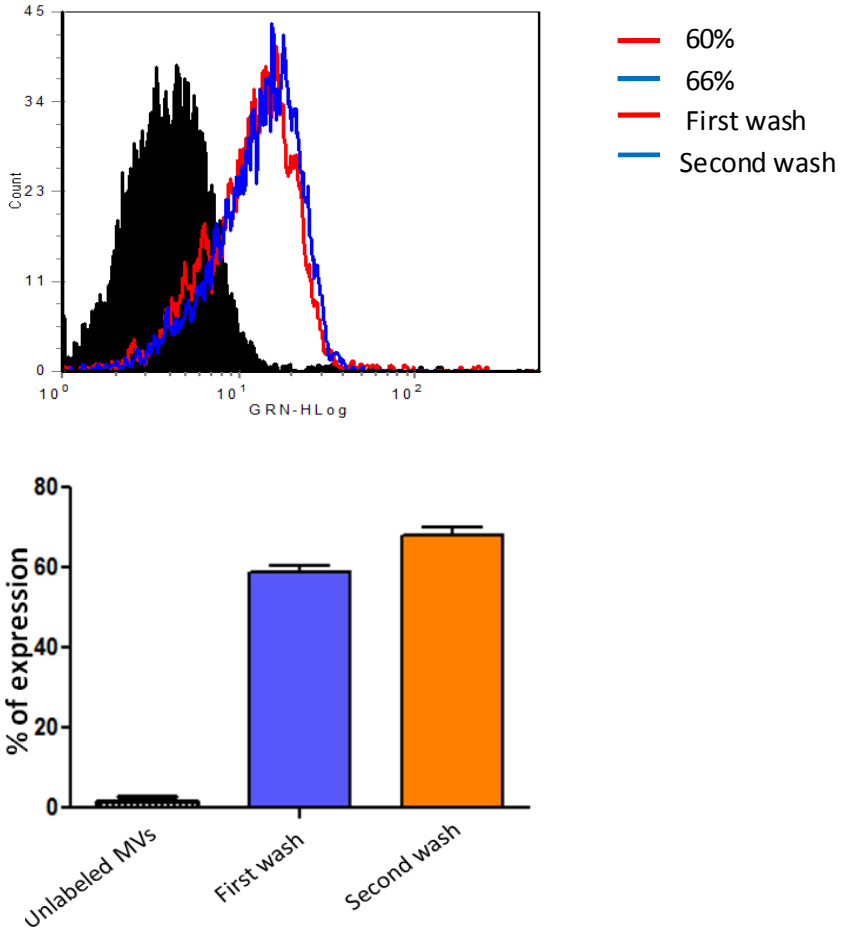

Figure 3.2 MVs labelled with Annexin V for the expression of phosphatidylserine that has translocated to the outer leaflet of MVs. (A) shows the flow cytometric analysis of MVs that labelled with Annexin V. MVs isolated from Jurkat cells were labelled with Annexin V according to manufacturer's instructions. Adding 5 µl of fluorochrome conjugated Annexin V was added to 100 µl of the MVs suspension and incubated at R.T for 30 min whilst protected from light. After incubation the sample was analyzed twice. After the first wash, 60 % of the MV population turned up positive with Annexin V and after the second wash by centrifugation at 25.000g and resuspension in 200 µl of 0.1 µm sterile filtered PBS, the percentage of Annexin V-positive MVs went up to 66% presumably due to the reduction of Annexin V-negative MVs after high speed centrifugation.

At this point it was important to establish the nature of those vesicles/particles which were counted as MVs by the flow cytometer but stained negative for Annexin V. **Figure 4.2** above illustrates the purification of MVs after staining with Annexin V. A Student's t-test between the two steps of centrifugation did not show a high significance. Overall my emphasis on these different ways of analysing samples was to make sure I was working with a pure population of biologically active MVs.

3.2.3 Flow cytometry shows loss of E-cadherin expression in PNT2 cells treated with T cell leukaemia-derived MVs

Prostate epithelial cells (5x 10^4/well) were seeded into 24 well plates in triplicate and grown in medium containing 10% FBS and treated with 30 µg/ml of Jurkat MVs. Cells were incubated for 5 days in (37^{o}C and 5% CO_2). Cells were monitored every 24 hrs using a phase contrast microscope and pictures were taken from both panels of control, untreated PNT2 cells and PNT2 cells treated with blood cancer MVs. On day 5, cells were washed and detatched by trypsin/EDTA, fixed with 4% PFA for 15 min then labelled with anti-E-cadherin antibody. The expression of E-cadherin was measured in both control and MV-treated cells using the Guava EasyCyte. E-cadherin protein is a cell junctional protein which is expressed on the cytoplasmic membrane of all epithelial cells. E-cadherin has long been known as an epithelial cell marker and its expression is significantly reduced in carcinomas of epithelial origin.

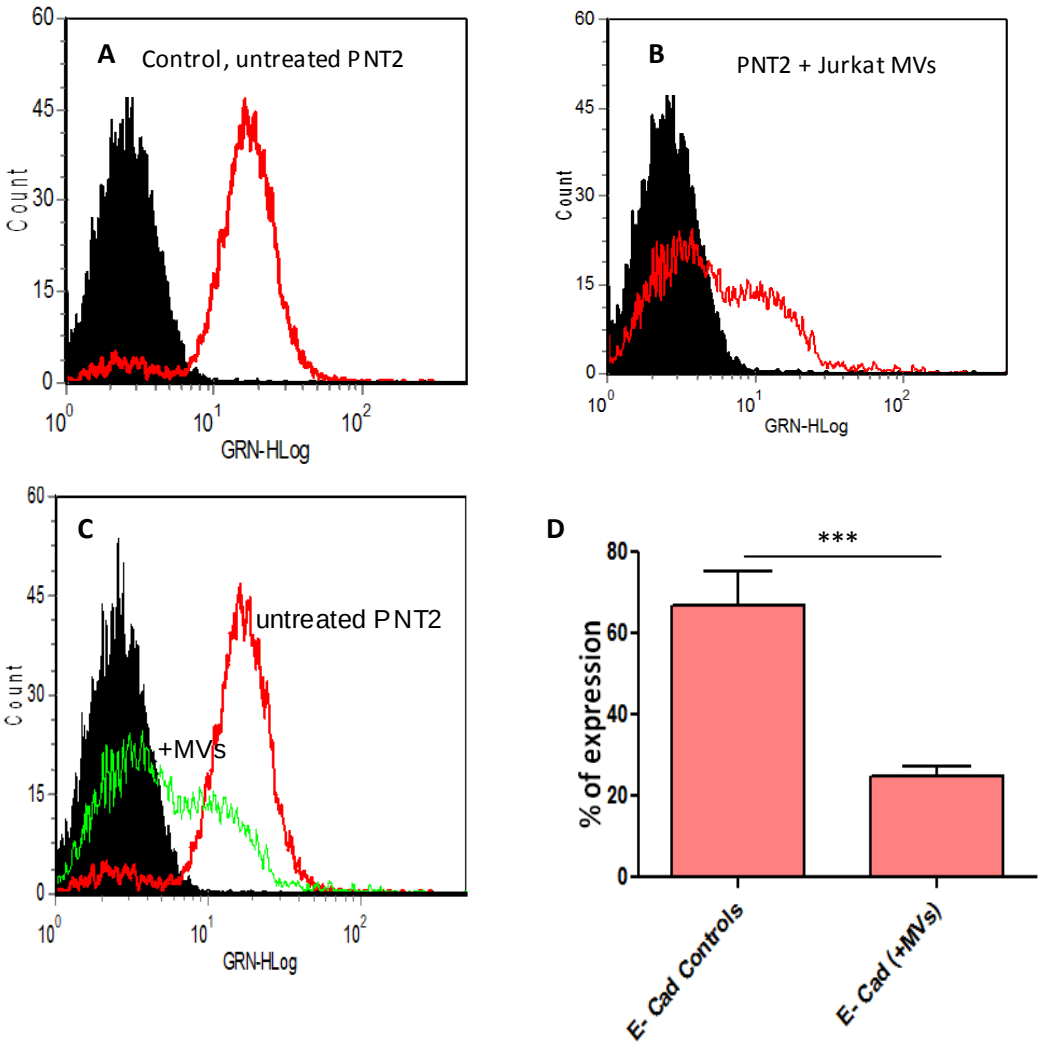

Figure 3.3 Decreased expression of E-Cadherinon PNT2 cells treated with Jurkat MVs. This figure shows the effects of Jurkat cell MVs on E-cadherin expression of PNT2 cells. **(A)** The expression of E-cadherin in PNT2 cells under basal condition. (B) E-cadherin expression in PNT2 cells treated with 30 µg/ml Jurkat MVs. **(C)** Combination of **(A)** and **(B)**. **(D)** Shows the percentage reduction of E-cadherin in PNT2 cells treated with Blood cancer cells derived MVs. ****P<0.001*.

The adhesion molecule E-cadherin is generally recognised as an epithelial marker. Loss of this junctional protein causes epithelial cells to reorganise their cytoskeletal proteins and consequently gain a mesenchymal phenotype. Loss of E-cadherin further causes epithelial cells to grow apart and no longer establish an epithelial sheet, this being an important hallmark of epithelial cell lines. Reduction of E-cadherin protein expression in PNT2 cells from 69% in the control panel to 23% in Jurkat MV-treated cells caused loss of cell-cell contact. This reduction in almost all cases changes the phenotypic characteristic of epithelial cells, such that loss of adhesion molecules in PNT2 cells can be accompanied by a gain of mesenchymal characteristics. In order to determine whether PNT2 cells had acquired mesenchymal characteristics, PNT2 cells were observed under the microscope for morphological changes and the cells were further stained with anti-E-cadherin antibody for fluorescent microscopic analysis.

3.2.4 Fluorescent microscopic analysis shows loss of E-cadherin expression in PNT2 cells treated with T cell leukaemia-derived MVs

To determine whether loss of E-cadherin protein expression in PNT2 cells causes EMT, I grew the PNT2 cells on coverslips in 24-well plates. The cells ($5x10^5$/well) were seeded for 14 hours in medium supplemented with 10% MV-free FBS. Cells were then washed twice with serum-free RPMI, fresh complete growth medium being added into each well and 30 µg Jurkat MVs (protein quantification method 2.3.6) added into each well except control group. The experiment was incubated for 5 days at 37^oC, 5% CO_2. The cells were then washed three times with ice cold PBS and fixed with 4% PFA and labelled with monoclonal anti-E-cadherin antibody. In order to stain the nucleus, the coverslips were removed and stained with DAPI- VECTASHIELD medium.

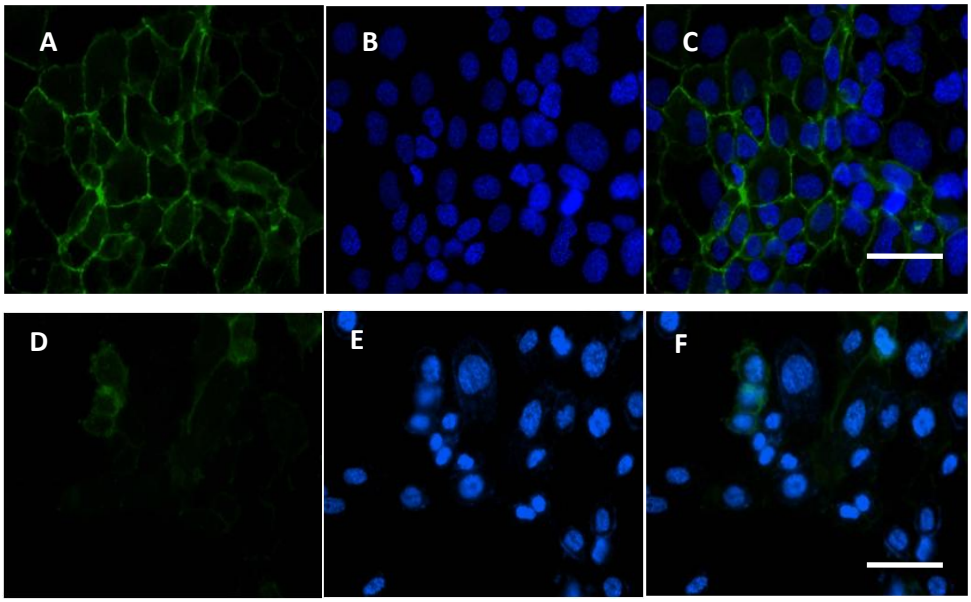

Figure 3.4 PNT2 cells treated with T cell leukaemia-derived MVs show marked loss of E-cadherin expression as determined by fluorescent microscopy. (A), expression level of E-cadherin in control, untreated PNT2 cells. **(B),** nuclear staining using DAPI-Vectashield. **(C),** combination of **(A)** and **(B)**. **(D),** expression level of E-cadherin on PNT2 cells treated with 30 µg/ml Jurkat MV for 5 days. **(E),** shows nuclear staining and **(F),** is combined **(D)** and **(E). Fig.3.4** illustrates the level of E-cadherin molecules and also the anatomical location of this protein on control PNT2 cells. Comparing **(A)** with **(D)** treatment groups, E-cadherin expression significantly decreased in MV-treated cells. Scale bar = 50µm.

3.2.5 Flow cytometric analysis of Vimentin expression following treatment of PNT2 cells with Jurkat MVs

Vimentin is as an intermediate filament protein which is an important part of the cytoskeleton present in almost all mammalian cells. It is mainly in the cytoplasm and partially in the nucleus of cells, and is recognised as a mesenchymal marker. The experiment for Vimentin expression in PNT2 cells was setup the same way as for E-cadherin. The only difference is that as Vimentin is a cytoskeletal protein expressed mainly in the cytoplasm, after fixing cells with 4% PFA, cells were permeabilized with Triton X-100 (0.1%) and then labelled with monoclonal anti-Vimentin antibody.

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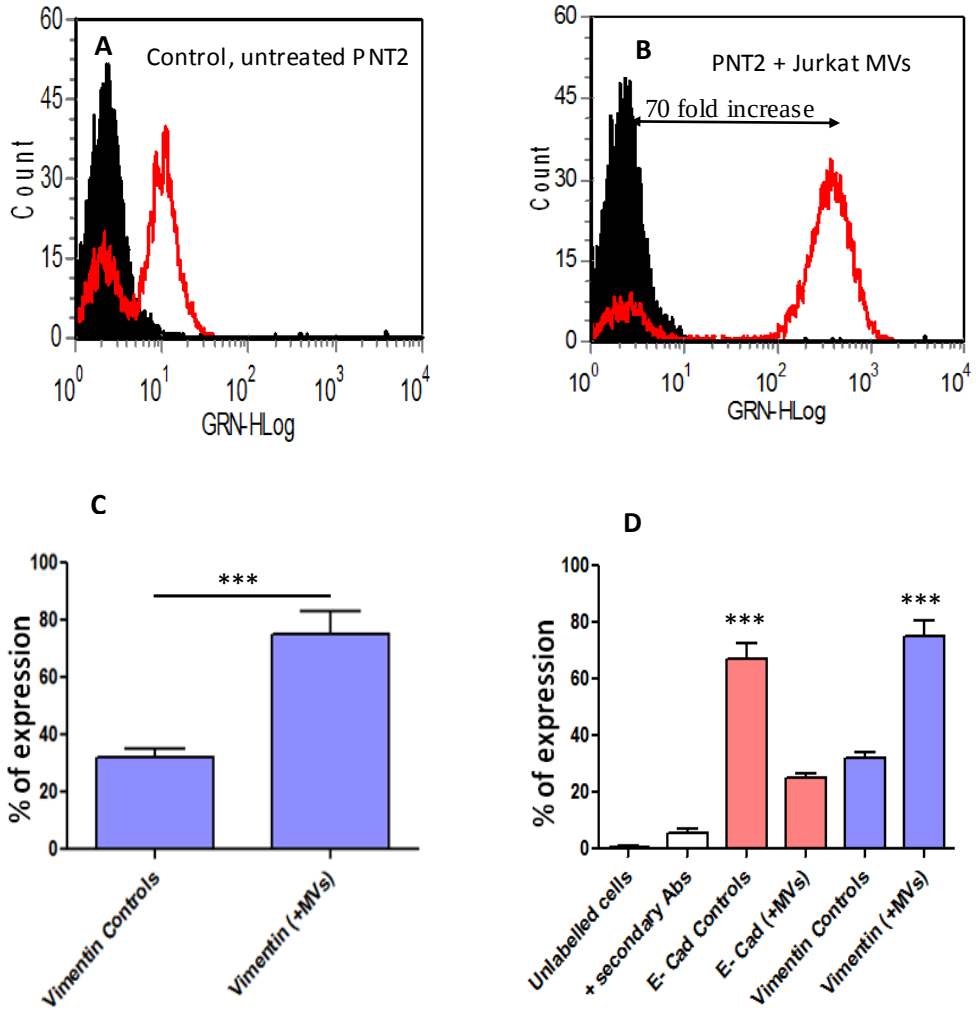


Figure 3.5 Expression of Vimentin is significantly up regulated on PNT2 cells upon treatment with T cell leukaemia (Jurkat) MVs. (A), Histogram showing Vimentin expression on untreated control PNT2 cells by flow cytometry. (B), Histogram showing Vimentin expression on PNT2 cells treated with Jurkat MVs (30 µg/ml). (C), Bar chart showing expression level of Vimentin in (A) and (B). (D), Summary of expression level in PNT2 cells of Vimentin and E-cadherein upon addition of Jurkat MVs, together with controls panels. ****P<0.001*.

The results in **Fig.3.5** indicate that normal prostate epithelial cell lines cultured with MVs derived from T lymphocyte cancer cells acquire phenotypic changes. The PNT2 cells appeared mesenchymal-like, and at the molecular level cells in the experimental group expressed high levels of the mesenchymal marker, Vimentin **(C)** and significantly low levels of E-cadherin **(D)**. This data indicates that MVs derived from leukaemic T lymphocytes induce EMT in normal prostate epithelial cell lines. However, it is still not clear that the phenotypic changes taking place are necessarily normal developmental EMT, as opposed to inflammatory or tumorigenic EMT. If we consider the background of the MVs used (derived from a T cell leukaemia) it could probably be either of them.

3.2.6 Fluorescent microscopic analysis shows significantly more Vimentin to be expressed in PNT2 cells treated with T cell leukaemia-derived MVs

Following measurement of the cytoskeletal protein, Vimentin in PNT2 cells by flow cytometry the cellular location and extent of expression of this intermediate filament protein was assessed using immunofluorescent labelling. The method was as described (2.2.6), except that permeabilization of the target cells was carried out using Triton X-100 (0.1%). Vimentin is a mesenchymal marker being expressed only in mesenchymal cells, and it is the most important cytoskeletal protein participating in tumour progression and metastasis, malignant cancer cells

expressing higher levels than benign tumour cells. Vimentin is closely associated with cell motility both in normal and cancer cells.

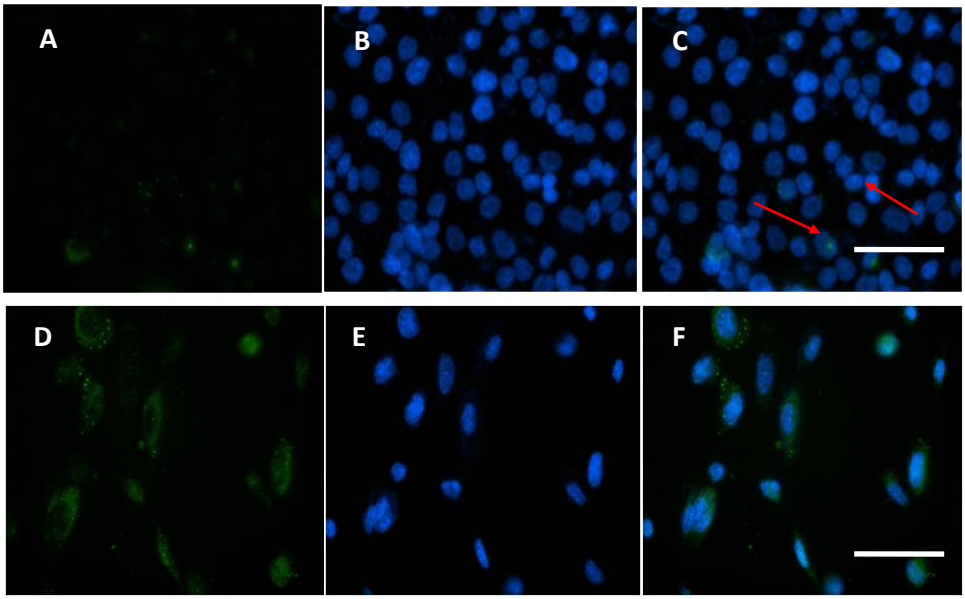


Figure 3.6 Expression of the cytoplasmic intermediate filament protein, Vimentin, is significantly increased in normal prostate epithelial cells treated with blood cancer cell-derived MVs. (A), Vimentin expression on untreated, control PNT2 cells as assessed by fluorescent microscopy. **(B),** shows DAPI-Vectashield staining of control PNT2 cell nudei. **(C),** is the combined image of **(A)** and **(B)**. **(D),** shows increased fluorescence with anti-Vimentin MAb on PNT2 cells treated with 30 µg/ml Jurkat-derived MVs. **(E)** indicates the nuclei of the cells in **(D)** and **(F)** represents the combined image of **(D)** and **(E)**.Scale bar = 50µm.

Fig.3.6 shows the amount of expression of the cytoskeletal protein, Vimentin, in PNT2 untreated, control cells and in the presence of blood cancer cell derived-MVs. The expression of Vimentin is significantly lower in normal epithelial cells (red arrows in **Fig.4.6.A&C)** compared with the amount of Vimentin expressed in PNT2 cells treated with Jurkat cell-derived MVs **(Fig.4.6.D&F)**. Only epithelial cells undergoing EMT in cancer or fibrosis, transforming into mesenchymal-like cells, express the intermediate filament protein, Vimentin. These results demonstrate that cancer cell-derived MVs actively participate in the EMT process. MVs, as intercellular vectors carrying cytokines, receptor proteins and miRNAs are able to induce changes in recipient cells. Indeed previous findings at CMIRC showed MVs able to induce terminal differentiation of monocytic cells through the delivery of TGF-β, tethered to the surface of the MVs (186).

4.2.7 Anti-TGF-β1pre-exposure of Jurkat MVs reduces the down regulation of expression of E-cadherin upon addition to PNT2 cells

TGF-β1 is an important inducer of EMT especially in the inflammatory and cancer microenvironment. In an attempt to see whether TFG-β1 on Jurkat cells can participate in the process of EMT of prostate epithelial cells, MVs were treated with anti-TGF-β1 antibody (15μg/ml),incubated for 2 hrs and centrifuged at 25,000 *g* for 2hrs to remove any non-specifically bound antibody. The MVs were resuspended in MV-free, sterile, PBS and the experiment was set up as before (3.2) in 24-well plates, the PNT2 cells being exposed to T cell leukaemia (Jurkat) MVs for 5 days. There now was a reduced % of cells expressing E-cadherin upon treating with MVs pre-incubated with MAb, anti-TGF-β1 **(Fig. 4.7A)**? The reduction of expression with MVs (66%) was reduced to only a 40% reduction when TGF-β was blocked with antibody **(Fig. 4.7, B and C).**

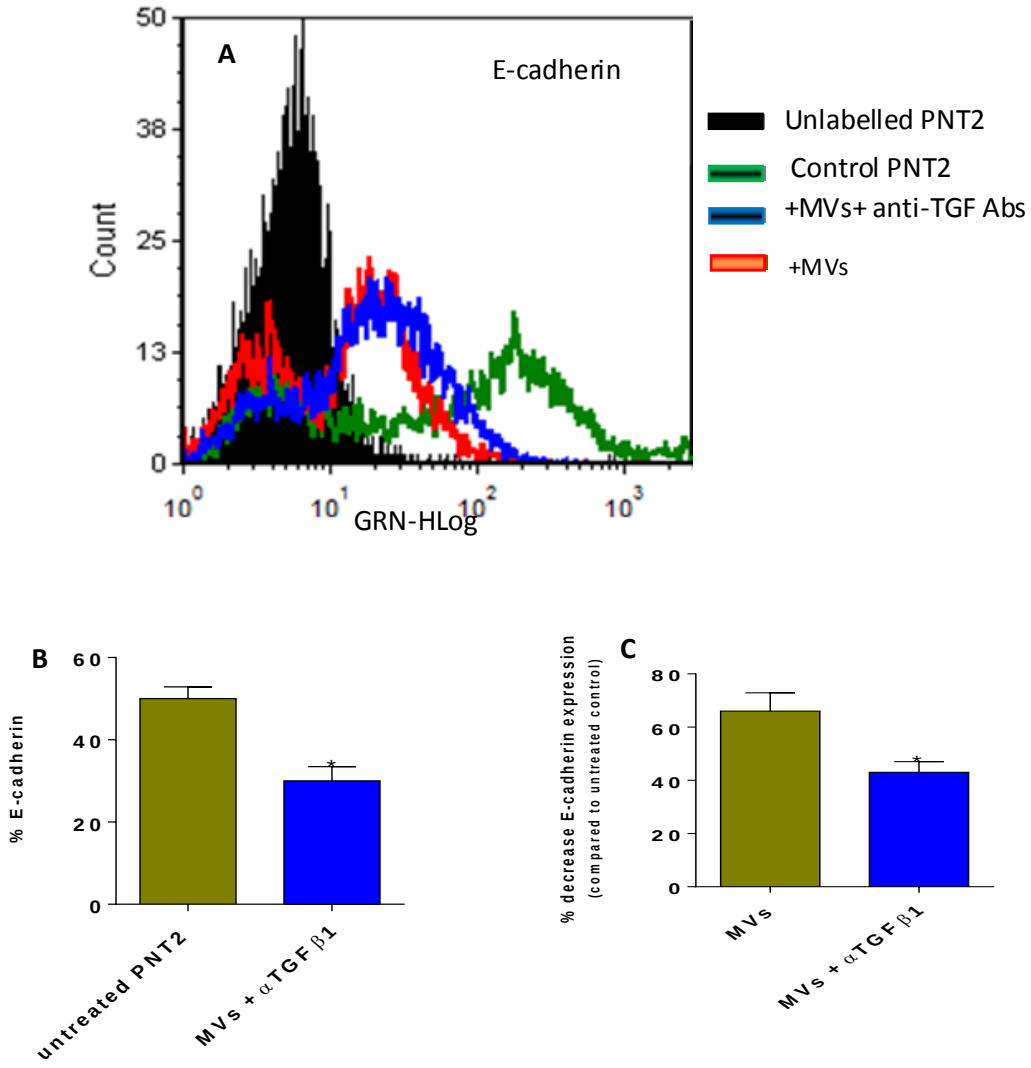


Figure 3.7 Flow cytometric analysis of reduction in E-cadherin expression following treatment of PNT2 cells with Jurkat MVs pr-incubated with anti-TGF-β1 (A) shows the histogram from the flow cytometry analysis of E-cadherin expression on PNT2 cells, 5 days following addition of T leukaemia cell-derived MVs where TGF-β1 had been pr-blocked with MAb against TGF-β1. In **(B)** the level of expression of E-cadherin is expressed as the % of positive cells. (C) Shows the percentage reduction of E-cadherin in the presence or not of anti-TGFβ1. ***p<0.001; **p<0.01; *p=0.05.

That EMT was still occurring, even in the absence of at least some of the MV-derived TGF-β1 was confirmed by microscopy analysis of the PNT2 cells following addition of Jurkat MVs in terms of maintenance of mesenchymal morphology **(Fig. 3.8)** and continued decreased expression of E-cadherin (albeit not as reduced, as determined quantitatively, **Fig. 3.7)**. It is through the extracellular domain of E-cadherin that neighbouring epithelial cells adhere together to form junctional complexes and when PNT2 cells are cultured with MVs, and by microscopy analysis it is clear that this junctional complex is degraded. However the morphology of the PNT2 cells is not completely disturbed. At the microscopic level Jurkat MVs in the presence of anti-TGFβ1 also showed reduced expression of E-cadherin on PNT2 but it is not possible to make quantitative comparisons as to the degree of reduction compared to that achieved by MVs alone **(Fig.3.4)**.

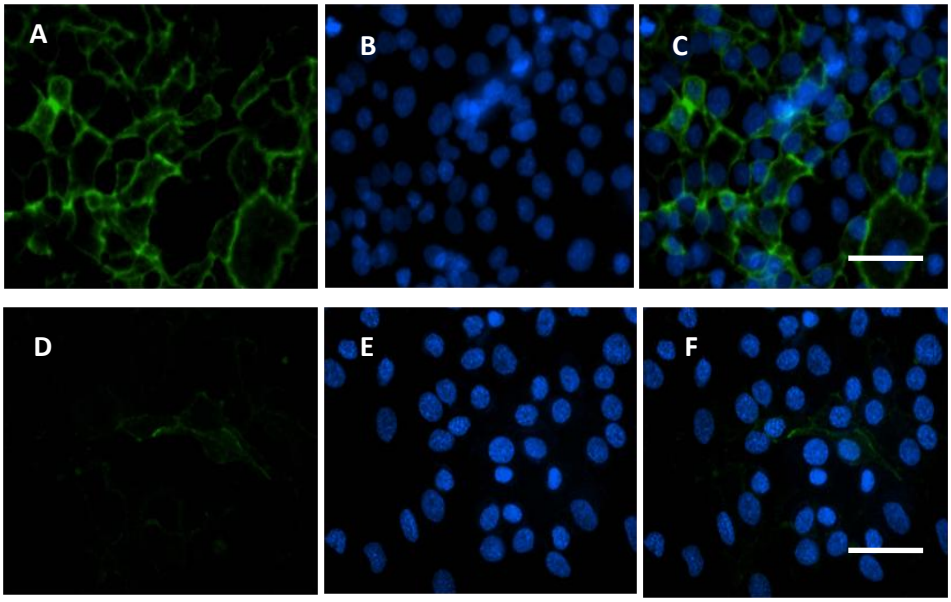


Figure 3.8 PNT2 prostate cells express E-cadherin on the cell surface as shown by fluorescent microscopy but this is reduced upon addition of Jurkat cell-derived MVs pre-incubated with MAb, anti-TGF-β1. (A), E-cadherin expression at the cellular junctions of untreated, control PNT2 cells as assessed by fluorescent microscopy. **(B),** shows DAPI-Vectashield staining of control PNT2 cell nuclei. **(C),** is the combined image of **(A)** and **(B)**. **(D),** shows increased fluorescence with anti-E-cadherin MAb on PNT2 cells treated with 30 µg/ml Jurkat-derived MVs. **(E)** indicates the nuclei of the cells in **(D)** and **(F)** represents the combined image of **(D)** and **(E)**. Scale bar = 50µm.

3.2.8 Jurkat MVs pre-exposed to anti-TGF-β reduce the up regulation of expression of Vimentin upon addition to PNT2 cells

The role of TGF-β1 in the expression of the intermediate filament Vimentin is demonstrated during wound healing as well as cancer progression and metastasis. During inflammation, cytokines released by various immune cells participate in fibrotic EMT. In the case of carcinomas of epithelial origin, the EMT signalling pathway is used by cancer cells to invade the local microenvironment and to metastasize to distant locations to establish secondary tumours. There is always a strong positive correlation between the level of Vimentin expression by cancer cells and prognosis; more aggressive types of cancer cells expressing higher levels of Vimentin than benign epithelial tumours. In order to examine the possible role of TGF-β1 present on MVs in causing EMT, MVs were treated with monoclonal anti-TGF-β1 before adding them as an activator of EMT signalling to prostate epithelial cells.

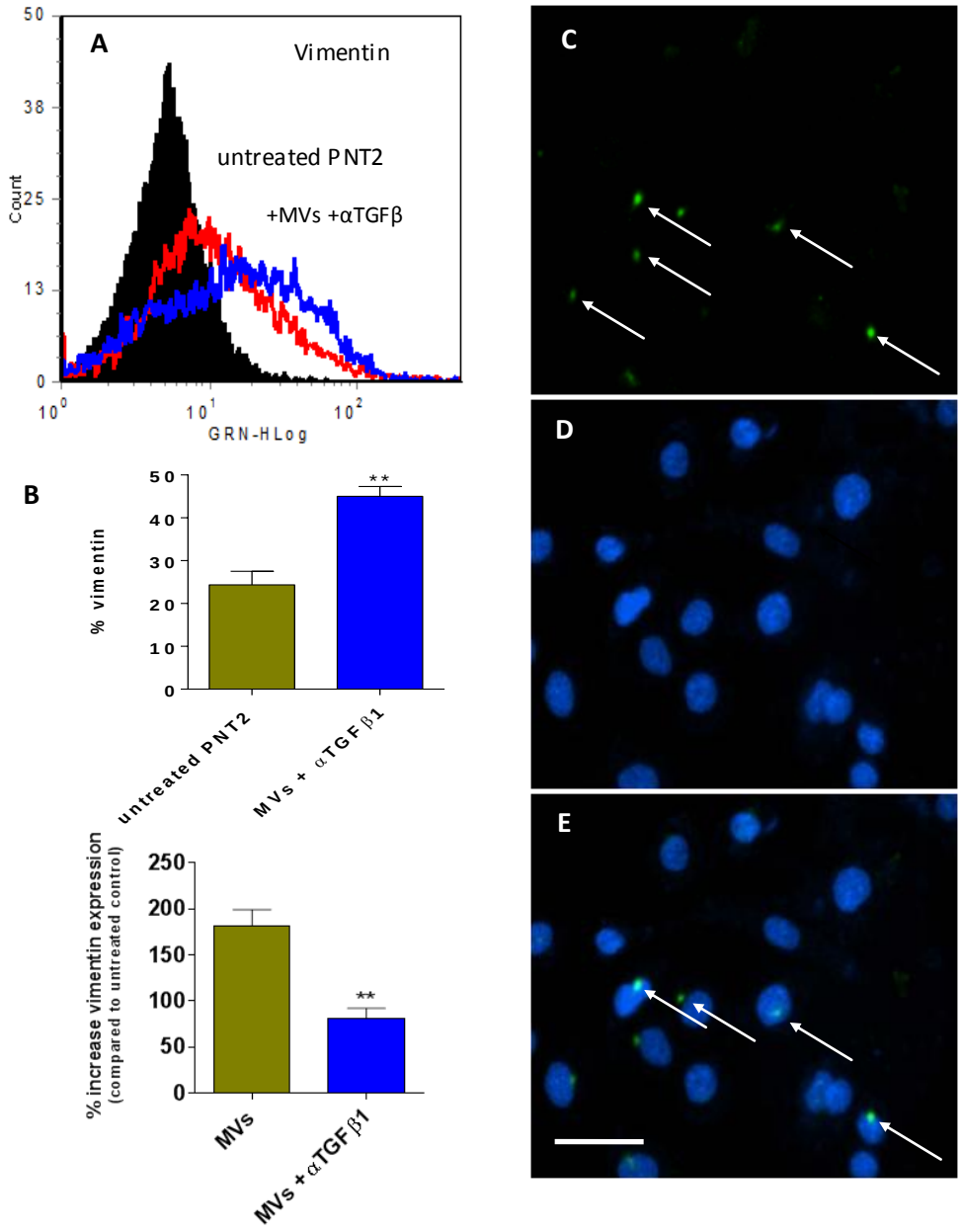


Figure 3.9 The increased expression of Vimentin on PNT2 cells upon exposure to Jurkat MVs was decreased if pre-exposed to anti-TGFβ MAb (A), expression levels of Vimentin in PNT2 cells treated with Jurkat cell derived-MVs and with Jurkat MVs pre-treated with anti-TGFβ1 MAb. **(B)**, bar charts showing expression level of Vimentin in control, untreated PNT2 cells and in PNT2 cells treated with Jurkat MVs pre-exposed to MAb against TGF-β1. The second graph compares the % increase between MV alone treatment and MV pre-exposed to anti-TGFβ1. **(C)**, Expression level of Vimentin on PNT2 cells treated with leukaemia cell derived-MV pre-treated with MAb against TGF-β1. **(D)**, Nuclear staining of using DAPI-Vectashield. **(E)**, Combination of **(C)** and **(D)**. Scale bar is= 50µm ***p<0.001; **p<0.01; *p=0.05.

Having shown Jurkat cell derived-MVs to induce EMT in normal PNT2 epithelial cells, in the following experiments I pre-incubated MVs with MAb against TGF-β1 in order to attempt to neutralize any effect of TGF-β on inducing EMT. I found that the % expression of Vimentin was reduced from a 150% increase with MVs compared to untreated control to 80% when using MVs pre-incubated with anti-TGFβ1 implying that despite blocking microvesicular TGF-β1 EMT is still being maintained but at lower level and that TGF-β1 may be playing a partial role. Vimentin expression was still clearly detectable in the PNT2 cells treated with blood cancer cell derived-MV using fluorescence microscopy analysis (white arrows **(Fig.3.9 C** and **E).** According to these results I propose that MVs derived from leukaemia blood cancer cells induce EMT in normal prostate epithelial cells and that this may partially be due to the effect of TGF-β that is carried by these MVs.

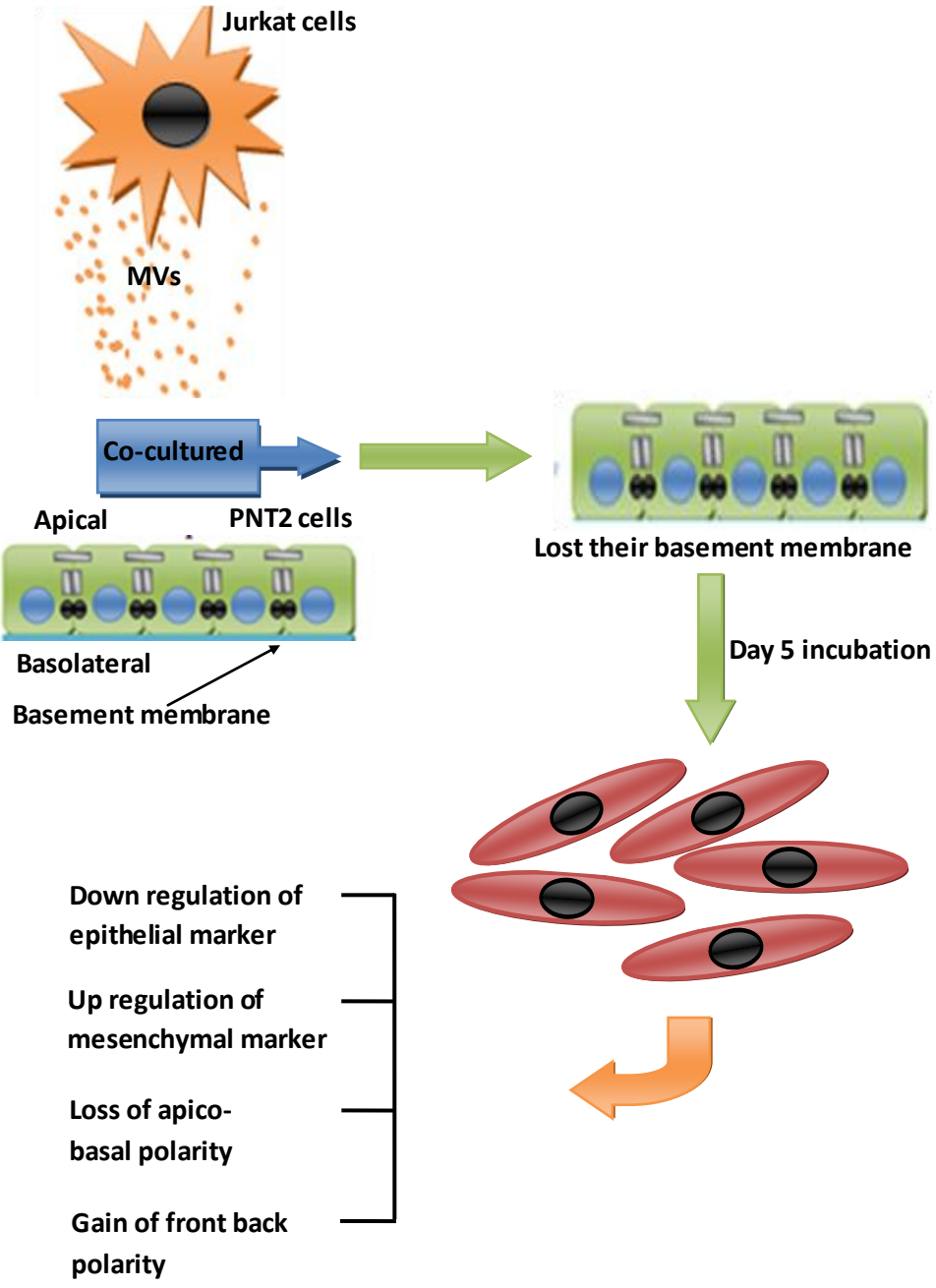
3.3 Summary

EMT is a physiological process in which epithelial cells are disseminated from the surrounding epithelial tissue and acquire mesenchymal-like properties. There are three types of EMT identified so far that depend on the type of inducer namely whether it is physiological, fibrotic or cancerous. Cells undergoing EMT are capable of migrating from the epithelial layer to distant sites and the transformation of epithelial cells to mesenchymal-like cells can be temporary or permanent depending on the inducers. There is much evidence in the literature suggesting that immune cells such as CD8 T positive cells can produce EMT mediators. In this chapter I have tried to ascertain whether MVs derived from T lymphocyte cancer cells (Jurkat cells) carry EMT mediators from the parent cells that have the potential to induce EMT in normal prostate epithelial cells. PNT2 cells were therefore grown in the presence of Jurkat cell-derived MVs for up to 5 days. The influence of blood cancer cell derived MVs was determined by the measurement of the epithelial marker (E-cadherin) and mesenchymal marker (Vimentin) on control, untreated PNT2 cells and MV-treated PNT2 cells. EMT was confirmed by:

1. Significant reduction in the expression of epithelial marker (E-cadherin);
2. Significant increase in the production of mesenchymal marker (Vimentin).
3. Morphological changes that occur during EMT in epithelial cells, gaining mesenchymal characteristics.

In order to understand the mechanism of the observed EMT, I investigated the TFG-β signalling to see if this cytokine carried by these MVs could cause EMT in PNT2 cells. It is one of the most important cytokines produced by T cells and is present in MVs. Furthermore, TGF-β is also believed to be one of the causes of EMT in the cases of fibrosis and carcinoma of epithelial origin. According to these results, by blocking TGF-β signalling the MVs still causes reduction of epithelial marker (E-cadherin) and increase expression of mesenchymal marker (Vimentin) but to a lesser extent than with MVs alone. It is important to consider that the TGF-β may not have been activated fully on delivery of the Jurkat MVs to PNT2 cells as it is in a latent complex which needs either contact with integrins or activation by plasmin to release the active homodimer. If this process was limited the blocking anti-TGF-β may not have been fully able to block any TGF-β-mediated effects on EMT. The process of TGF-β activation on MVs is part of ongoing work at Cellular & Molecular Immunology Research Centre.

3.4. Schematic diagram of immune cell derived MVs induce EMT in PNT2 cells



4. Causes and functional consequences of EMT in normal epithelial cells induced by leukaemia cell derived-MVs

4.1 Introduction

Epithelial Mesenchymal Transition is a highly specialized physiological process that is defined by loss of epithelial characteristics and acquisition of a mesenchymal phenotype and is regulated by various transcription factors including members of the *Zeb*, *Snail* and *Twist* families. The functional consequences of EMT generally depend on the EMT regulators and this phenotypic process is not always permanent. During morphogenesis primary epithelial cells undergo EMT to induce morphogenesis such as neural crest formation and heart valve formation. Therefore, the functional consequences of this type of EMT are purely physiological taking place for developmental purposes. The EMT involved during the process of wound healing is termed fibrotic EMT. In this process keratinocyte cells at the edge of the wound transform to mesenchymal-like cells. This type of EMT is also a physiological function of cells in the epithelial tissue during cell injury and inflammation. In the case of epithelial cell injury and inflammation, fibrotic EMT is activated to close the gap and prevent pathogens getting deeper into tissues and organs in order to prevent further damage. The purpose of this type of EMT is therefore protective. The EMT taking place during cancer progression is called cancerous EMT and is a pathological process which takes place in carcinoma of epithelial origin. Cancer cells are believed to highjack this physiological process, firstly, to disseminate from the primary tumour and invade tumour microenvironment and secondly, to get into blood circulation and the lymphatic system in order to establish a secondary tumour in the distant organ.

In this study, I was trying to demonstrate the type of EMT induced by cancer cell-derived MVs on normal prostate epithelial cells that was performed in the first chapter of this thesis. I therefore performed a range of assays to assess the functional consequences of this particular MV-induced cancerous EMT.

4.2 Results

4.2.1 Cytosolic calcium measurement

Calcium is believed to play an important role as a secondary messenger in multiple intracellular pathways. The role of calcium signalling in the induction of EMT in breast cancer cells has been well documented over the past years. Here in the second chapter of this thesis, I have examined whether cancer cell-derived MVs induce EMT associated with a remodelling of $Ca2^{+}$ in the normal prostate epithelial cell lines. To assess the cause of EMT in PNT2 cells treated with Jurkat cell-derived MVs, epithelial cells were exposed to MVs pre-treated by Ca^{2} chelation (BAPTA-AM). As well as this experiment being set up (3.3.5) the level of intracellular Ca^{2} was also assessed by ion chromatography (3.3.3.2). The mechanism of ion chromatography is based on the elution time, or time it takes for the ion to move through the column, varying for each ionic species as they elute from the column separately (with increasing ionic strength of the eluent). The concentration of ions moving through the column at a particular time is represented by the height and the breadth of the peaks and can be correlated to the concentration of a particular species, such as Ca^{2} for example, **(Fig.4.1B)** in the sample solution. **Fig.4.1 A&B** display the typical data output from an ion chromatography run for standard curve preparation.

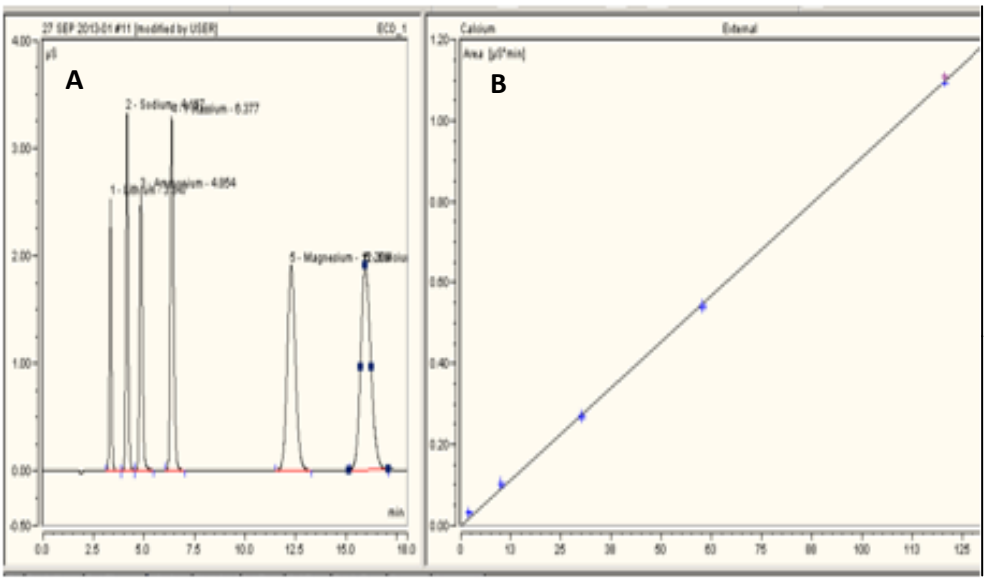


Figure 4.1 Standard curves of six cations from an ion chromatography. (A), represents the 6 separate ions from a known sample solution. **(B),** Ca^2 standard curve of absorbance in linear function with respect to µg/ ml concentration.

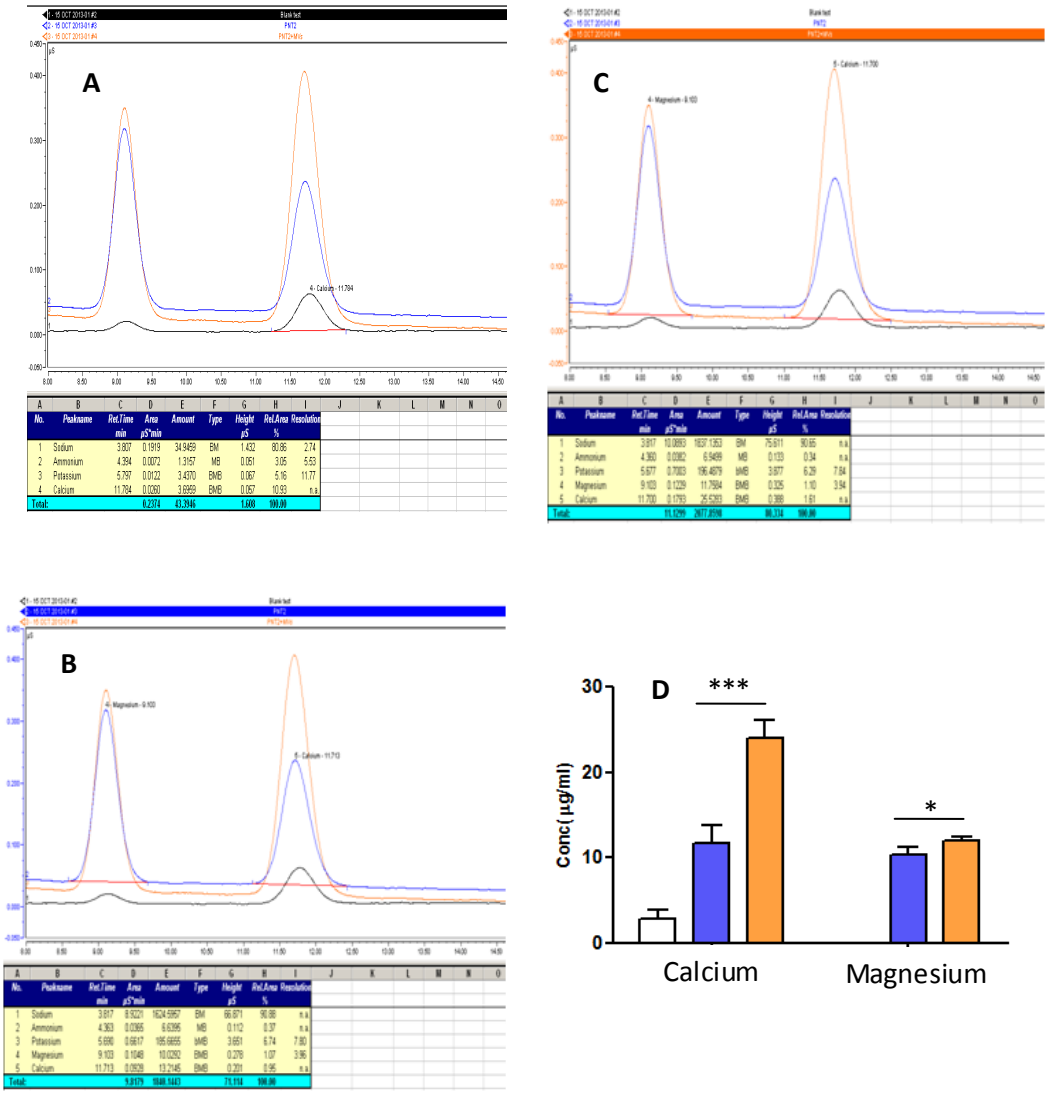


Figure 4.2 Ca^{2} and Mg^{2+} concentrations are increased in PNT2 cells treated with leukaemia cancer cell derived-MVs. (A), shows the concentration of Ca^{2} and Mg in µg/ml in blank control. **(B),** is the concentration of Ca^{2} and Mg^{2+}in µg/ml in PNT2 untreated control cells. **(C),** shows the concentration of Ca^{2} and Mg^{2+} in PNT2 cells treated with Jurkat cell derived-MVs. **(D)** is a summary of concentration levels ofCa^{2} and Mg^{2+} upon addition of Jurkat MVs, together with blank control and PNT2 untreated control cells. ****P<0.001, **P<0.01, *P<0.05.*

Measuring the level of six cations; lithium, sodium, ammonium, Potassium, Magnesium and calcium in blank control, untreated PNT2 cells and PNT2 cells treated with leukaemia cell derived-MV which was measured by ion chromatography. Double distilled water was used here as a blank control that was added onto the cells pellets, both in control cells and treated panels before lysing them. This result demonstrates that PNT2 cells exposed to Jurkat cell derived-MVs causes changes in the cytosolic concentrations of sodium, potassium, magnesium and calcium, changes in calcium appearing to be most significant. The cytosolic concentration of calcium in PNT2 control, untreated cells **(Fig.4.2 B)** was 10. 2145 µg/ml after blank correction and increased to 22.5283µg/ml **(Fig.12.C)**, in PNT2 cells were treated with Jurkat cell derived-MVs. The difference between control PNT2 cells and treated PNT2 cells, in terms of potassium cations is 1.5µg/ml and sodium is 200µg/ml. Therefore, 45 min treatment of PNT2 cells with leukaemia cell derived-MVs causes increased cytosolic concentrations of these cations. According to these results, Jurkat cell derived-MVs increased the cytosolic concentration of calcium, magnesium, sodium, and potassium in PNT2 cells. The results were highly significant, with a decreasing order of significance from calcium, magnesium, sodium through to potassium (****P<0.001, *P<0.05*). This experiment was carried out to examine if the changes in concentration of cytosolic cations occur after adding MVs to the PNT2 cells. Although the concentration of the other four cations increased upon addition, the result was highly significant for cytosolic calcium ions only (***P<0.01*). This could be an indication of phenotypic changes in PNT2 cells treated with blood cancer cells-MVs that

is probably associated with remodelling of calcium influx pathways. MVs caused an increases of cytosolic magnesium ions in the experiment panel and the result was significant (*$P<0.05$). The correlation between cytosolic concentrations of these cations especially calcium and EMT are well documented in epithelial cells. Epithelial growth factor induces EMT in epithelial cells in the presence of high level of calcium in the cytoplasm of the target cells. Following on from this it was important to find out if Jurkat cell derived-MVs cause EMT in PNT2 cells by increasing the cytosolic calcium concentration.

4.2.2 Blocking intracellular calcium

In order to determine the effect of cytosolic calcium concentration on EMT, a calcium chelator (BAPTA-AM) was used to chelate intracellular calcium in PNT2 cells treated with blood cancer MVs. The experiment was set up the same way as before (section 3.3.5), except that the PNT2 cells were pre-incubated with BAPTA-AM. Before the PNT2 cells were exposed to blood cancer MVs, the cells were seeded for 24 hrs, and then washed twice with serum-free medium. BAPTA-AM (0.2µM) was then added to each well except the control group. The plate was then incubated for 15 min and then washed three times with serum-free medium. Fresh complete growth medium was added into each well plate together with 30µg/ml of Jurkat cell derived-MVs into the experiment panels.

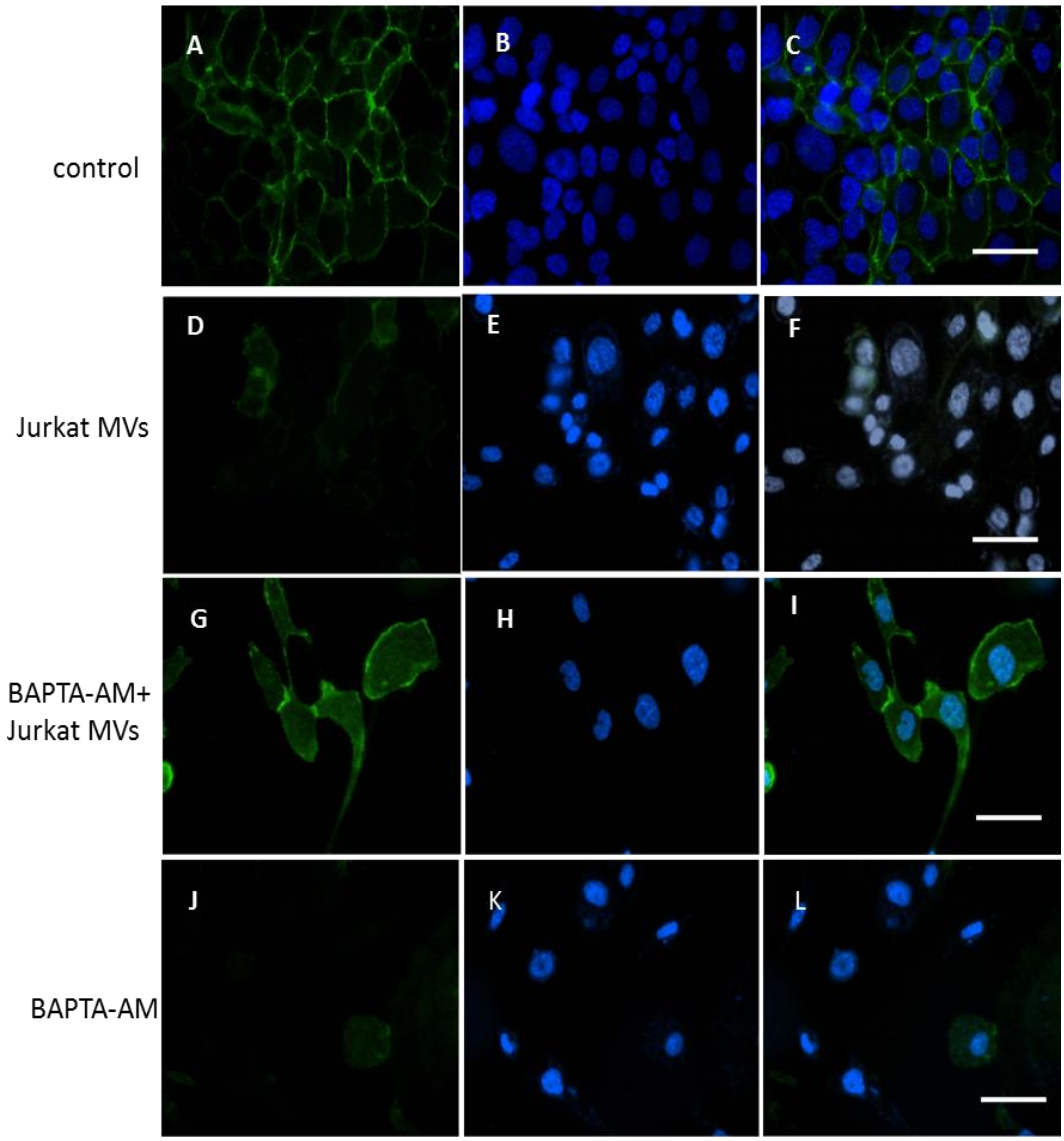


Figure 4.3 Fluorescence microscopic analysis of PNT cells pre-treated with BAPTA-AM exposed to leukaemia cell derived-MVs acquire phenotypic changes. (A), Expression level of E-cadherin on control, untreated PNT2 cells. **(B),** Nuclear staining using DAPI- Vectashield. **(C),** Combination of **(A)** and **(B)**. **(D),** Expression level of E-cadherin on PNT2 cells treated with Jurkat MVs. **(E)** Nuclear staining using DAPI-Vectashield. **(F)** Combination of **(A)** and **(B)**. **(G)** pre-treated with BAPTA-AM and then exposed to 30 µg/ml leukaemia cell derived-MVs**. (H),** Nuclear staining using DAPI-Vectasheild. **(I),** Combination of **(G)** and **(H)**. **(J)**, Expression level of E-cadherin on PNT2 cells treated with BAPTA-AM. **(K),** Nuclear staining using DAPI-Vectashield. **(L),** Combination of **(J)** and **(K).**

Fig.4.3 demonstrates the effect of cytosolic calcium concentration on PNT2 cell morphology and E-cadherin protein expression. In this experiment prior to exposure of PNT2 cells to Jurkat cell derived-MVs, the cells were incubated with BAPTA-AM. Leukaemia cell derived MVs caused EMT in PNT2 cells, as we can see **(Fig.4.3 G** and **I**, compared to untreated controls, **Fig. 4.3, A** and **C**),PNT2 cells becoming stretched out, no longer showing the normal, uniform, round-shaped phenotype (classical epithelial sheet) of epithelial cells. However, although surface expression levels of E-cadherin had not diminished, there now was more of a cytoplasmic localization of E-cadherin implying an inhibition of calcium-mediated recycling (endocytosis and exocytosis) of E-cadherin. The morphology of the cells was however still altered (as for the Jurkat MV-treated PNT2 cells) acquiring a stretched out, more mesenchymal-like shape, suggesting partial EMT. PNT2 cells treated with BAPTA-AM only, maintained their morphology **(Fig.4.3 J and L),** but E-cadherin molecules appeared mainly in the cytosol of the target PNT2 cells. According to these findings, Jurkat MV-derived increased in cytosolic calcium might probably constitute one of the elements in the EMT process of normal prostate epithelial cells. As Ca^{2+}-mediated MMPs cleavage of the extracellular domain of E-cadherin is well described in EMT(187) and both MMP9 and Ca^{2+} are carried by MVs, this is an aspect of MV-mediated EMT investigated later in this chapter.

4.2.3 Transformed PNT2 cells (tPNT2) cells acquire EMT at the expense of cell proliferation

In this experiment I compared the proliferation and differentiation rate of the PNT2 and tPNT2 cells in a time-dependent manner. **Fig.4.4** shows the microscopic analysis of control, untreated PNT2 cells and PNT2 cells treated with 30 µg/ml leukaemia cell derived-MVs. **Fig.4.4 A,B** and C shows the typical features of normal epithelial cells which look round, globular and tend to grow together as an epithelial sheet.The proliferation rate of these control, untreated PNT2 cells was normal as by day 5 they appeared to be confluent. **Fig.4.4 D, E** and **F** demonstartes the role of leukaemia cell derived-MV on the phenotypic appearance of and proliferation rate of PNT2 cells. These cells have lost their epithelial characteristics, cells in the experiment panel (F) compared to untreated controls **(C)** looking stretched out and no longer growing together. Furthermore, to compare the proliferation rate of control and treated cells, it can be clearly seen that epithelial cells treated with Jurkat MVs proliferate significanty less than control untreated PNT2 cells **(Fig. 4.4 G).** Reduction in cell proliferation (which was not apparent until after the first 48hrs) might be due to the differentiation of PNT2 cells. In fact, Epithelial cells treated with T cell leukaemia derived-MVs differentiate into mesenchymal-like cells at the cost of their proliferation.The effect of Jurkat MVs on PNT2 proliferation and differentiation begin from day three onwards and reached a maximum by day five.

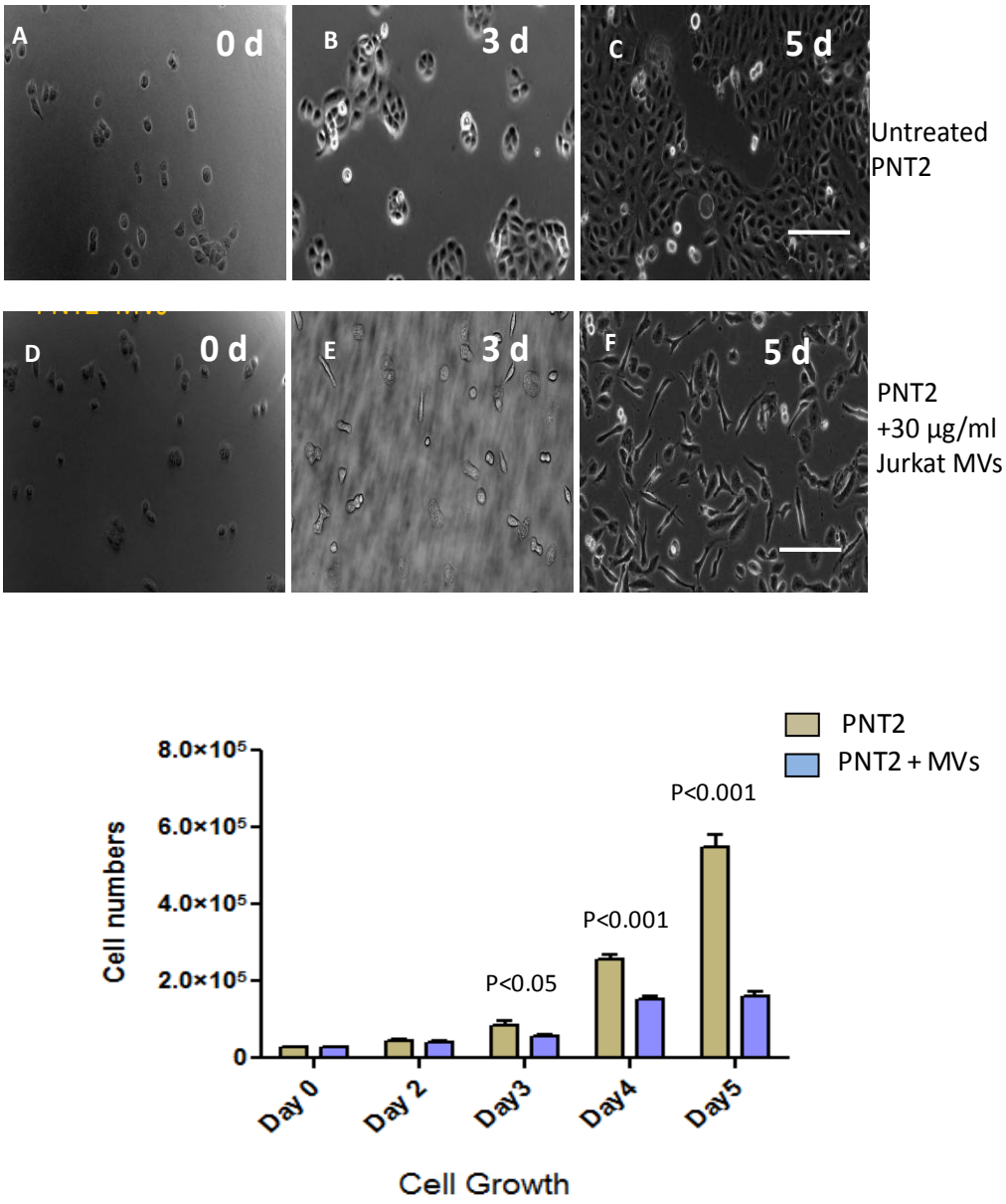


Figure 4.4 Jurkat cells derived-MVs causes significant reduction of cell proliferation in normal prostate epithelial cells. (A), shows the morphology of control, untreated PNT2 cells on day zero, day 3 **(B)** and day 5 **(C)**.The morphology of PNT2 cells treated with leukaemia cell derived-MVs is shown on day zero (**D**), day 3 **(E)** and day 5 **(F)**. **(G),** Summary of PNT2 cell proliferation and differentiation upon addition of Jurkat MVs, together with controls. **P<0.05,**P<0.001, ***P<0.001. Scal bar= 200µm.*

4.2.4 Serum starved tPNT2 cells resist apoptotic signals

Having shown Jurkat MVs to appear to induce EMT in benign, prostate, PNT2 cells, the aim now was to see how these Jurkat MV treated PNT2 (tPNT2) cells react to 48 hrs serum starvations, compared to normal prostate epithelial cells. An Apoptotic assay was carried out using Guava Nexin (Millipore) according to the manufacturer's instructions, using Guava EasyCyte technology. Apoptosis is a highly organised process of programmed cell death which ensures normal development and homeostasis of multi-cellular organisms. This process provides a balance between cell proliferation and differentiation and removal of unwanted cells. **Fig. 4.5 A, B** show the Guava EasyCyte analysis of apoptosis, summarised in **Fig. 4.5 C** and **D**. PNT2 cells exposed to serum starvation for 48 hrs lose the asymmetry of their cell membrane phospholipids resulting in the exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane, which binds to annexin V (X-axis) and 7-AAD a fluorescent compound with strong affinity for cellular DNA (Y-axis). The normal epithelial cells **(Fig. 4.5 A)**, exhibited more late apoptosis (15%) following 48 hrs serum starvation than transformed PNT2 **(Fig. 4.5 B)** cells, exhibiting just 7.5% late apoptosis. Similarly, while the viability of PNT2 serum starved cells fell to 84%, it only fell to 90% for Jurkat MV treated PNT2 cells.

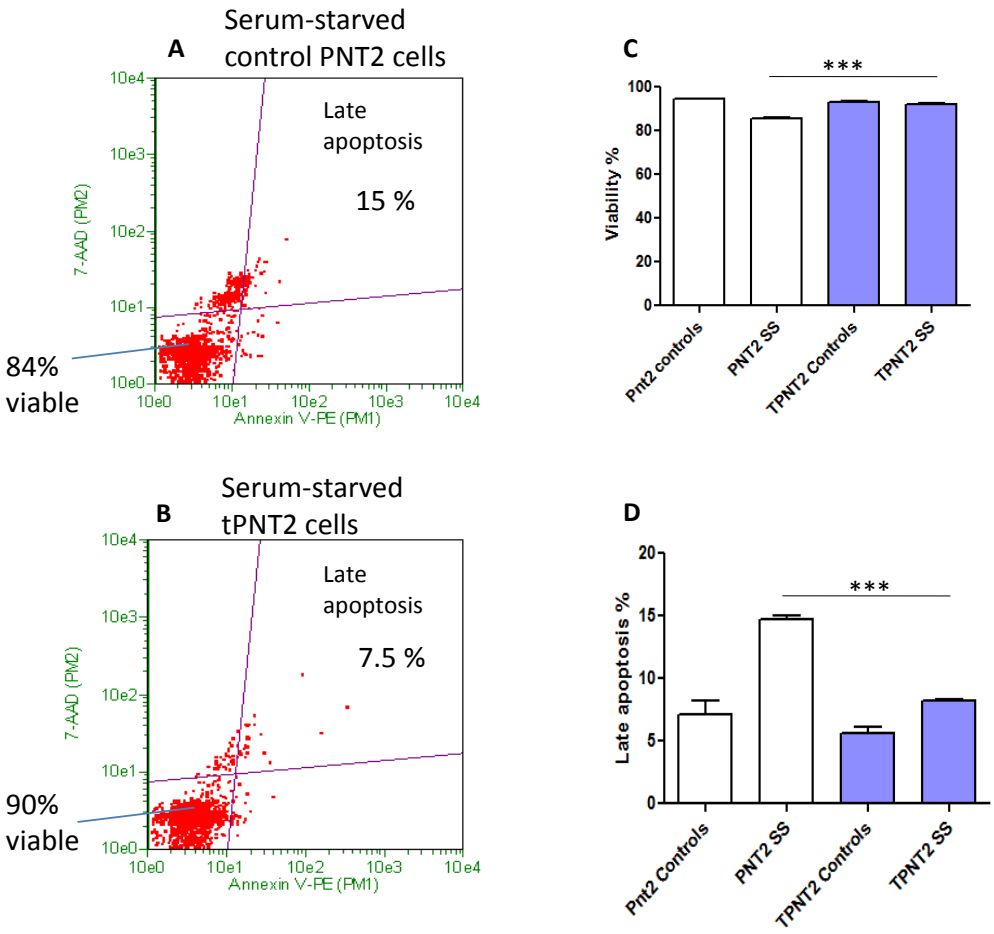


Figure 4.5 Normal prostate epithelial cells treated with Jurkat MVs show resistance against apoptosis under stress conditions (A), Apoptotic assay of control untreated PNT2 cells exposed to serum starvation using Guava EasyCyte. **(B),** Apoptotic assay of tPNT2 cells using Guava EasyCyte analysis. **(C),** shows the summary of cell viability of PNT2 and tPNT2 cells under stress condition for 48 hrs. **(D),** shows the % of late apoptotic PNT2 and tPNT2 cells after 48 hrs exposure to serum starvation.

4.2.5 tPNT2 cells show significantly reduced sensitivity to cytotoxic agent

This assay was set up to address the functional consequences of EMT induced by Jurkat cell derived-MVs. To begin with, any difference in sensitivity to a cytotoxic drug, such as docetaxel, was compared between Jurkat MV treated PNT2 and normal PNT2 cells. This assay has been applied to preclinical screening of new anti-neoplastic agents, cytogenetic analysis of human tumour specimens, and the identification of growth factors. Essentially PNT2 cells were treated with anti-cancer drug for 48 hrs and then viability and status of apoptosis determined as described before using Guava Nexin. PNT2 cells **(Fig.4.6 A)**, exhibited significantly higher susceptibility to docetaxel than Jurkat MV treated cells PNT2 **(Fig.4.6 B)** cells, showing 15% of the population (PNT2) in late apoptosis as opposed to 7.5 % (tPNT2 cells). Looking at cell viability it is clear that the Jurkat MV treated PNT2 cells are more resistant to the effect of docetaxel, viability having only reduced to 70% compared to 60% with control untreated PNT2 cells. Furthermore, this resistance to a chemotherapeutic drug is a clear indication of one of the functional consequences of EMT and interestingly, the mechanism bringing about such changes is normally through genetic or epigenetic changes.

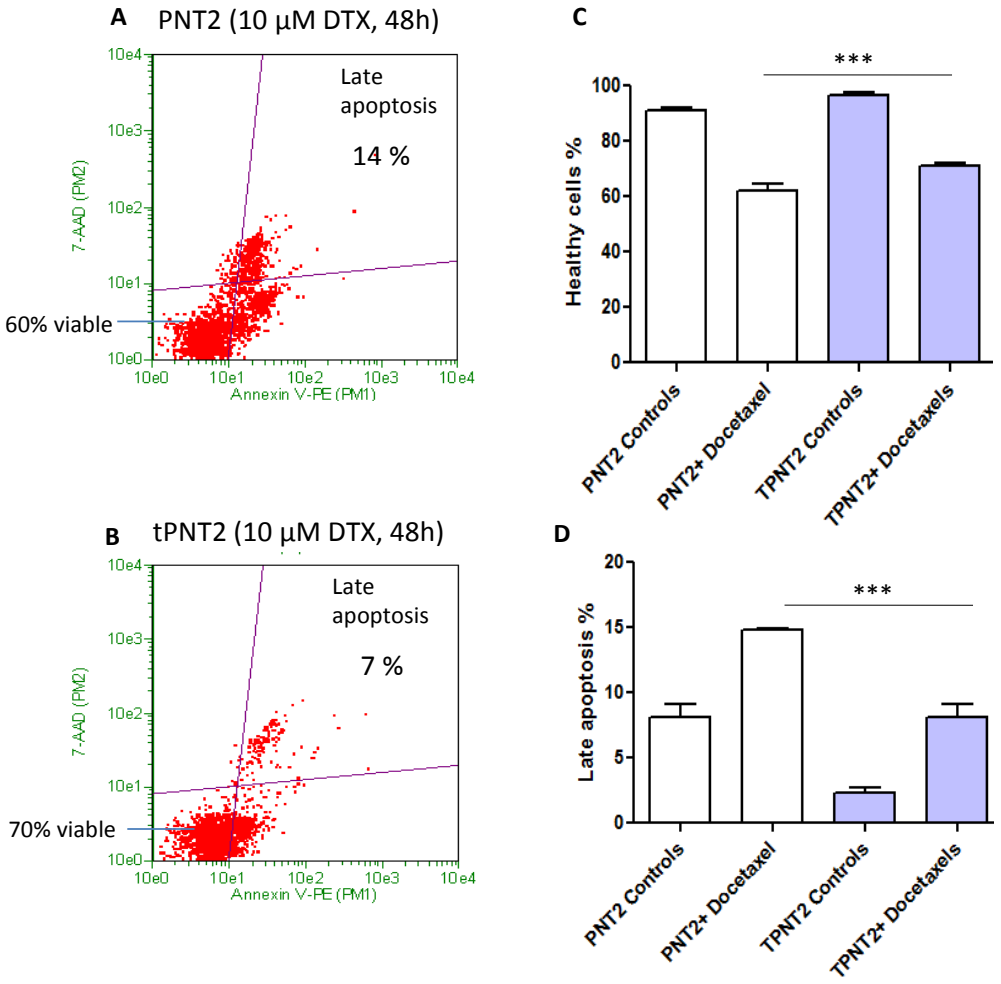


Figure 4.6 Transformed PNT2 cells showed resistance upon 48 hrs treatment with docetaxel (10 µM). A), Apoptotic assay of control untreated PNT2 cells using Guava Easy Cyte technique. **(B),** Apoptotic assay of Jurkat MV treated PNT2 cells using Guava EasyCyte analysis. **(C),** shows the summary of cell viability of PNT2 and Jurkat MV treated PNT2 cells control untreated cells and PNT2 and tPNT2 cells treated with 10µM docetaxel for 48 hrs. **(D),** shows the % of late apoptotic PNT2 and tPNT2 cells after 48 hrs treatment with docetaxel.

4.2.6 PNT2 cells treated with Jurkat MVs show resistance to docetaxel

This experiment was carried out to observe the cytotoxity effect of docetaxel on PNT2 cells and tPNT2 cells using 7-AAD staining and fluorescence microscopy. 7-AAD is a ready-to-use solution for the exclusion of non-viable cells in fluorescent microscopic analysis. This dye can pass through the membrane of damaged cells but not through intact cell membranes of normal, viable cells. It is a fluorescent intercalator with a high affinity for DNA. In this study, PNT2 cells treated with docetaxel showed greater vulnerability in their cell membrane compared to tPNT2 cells. **Fig.4.7** shows fluorescent micrographs of PNT2 cells **(Fig. A, B, C)** and tPNT2 **(Fig.4.7 C, D, E)** after exposure to 10 µM docetaxel for 48 hrs. Normal prostate epithelial cells treated with docetaxel for 48 hrs **(Fig.5.7B)** shows more cells with damaged plasma membrane compared to tPNT2 cells **(Fig.4.7 E)**. Resistance to chemotherapy demonstrates an important functional consequence of EMT as demonstrated in tPNT2 cells. PNT2 cells, as well as acquiring morphological changes after treatment with leukaemia cell derived-MVs, show increased resistance against apoptotic signals. This anti-apoptotic effect in tPNT2 cells in fact demonstrates that leukaemia cell derived-MVs not only induced phenotypic changes in PNT2, but presumably transferred certain cancer cell elements into PNT2 cells which capable of interfering with apoptotic signalling pathways responsible for the regulation of apoptosis in normal prostate epithelial cells.

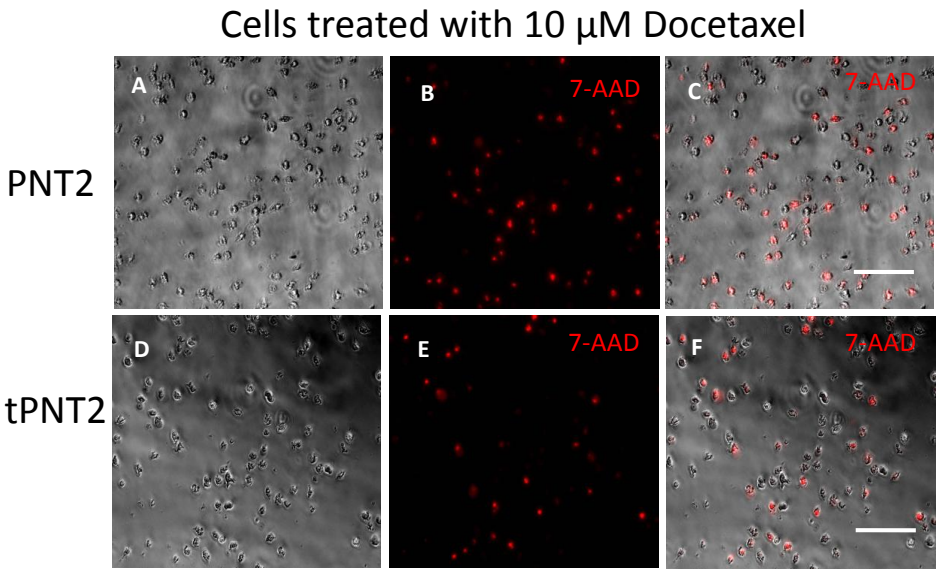
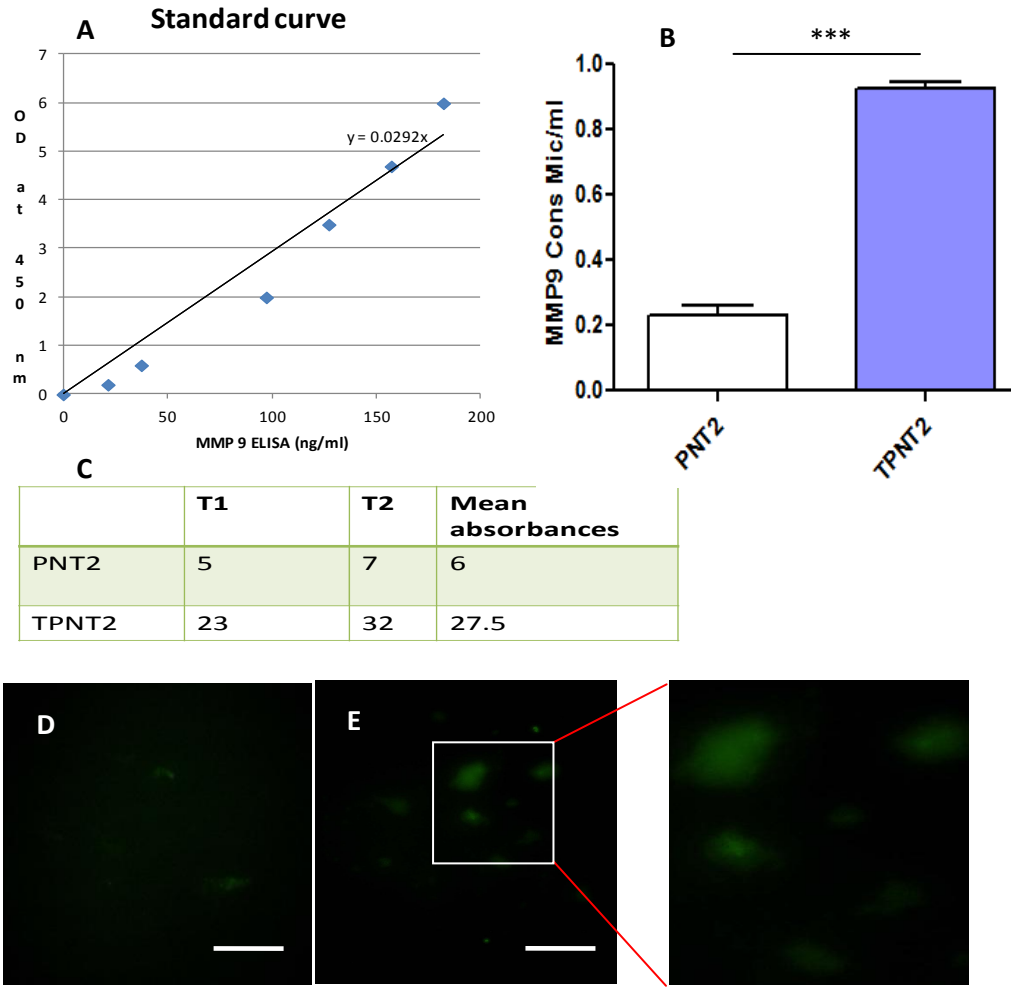


Figure 4.7 tPNT2 cells show reduced nudear staining (7-AAD) indicating reduced late apoptosis and greater resistance to Docetaxel compared to control, untreated PNT2 cells. (A), Phase contrast image of PNT2 cells treated with 10 µM docetaxel. **(B),** PNT2 cells treated with docetaxel and stained with7-AAD red dye. **(C),** combination of **(A)** and **(B). (D),** Phase contrast image of tPNT2 cells treated with 10 µM docetaxel. **(E),** tPNT2 cells treated with docetaxel and stained with7-AAD red dye. **(F),** combination of **(D)** and **(E)**. Scale bar is 50µm.

4.2.7 Enzyme Liked Immuosorbent Assay confirmed significant level of MMP9 expression in tPNT2 cells

MMP9 is a member of the family of structural and functional related endopeptidases. This enzyme belongs to the zinc-metalloproteinase family involved in the degradation of the extracellular matrix in normal physiological processes such as bone development and wound healing, but also in pathological conditions such inflammation and cancer metastasis. In this study I focused on the expression of this specific matrix enzyme, because it is involved in the development of malignant tumour including invasion of the tumour microenvironment, abnormal growth, angiogenesis and cancer metastasis. Here, I was trying to determine whether Jurkat MVs cause EMT in PNT2 cells due to the transfer of some oncogenic factors to the target cells. MMP9 was measured from the cell culture supernatant of PNT2 and tPNT2 cells. The intention was thereby to examine further the functional consequences of EMT in tPNT2 cells, in this case by measuring MMP9. tPNT2 cells express high level of MMPs **(Fig.4.8 B and E)** compared to control, untreated PNT2 cells. MMPs play a significant role in the occurrence of cancer, especially in the remodelling of tissue that occurs with the invasion of prostate tumours. This result in fact strongly suggests that PNT2 cells treated with leukaemia cell derived-MVs express significantly high levels of this degrading enzyme compared to control, untreated PNT2 cells and this could confirm one of the functional consequences of EMT induced by Jurkat MVs on target prostate cells.



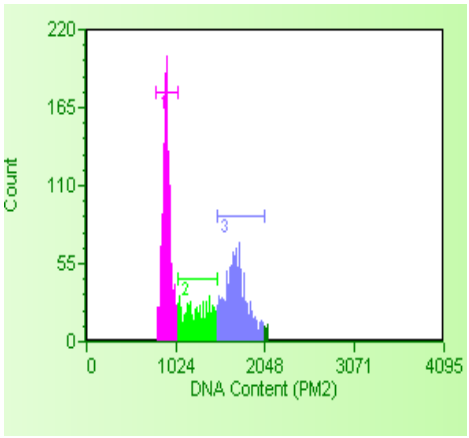
	T1	T2	Mean absorbances
PNT2	5	7	6
TPNT2	23	32	27.5

Figure 4.8 tPNT2 cells express a significantly high level of MMP9 as measured by ELISA. (A), MMP 9 standard curve in linear function of absorbance against concentration (µg/ml). **(B)** Expression level of MMP9 in normal PNT2 and tPNT2 cells. **(C)**, Mean absorbance of ELISA for PNT2 and tPNT2 cells using FLUOstar Omega plate reader. **(D),** Fluorescence microscopy image of MMP9 in PNT2 cells, and **(E)**, Fluorescence microscopy image of MMP9 in tPNT2 cells.*P<0.05, **P<0.01, ***P<0.001, Scale bar is 50µm.

4.2.8 Cell Cycle analysis shows significant number of tPNT2 cells arrested in the G2 phase of the cell cycle

In this experiment I tried to determine another of the functional consequences of EMT in tPNT2 cells at the DNA level. This study in fact, confirmed that leukaemia cell derived-MVs cause EMT through the dysregulation of the cell cycle in tPNT2 cells. **Fig.4.9** A and **B** show the DNA contents of PNT2 cells in each stage of the cell cycle and the pattern seems to follow a normal distributions (G0/G1=41%, S=20% and 36%). **Fig.4.9 C** and **D** show the percentage of cell distribution for tPNT2 cells. The pattern in tPNT cells does not appear normal by any mean. **Fig.4.9 D** shows only 11% of the total tPNT2 cells to be in G0/G1. Percentages of cells in the S phases of both PNT2 and tPNT2 cells are almost the same, 20% and 19% respectively but the percentage of tPNT2 cells in G2/M phases of the cell cycle is significantly high (69%) compared to PNT2 cells (36%). The G2 phase is one the several checkpoint in the mammalian cell cycle at which the progression of a cell to the next stage in the cycle can be halted until conditions are favourable. Therefore, the most important function of the G2 phase is to ensure chromosomes are accurately replicated without any mistakes. Accordingly a high percentage of tPNT2 cells were found to be arrested in G2/M which in fact means exposure of normal prostate epithelial cells to leukaemia cell derived-MVs causes DNA damage of the target PNT2 cells.

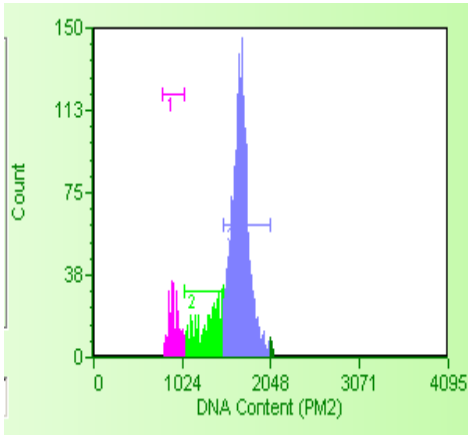
A



B

DNA Histogram Results	M1	M2	M3	M4
% Total	41.5	20.9	36.5	0.0
PM2 Mean:	925	1273	1732	0
PM2 Median:	922	1278	1722	0
PM2 %CV	5.0	10.5	7.3	0.0
Count	1355	682	1192	0

C



D

DNA Histogram Results	M1	M2	M3	M4
% Total	11.0	18.9	69.2	0.0
PM2 Mean:	931	1311	1708	0
PM2 Median:	926	1335	1704	0
PM2 %CV	5.9	10.1	6.0	0.0
Count	301	515	1886	0

E

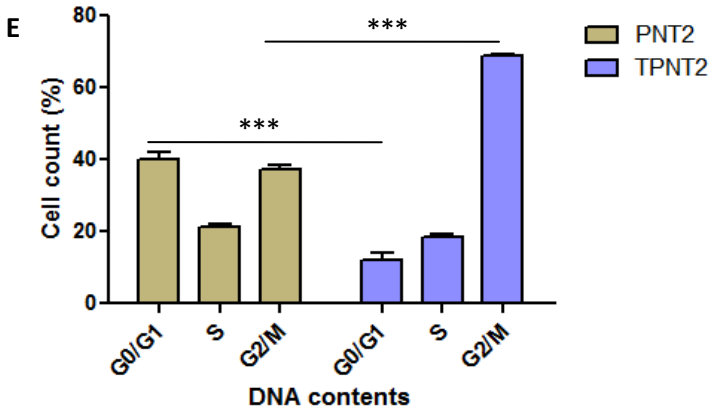


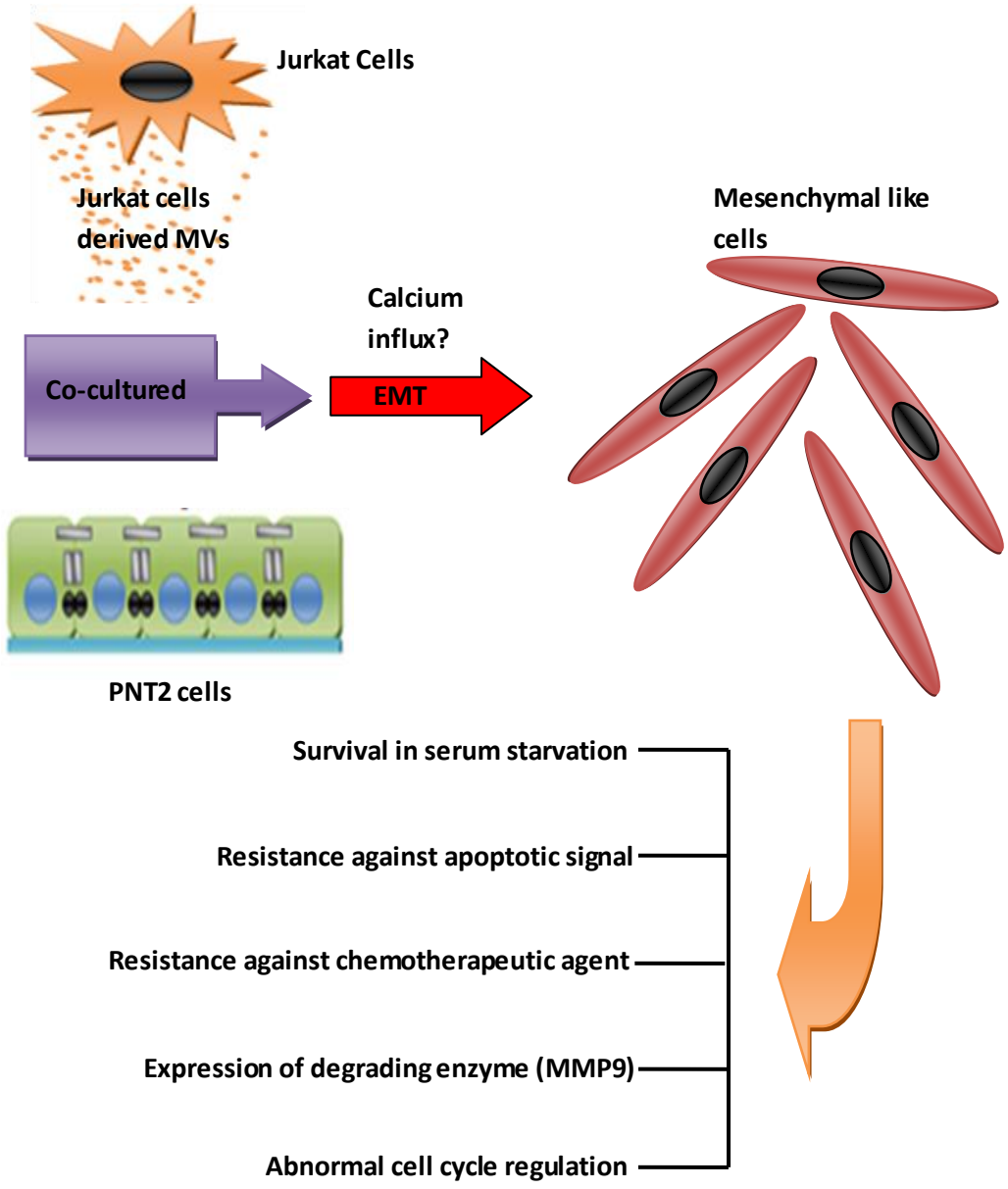
Figure 4.9 High numbers of tPNT2 cells are arrested in the G2/M phase of the cell cycle. **(A)** Shows normal histogram pattern of PNT2 cells in the G0/G1, S and G2/M phases of the cell cycle. **(B),** show the DNA content of PNT2 cells in each stage of the cell cycle. **(C)** Is the histogram pattern of tPNT2 cells in the G0/G1, S and G2/M phases of the cell cycle. **(D)** Indicates DNA content of tPNT2 cells in each stage of the cell cycle. **(E)** Shows the summary of the **%** of PNT2 and tPNT2 cell distributions in each phase of the cell cycle.

4.3 Summary

The first results chapter of this thesis concentrated on the morphological and markers of EMT that I was able to observe could be induced in prostate cells upon exposure to T cell leukaemia-derived MVs. Having made this important observation the second results chapter has set about understanding the causes and biological consequences of EMT in normal prostate epithelial cell lines (after exposure to Jurkat-derived MVs). The cause of MVs-induced EMT in the epithelial cells was partially answered in the first part of the result section and in this results chapter I studied the role Ca^{2+} signalling plays in PNT2 cells treated with Jukat MVs that acquired EMT. According to this finding, cytosolic Ca^{2+} concentration was increased **(Fig.4.3)** in PNT2 cells treated with blood cancer cell MVs. However, the increased cytosolic Ca^{2+} concentration causes a likely endocytosis of E-cadherin **(Fig.4.3)** from the cellular junction into the cytoplasm of PNT2 cells that have been treated with Jurkat MVs. This internalisation of junctional protein might be one of the reasons for the epithelial cells to grow apart from each other and loss their epithelial characteristics. In order to answer these questions, a series of immunocytochemistry assays and ELISA were performed accordingly. According to these results, tPNT2 cells no longer behave like normal prostate epithelial cells and also PNT2 cells that acquired EMT show more resistance against apoptotic signals under stress conditions such as serum starvation and chemotherapies **(Fig.4.5, 4.6)**.

Results obtained by MMP9 ELISA confirmed that epithelial cells exposed to leukaemia cell derived-MVs express high levels of degrading enzyme **(Fig.4.8)** compare to normal PNT2 cells. This can be a clear indication that, MVs derived from cancer cells are responsible for horizontal transfer of some oncogenic protein in the recipient cells as would be indeed from transformed prostate cancer cells to normal prostate cells. Furthermore, results from the cell cycle analysis show that, a high proportion of the cell population in the transformed PNT2 cells are arrested in the G2 phase of the cell cycle **(Fig.4.9 C** compared to normal prostate epithelial cell lines **Fig.4.9 A)**. Therefore, according to this study I postulated that, leukaemia cell derived-MVs induce the cancerous type of EMT in normal prostate epithelial cell lines. This influence of MVs on epithelial cells can be clearly due to its bioactive molecules which they may inherit from their parental blood cancer cells.

4.6 Schematic diagram of causes and functional consequences of EMT induced by leukaemia cell derived-MVs on PNT2 cells



5. Non-Small Lung Cancer Cell (A549) microvesicles (tMVs) affect normal lung fibroblast cells (MRC5) in the tumour microenvironment

5.1 Introduction

Non-Small Cell Lung Cancer (NSCLC) cells are adenocarcinomas of human alveolar basal epithelial cells. It is the leading cause of cancer related death all over the world. The tumour microenvironment is believed to have a significant influence on cancer cell growth, progression, and metastasis and on the treatment outcome. These influences are mainly due the ability of the microenvironment to change its landscape in response to stress signals. Fibroblasts are resident stromal cells that function as supportive cells in tissue organ. During wound healing, chronic inflammation and malignancy these cells become activated. The central feature of fibroblast activation, upon which they are known as myofibroblasts, is the expression of α-SMA protein. In this study I tried to determine the role of NSCLC cell-(A549-) derived microvesicles (tMVs) on normal lung fibroblast cells (MRC5). Non-Small Cell Lung Cancer cell supernatant was used as a source of tMVs for the treatment of MRC5 target cells. I aimed to test the various concentrations with different time exposures of tMVs on the MRC5 cells. Lung fibroblast cells were labelled with anti-α-SMA antibody and an anti-Fibroblast Growth Factor (FGF) antibody. The analysis was carried using Guava EasyCyte technology, fluorescence microscopy imaging and the FLUOstar Omega plate reader. In the second attempt MVs were isolated from the supernatant of the activated form of fibroblasts and labelled with anti-α-SMA antibody. MRC5 cells were co-cultured with myofibroblast-derived MVs for 5-7 days incubation periods and then assessed for FGF and α-SMA expression.

Statistical comparisons on data between the two groups of MRC5, untreated, control cells and MRC5 cells treated with tMVs were performed using GraphPad Prism 5 software. The differences were considered to be significant when $*P<0.05$.

5.2 Results

5.2.1 MRC5 cell treated with tMVs acquire phenotypic changes as observed by phase contrast microscopy

Upon treatment of MRC5 cells with 50 µg/ml A549-derived tMVs, there was a clear phenotypic change in the cells **(Fig.5.1)**. Control untreated MRC5 cells **(Fig 5.1A)** exhibit the normal characteristics of primary lung fibroblasts including uniformly elongated and stretched cells, whilst MRC5 cells were treated with A549 cell derived tMVs **(Fig.5.1B)**, showed loss of fibroblast features and were no longer uniformly elongated having now acquired irregular shape. However, in order to find out whether addition of A549 tMVs to the MRC5 cells lead to the activation of these fibroblasts, I labelled the MRC5 cells to ascertain α-SMA expression at the protein level as this is a specific marker for fibroblast activation. The aim, therefore, was to demonstrate the role of A549 tMVs on fibroblast activation at the molecular level by monitoring the expression of the cytoskeletal protein, α-SMA, using immunocytochemistry. Alpha-smooth muscle actin, as a specific marker for myofibroblasts, plays a central role in myofibroblast contraction. Myofibroblasts are capable of regulating tissue fibrosis through the action of their contractility. At the genetic level, a number of transcription factors are believed to be involved in the expression of α-SMA such as the DPP homologue 2/3, myocardin-related transcription factors A/B (MRTF-A/B) and CCAAT/enhancer-binding protein beta (CEBP-β); high expression of miR-21 is another contributor of fibroblast activation in lung tissue.

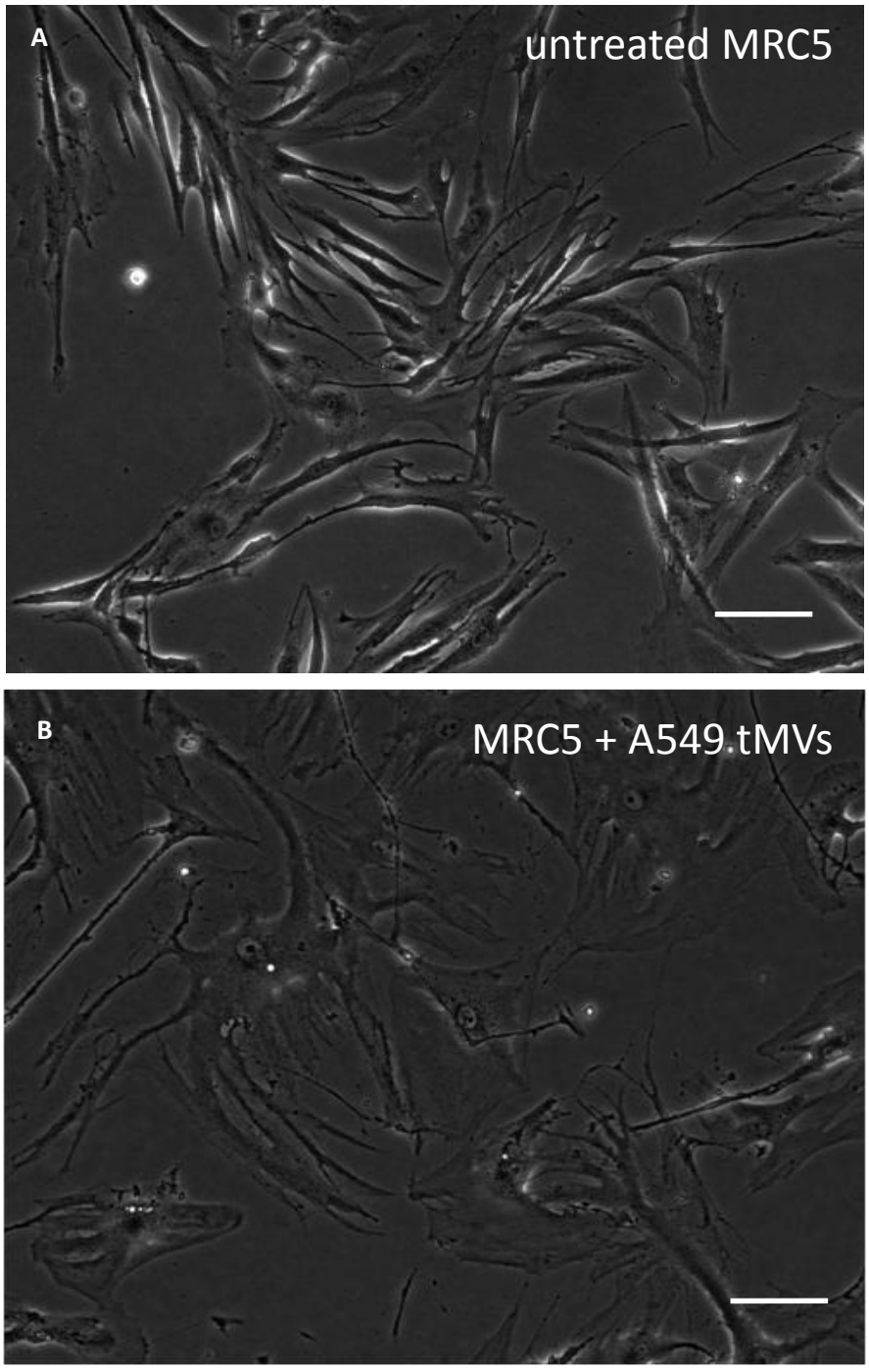


Figure 5.2.1 A549 tMVs alter the morphology of MRC5 cells. (A), MRC5 control, untreated cells**. (B),** MRC5 cells treated with A549 tMVs. Scale bar is 50µm.

5.2.2 MRC5 cells treated with A549tMVs express significantly higher levels of α-SMA protein as observed using Guava EasyCyte flow cytometer

In this study MRC5 cells were treated with A549 tMVs for 5 and 7 days and then both MRC5 control, untreated cells and MRC5 cells in the experiment group were labelled with mAb against α-SMA to confirm fibroblast activation (to myofibroblasts). My aim here was to try to determine firstly whether A549 tMVs have any effect on MRC5 cells at the molecular level and secondly whether any such effect of tMVs on fibroblasts was dose dependent. Therefore, MRC5 cells were treated with two different concentrations 30 µg/ ml and 50µg/ ml of A549 tMVs for 5 and 7 days. The percentage of α-SMA protein expression is significantly higher in the MRC5 cells treated with 50µg/ml A549 tMVs for 7 days compared to cells given 30µg/ml for 5days and control, untreated MRC5 cells **(Fig.5.2).** This study shows that the effect of A549 tMVs on MRC5 activation is dose and time dependent.

Whereas the percentage of α-SMA expression in the panel treated panel with 30 µg/ml is about 6% greater compared to cells under basal conditions (*$*P<0.5$*), exposing MRC5 cells to 50µg/ml tMVs for 7 days increased the expression of the myofibroblast marker on the MRC5 cells by a highly significant 60% (*$***P<0.001$*).

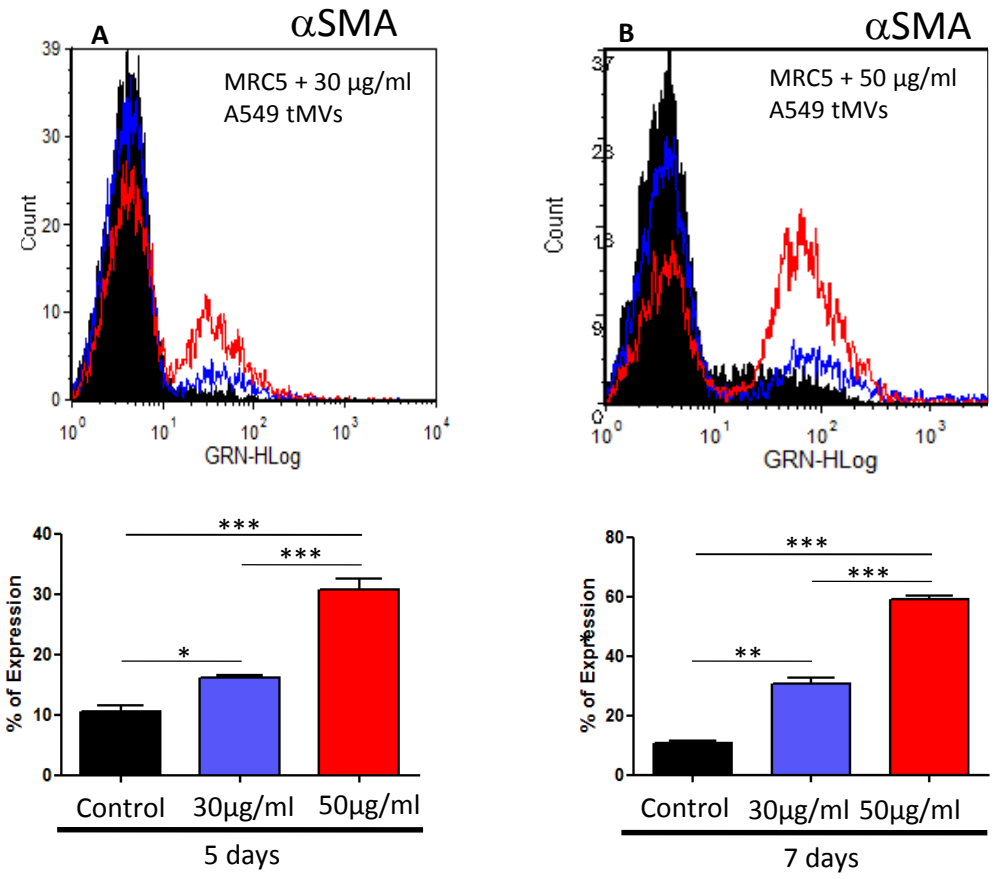


Figure 5.2.2 MRC5 cells treated with A549 tMVs express significantly higher levels of α-SMA. (A), Expression level of α-SMA in MRC5 cells treated with 30 µg/ml of A549 tMVs. **(B),** Expression level of α-SMA protein on MRC5 cells treated with 50µg/ml of A549 TMVs. **(C),** Summary of expression level of α-SMA on MRC5 cells treated with A549 TMVs.(-) Percentage of expression of α-SMA in MRC5 control, untreated MRC5 cells. **(-)** Percentage of expression of α-SMA in MRC5 treated cells with 30 µg/ml of A549 tMVs. **(-)** Percentage of expression inMRC5 cells treated with 50µg/ml of A549tMVs. **P<0.05, **P<0.1, ***P<0.001.*

5.2.3 MRC5 cells treated with A549 tMVs express higher level of α-SMA as observed using fluorescence microscopy

As before, fibroblasts were treated with A549 tm's, this time just at the 50 µg/ml dose for 5 and 7 days **(Fig.5.4.3 D, F and G,I),** and the expression of α-SMA protein compared to the MRC5 control, untreated cells **(Fig. 5.4.3 A and C)** using fluorescence microscopy. According to these experiments, MRC5 cells treated with 50µg/ml of tm's for 7 days expressed significantly high levels of α-SMA protein compared to cells treated with 50 µg/ml of A549 tm's for just 5 days (and control, untreated cells) as determined by fluorescence microscopy. This experiment demonstrates that A549 cells, through the interactions of their MVs, can have an influence on their surrounding ECM, including primary lung fibroblasts which may become activated, expressing greatly increased levels of α-SMA.

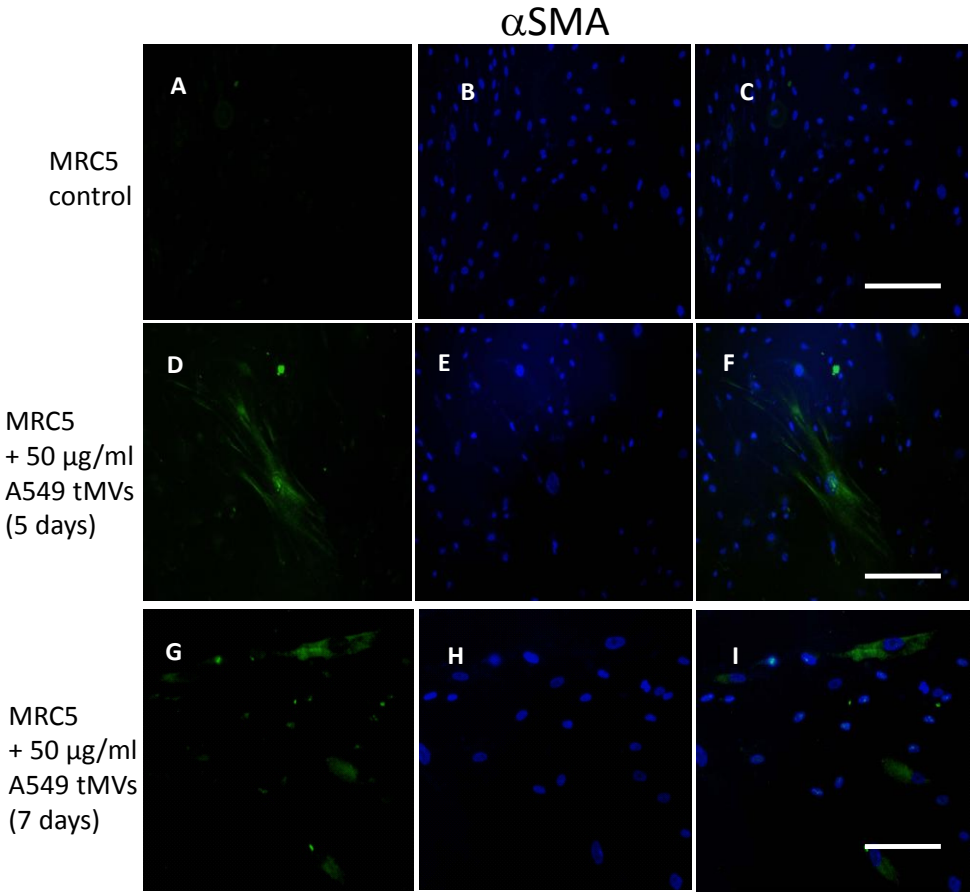


Figure 5.2.3 MRC5 cells express high levels of α-SMA protein after treatment with A549 tMVs using fluorescence microscopy. **(A)**, expression level of α-SMA protein in MRC5 control, untreated cells. **(B)** Nuclear staining using DAPI-Vectashield. **(C)**, Combination of **(A)** and **(B)**. **(D)**, Expression level of α-SMA protein in MRC5 cells treated with 50µg/ml A549 tMVs for 5 days. **(E),** Nuclear staining using DAPI-Vectashield. **(F)**, Combination of **(D)** and **(E)**. **(G)** Expression level of α-SMA protein in MRC5 cells treated with 50 µg/ml of A549 tMVs for 7 days. **(H),** Nuclear staining using DAPI-Vectashield. **(I),** Combination of **(G)** and **(H).** The scale bar is 200µm.

5.2.4 A549 tm's increase the expression level of Fibroblast Growth Factor-1 in primary lung fibroblasts as determined using the Guava EasyCyte flow cytometer

Another parameter that was deemed interesting to monitor in fibroblast activation by A549-derived tMVs was the expression of Fibroblast Growth Factor-1 (FGF-1). FGFs are multifunctional proteins that have been shown to play important roles in the process of proliferation and differentiation of a variety of cells and tissues. Therefore, in the following experiments where primary lung fibroblasts treated with A549 tMVs acquired different morphological characteristics labelled these fibroblasts with anti-FGF-1 mAb to monitor its expression, before and after treatment with A549 tMVs using standard immunocytochemistry. **Fig.5.4.4 A** shows the immunolabelling of MRC5 cells with anti-FGF-1 antibody before and after treatment with tMVs. The result was analysed by flow cytometer showing unlabelled cells (-), control untreated MRC5 cells (-), and MRC5 cells treated with A549 tMVs (-). Accordingly, primary lung fibroblasts treated with A549 tMVs expressed more FGF-1protein than control, untreated MRC5 cells. **Fig.5.4 B** is the summary of FGF expression in MRC5 control untreated cells and MRC5 cells were treated with 50µg/ml of A549 TMVs for 5 and 7 days. According to this finding, 5 days treatment of MRC5 cells with 50µg/ml of A549 TMVs has little effects on FGF expression $P>0.5$, However, when the incubation period was increased to 7 days, the expression of FGF was increased to a significant level ($*P<0.05$).

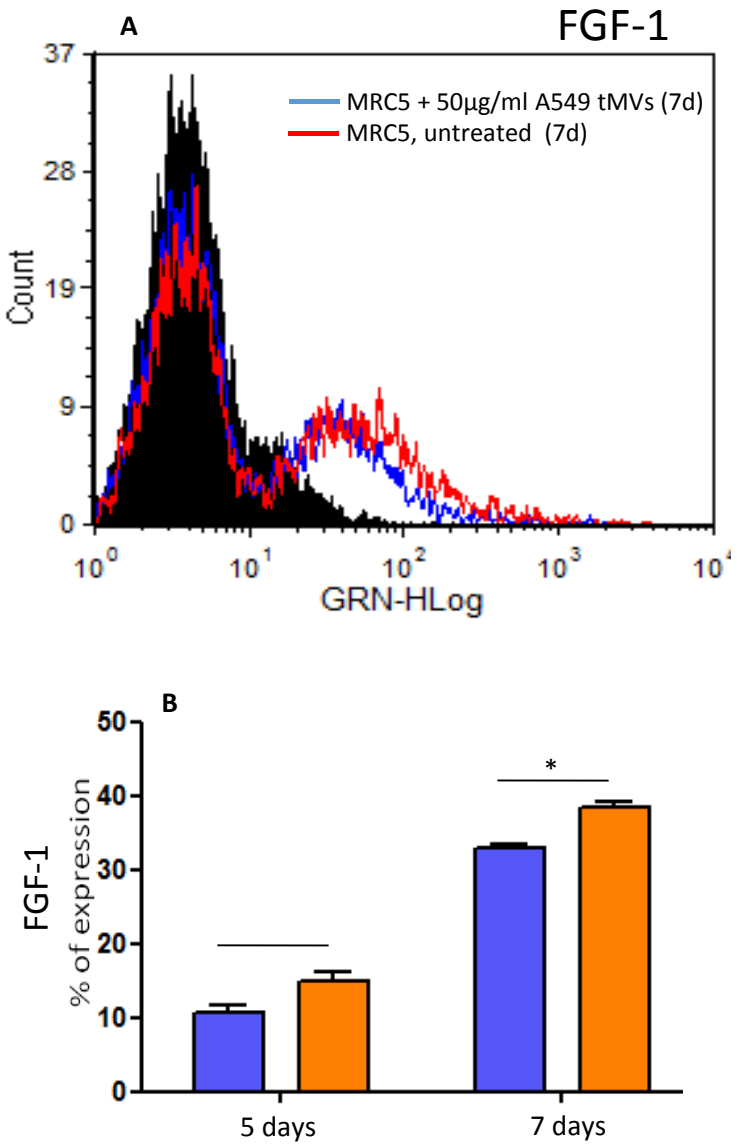


Figure 5.2.4 Primary lung fibroblasts treated with A549 tMVs express increased FGF-1 protein. (A), Expression level of FGF-1 in MRC5 control, untreated MRC5 cells (—) and expression level in MRC5 cells treated with 50µg/ml of A549 tMVs (—). **(B),** Summary of FGF-1 expression in MRC5 cells treated A549 tMVs. **P<0.05.*

5.2.5 A549 tMVs increase the expression level of FGF-1 in MRC5 cells as observed by fluorescence microscopy

To ascertain the cellular location of the up regulated FGF-1 on MRC5 cells upon treatment with A549 tMVs, I used fluorescence microscopy. **Fig. 5.4.5** shows the immunostaining of MRC5 cells with anti-FGF-1 antibody. **(A, B and C)** are the results from normal lung fibroblast cells without treatment with A549 CD- MVs. **(A)** the FITC labelled control untreated fibroblast cells that shows no FGF expression. **(B)** Nuclear staining of MRC5 cells under basal condition with DAPI Vectashield. (C) Merge image of FITC and DAPI in control untreated cells. **(D)** FITC labelled CD- MVs treated MRC5 cells that shows some FGF expression activity. **(E)** The nuclear staining of MRC5 cells in the experiment panel carried out by DAPI vectashield. **(F)** Merge image of FITC and DAPI staining of MRC5 cells treated with CD- MVs. According to this experiment, activated form of MRC5 cells that induced by non- small lung cancer MVs exhibit more FGF expression compare to primary fibroblast cells.

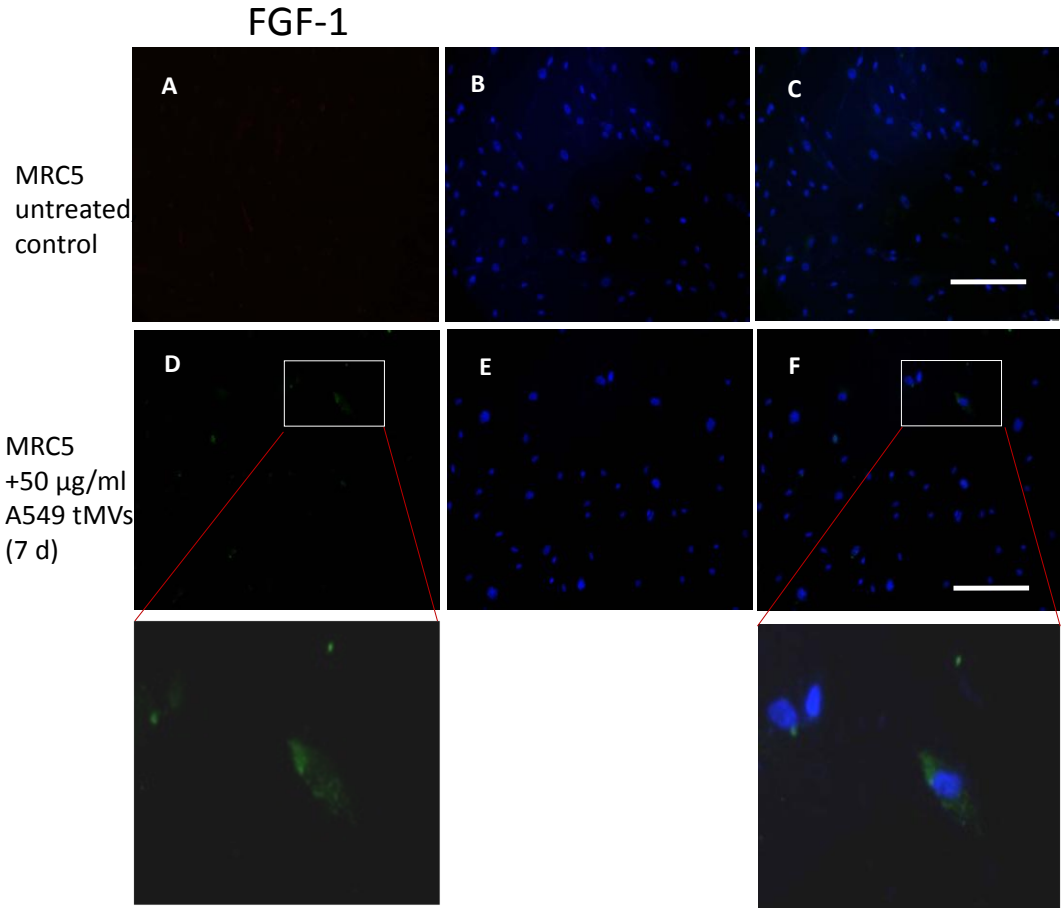


Figure 5.2.5 Primary lung fibroblasts express raised levels of FGF-1 after treatment with lung carcinoma derived-(A549-) MVs. (A), Expression level of FGF-1 in MRC5 control, untreated cells. **(B),** Nuclear staining with DAPI-Vectashield. **(C)** Combination of **(A)** and **(B). (D),** Expression level of FGF-1 in primary lung fibroblasts treated with 50µg/ml A549 tMVs. **(E)**, Nuclear staining with DAPI-Vectashield. **(F),** Combination of **(D)** and **(E).** Scale bar = 200µm.

5.2.6 Primary lung fibroblasts (MRC5) treated with A549 tMVs release MVs with significantly raised expression levels of α-SMA

Having shown that human lung fibroblasts (MRC5) cells treated with human Non-Small Lung Cancer Cell-(A549-) derived MVs become activated, expressing raised levels of α-SMA, I now sought to ascertain whether the MVs released from such activated MRC5 cells (after treatment with A549 tMVs) themselves carry α-SMA protein. Therefore, MRC5 cells were treated with 50µg/ml A540-derived tMVs for 7 days, washed once with DMEM (serum-free) and then incubated for 12 hrs in DMEM supplied with 10% FBS (MV and exosome-free). After 12 hrs, MRC5 cell culture supernatant was collected and MVs isolated according to the protocol described previously (3.3.1). Isolated MRC5-derived MVs were then labelled with anti-α-SMA mAb and analysed using the Guava EasyCyte flow cytometer. This result clearly demonstrates that treatment of MRC5 cells with A549-derived tMVs produces significantly high numbers of MVs (75%-positive) expressing α-SMA **(Fig. 5.6 E, F),** compared to MVs released from control, untreated MRC5 cells (20%-positive) **(Fig. 5.6 C, D)**; **Fig. 5.6.A** and B show the negative control in this experiment (0.68%-α-SMA-positive). It is also noteworthy that the total amount of protein in MVs derived from activated fibroblasts is twice as high **(Fig.5.6 H)** as MVs derived from fibroblasts **(Fig.5.6 H)**. This study in fact confirmed the standard definition of MVs which almost all scientists have been emphasising, namely, to paraphrase, that 'the constituents of MVs depend on the nature of their parental cells.' The constant release of these subpopulations of MVs from the activated fibroblasts along with the A549 cancer cell-derived tMVs is very likely to be

able to change the landscape of the tumour microenvironment and help towards the invasion of its microenvironment along with intravasation, extravasation and metastasis to other distant organs.

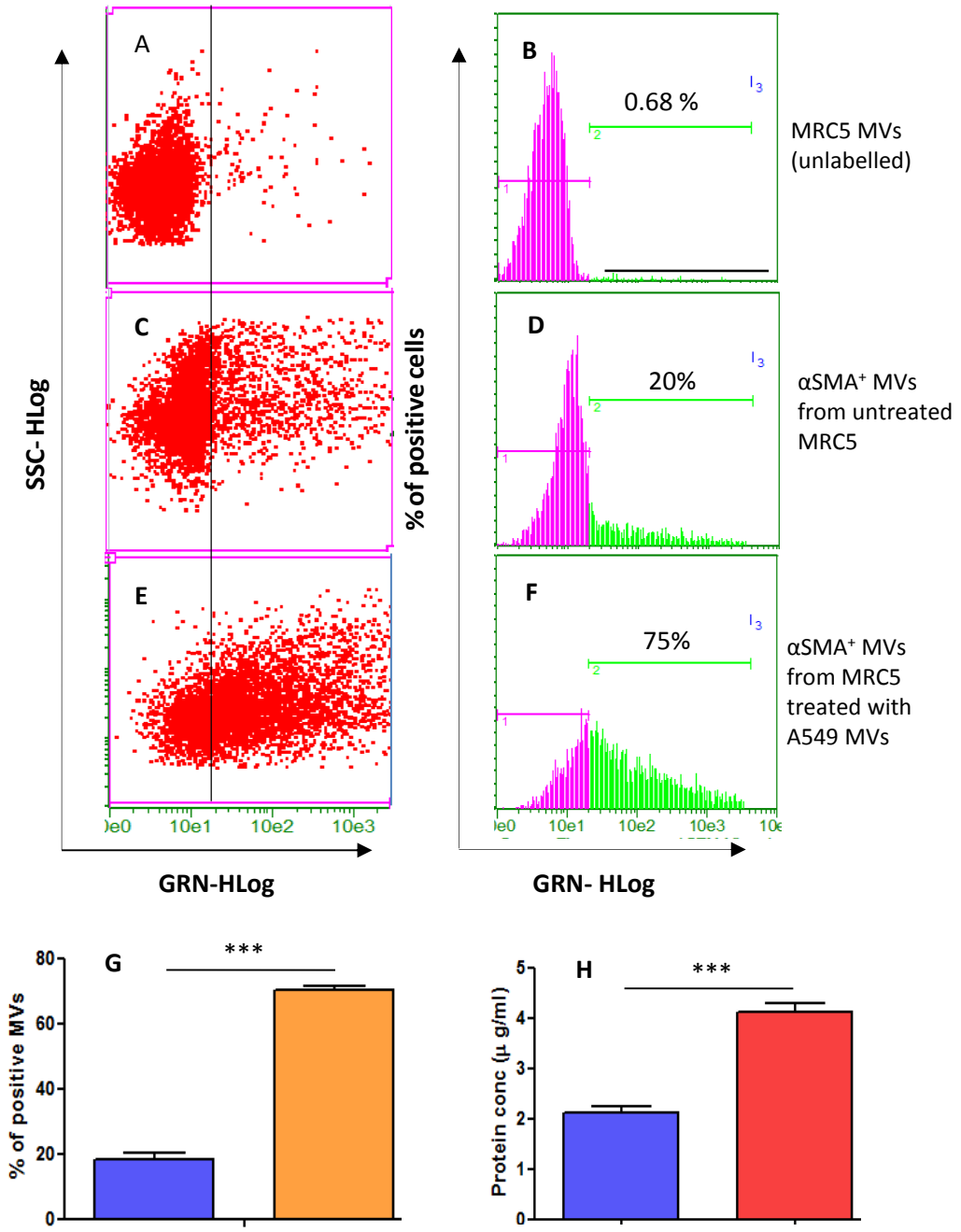


Figure 5.2.6 Primary lung fibroblasts treated with A549 tMVs release MVs expressing high levels of α-SMA. **(A and B),** unlabeled MRC5 cell-derived MVs. **(C and D),** Expression level of α-SMA protein in control, untreated primary lung fibroblast cell-derived MVs. **(E and F),** Expression level of α-SMA protein on MVs derived from MRC5 cells treated with A549 tMVs. **(G),** Summary of expression level of α-SMA protein in MVs released from activated MRC5 cells. **(H),** total protein expression level in MVs derived from A549 tMV-treated MRC5 cells and control, untreated MRC5 cells. ****P<0.001.*

5.2.7 MRC5 cells were treated with A549 TMVs expressed high number of α-SMA positive MVs using fluorescence microscopy

In this study MVs were isolated from myofibroblasts and stained with MAb against α-SMA cytoskeleton protein using fluorescence microscopy. This study demonstrates that MVs derived from the myofibroblasts carrying α-SMA cytoskeleton protein and I noticed that, there are some factors which could affect the enfluence of MVs on the target cells. According to this results, FBS MVs present in the DMEM medium **(Fig.5.4.7 I)** casuses clamping of activated fibroblasts MVs, this can indeed generally decrease the enfluence of MVs on the target cells and I found from my previous study, the results are not included here, the effect of A549 TMVs on the primary fibroblasts that were grown in DMEM medium (+FBSMVs) was significantly lower than (MV free FBS) medium. This is a clear indication of the influence of microparticles such as MVs and exosome that are present in growth medium can prevent or to a lesser extend can reduce the effect of extra cellular vesicles on the target cells and this can be explained from the **(Fig.5.4.7 G),** which is the myofibroblasts derived-MVs in DMEM serum free, and MVs are present indiviually without clumping. **(Fig.5.7H)** Activated fibroblast derived-MVs in DMEM medium supplied with 10 % FBS (MV free), MVs were clumped but to a lsser extent comparing to **(Fig. 5.4.7 I),** wich is activated fibroblasts MVs were added in DMEM medium with 10% FBS(+MVs), once MVs were clumped the number of target cells with MVs are decrreased as results of their aggregations .available Scale bare= 200µm.

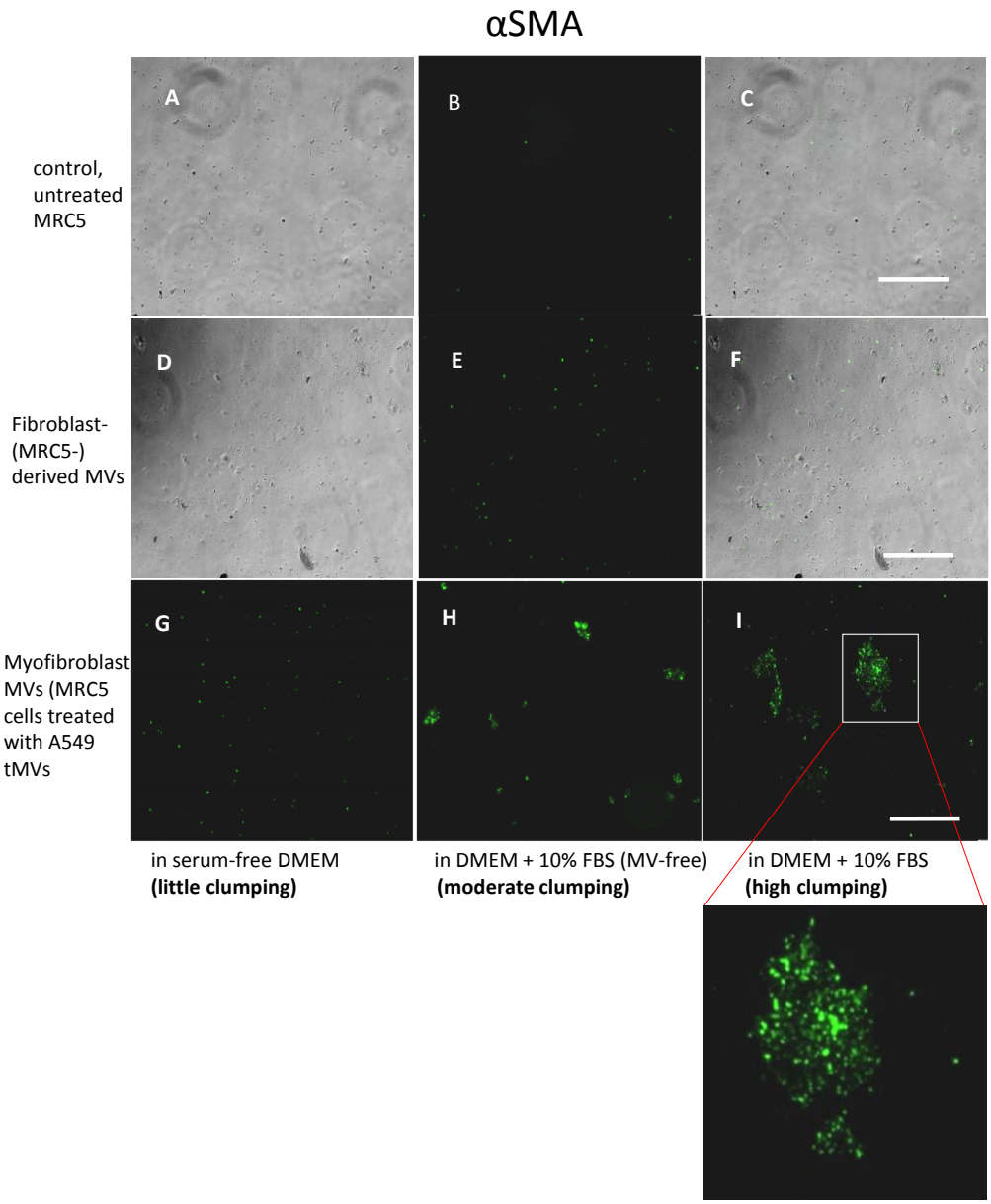


Figure 5.2.7 Activated fibroblast- (myofibroblast-) derived MVs are highly positive for α-SMA protein as observed by fluorescent microscopy. (A), phase contrast image of MVs derived from control, untreated MRC5 cells. **(B),** Expression of α-SMA on primary fibroblast-(MRC5-) derived MVs. **(C),** Combination of **(A)** and **(B). (D),** phase contrast image of MVs derived from MRC5 after treatment with 50µg/ml of A549 tMVs. **(E),** Expression level of α-SMA protein on activated fibroblast-derived MVs. **(F),** Combination of **(D)** and **(E). (G),** α-SMA labelled MVs from activated fibroblasts (A549 tMV-treated) myofibroblasts in serum-free DMEM medium. **(H),** Alpha-SMA labelled MVs in DMEM medium supplied with 10 % FBS (MVs-free). **(I)** Alpha-SMA labelled MVs in DMEM supplied with 10 % (+MVs). Scale bar is 200µm.

5.2.8 Transformation of primary fibroblasts with activated fibroblast derived-MVs

Activated fibroblasts or myofibroblasts, in this case MRC5, lung fibroblasts, treated with NSCLC-MVs, release two subpopulations of MVs, those that stain positive and those that stain negative for α-SMA. In the following series of experiments I intended to find which population of MRC5-derived MVs, after treatment with A549 tMVs, can in turn alter MRC5 cells. Images in **Fig.5.4.8** were taken 72 hrs after adding the myofibroblast MVs. The boxed area shows MV attachment to MRC5, these MVs being positive for α-SMA **(Fig 5.4.8 C)**; a second subpopulation of myofibroblast MVs is also seen attached to another MRC6 cells that is negative for α-SMA **(Fig.5.8 C** red arrow). Comparing the interaction of α-SMA-positive and -negative MVs with MRC5 cells, it would appear that only α-SMA-positive myofibroblast MVs can significantly alter the morphology of MRC5 cells. Such a cell (boxed in **Fig. 5.4.8 C**) appears overgrown, its size seeming at least three times bigger than the normal fibroblast; this cell appears 'transformed.' **Fig. 5.4.8 D** shows MRC5 cells treated with myofibroblast derived-MVs for 7 days and the now green fluorescence diffused throughout the cells demonstrates fusion of labelled MVs with the target cells **(Fig. 5.4.8 E, F)**. Normal fibroblast cells generally look stretched out and exhibit front and back polarity, this morphological feature being no longer visible in the cell that interacted with α-SMA-positive MVs.

These findings demonstrate the influence of ‘cancer’ cell-derived MVs on the microenvironment, because only MVs derived from the activated fibroblasts, as a result of treatment with 50µg/ml of tMVs, could cause ‘transformation’ of MRC5 cells.

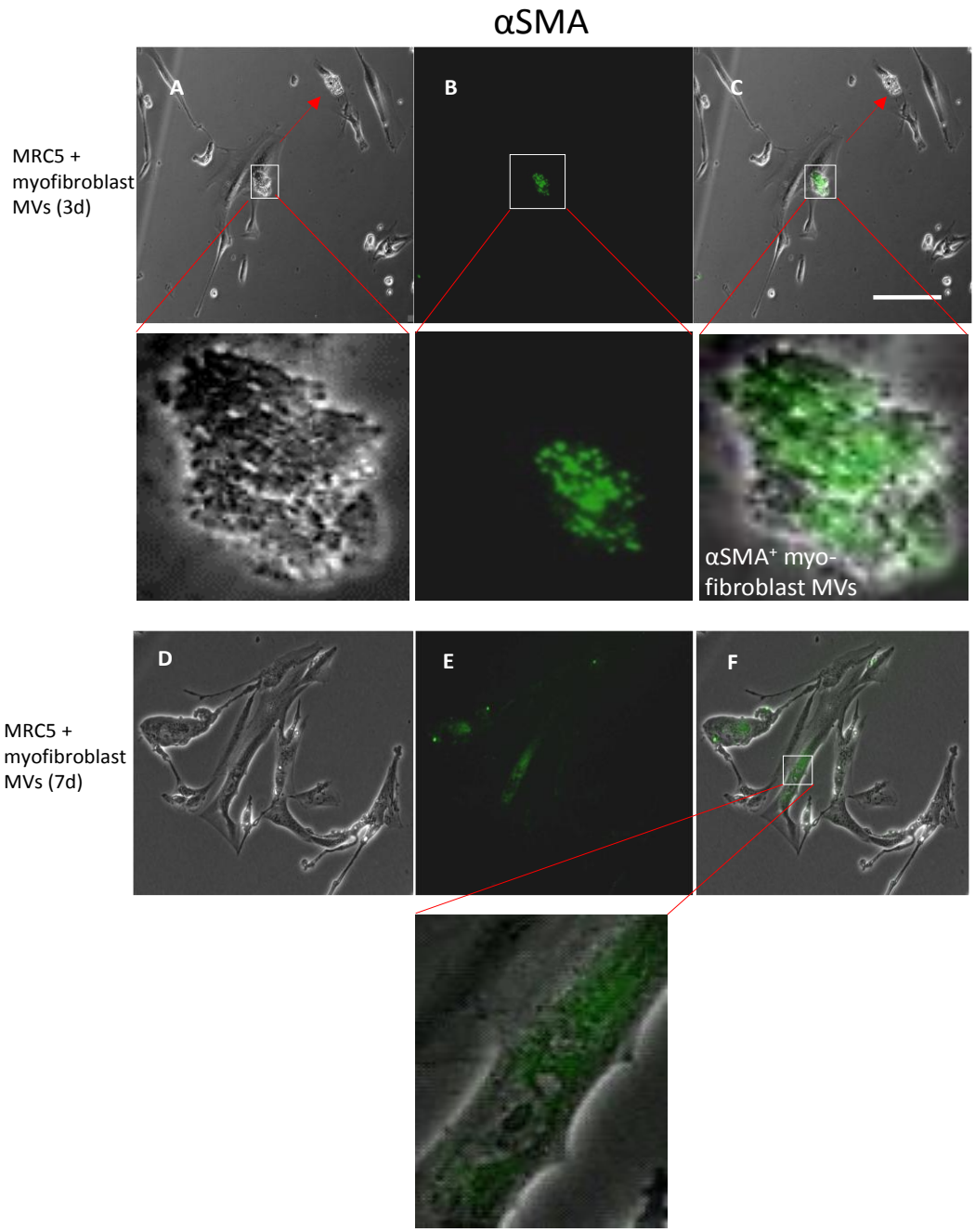


Figure 5.2.8 α-SMA-positive MVs fuse with the plasma membrane of primary lung fibroblasts, MRC5. (A), Phase contrast image of MRC5 cells treated for 3d with myofibroblast derived-MVs. **(B),** α-SMA-positive myofibroblast derived-MVs attached to primary lung fibroblasts. **(C),** Combination of (A) and (B). **(D)** MRC5 cells treated for 7d with α-SMA-positive MVs derived from the activated fibroblast cells. **(E)** Expression level of α-SMA protein. **(F)** Combination of **(D)** and **(E).** Scale bar is 200µm.

5.2.9 MRC5 cells were treated with myofibroblasts derived-MVs expressed high level of α-SMA using fluorescent microscopic analysis

In section 5.2.8 I showed that myofibroblast MVs expressing α-SMA appeared to 'transform' primary lung fibroblasts. To further confirm this, the following experiments were conducted to look for the concomitant expression of α-SMA as a measure of the observed 'transformed' morphology. MRC5 cells were treated with 50 µg/ml of myofibroblast-derived MVs for 7 days then stained against α-SMA protein to detect the expression of this cytoskeletal protein. This study clearly demonstates that MVs derived from activated fibroblast cells, likely carrying certain elements of cancer cells (inherited from parent cells that were treated with A549 carcinoma cell MVs and which activated primary lung fibroblasts into myofibroblasts (activated fibroblasts). The activation of these mesenchymal cells were confirmed by the phenotypic changes of the target fibroblasts and also at the molecular level by the expression of α-SMA protein on the recipient MRC5 cells. This study therefore demonstrates the influence carcinomas have on their microenvironment. It would thus appear that carcinomas are able to govern the landscape of of the tumour microenvironment through the release of MVs. The transformation of primary fibroblasts into myofibroblasts was started by the release of A549 cell-derived MVs and then these activated fibroblasts act as a cofactor to increase the process of fibroblast activation and also participate in the regulation of the tumour microenvironment.

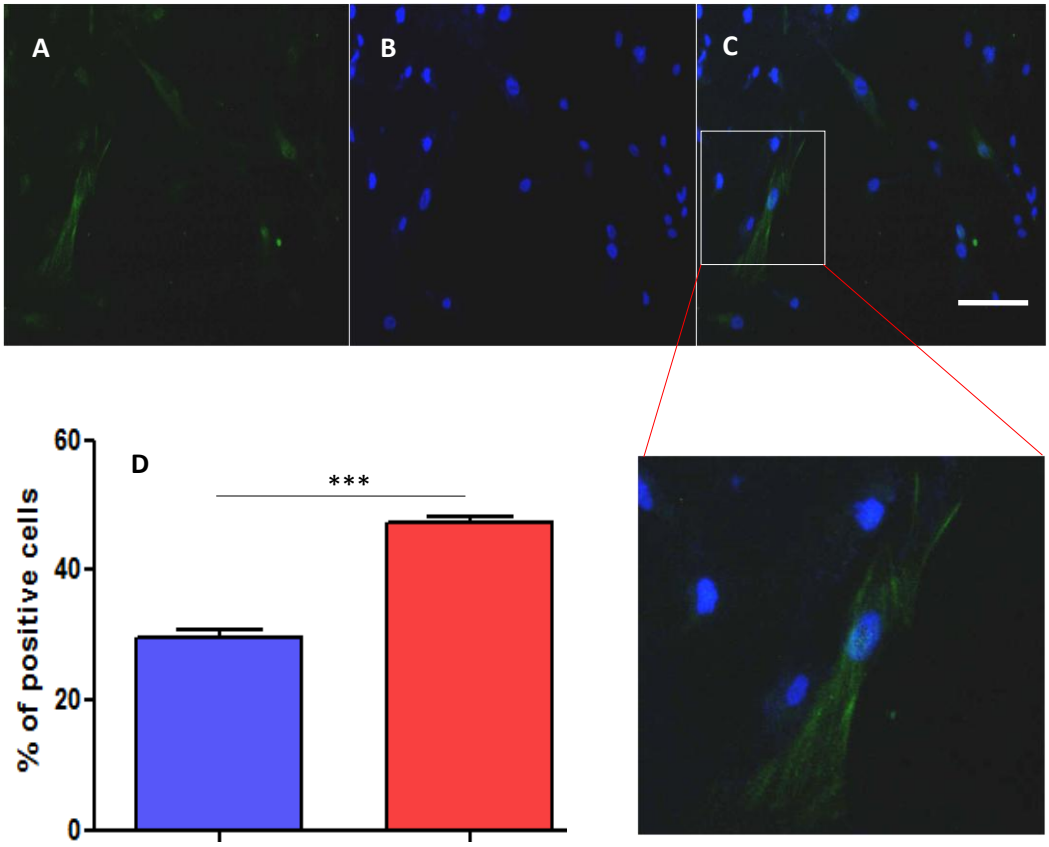
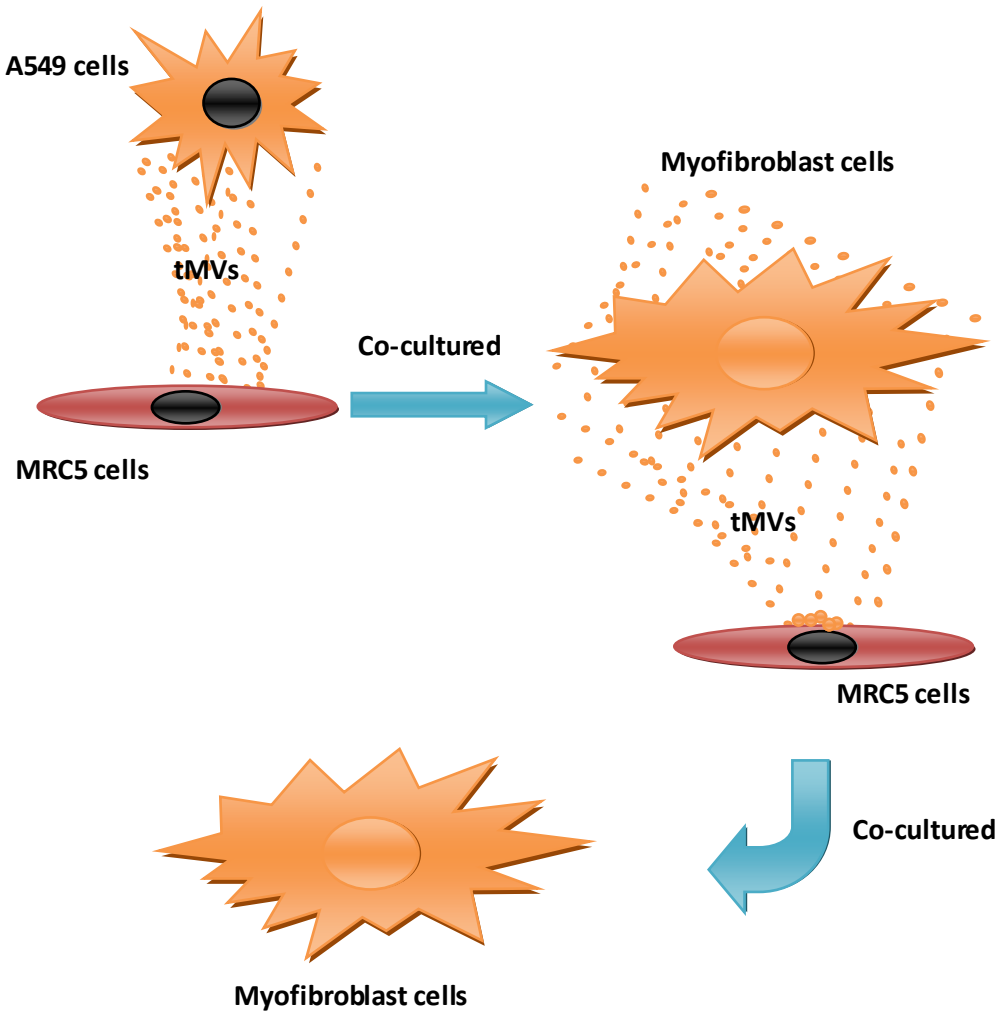


Figure 5.2.9 MRC5 cells treated with myofibroblast-derived MVs express significantly high levels of α-SMA protein as observed by fluorescence microscopy. (A), Expression level of α-SMA protein in MRC5 cells. **(B),** Nuclear stainingwith DAPI-Vectashield. **(C),** Combination of **(A)** and **(b). (D),** Summary of the expression level of α-SMA cytoskeleton protein in MRC5 cells treated with 50µg/ml of myofibroblast-derived MVs (red bar) and 50 µg/ml primary lung fibroblast-derived MVs (blue bar). Scale bar is 200 µm, ****P<0.001.*

5.3 Summary

This study was performed to determine the influence of carcinoma cell-derived (A549-) MVs on primary lung fibroblast cells and also to have an insight into the mechanism of interaction between tMVs and the MRC5, primary, human fibroblast target cells. According to these findings, MVs derived from the Non-Small Cell Lung Cancer cells; result in major phenotypic changes of primary lung fibroblast cells, causing them to become activated fibroblast cells, known as myofibroblast cells. The transformation of fibroblast cells into myofibroblast cells can be confirmed by the expression of a myofibroblast marker, α-SMA, and a cytoskeletal protein in the target cells. In this study, the expression level of the myofibroblast marker was measured in both fibroblast cells under basal conditions and in fibroblast cells treated with tMVs using various technologies including the Guava EasyCyte flow cytometer (Millipore), fluorescent microscope (Olympus IX81) and FLUOstar Omega plate reader. FGF-1 expression levels were also measured in both control untreated fibroblast cells and tMV-treated cells. In later experiments I attempted to gain an insight into the nature of the MVs derived from the activated form of the fibroblast cells collected from MRC5 cells after treatment with tMVs. Further work was also performed to examine the influence of MVs derived from the activated form of fibroblast cells on the primary fibroblast cells. The results obtained from this study demonstrated that only those populations of MVs that were positive for α-SMA cytoskeletal proteins were capable of fibroblast transformation into myofibroblast cells.

5.4 Schematic diagram of tMVs inducing fibroblast activation



6. Discussion

6. Discussion

6.1 Limitations of MV analysis

Microvesicles (MVs) are small membrane enclosed vesicles released into the extracellular environment by most mammalian cell types under normal and pathological conditions. Microvesiculation is observed both under *in vitro* and *in vivo* conditions and its process does not affect cell viability (188). MVs are generated by an external budding from the cell surface and are detected in a variety of biological fluids such as blood, urine, spinal cerebral fluids, saliva and ascites, and MVs play crucial roles in intercellular communication (189). Apart from participation in physiological cell signalling, there are strong suggestions that these vesicles can be useful in novel treatment modalities; for example MVs are considered as potential carriers of therapeutic drugs that could elicit anti-tumour responses.

The advances in research on MVs in cancer therapeutics are extraordinary (190). However, there are major concerns regarding an efficient and standardized method of MV isolation, purification, concentration, size measurement and molecular content. These issues in fact limit the capacity of MVs in the clinical diagnostic and prognostic fields for a variety of diseases, and certainly in the cancer therapeutics field (191). To overcome this, a reliable and re-producible MV isolation/characterization protocol is still needed. This will also enable accurate genomic, proteomic and lipidomic characterization carried out on a variety of cell types including blood cells, epithelial cells, endothelial cells, muscles cells, and neuronal cells both under normal and different pathological conditions such as systemic, inflammatory conditions and cancers. MV characterization in fact,

is a major challenge and none of the available techniques including flow cytometry can reliably distinguish the size of MVs and also define them at the single vesicle level.

Fig.3.1 in the results section presents the flow cytometric analysis of MVs using the Guava EasyCyte instrument. This flow cytometer enables MVs to be characterized on the basis of the spectral properties of the fluorescence signal that defines the morphology and specific sorting. Analysis of MVs is achieved through the hydrodynamic focusing where the suspended cells move through a compressed channel until they encounter the laser. The emitted scatter and fluorescence is captured and measured by a detector which is located in front of the laser. The intensity of light is reported as forward scatter (FSC) and side light scatter (SSC). The quantity of forward scatter light is proportional to diameter and side scatter light is related to the morphology of the MVs. The heterogeneity of MVs is determined by the filter that captured the appropriate range of the emission peak. This scattering requires the use of calibration beads of known size and numbers to identify the heterogeneous population of MVs.

Fig.4.2 A&B defines this heterogeneous population of MVs labelled with fluorescence labelled annexin V. Annexins constitute a family of phospholipid binding proteins that are predominantly located on the cytosolic face of the plasma membrane of healthy cells. During microvesiculation phosphatidylserine loses its asymmetric distribution in the phospholipids' bilayer and translocates to the extracellular leaflet of the plasma membrane where its presence can be detected by fluorescence labelled Annexin V. Jurkat (T cell leukaemia) MVs were isolated and

analyzed by fluorescence activated cell sorting **(Fig.3.2 A)**, and expression of Annexin V was analyzed separately **(Fig.4.2 B)**. From the fluorescent staining results that were carried out by FACS, 60 % of the MV population was positive after the first wash of the sample labelled with Annexin V, a level which increased to 66% after the sample was washed a second time. A marked reduction on the total number of MVs was noticed after the second centrifugation. Increased percentage of Annexin V positive MVs after the second wash can be due to the degradation of Annexin V negative MVs which happened after the second centrifugation. It appears that, translocation of PS to the outer membrane bilayer probably protects MVs from degradation. However the question yet to be answered is the nature of the remaining 36-40% of these particles which were gated in the area 0.1 - 1µm on the FSC region. There is evidence in the literature suggesting that around 30-35% of MVs do not possess PS on their membrane. Therefore, it has become a high priority to find an alternative marker for greater specificity and sensitivity than Annexin V for MVs characterization. The basis of this section of the discussion about MV characterization is to consider that the results obtained from this experiment are purely due the effect of a heterogeneous population of T-lymphocyte cancer cell derived MVs.

6.2 Leukaemia cell derived-MVs and EMT

Another important question that needs to be addressed is why was the EMT observed obtained with T cell derived MVs (192). Furthermore, CD8 positive T cells were found to be capable of inducing tumours with EMT characteristics possessed by breast cancer stem cells (193,194). Here in the first section of the results chapter I posed the question whether “MVs derived from the leukaemia cancer cells are capable of inducing EMT in normal prostate epithelial cells’’.

To determine the effect of leukaemia cell derived-MVs on prostate epithelial cells and prospective epithelial to mesenchymal transition change, the EMT primarily needs to be defined at the cellular, molecular and genetic levels. EMT is a process in which epithelial cells with apico-basal polarity that are attached to the basal membrane differentiate into fibroblast like epithelial cells (195). The top-bottom polarity which is the most characteristic phenomenon of epithelial cells is disrupted as a result of the disassembly of cell-to-cell contacts at adherent junction (E-cadherin) (196). Furthermore the EMT process involves the expression of mesenchymal proteins such as Vimentin and fibronectin (197).

The effects of Jurkat cell-derived MVs on PNT2 cells were examined in the context of firstly, the top-bottom polarity and secondly, on the expression of the epithelial marker (E-cadherin) and mesenchymal marker (Vimentin). **Fig.3.3** in the result section presented the effects of Jurkat cell derived-MVs on the EMT process in PNT2 cells. This result obtained from the flow cytometric analysis shows the molecular changes that take place after PNT2 cells were treated with MVs. Comparing control untreated groups

with experimental panels clearly demonstrates the down regulation of the E-cadherin protein, the hallmark of epithelial cell lines, in the MV treated cells. This junctional protein consists of an extracellular domain which is in close contact with neighbouring epithelial cells that form the epithelial sheet and a single pass trans-membrane domain. The intracellular domain of E-cadherin consists of two well conserved catenin binding sites (198). The cytoplasmic tails of E-cadherin are bound to the actin cytoskeleton protein of epithelial cells through the α and β catenin (199). Therefore, alteration in the expression of this glycoprotein can disturb the morphology of epithelial cells and probably affects intracellular signalling events leading to gene expression. **Fig.3.4** illustrates the microscopic analysis of epithelial cells treated with Jurkat cell-derived MVs. According to this result, **(Fig.3.4 D)** leukaemia cell derived-MVs down regulate the expression of E-cadherin on the epithelial cellular junction. This molecular alteration is accompanied by cytoskeleton rearrangement of epithelial cells that eventually leads to the mesenchymal phenotype. Comparing the treated cells with control untreated cells at the morphological level, cells in the experimental group lose their top-bottom polarity and acquire front–back polarity. The treated group looks stretched out and motile compared with control **(Fig.3.4 C)** exhibiting more classical round shapes with apico-basal polarity.

According to the results obtained from flow cytometry and fluorescent microscopy, PNT2 cells that are cultured with leukaemia cell derived-MVs alter their expression of E-cadherin molecules significantly. It is already well known that E-cadherin participates in intracellular signalling events (200). One of the signalling pathways partially regulated by this cadherin molecule

is the Wnt signalling pathway. The cytoplasmic domain of E-cadherin is bound to the actin cytoskeleton protein through the α and β-catenin (201) and the Wnt signalling pathway is regulated by multiple interacting signalling proteins. In the absence of Wnt, β-catenin is phosphorylated by glycogen synthase kinase in a complex composed of Axin protein and adenomatous polyposis coli protein that causes proteasomal degradation of β-catenin (202). In the present of Wnt, the complex of Axin protein, adinomatous polyposis coli protein and glycogen synthase kinaseis not formed as glycogen synthase kinase is displaced from this complex. This displacement in turn causes reduction in the phosphorylation of β-catenin and stabilisation of β-catenin (203). The data presented in this thesis suggests for the first time that one of the mechanisms leukaemia cell derived-MVs may modulate the Wnt signalling pathway is through the cleavage of E-cadherin that occurs in the presence of proteases delivered by MVs.

EMT is defined as a reduction of epithelial marker and increase of mesenchymal markers. Therefore, PNT2 cells co-cultured with Jurkat MVs were examined in the context of the mesenchymal marker (Vimentin). **Fig.3.5 B** is the result from a FACS analysis which shows a significant increase in the expression of Vimentin comparing to control group **(Fig.3.5 A)**. These results were confirmed by fluorescence microscopic analysis. **Fig.3.6** shows the expression level of Vimentin in PNT2 cells treated with leukaemia cell derived-MVs. Vimentin expression increased to a significant level **(Fig.3.6 D)** compared with control untreated PNT2 cells **(Fig.3.6. A)**. Vimentin is type three intermediate filaments that help to provide cellular

structure and movement to mesenchymal cells. This protein is often found in mesenchymal cells and has gained much importance as a canonical marker of EMT (204). EMT is in fact characterized by the expression of Vimentin filaments in epithelial cells which is normally express only keratin filaments (205). This protein is encoded by a single gene that is located on chromosome *10p*. The expression of Vimentin is believed to be regulated by three different promoter elements. Association of Vimentin promoter with these elements suggests that the Vimentin gene is subjected to a complex control (206). As a cytoskeleton structural protein, Vimentin is believed to be restricted into the cytoplasm of mesenchymal cells, however, this protein can be partially found in the nucleus and cytoplasmic membrane. Therefore, this intermediate filament may participates in certain DNA and RNA mediated events (207).

The expression patterns of Vimentin in normal cells and cancerous tissues are evaluated as a factor for diagnosis and prognosis (208). In prostate cancer, Vimentin expression is highly detectable in metastatic form and almost undetectable in benign tumour (209). Vimentin expression is also associated with prostate cancer cell motility. Knocking down the expression of this filament significantly decreases cells' motility and invasiveness in highly aggressive prostate cancer cell lines (210).

6.3 Causes and consequences of EMT

One the most fundamental questions yet be answered is the nature of the elements in Jurkat cell MVs responsible for this phenotypic change in epithelial cell lines? I tried to answer this question by examining the role of TGF-β signalling pathway on the EMT process as induced by Jurkat MVs. **Fig.3.7 D** presents the epithelial cellular morphology and the E-cadherin expression in the MV-treated PNT2 cells, comparing them with control untreated PNT2 cells **(Fig.3.7A)**. The experimental panel shows a significant reduction in E-cadherin protein and in the context of morphological changes the test group no longer grew as an epithelial sheet. **Fig.3.8** again confirmed the morphological changes of epithelial cells into mesenchymal-like cells and expression of Vimentin filament. According to this result the expression of Vimentin protein decreased after the blocking the TGF-β signalling pathway but it was still detectable in the cytosol of PNT2 cells which were treated with leukaemia cell derived-MVs.

6.3.1 Leukaemia cell derived-MVs cause endocytosis of E-cadherin in target PNT2 cells

In the second attempt after I found that PNT2 cells exposed to Jurkat cells MV causes increases of the cytosolic concentration of Ca^{2+} at a highly significant level, as well as of magnesium, potassium and sodium on the target cells **(Fig.4.2)**. I determined the effect of cytosolic Ca^{2+} concentration on the EMT process when PNT2 cells were treated with leukaemia cell derived-MVs pre-treated with the calcium chelator, BAPTA-AM. Prior to

treatment with leukaemia cell derived-MVs, which causes EMT, PNT2 cells treated with calcium chelator **(Fig.4.3 C)**, instead resulted in the degradation of E-cadherin as this molecule was endocytosed into the cytoplasm of target cells. According to this finding, increasing cytosolic Ca^{2+} concentration by Jurkat MVs might be one of the elements that participate in cytosolic E-cadherin degradation on transformed PNT2 cells. Calcium plays an important factor in a variety of physiological roles such as neurotransmission and cardiac and pathological contractility and also in pathological conditions such as cancer invasion and metastasis (211). This study described for the first time a mechanism whereby induction of EMT in normal prostate epithelial cells was linked with increased intracellular concentrations of Ca^{2+} after treatment with leukaemia cell derived-MVs. However, further work is required to answer the question of how leukaemia cell derived-MVs cause increased intracellular Ca^{2+} concentrations in the target PNT2 cells, for example, in other words whether these vesicles are carrying Ca^{2+} ions or carrying some Ca^{2+} mobilizing agent which causes activation of Ca^{2+} channels from the cytoplasmic membrane or from ER of the target PNT2 cells. The Ca^{2+}-permeable ion channel TRPM7 is believed to be one of the EMT regulators and is also involved in cell migration (212). However, there is evidence in the literature suggesting that, silencing the TRPM7 did not change the expression of Vimentin in response to cytosolic calcium concentration in breast cancer cells and neither of EMT induction and cell migration; the role of Ca^{2+} signalling is critically important in apoptotic resistance (213). Therefore, blocking the interaction of cancer cell

derived-MVs with the target cells could offer a new therapeutic approach for combating phenotypic changes in normal prostate epithelial cell line.

6.3.2 PNT2 cells acquire EMT at the expense of their proliferation

Comparing cell proliferation between PNT2 and tPNT2 (so-called Jurkat MV treated PNT2 cells), I found that PNT2 cells treated with 30 µg/ml of Jurkat derived-MVs **(Fig.4.4.D,E and F)** growth was significantly less than control PNT2 cells **(Fig.4.4. A, B and C)**. Reduction in PNT2 cell proliferation was clearly noticed after 72hrs incubation periods and interestingly this was accompanied with PNT2 cell differentiation. There is evidence suggesting that E-caderin-mediated cell-cell adhesion generates a cell survival signal, the loss of E-caherin and cell-cell contact by the EMT induction likely reducing the anchorage independent growth ability of cells. Furthermore, it is logical to consider that invading cancer cells transiently lose their cell proliferation activity. This study suggests that mesenchymal-epithelial transition (MET) under different microenvironment is required for the cancer cells to grow again (214). Some researchers blamed histone deacetylases (HDACs)-mediated epigenetic mechanisms as having a central role in controlling cell proliferation and differentiation in a variety of cells and pathogenesis of various diseases (215). Multiple studies propose that HDACs are the vital target in a variety of diseases including inflammatory conditions, metabolic diseases and malignancy (216). PI3K/AKt and MAPK signalling pathways are another important signal transducers for cell proliferation and differentiation and also play a key role in the regulation of cancer invasion and metastasis (217). Jurkat-MVs induce EMT in normal

prostate epithelial cell lines and halt their proliferation activity through the HDACs, PI3K/AKt or MAPK signalling pathways.

6.3.3 TPNT2 cells resist programmed cell death induced by serum starvation and chemotherapeutic agents

Serum starvation and docetaxel-induced apoptosis in normal prostate epithelial cell lines **(Fig.4.4 A)** and tPNT2 cells produce considerably high levels of resistance against apoptotic signalling which was activated in response to serum starvation **(Fig.4.4 B)**. EMT is well documented to be associated with death resistance and this phenotypic change could potentially play as a pathway to escape programmed cell death under physiological and pathological conditions (218). Snail functions as a transcription factor to down regulate the expression of pro-apoptotic genes such as Bax, Bid, Pten and Puma which can take place in epithelial cells that acquire mesenchymal phenotypes (219). Another group found that TGF-β induced EMT with the acquisition of apoptotic resistance in cholangiocytes (220). TGF-β1 signalling is believed to up-regulate survivin to inhibit apoptosis during EMT. Survivin is a member of the Inhibitor of Apoptosis Protein (IAP) family and is a key regulator of mitosis and programmed cell death and it also regulates the G2/M phase of the cell cycle by associating with mitotic spindle microtubules and by direct inhibition of caspase-3 and 7 (221). Depletion of survivin using siRNA significantly enhanced TGF-β1 induced apoptosis. However, I used leukaemia cell derived-MV as a stimulus to induce multiple distinct cellular

responses such as induction of apoptosis, cell proliferation, differentiation, MMP-9 expression and cell cycle analysis in the PNT2 cells which has not hitherto been studied in great detail yet.

6.3.4 MMP-9 expression in TPNT2 cells

TPNT cells expressed high levels of MMP-9 **(Fig.4.8 B and E)**. MMPs degrade the ECM, cell-ECM and cell-cell structures, thereby causing detachment of epithelial cells from surrounding tissues (222). These enzymes serve a path-clearing role in facilitating the movement of cells or groups of cells through the ECM (223). This also results in cleavage of adhesion proteins such as E-cadherin which leads to the release of individual epithelial cells from the epithelial sheets and initiates outside-in signalling pathways that cause changes in gene transcription patterns. MMP-9 is believed to dock to the hyaluronan receptor CD44, mediating proteolytic activation of TGF-β and promotion of tumour invasion and metastasis and angiogenesis in carcinoma models. MMPs are linked with EMT in cancer progression through three distinct mechanisms, (A), elevated levels of MMPs can directly causes EMT in the tumour microenvironment, (B), Cancer cells undergoing EMT produce high levels of MMPs which facilitates cell invasion and metastasis, and (C), EMT can generate activated stromal cells that drive cancer progression by MMP production (224). Within this contest, TPNT2 cells expressing high levels of MMP-9 after EMT induction, using leukaemia cell derived-MV as a stimulus, may transform into cancer cells. Statements A and C which quoted that

'high level MMPs induce EMT and EMT activates stromal cell' could not be true, because, in both conditions I have PNT2 controls and as for statement C, there no stromal cells to get activated and yet MMP-9 is produced by TPNT2 cells.

6.3.5 TPNT2 and cell cycle regulation

Figs. 4.9 C and **D** show misregulation of the cell cycle in tPNT2 cells and this was obviously clear on comparison with normal prostate epithelial cells. The significant change was the high percentage (69%) of TPNT cells arrested in the G2/M phases of the cell cycle. Cell cycle phase is believed to be a key event in the determination of EMT and apoptotic pathways (225). Treatment of epithelial cells with TGF-β induced apoptosis of the target epithelial cells in the G2/M phase of the cell cycle and if these cell are in the G1/S phase of the cell cycle they transfer to EMT (226). In my study however, there is a very small population (11%) of TPNT2 cells present in G1/S and 69% of TPNT cells were arrested in G2/M phase of the cell cycle. Most interestingly, these cells produced significantly high levels of resistance against apoptotic signals. This study could suggest a rethink about the role of TGF-β present on leukaemia cell derive-MVs causing EMT on PNT2 cells. G2/M phase of the cell cycle serves as a checkpoint for cells with DNA damage to prevent them from entering mitosis with genomic DNA damage. The activity of CDK1 complex is crucial in regulating the G2-phase of transition. Almost all researchers agree that these cells with DNA damage are arrested in the G2/ phases of the cell cycle, so that treatment

of PNT2 cells with Jurkat MVs causes 69% arrest in the G2/M phase of the cell cycle meaning that treatment of these cells with leukaemia cell derived-MVs causes DNA damage to the target TPNT2 cells.

6.4 Carcinoma cell derived-MVs and the tumour microenvironment

6.4.1 A549 cell derived-MVs transform fibroblasts to myofibroblasts

Treatment of Non-Small lung cancer cells derived-MVs caused transformation of primary lung fibroblasts into myofibroblasts **(Figs.6.2** and **6.3)**. Transformation of primary fibroblasts was confirmed by the expression of α-SMA cytoskeleton protein. Alpha-SMA protein is the central feature of activated fibroblasts which is the central mechanism of the tissue repair process and also it is a response from the microenvironment to the epithelial tumour (159,227). Using α-SMA expression as a marker I found there to be a sub-population of cancer-associated fibroblasts in the tumour stroma. However, in the complex microenvironment of wound healing and tumour it cannot be used because smooth muscle cells, which are abundant in wound healing and tumour stroma, also express α-SMA protein (160). The consequences of this phenotypic change in tumour stroma lead to tumour development and cancer metastasis. Myofibroblasts are reported to produce significantly high levels of a variety of growth factors such as HGF, EGF, VEGF, FGF and cytokines such TGF-β1 and pro-inflammatory cytokines including CXCL 14, IL-1, IL-6 and IL-8 which promote tumour progression and metastasis (228,229). Furthermore, myofibroblastsare involved in the remodelling of the ECM and foster cancer invasion by

producing high levels of MMPs such as MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, and MMP-14 (230). In addition to changing the chemical composition of stroma, the stiffening action as a result of stress fibre production has an important role for tumour production (231).

6.4.2 Myofibroblasts and Fibroblast Growth Factor expression

One of the consequences of myofibroblast activation is the excessive production of fibroblast growth factor (FGF) which cooperates with other GFs, cytokines and pro-inflammatory cytokines which collectively facilitate tumour invasion and metastasis (228). Treatment of MRC5 cells with lung carcinoma cell derived-MVs in **Fig.5.5** demonstrated that myofibroblasts expressed high levels of FGF compared to primary fibroblasts. FGF signalling is involved in many physiological developmental processes including morphogenesis in organs such as kidneys, lung, and mammary glands (232). Amplification of FGF ligands and receptors is observed in human breast and lung cancer samples, the FGF ligands being located on chromosome *11q13* which are amplified in 15% of human breast cancers and FGFR genes which are located on chromosome *8p12*, amplified in 10% of human breast cancers (233). Recent studies suggest that ectopic expression of FGFR1 correlates with increased protein expression and that blocking FGFR activity in breast cancer leads to decreased growth and survival of target cells (233). Immunohistochemical analysis in human breast cancer demonstrated increased expression of FGF in 62% of basal like breast cancer tissue (234). Within this context, treatment of primary

lung fibroblasts with 50 µg/ml Non-small lung cancer cells derived-MVs for 7 days significantly increased the expression of FGF on target MRC5 cells. However, further work is needed to be done to understand the mechanism of increased expression of FGF in myofibroblasts, whether it is due to the FGF receptor transfer or FGF ligand transfer from A549 cancer cells through A549 cell derived-MVs to primary lung fibroblasts.

6.4.3 Lung cancer cells remodelling the ECM through their MV release

Stroma surrounding the solid tumour represents an active element in the process of tumour development (235,236). Myofibroblasts are the main cell type in the ECM involved in the development of reactive stroma (237). Cancer cells have the ability to induce fibroblasts to active phenotype myofibroblasts (238). In this study I have shown that MVs derived from myofibroblasts can induce normal fibroblasts to change into reactive myofibroblasts **(Fig.5.6)**. The current study also examined some elements present in the tumour microenvironment such as MVs secreted from immune cells that could possibly block the effects of cancer cell derived MVs on stromal cells **(Fig.5.7)**. According to this study, the effects of myofibroblast MVs on primary lung fibroblasts were positively correlated with the presence of α-SMA myfibroblast MVs **(Fig.5.8)**. It may also be that ECM mediated cell-cell communication between tissues from the same or different organs could deliver bioactive molecules (239). However, the mechanism for this interaction has not yet been explained in much detail. I tried to observe the interaction of MVs and target cells for 7 consecutive

days and found that the delivering of bioactive molecules most likely takes place by the fusion of MVs with target cells **(Fig.5.8F)**. The most important question yet to be answered is whether these MVs are used by cells as a source of lipid bilayer to renew their membrane, or MVs as biologically active molecules establish a ligand-receptor partnership and cause these changes. An intensive research at cellular and molecular level needs to be carried out to answer this question.

7. References

1. Combes, V., F. El-Assaad, D. Faille, R. Jambou, N. H. Hunt, and G. E. Grau. 2010. Microvesiculation and cell interactions at the brain-endothelial interface in cerebral malaria pathogenesis. *Prog. Neurobiol.* 91: 140-151.

2. Gyorgy, B., T. G. Szabo, M. Pasztoi, Z. Pal, P. Misjak, B. Aradi, V. Laszlo, E. Pallinger, E. Pap, A. Kittel, G. Nagy, A. Falus, and E. I. Buzas. 2011. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell Mol. Life Sci.* 68: 2667-2688.

3. Lee, T. H., E. D'Asti, N. Magnus, K. Al-Nedawi, B. Meehan, and J. Rak. 2011. Microvesicles as mediators of intercellular communication in cancer--the emerging science of cellular 'debris'. *Semin. Immunopathol.* 33: 455-467.

4. CHARGAFF, E., and R. WEST. 1946. The biological significance of the thromboplastic protein of blood. *J. Biol. Chem.* 166: 189-197.

5. Gyorgy, B., T. G. Szabo, M. Pasztoi, Z. Pal, P. Misjak, B. Aradi, V. Laszlo, E. Pallinger, E. Pap, A. Kittel, G. Nagy, A. Falus, and E. I. Buzas. 2011. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell Mol. Life Sci.* 68: 2667-2688.

6. Yuana, Y., R. M. Bertina, and S. Osanto. 2011. Pre-analytical and analytical issues in the analysis of blood microparticles. *Thromb. Haemost.* 105: 396-408.

7. Gummadi, S. N., and A. K. Menon. 2002. Transbilayer movement of dipalmitoylphosphatidylcholine in proteoliposomes reconstituted from detergent extracts of endoplasmic reticulum. Kinetics of transbilayer transport mediated by a single flippase and identification of protein fractions enriched in flippase activity. *J. Biol. Chem.* 277: 25337-25343.

8. Hrafnsdottir, S., and A. K. Menon. 2000. Reconstitution and partial characterization of phospholipid flippase activity from detergent extracts of the Bacillus subtilis cell membrane. *J. Bacteriol.* 182: 4198-4206.

9. Vega, F. M., and A. J. Ridley. 2008. Rho GTPases in cancer cell biology. *FEBS Lett.* 582: 2093-2101.

10. Hashimoto, S., Y. Onodera, A. Hashimoto, M. Tanaka, M. Hamaguchi, A. Yamada, and H. Sabe. 2004. Requirement for Arf6 in breast cancer invasive activities. *Proc. Natl. Acad. Sci. U. S. A* 101: 6647-6652.

11. Muralidharan-Chari, V., J. Clancy, C. Plou, M. Romao, P. Chavrier, G. Raposo, and C. D'Souza-Schorey. 2009. ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles. *Curr. Biol.* 19: 1875-1885.

12. Wurdinger, T., N. N. Gatson, L. Balaj, B. Kaur, X. O. Breakefield, and D. M. Pegtel. 2012. Extracellular vesicles and their convergence with viral pathways. *Adv. Virol.* 2012: 767694.

13. Liao, C. F., S. H. Lin, H. C. Chen, C. J. Tai, C. C. Chang, L. T. Li, C. M. Yeh, K. T. Yeh, Y. C. Chen, T. H. Hsu, S. C. Shen, W. R. Lee, J. F. Chiou, S. F. Luo, and M. C. Jiang. 2012. CSE1L, a novel microvesicle membrane protein, mediates Ras-triggered microvesicle generation and metastasis of tumor cells. *Mol. Med.* 18: 1269-1280.

14. D'Souza-Schorey, C., and J. W. Clancy. 2012. Tumor-derived microvesicles: shedding light on novel microenvironment modulators and prospective cancer biomarkers. *Genes Dev.* 26: 1287-1299.

15. Andersen, K. B., S. Levinsen, W. E. Svendsen, and F. Okkels. 2009. A generalized theoretical model for "continuous particle separation in a microchannel having asymmetrically arranged multiple branches". *Lab Chip.* 9: 1638-1639.

16. Dragovic, R. A., C. Gardiner, A. S. Brooks, D. S. Tannetta, D. J. Ferguson, P. Hole, B. Carr, C. W. Redman, A. L. Harris, P. J. Dobson, P. Harrison, and I. L. Sargent. 2011. Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis. *Nanomedicine.* 7: 780-788.

17. Miyazaki, Y., S. Nomura, T. Miyake, H. Kagawa, C. Kitada, H. Taniguchi, Y. Komiyama, Y. Fujimura, Y. Ikeda, and S. Fukuhara. 1996. High shear stress can initiate both platelet aggregation and shedding of procoagulant containing microparticles. *Blood* 88: 3456-3464.

18. Connor, D. E., T. Exner, D. D. Ma, and J. E. Joseph. 2010. The majority of circulating platelet-derived microparticles fail to bind annexin V, lack phospholipid-dependent procoagulant activity and demonstrate greater expression of glycoprotein Ib. *Thromb. Haemost.* 103: 1044-1052.

19. Rubin, O., D. Crettaz, J. D. Tissot, and N. Lion. 2010. Pre-analytical and methodological challenges in red blood cell microparticle proteomics. *Talanta* 82: 1-8.

20. van der Pol, E., A. G. Hoekstra, A. Sturk, C. Otto, T. G. van Leeuwen, and R. Nieuwland. 2010. Optical and non-optical methods for detection and characterization of microparticles and exosomes. *J. Thromb. Haemost.* 8: 2596-2607.

21. Chaput, N., C. Flament, S. Viaud, J. Taieb, S. Roux, A. Spatz, F. Andre, J. B. LePecq, M. Boussac, J. Garin, S. Amigorena, C. Thery, and L. Zitvogel. 2006. Dendritic cell derived-exosomes: biology and clinical implementations. *J. Leukoc. Biol.* 80: 471-478.

22. Cerri, C., D. Chimenti, I. Conti, T. Neri, P. Paggiaro, and A. Celi. 2006. Monocyte/macrophage-derived microparticles up-regulate inflammatory mediator synthesis by human airway epithelial cells. *J. Immunol.* 177: 1975-1980.

23. Cocucci, E., G. Racchetti, M. Rupnik, and J. Meldolesi. 2008. The regulated exocytosis of enlargeosomes is mediated by a SNARE machinery that includes VAMP4. *J. Cell Sci.* 121: 2983-2991.

24. van, N. G., G. Raposo, C. Candalh, M. Boussac, R. Hershberg, N. Cerf-Bensussan, and M. Heyman. 2001. Intestinal epithelial cells secrete exosome-like vesicles. *Gastroenterology* 121: 337-349.

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

26. Frank, R., and R. Hargreaves. 2003. Clinical biomarkers in drug discovery and development. *Nat. Rev. Drug Discov.* 2: 566-580.

27. Dinger, M. E., T. R. Mercer, and J. S. Mattick. 2008. RNAs as extracellular signaling molecules. *J. Mol. Endocrinol.* 40: 151-159.

28. Skog, J., T. Wurdinger, R. S. van, D. H. Meijer, L. Gainche, M. Sena-Esteves, W. T. Curry, Jr., B. S. Carter, A. M. Krichevsky, and X. O. Breakefield. 2008. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat. Cell Biol.* 10: 1470-1476.

29. Hong, B. S., J. H. Cho, H. Kim, E. J. Choi, S. Rho, J. Kim, J. H. Kim, D. S. Choi, Y. K. Kim, D. Hwang, and Y. S. Gho. 2009. Colorectal cancer cell-derived microvesicles are enriched in cell cycle-related mRNAs that promote proliferation of endothelial cells. *BMC. Genomics* 10: 556.

30. Bravo-Cordero, J. J., R. Marrero-Diaz, D. Megias, L. Genis, A. Garcia-Grande, M. A. Garcia, A. G. Arroyo, and M. C. Montoya. 2007. MT1-MMP proinvasive activity is regulated by a novel Rab8-dependent exocytic pathway. *EMBO J.* 26: 1499-1510.

31. Taraboletti, G., S. D'Ascenzo, I. Giusti, D. Marchetti, P. Borsotti, D. Millimaggi, R. Giavazzi, A. Pavan, and V. Dolo. 2006. Bioavailability of VEGF in tumor-shed vesicles depends on vesicle burst induced by acidic pH. *Neoplasia.* 8: 96-103.

32. Mallegol, J., N. G. Van, C. Lebreton, Y. Lepelletier, C. Candalh, C. Dugave, J. K. Heath, G. Raposo, N. Cerf-Bensussan, and M. Heyman. 2007. T84-intestinal epithelial exosomes bear MHC class II/peptide

complexes potentiating antigen presentation by dendritic cells. *Gastroenterology* 132: 1866-1876.

33. Denzer, K., M. J. Kleijmeer, H. F. Heijnen, W. Stoorvogel, and H. J. Geuze. 2000. Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J. Cell Sci.* 113 Pt 19: 3365-3374.

34. Werner, N., and G. Nickenig. 2006. [Stem cells in cardiovascular medicine]. *Dtsch. Med. Wochenschr.* 131: 1438-1440.

35. Preston, R. A., W. Jy, J. J. Jimenez, L. M. Mauro, L. L. Horstman, M. Valle, G. Aime, and Y. S. Ahn. 2003. Effects of severe hypertension on endothelial and platelet microparticles. *Hypertension* 41: 211-217.

36. Amabile, N., C. Heiss, W. M. Real, P. Minasi, D. McGlothlin, E. J. Rame, W. Grossman, M. T. De, and Y. Yeghiazarians. 2008. Circulating endothelial microparticle levels predict hemodynamic severity of pulmonary hypertension. *Am. J. Respir. Crit Care Med.* 177: 1268-1275.

37. Tetta, C., A. L. Consiglio, S. Bruno, E. Tetta, E. Gatti, M. Dobreva, F. Cremonesi, and G. Camussi. 2012. The role of microvesicles derived from mesenchymal stem cells in tissue regeneration; a dream for tendon repair? *Muscles. Ligaments. Tendons. J.* 2: 212-221.

38. Bruno, S., C. Grange, F. Collino, M. C. Deregibus, V. Cantaluppi, L. Biancone, C. Tetta, and G. Camussi. 2012. Microvesicles derived from mesenchymal stem cells enhance survival in a lethal model of acute kidney injury. *PLoS. One.* 7: e33115.

39. Ranghino, A., V. Cantaluppi, C. Grange, L. Vitillo, F. Fop, L. Biancone, M. C. Deregibus, C. Tetta, G. P. Segoloni, and G. Camussi. 2012. Endothelial progenitor cell-derived microvesicles improve neovascularization in a murine model of hindlimb ischemia. *Int. J. Immunopathol. Pharmacol.* 25: 75-85.

40. Cantaluppi, V., S. Gatti, D. Medica, F. Figliolini, S. Bruno, M. C. Deregibus, A. Sordi, L. Biancone, C. Tetta, and G. Camussi. 2012. Microvesicles derived from endothelial progenitor cells protect the kidney from ischemia-reperfusion injury by microRNA-dependent reprogramming of resident renal cells. *Kidney Int.* 82: 412-427.

41. Gross, J. C., and M. Boutros. 2013. Secretion and extracellular space travel of Wnt proteins. *Curr. Opin. Genet. Dev.* 23: 385-390.

42. Fuccillo, M., M. Rutlin, and G. Fishell. 2006. Removal of Pax6 partially rescues the loss of ventral structures in Shh null mice. *Cereb. Cortex* 16 Suppl 1: i96-102.

43. Qazi, K. R., P. P. Torregrosa, B. Dahlberg, J. Grunewald, A. Eklund, and S. Gabrielsson. 2010. Proinflammatory exosomes in bronchoalveolar lavage fluid of patients with sarcoidosis. *Thorax* 65: 1016-1024.

44. Gomes, C., S. Keller, P. Altevogt, and J. Costa. 2007. Evidence for secretion of Cu,Zn superoxide dismutase via exosomes from a cell model of amyotrophic lateral sclerosis. *Neurosci. Lett.* 428: 43-46.

45. Pap, E., E. Pallinger, M. Pasztoi, and A. Falus. 2009. Highlights of a new type of intercellular communication: microvesicle-based information transfer. *Inflamm. Res.* 58: 1-8.

46. Gawaz, M., F. J. Neumann, I. Ott, A. Schiessler, and A. Schomig. 1996. Platelet function in acute myocardial infarction treated with direct angioplasty. *Circulation* 93: 229-237.

47. Goichot, B., L. Grunebaum, D. Desprez, S. Vinzio, L. Meyer, J. L. Schlienger, M. Lessard, and C. Simon. 2006. Circulating procoagulant microparticles in obesity. *Diabetes Metab* 32: 82-85.

48. Baran, J., M. Baj-Krzyworzeka, K. Weglarczyk, R. Szatanek, M. Zembala, J. Barbasz, A. Czupryna, A. Szczepanik, and M. Zembala. 2010. Circulating tumour-derived microvesicles in plasma of gastric cancer patients. *Cancer Immunol. Immunother.* 59: 841-850.

49. Andrews, R. K., E. E. Gardiner, Y. Shen, and M. C. Berndt. 2004. Platelet interactions in thrombosis. *IUBMB. Life* 56: 13-18.

50. Sadallah, S., C. Eken, P. J. Martin, and J. A. Schifferli. 2011. Microparticles (ectosomes) shed by stored human platelets downregulate macrophages and modify the development of dendritic cells. *J. Immunol.* 186: 6543-6552.

51. Koppler, B., C. Cohen, D. Schlondorff, and M. Mack. 2006. Differential mechanisms of microparticle transfer toB cells and monocytes: anti-inflammatory propertiesof microparticles. *Eur. J. Immunol.* 36: 648-660.

52. Andreola, G., L. Rivoltini, C. Castelli, V. Huber, P. Perego, P. Deho, P. Squarcina, P. Accornero, F. Lozupone, L. Lugini, A. Stringaro, A. Molinari, G. Arancia, M. Gentile, G. Parmiani, and S. Fais. 2002. Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. *J. Exp. Med.* 195: 1303-1316.

53. Huber, V., S. Fais, M. Iero, L. Lugini, P. Canese, P. Squarcina, A. Zaccheddu, M. Colone, G. Arancia, M. Gentile, E. Seregni, R. Valenti, G. Ballabio, F. Belli, E. Leo, G. Parmiani, and L. Rivoltini. 2005. Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape. *Gastroenterology* 128: 1796-1804.

54. Lee, H. M., E. J. Choi, J. H. Kim, T. D. Kim, Y. K. Kim, C. Kang, and Y. S. Gho. 2010. A membranous form of ICAM-1 on exosomes efficiently blocks leukocyte adhesion to activated endothelial cells. *Biochem. Biophys. Res. Commun.* 397: 251-256.

55. Kim, H. K., K. S. Song, Y. S. Park, Y. H. Kang, Y. J. Lee, K. R. Lee, H. K. Kim, K. W. Ryu, J. M. Bae, and S. Kim. 2003. Elevated levels of circulating platelet microparticles, VEGF, IL-6 and RANTES in patients with gastric cancer: possible role of a metastasis predictor. *Eur. J. Cancer* 39: 184-191.

56. Mathivanan, S., H. Ji, and R. J. Simpson. 2010. Exosomes: extracellular organelles important in intercellular communication. *J. Proteomics.* 73: 1907-1920.

57. Lee, T. H., E. D'Asti, N. Magnus, K. Al-Nedawi, B. Meehan, and J. Rak. 2011. Microvesicles as mediators of intercellular communication in cancer--the emerging science of cellular 'debris'. *Semin. Immunopathol.* 33: 455-467.

58. Henderson, M. C., and D. O. Azorsa. 2012. The genomic and proteomic content of cancer cell-derived exosomes. *Front Oncol.* 2: 38.

59. Fontana, S., L. Saieva, S. Taverna, and R. Alessandro. 2013. Contribution of proteomics to understanding the role of tumor-derived

exosomes in cancer progression: state of the art and new perspectives. *Proteomics.* 13: 1581-1594.

60. Graner, M. W., D. A. Raynes, D. D. Bigner, and V. Guerriero. 2009. Heat shock protein 70-binding protein 1 is highly expressed in high-grade gliomas, interacts with multiple heat shock protein 70 family members, and specifically binds brain tumor cell surfaces. *Cancer Sci.* 100: 1870-1879.

61. Kim, C. W., H. M. Lee, T. H. Lee, C. Kang, H. K. Kleinman, and Y. S. Gho. 2002. Extracellular membrane vesicles from tumor cells promote angiogenesis via sphingomyelin. *Cancer Res.* 62: 6312-6317.

62. Millimaggi, D., M. Mari, S. D'Ascenzo, E. Carosa, E. A. Jannini, S. Zucker, G. Carta, A. Pavan, and V. Dolo. 2007. Tumor vesicle-associated CD147 modulates the angiogenic capability of endothelial cells. *Neoplasia.* 9: 349-357.

63. Gurdon, J. B., and J. A. Byrne. 2003. The first half-century of nuclear transplantation. *Proc. Natl. Acad. Sci. U. S. A* 100: 8048-8052.

64. Rak, J., and A. Guha. 2012. Extracellular vesicles--vehicles that spread cancer genes. *Bioessays* 34: 489-497.

65. Quesenberry, P. J., and J. M. Aliotta. 2010. Cellular phenotype switching and microvesicles. *Adv. Drug Deliv. Rev.* 62: 1141-1148.

66. Kalluri, R., and E. G. Neilson. 2003. Epithelial-mesenchymal transition and its implications for fibrosis. *J. Clin. Invest* 112: 1776-1784.

67. Shook, D., and R. Keller. 2003. Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development. *Mech. Dev.* 120: 1351-1383.

68. Thiery, J. P., and J. P. Sleeman. 2006. Complex networks orchestrate epithelial-mesenchymal transitions. *Nat. Rev. Mol. Cell Biol.* 7: 131-142.

69. Samatov, T. R., A. G. Tonevitsky, and U. Schumacher. 2013. Epithelial-mesenchymal transition: focus on metastatic cascade, alternative splicing, non-coding RNAs and modulating compounds. *Mol. Cancer* 12: 107.

70. Nakaya, Y., and G. Sheng. 2013. EMT in developmental morphogenesis. *Cancer Lett.* 341: 9-15.

71. Taylor, M. A., J. G. Parvani, and W. P. Schiemann. 2010. The pathophysiology of epithelial-mesenchymal transition induced by transforming growth factor-beta in normal and malignant mammary epithelial cells. *J. Mammary. Gland. Biol. Neoplasia.* 15: 169-190.

72. Mani, S. A., W. Guo, M. J. Liao, E. N. Eaton, A. Ayyanan, A. Y. Zhou, M. Brooks, F. Reinhard, C. C. Zhang, M. Shipitsin, L. L. Campbell, K. Polyak, C. Brisken, J. Yang, and R. A. Weinberg. 2008. The epithelial-

mesenchymal transition generates cells with properties of stem cells. *Cell* 133: 704-715.

73. Kalluri, R., and R. A. Weinberg. 2009. The basics of epithelial-mesenchymal transition. *J. Clin. Invest* 119: 1420-1428.

74. Chea, H. K., C. V. Wright, and B. J. Swalla. 2005. Nodal signaling and the evolution of deuterostome gastrulation. *Dev. Dyn.* 234: 269-278.

75. Sauka-Spengler, T., and M. Bronner-Fraser. 2008. A gene regulatory network orchestrates neural crest formation. *Nat. Rev. Mol. Cell Biol.* 9: 557-568.

76. Mercado-Pimentel, M. E., and R. B. Runyan. 2007. Multiple transforming growth factor-beta isoforms and receptors function during epithelial-mesenchymal cell transformation in the embryonic heart. *Cells Tissues. Organs* 185: 146-156.

77. Yang, J., and R. A. Weinberg. 2008. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev. Cell* 14: 818-829.

78. Onder, T. T., P. B. Gupta, S. A. Mani, J. Yang, E. S. Lander, and R. A. Weinberg. 2008. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res.* 68: 3645-3654.

79. Wynn, T. A. 2007. Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. *J. Clin. Invest* 117: 524-529.

80. Iwano, M., D. Plieth, T. M. Danoff, C. Xue, H. Okada, and E. G. Neilson. 2002. Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J. Clin. Invest* 110: 341-350.

81. Wendt, M. K., T. M. Allington, and W. P. Schiemann. 2009. Mechanisms of the epithelial-mesenchymal transition by TGF-beta. *Future. Oncol.* 5: 1145-1168.

82. Moody, S. E., D. Perez, T. C. Pan, C. J. Sarkisian, C. P. Portocarrero, C. J. Sterner, K. L. Notorfrancesco, R. D. Cardiff, and L. A. Chodosh. 2005. The transcriptional repressor Snail promotes mammary tumor recurrence. *Cancer Cell* 8: 197-209.

83. Lee, J. M., S. Dedhar, R. Kalluri, and E. W. Thompson. 2006. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *J. Cell Biol.* 172: 973-981.

84. Maeda, M., K. R. Johnson, and M. J. Wheelock. 2005. Cadherin switching: essential for behavioral but not morphological changes during an epithelium-to-mesenchyme transition. *J. Cell Sci.* 118: 873-887.

85. Zeisberg, E. M., O. Tarnavski, M. Zeisberg, A. L. Dorfman, J. R. McMullen, E. Gustafsson, A. Chandraker, X. Yuan, W. T. Pu, A. B. Roberts, E. G. Neilson, M. H. Sayegh, S. Izumo, and R. Kalluri. 2007. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nat. Med.* 13: 952-961.

86. Zeisberg, E. M., S. Potenta, L. Xie, M. Zeisberg, and R. Kalluri. 2007. Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. *Cancer Res.* 67: 10123-10128.

87. Tsuji, T., S. Ibaragi, and G. F. Hu. 2009. Epithelial-mesenchymal transition and cell cooperativity in metastasis. *Cancer Res.* 69: 7135-7139.

88. Brabletz, T., A. Jung, S. Spaderna, F. Hlubek, and T. Kirchner. 2005. Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. *Nat. Rev. Cancer* 5: 744-749.

89. Cavallaro, U., and G. Christofori. 2004. Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. *Nat. Rev. Cancer* 4: 118-132.

90. Yoshimura, M., Y. Ihara, Y. Matsuzawa, and N. Taniguchi. 1996. Aberrant glycosylation of E-cadherin enhances cell-cell binding to suppress metastasis. *J. Biol. Chem.* 271: 13811-13815.

91. Van, R. F., and G. Berx. 2008. The cell-cell adhesion molecule E-cadherin. *Cell Mol. Life Sci.* 65: 3756-3788.

92. Hennig, G., J. Behrens, M. Truss, S. Frisch, E. Reichmann, and W. Birchmeier. 1995. Progression of carcinoma cells is associated with alterations in chromatin structure and factor binding at the E-cadherin promoter in vivo. *Oncogene* 11: 475-484.

93. Mizejewski, G. J. 1999. Role of integrins in cancer: survey of expression patterns. *Proc. Soc. Exp. Biol. Med.* 222: 124-138.

94. Desgrosellier, J. S., and D. A. Cheresh. 2010. Integrins in cancer: biological implications and therapeutic opportunities. *Nat. Rev. Cancer* 10: 9-22.

95. Jeon, E. S., J. H. Kim, H. Ryu, and E. K. Kim. 2012. Lysophosphatidic acid activates TGFBIp expression in human corneal fibroblasts through a TGF-beta1-dependent pathway. *Cell Signal.* 24: 1241-1250.

96. Sarrazy, V., A. Koehler, M. Chow, E. Zimina, C. X. Li, H. Kato, C. A. Caldarone, and B. Hinz. 2014. Integrins alphavbeta5 and alphavbeta3 promote latent TGF-beta1 activation by human cardiac fibroblast contraction. *Cardiovasc. Res.*

97. Dey, P., J. Togra, and S. Mitra. 2014. Intermediate filament: Structure, function, and applications in cytology. *Diagn. Cytopathol.*

98. Sarrio, D., S. M. Rodriguez-Pinilla, D. Hardisson, A. Cano, G. Moreno-Bueno, and J. Palacios. 2008. Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. *Cancer Res.* 68: 989-997.

99. Vuoriluoto, K., H. Haugen, S. Kiviluoto, J. P. Mpindi, J. Nevo, C. Gjerdrum, C. Tiron, J. B. Lorens, and J. Ivaska. 2011. Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer. *Oncogene* 30: 1436-1448.

100. Gjerdrum, C., C. Tiron, T. Hoiby, I. Stefansson, H. Haugen, T. Sandal, K. Collett, S. Li, E. McCormack, B. T. Gjertsen, D. R. Micklem, L. A. Akslen, C. Glackin, and J. B. Lorens. 2010. Axl is an essential epithelial-to-mesenchymal transition-induced regulator of breast cancer metastasis and patient survival. *Proc. Natl. Acad. Sci. U. S. A* 107: 1124-1129.

101. Vuoriluoto, K., G. Hognas, P. Meller, K. Lehti, and J. Ivaska. 2011. Syndecan-1 and -4 differentially regulate oncogenic K-ras dependent cell invasion into collagen through alpha2beta1 integrin and MT1-MMP. *Matrix Biol.* 30: 207-217.

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

103. Wei, J., G. Xu, M. Wu, Y. Zhang, Q. Li, P. Liu, T. Zhu, A. Song, L. Zhao, Z. Han, G. Chen, S. Wang, L. Meng, J. Zhou, Y. Lu, S. Wang, and D. Ma. 2008. Overexpression of vimentin contributes to prostate cancer invasion and metastasis via src regulation. *Anticancer Res.* 28: 327-334.

104. Sethi, S., J. Macoska, W. Chen, and F. H. Sarkar. 2010. Molecular signature of epithelial-mesenchymal transition (EMT) in human prostate cancer bone metastasis. *Am. J. Transl. Res.* 3: 90-99.

105. Halbleib, J. M., and W. J. Nelson. 2006. Cadherins in development: cell adhesion, sorting, and tissue morphogenesis. *Genes Dev.* 20: 3199-3214.

106. Fuxe, J., T. Vincent, and H. A. Garcia de. 2010. Transcriptional crosstalk between TGF-beta and stem cell pathways in tumor cell invasion: role of EMT promoting Smad complexes. *Cell Cycle* 9: 2363-2374.

107. Gavert, N., and A. Ben-Ze'ev. 2008. Epithelial-mesenchymal transition and the invasive potential of tumors. *Trends Mol. Med.* 14: 199-209.

108. Yook, J. I., X. Y. Li, I. Ota, E. R. Fearon, and S. J. Weiss. 2005. Wnt-dependent regulation of the E-cadherin repressor snail. *J. Biol. Chem.* 280: 11740-11748.

109. Ikenouchi, J. 2007. [Molecular mechanisms in the formation of discrete apical and basolateral membrane domains in polarized epithelial cells]. *Tanpakushitsu Kakusan Koso* 52: 1863-1870.

110. Wang, X., J. Nie, Q. Zhou, W. Liu, F. Zhu, W. Chen, H. Mao, N. Luo, X. Dong, and X. Yu. 2008. Downregulation of Par-3 expression and disruption of Par complex integrity by TGF-beta during the process of epithelial to mesenchymal transition in rat proximal epithelial cells. *Biochim. Biophys. Acta* 1782: 51-59.

111. Ozdamar, B., R. Bose, M. Barrios-Rodiles, H. R. Wang, Y. Zhang, and J. L. Wrana. 2005. Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. *Science* 307: 1603-1609.

112. Zohn, I. E., Y. Li, E. Y. Skolnik, K. V. Anderson, J. Han, and L. Niswander. 2006. p38 and a p38-interacting protein are critical for downregulation of E-cadherin during mouse gastrulation. *Cell* 125: 957-969.

113. Spaderna, S., O. Schmalhofer, M. Wahlbuhl, A. Dimmler, K. Bauer, A. Sultan, F. Hlubek, A. Jung, D. Strand, A. Eger, T. Kirchner, J. Behrens, and

T. Brabletz. 2008. The transcriptional repressor ZEB1 promotes metastasis and loss of cell polarity in cancer. *Cancer Res.* 68: 537-544.

114. Spaderna, S., O. Schmalhofer, F. Hlubek, A. Jung, T. Kirchner, and T. Brabletz. 2007. Epithelial-mesenchymal and mesenchymal-epithelial transitions during cancer progression. *Verh. Dtsch. Ges. Pathol.* 91: 21-28.

115. Buck, M. B., and C. Knabbe. 2006. TGF-beta signaling in breast cancer. *Ann. N. Y. Acad. Sci.* 1089: 119-126.

116. Moreno-Bueno, G., F. Portillo, and A. Cano. 2008. Transcriptional regulation of cell polarity in EMT and cancer. *Oncogene* 27: 6958-6969.

117. Zavadil, J., and E. P. Bottinger. 2005. TGF-beta and epithelial-to-mesenchymal transitions. *Oncogene* 24: 5764-5774.

118. Buijs, J. T., N. V. Henriquez, P. G. van Overveld, G. van der Horst, D. P. ten, and G. van der Pluijm. 2007. TGF-beta and BMP7 interactions in tumour progression and bone metastasis. *Clin. Exp. Metastasis* 24: 609-617.

119. Shirakihara, T., M. Saitoh, and K. Miyazono. 2007. Differential regulation of epithelial and mesenchymal markers by deltaEF1 proteins in epithelial mesenchymal transition induced by TGF-beta. *Mol. Biol. Cell* 18: 3533-3544.

120. Valcourt, U., M. Kowanetz, H. Niimi, C. H. Heldin, and A. Moustakas. 2005. TGF-beta and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition. *Mol. Biol. Cell* 16: 1987-2002.

121. Barcellos-Hoff, M. H., and R. J. Akhurst. 2009. Transforming growth factor-beta in breast cancer: too much, too late. *Breast Cancer Res.* 11: 202.

122. Xiao, D., and J. He. 2010. Epithelial mesenchymal transition and lung cancer. *J. Thorac. Dis.* 2: 154-159.

123. Thiery, J. P., H. Acloque, R. Y. Huang, and M. A. Nieto. 2009. Epithelial-mesenchymal transitions in development and disease. *Cell* 139: 871-890.

124. Thuault, S., E. J. Tan, H. Peinado, A. Cano, C. H. Heldin, and A. Moustakas. 2008. HMGA2 and Smads co-regulate SNAIL1 expression during induction of epithelial-to-mesenchymal transition. *J. Biol. Chem.* 283: 33437-33446.

125. Vincent, T., E. P. Neve, J. R. Johnson, A. Kukalev, F. Rojo, J. Albanell, K. Pietras, I. Virtanen, L. Philipson, P. L. Leopold, R. G. Crystal, A. G. de Herreros, A. Moustakas, R. F. Pettersson, and J. Fuxe. 2009. A SNAIL1-

SMAD3/4 transcriptional repressor complex promotes TGF-beta mediated epithelial-mesenchymal transition. *Nat. Cell Biol.* 11: 943-950.

126. Heldin, C. H., M. Landstrom, and A. Moustakas. 2009. Mechanism of TGF-beta signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition. *Curr. Opin. Cell Biol.* 21: 166-176.

127. Moustakas, A., and C. H. Heldin. 2009. The regulation of TGFbeta signal transduction. *Development* 136: 3699-3714.

128. Hall, A. 2005. Rho GTPases and the control of cell behaviour. *Biochem. Soc. Trans.* 33: 891-895.

129. Zavadil, J., M. Bitzer, D. Liang, Y. C. Yang, A. Massimi, S. Kneitz, E. Piek, and E. P. Bottinger. 2001. Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta. *Proc. Natl. Acad. Sci. U. S. A* 98: 6686-6691.

130. Konigshoff, M., and O. Eickelberg. 2010. WNT signaling in lung disease: a failure or a regeneration signal? *Am. J. Respir. Cell Mol. Biol.* 42: 21-31.

131. Konigshoff, M., M. Kramer, N. Balsara, J. Wilhelm, O. V. Amarie, A. Jahn, F. Rose, L. Fink, W. Seeger, L. Schaefer, A. Gunther, and O. Eickelberg. 2009. WNT1-inducible signaling protein-1 mediates pulmonary

fibrosis in mice and is upregulated in humans with idiopathic pulmonary fibrosis. *J. Clin. Invest* 119: 772-787.

132. Timmerman, L. A., J. Grego-Bessa, A. Raya, E. Bertran, J. M. Perez-Pomares, J. Diez, S. Aranda, S. Palomo, F. McCormick, J. C. Izpisua-Belmonte, and J. L. de la Pompa. 2004. Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. *Genes Dev.* 18: 99-115.

133. Karhadkar, S. S., G. S. Bova, N. Abdallah, S. Dhara, D. Gardner, A. Maitra, J. T. Isaacs, D. M. Berman, and P. A. Beachy. 2004. Hedgehog signalling in prostate regeneration, neoplasia and metastasis. *Nature* 431: 707-712.

134. Briegel, K. J. 2006. Embryonic transcription factors in human breast cancer. *IUBMB. Life* 58: 123-132.

135. Mani, S. A., W. Guo, M. J. Liao, E. N. Eaton, A. Ayyanan, A. Y. Zhou, M. Brooks, F. Reinhard, C. C. Zhang, M. Shipitsin, L. L. Campbell, K. Polyak, C. Brisken, J. Yang, and R. A. Weinberg. 2008. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 133: 704-715.

136. Thiery, J. P. 2003. Epithelial-mesenchymal transitions in development and pathologies. *Curr. Opin. Cell Biol.* 15: 740-746.

137. Shang, Y., X. Cai, and D. Fan. 2013. Roles of epithelial-mesenchymal transition in cancer drug resistance. *Curr. Cancer Drug Targets.* 13: 915-929.

138. Sarkar, S., G. Horn, K. Moulton, A. Oza, S. Byler, S. Kokolus, and M. Longacre. 2013. Cancer development, progression, and therapy: an epigenetic overview. *Int. J. Mol. Sci.* 14: 21087-21113.

139. Wendt, M. K., J. A. Smith, and W. P. Schiemann. 2010. Transforming growth factor-beta-induced epithelial-mesenchymal transition facilitates epidermal growth factor-dependent breast cancer progression. *Oncogene* 29: 6485-6498.

140. Tam, W. L., H. Lu, J. Buikhuisen, B. S. Soh, E. Lim, F. Reinhardt, Z. J. Wu, J. A. Krall, B. Bierie, W. Guo, X. Chen, X. S. Liu, M. Brown, B. Lim, and R. A. Weinberg. 2013. Protein kinase C alpha is a central signaling node and therapeutic target for breast cancer stem cells. *Cancer Cell* 24: 347-364.

141. Nathoo, N., A. Chahlavi, G. H. Barnett, and S. A. Toms. 2005. Pathobiology of brain metastases. *J. Clin. Pathol.* 58: 237-242.

142. Crawford, H. C., U. S. Krishna, D. A. Israel, L. M. Matrisian, M. K. Washington, and R. M. Peek, Jr. 2003. Helicobacter pylori strain-selective

induction of matrix metalloproteinase-7 in vitro and within gastric mucosa. *Gastroenterology* 125: 1125-1136.

143. Haberland, M., A. Johnson, M. H. Mokalled, R. L. Montgomery, and E. N. Olson. 2009. Genetic dissection of histone deacetylase requirement in tumor cells. *Proc. Natl. Acad. Sci. U. S. A* 106: 7751-7755.

144. Valdes, F., A. M. Alvarez, A. Locascio, S. Vega, B. Herrera, M. Fernandez, M. Benito, M. A. Nieto, and I. Fabregat. 2002. The epithelial mesenchymal transition confers resistance to the apoptotic effects of transforming growth factor Beta in fetal rat hepatocytes. *Mol. Cancer Res.* 1: 68-78.

145. Radisky, D., C. Hagios, and M. J. Bissell. 2001. Tumors are unique organs defined by abnormal signaling and context. *Semin. Cancer Biol.* 11: 87-95.

146. Coussens, L. M., B. Fingleton, and L. M. Matrisian. 2002. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* 295: 2387-2392.

147. Gabrilovich, D. I., S. Ostrand-Rosenberg, and V. Bronte. 2012. Coordinated regulation of myeloid cells by tumours. *Nat. Rev. Immunol.* 12: 253-268.

148. Condeelis, J., and J. W. Pollard. 2006. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* 124: 263-266.

149. Ohta, M., Y. Kitadai, S. Tanaka, M. Yoshihara, W. Yasui, N. Mukaida, K. Haruma, and K. Chayama. 2002. Monocyte chemoattractant protein-1 expression correlates with macrophage infiltration and tumor vascularity in human esophageal squamous cell carcinomas. *Int. J. Cancer* 102: 220-224.

150. Biswas, S. K., and A. Sodhi. 2002. In vitro activation of murine peritoneal macrophages by monocyte chemoattractant protein-1: upregulation of CD11b, production of proinflammatory cytokines, and the signal transduction pathway. *J. Interferon Cytokine Res.* 22: 527-538.

151. Heusinkveld, M., and S. H. van der Burg. 2011. Identification and manipulation of tumor associated macrophages in human cancers. *J. Transl. Med.* 9: 216.

152. Qian, B. Z., and J. W. Pollard. 2010. Macrophage diversity enhances tumor progression and metastasis. *Cell* 141: 39-51.

153. Condeelis, J., and J. W. Pollard. 2006. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* 124: 263-266.

154. Martin, M. L., and B. C. Blaxall. 2012. Cardiac intercellular communication: are myocytes and fibroblasts fair-weather friends? *J. Cardiovasc. Transl. Res.* 5: 768-782.

155. Yang, L., N. Chang, X. Liu, Z. Han, T. Zhu, C. Li, L. Yang, and L. Li. 2012. Bone marrow-derived mesenchymal stem cells differentiate to hepatic myofibroblasts by transforming growth factor-beta1 via sphingosine kinase/sphingosine 1-phosphate (S1P)/S1P receptor axis. *Am. J. Pathol.* 181: 85-97.

156. Grafi, G. 2009. The complexity of cellular dedifferentiation: implications for regenerative medicine. *Trends Biotechnol.* 27: 329-332.

157. Tan, J., X. Peng, G. Luo, B. Ma, C. Cao, W. He, S. Yuan, S. Li, J. A. Wilkins, and J. Wu. 2010. Investigating the role of P311 in the hypertrophic scar. *PLoS. One.* 5: e9995.

158. LeBleu, V. S., G. Taduri, J. O'Connell, Y. Teng, V. G. Cooke, C. Woda, H. Sugimoto, and R. Kalluri. 2013. Origin and function of myofibroblasts in kidney fibrosis. *Nat. Med.* 19: 1047-1053.

159. Tomasek, J. J., G. Gabbiani, B. Hinz, C. Chaponnier, and R. A. Brown. 2002. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat. Rev. Mol. Cell Biol.* 3: 349-363.

160. Hinz, B., S. H. Phan, V. J. Thannickal, A. Galli, M. L. Bochaton-Piallat, and G. Gabbiani. 2007. The myofibroblast: one function, multiple origins. *Am. J. Pathol.* 170: 1807-1816.

161. Moustakas, A., and C. H. Heldin. 2009. The regulation of TGFbeta signal transduction. *Development* 136: 3699-3714.

162. Sheppard, D. 2006. Transforming growth factor beta: a central modulator of pulmonary and airway inflammation and fibrosis. *Proc. Am. Thorac. Soc.* 3: 413-417.

163. Bork, P. 1993. The modular architecture of a new family of growth regulators related to connective tissue growth factor. *FEBS Lett.* 327: 125-130.

164. Gressner, O. A., and A. M. Gressner. 2008. Connective tissue growth factor: a fibrogenic master switch in fibrotic liver diseases. *Liver Int.* 28: 1065-1079.

165. Bogatkevich, G. S., A. Ludwicka-Bradley, C. B. Singleton, J. R. Bethard, and R. M. Silver. 2008. Proteomic analysis of CTGF-activated lung fibroblasts: identification of IQGAP1 as a key player in lung fibroblast migration. *Am. J. Physiol Lung Cell Mol. Physiol* 295: L603-L611.

166. Kreuger, J., M. Salmivirta, L. Sturiale, G. Gimenez-Gallego, and U. Lindahl. 2001. Sequence analysis of heparan sulfate epitopes with graded affinities for fibroblast growth factors 1 and 2. *J. Biol. Chem.* 276: 30744-30752.

167. Hanahan, D., and R. A. Weinberg. 2000. The hallmarks of cancer. *Cell* 100: 57-70.

168. Scott, R. W., S. Hooper, D. Crighton, A. Li, I. Konig, J. Munro, E. Trivier, G. Wickman, P. Morin, D. R. Croft, J. Dawson, L. Machesky, K. I. Anderson, E. A. Sahai, and M. F. Olson. 2010. LIM kinases are required for invasive path generation by tumor and tumor-associated stromal cells. *J. Cell Biol.* 191: 169-185.

169. Olumi, A. F., G. D. Grossfeld, S. W. Hayward, P. R. Carroll, T. D. Tlsty, and G. R. Cunha. 1999. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res.* 59: 5002-5011.

170. Carmeliet, P., and R. K. Jain. 2011. Molecular mechanisms and clinical applications of angiogenesis. *Nature* 473: 298-307.

171. Castells, M., B. Thibault, J. P. Delord, and B. Couderc. 2012. Implication of tumor microenvironment in chemoresistance: tumor-

associated stromal cells protect tumor cells from cell death. *Int. J. Mol. Sci.* 13: 9545-9571.

172. Orimo, A., P. B. Gupta, D. C. Sgroi, F. Arenzana-Seisdedos, T. Delaunay, R. Naeem, V. J. Carey, A. L. Richardson, and R. A. Weinberg. 2005. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 121: 335-348.

173. Mareel, M., M. J. Oliveira, and I. Madani. 2009. Cancer invasion and metastasis: interacting ecosystems. *Virchows Arch.* 454: 599-622.

174. Bissell, M. J., and W. C. Hines. 2011. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nat. Med.* 17: 320-329.

175. Bhowmick, N. A., E. G. Neilson, and H. L. Moses. 2004. Stromal fibroblasts in cancer initiation and progression. *Nature* 432: 332-337.

176. Frantz, C., K. M. Stewart, and V. M. Weaver. 2010. The extracellular matrix at a glance. *J. Cell Sci.* 123: 4195-4200.

177. Todorovic, V., and D. B. Rifkin. 2012. LTBPs, more than just an escort service. *J. Cell Biochem.* 113: 410-418.

178. Brabek, J., C. T. Mierke, D. Rosel, P. Vesely, and B. Fabry. 2010. The role of the tissue microenvironment in the regulation of cancer cell motility and invasion. *Cell Commun. Signal.* 8: 22.

179. Ungefroren, H., S. Sebens, D. Seidl, H. Lehnert, and R. Hass. 2011. Interaction of tumor cells with the microenvironment. *Cell Commun. Signal.* 9: 18.

180. Kakkad, S. M., M. Solaiyappan, B. O'Rourke, I. Stasinopoulos, E. Ackerstaff, V. Raman, Z. M. Bhujwalla, and K. Glunde. 2010. Hypoxic tumor microenvironments reduce collagen I fiber density. *Neoplasia.* 12: 608-617.

181. Levental, K. R., H. Yu, L. Kass, J. N. Lakins, M. Egeblad, J. T. Erler, S. F. Fong, K. Csiszar, A. Giaccia, W. Weninger, M. Yamauchi, D. L. Gasser, and V. M. Weaver. 2009. Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell* 139: 891-906.

182. Fukumura, D., and R. K. Jain. 2007. Tumor microvasculature and microenvironment: targets for anti-angiogenesis and normalization. *Microvasc. Res.* 74: 72-84.

183. Leight, J. L., M. A. Wozniak, S. Chen, M. L. Lynch, and C. S. Chen. 2012. Matrix rigidity regulates a switch between TGF-beta1-induced apoptosis and epithelial-mesenchymal transition. *Mol. Biol. Cell* 23: 781-791.

184. Thannickal, V. J., G. B. Toews, E. S. White, J. P. Lynch, III, and F. J. Martinez. 2004. Mechanisms of pulmonary fibrosis. *Annu. Rev. Med.* 55: 395-417.

185. Artinian, V., and P. A. Kvale. 2004. Cancer and interstitial lung disease. *Curr. Opin. Pulm. Med.* 10: 425-434.

186. Ansa-Addo, E. A., S. Lange, D. Stratton, S. Antwi-Baffour, I. Cestari, M. I. Ramirez, M. V. McCrossan, and J. M. Inal. 2010. Human plasma membrane-derived vesicles halt proliferation and induce differentiation of THP-1 acute monocytic leukemia cells. *J. Immunol.* 185: 5236-5246.

187. De, W. O., L. Derycke, A. Hendrix, M. G. De, F. Godeau, H. Depypere, and M. Bracke. 2007. Soluble cadherins as cancer biomarkers. *Clin. Exp. Metastasis* 24: 685-697.

188. Cocucci, E., G. Racchetti, and J. Meldolesi. 2009. Shedding microvesicles: artefacts no more. *Trends Cell Biol.* 19: 43-51.

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

190. Momen-Heravi, F., L. Balaj, S. Alian, A. J. Trachtenberg, F. H. Hochberg, J. Skog, and W. P. Kuo. 2012. Impact of biofluid viscosity on size and sedimentation efficiency of the isolated microvesicles. *Front Physiol* 3: 162.

191. Momen-Heravi, F., L. Balaj, S. Alian, J. Tigges, V. Toxavidis, M. Ericsson, R. J. Distel, A. R. Ivanov, J. Skog, and W. P. Kuo. 2012. Alternative methods for characterization of extracellular vesicles. *Front Physiol* 3: 354.

192. Reiman, J. M., K. L. Knutson, and D. C. Radisky. 2010. Immune promotion of epithelial-mesenchymal transition and generation of breast cancer stem cells. *Cancer Res.* 70: 3005-3008.

193. Al-Hajj, M., M. S. Wicha, A. Benito-Hernandez, S. J. Morrison, and M. F. Clarke. 2003. Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci. U. S. A* 100: 3983-3988.

194. Dontu, G., M. Al-Hajj, W. M. Abdallah, M. F. Clarke, and M. S. Wicha. 2003. Stem cells in normal breast development and breast cancer. *Cell Prolif.* 36 Suppl 1: 59-72.

195. Hollier, B. G., A. A. Tinnirello, S. J. Werden, K. W. Evans, J. H. Taube, T. R. Sarkar, N. Sphyris, M. Shariati, S. V. Kumar, V. L. Battula, J. I. Herschkowitz, R. Guerra, J. T. Chang, N. Miura, J. M. Rosen, and S. A.

Mani. 2013. FOXC2 expression links epithelial-mesenchymal transition and stem cell properties in breast cancer. *Cancer Res.* 73: 1981-1992.

196. Thomson, S., F. Petti, I. Sujka-Kwok, P. Mercado, J. Bean, M. Monaghan, S. L. Seymour, G. M. Argast, D. M. Epstein, and J. D. Haley. 2011. A systems view of epithelial-mesenchymal transition signaling states. *Clin. Exp. Metastasis* 28: 137-155.

197. Pain, M., O. Bermudez, P. Lacoste, P. J. Royer, K. Botturi, A. Tissot, S. Brouard, O. Eickelberg, and A. Magnan. 2014. Tissue remodelling in chronic bronchial diseases: from the epithelial to mesenchymal phenotype. *Eur. Respir. Rev.* 23: 118-130.

198. Paulson, A. F., M. S. Prasad, A. H. Thuringer, and P. Manzerra. 2014. Regulation of cadherin expression in nervous system development. *Cell Adh. Migr.* 8: 19-28.

199. Marie, P. J., E. Hay, D. Modrowski, L. Revollo, G. Mbalaviele, and R. Civitelli. 2014. Cadherin-mediated cell-cell adhesion and signaling in the skeleton. *Calcif. Tissue Int.* 94: 46-54.

200. McEwen, A. E., D. E. Escobar, and C. J. Gottardi. 2012. Signaling from the adherens junction. *Subcell. Biochem.* 60: 171-196.

201. MacDonald, B. T., K. Tamai, and X. He. 2009. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev. Cell* 17: 9-26.

202. Schambony, A., M. Kunz, and D. Gradl. 2004. Cross-regulation of Wnt signaling and cell adhesion. *Differentiation* 72: 307-318.

203. Verheyen, E. M., and H. Clevers. 2010. Wnts as self-renewal factors: mammary stem cells and beyond. *Cell Stem Cell* 6: 494-495.

204. Satelli, A., and S. Li. 2011. Vimentin in cancer and its potential as a molecular target for cancer therapy. *Cell Mol. Life Sci.* 68: 3033-3046.

205. Chaffer, C. L., J. P. Brennan, J. L. Slavin, T. Blick, E. W. Thompson, and E. D. Williams. 2006. Mesenchymal-to-epithelial transition facilitates bladder cancer metastasis: role of fibroblast growth factor receptor-2. *Cancer Res.* 66: 11271-11278.

206. Farach, A. M., and D. S. Galileo. 2008. O-GlcNAc modification of radial glial vimentin filaments in the developing chick brain. *Brain Cell Biol.* 36: 191-202.

207. Moisan, E., and D. Girard. 2006. Cell surface expression of intermediate filament proteins vimentin and lamin B1 in human neutrophil spontaneous apoptosis. *J. Leukoc. Biol.* 79: 489-498.

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

209. Singh, S., S. Sadacharan, S. Su, A. Belldegrun, S. Persad, and G. Singh. 2003. Overexpression of vimentin: role in the invasive phenotype in an androgen-independent model of prostate cancer. *Cancer Res.* 63: 2306-2311.

210. Sethi, S., J. Macoska, W. Chen, and F. H. Sarkar. 2010. Molecular signature of epithelial-mesenchymal transition (EMT) in human prostate cancer bone metastasis. *Am. J. Transl. Res.* 3: 90-99.

211. Berridge, M. J., P. Lipp, and M. D. Bootman. 2000. Signal transduction. The calcium entry pas de deux. *Science* 287: 1604-1605.

212. Wei, C., X. Wang, M. Chen, K. Ouyang, L. S. Song, and H. Cheng. 2009. Calcium flickers steer cell migration. *Nature* 457: 901-905.

213. Polyak, K., and R. A. Weinberg. 2009. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat. Rev. Cancer* 9: 265-273.

214. Christiansen, J. J., and A. K. Rajasekaran. 2006. Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. *Cancer Res.* 66: 8319-8326.

215. Tang, J., H. Yan, and S. Zhuang. 2013. Histone deacetylases as targets for treatment of multiple diseases. *Clin. Sci. (Lond)* 124: 651-662.

216. Henderson, C., M. Mizzau, G. Paroni, R. Maestro, C. Schneider, and C. Brancolini. 2003. Role of caspases, Bid, and p53 in the apoptotic response triggered by histone deacetylase inhibitors trichostatin-A (TSA) and suberoylanilide hydroxamic acid (SAHA). *J. Biol. Chem.* 278: 12579-12589.

217. Willems, L., J. Tamburini, N. Chapuis, C. Lacombe, P. Mayeux, and D. Bouscary. 2012. PI3K and mTOR signaling pathways in cancer: new data on targeted therapies. *Curr. Oncol. Rep.* 14: 129-138.

218. Valdes, F., M. M. Murillo, A. M. Valverde, B. Herrera, A. Sanchez, M. Benito, M. Fernandez, and I. Fabregat. 2004. Transforming growth factor-beta activates both pro-apoptotic and survival signals in fetal rat hepatocytes. *Exp. Cell Res.* 292: 209-218.

219. Kurrey, N. K., S. P. Jalgaonkar, A. V. Joglekar, A. D. Ghanate, P. D. Chaskar, R. Y. Doiphode, and S. A. Bapat. 2009. Snail and slug mediate radioresistance and chemoresistance by antagonizing p53-mediated

apoptosis and acquiring a stem-like phenotype in ovarian cancer cells. *Stem Cells* 27: 2059-2068.

220. Liu, J., A. N. Eischeid, and X. M. Chen. 2012. Col1A1 production and apoptotic resistance in TGF-beta1-induced epithelial-to-mesenchymal transition-like phenotype of 603B cells. *PLoS. One.* 7: e51371.

221. Olie, R. A., A. P. Simoes-Wust, B. Baumann, S. H. Leech, D. Fabbro, R. A. Stahel, and U. Zangemeister-Wittke. 2000. A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. *Cancer Res.* 60: 2805-2809.

222. Ordonez, G. R., X. S. Puente, V. Quesada, and C. Lopez-Otin. 2009. Proteolytic systems: constructing degradomes. *Methods Mol. Biol.* 539: 33-47.

223. Egeblad, M., and Z. Werb. 2002. New functions for the matrix metalloproteinases in cancer progression. *Nat. Rev. Cancer* 2: 161-174.

224. Sternlicht, M. D., A. Lochter, C. J. Sympson, B. Huey, J. P. Rougier, J. W. Gray, D. Pinkel, M. J. Bissell, and Z. Werb. 1999. The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell* 98: 137-146.

225. Tian, H. Y., K. H. Zhang, X. Gao, W. W. Lei, L. Zhang, M. L. Yu, J. G. Song, and F. K. Zhao. 2009. Comparative proteomic analysis of cell cycle-dependent apoptosis induced by transforming growth factor-beta. *Biochim. Biophys. Acta* 1794: 1387-1397.

226. Yang, Y., X. Pan, W. Lei, J. Wang, and J. Song. 2006. Transforming growth factor-beta1 induces epithelial-to-mesenchymal transition and apoptosis via a cell cycle-dependent mechanism. *Oncogene* 25: 7235-7244.

227. Dvorak, H. F. 1986. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N. Engl. J. Med.* 315: 1650-1659.

228. Fiaschi, T., A. Marini, E. Giannoni, M. L. Taddei, P. Gandellini, D. A. De, M. Lanciotti, S. Serni, P. Cirri, and P. Chiarugi. 2012. Reciprocal metabolic reprogramming through lactate shuttle coordinately influences tumor-stroma interplay. *Cancer Res.* 72: 5130-5140.

229. Joyce, J. A., and J. W. Pollard. 2009. Microenvironmental regulation of metastasis. *Nat. Rev. Cancer* 9: 239-252.

230. Lu, P., K. Takai, V. M. Weaver, and Z. Werb. 2011. Extracellular matrix degradation and remodeling in development and disease. *Cold Spring Harb. Perspect. Biol.* 3.

231. DuFort, C. C., M. J. Paszek, and V. M. Weaver. 2011. Balancing forces: architectural control of mechanotransduction. *Nat. Rev. Mol. Cell Biol.* 12: 308-319.

232. Dieci, M. V., M. Arnedos, F. Andre, and J. C. Soria. 2013. Fibroblast growth factor receptor inhibitors as a cancer treatment: from a biologic rationale to medical perspectives. *Cancer Discov.* 3: 264-279.

233. Turner, N., A. Pearson, R. Sharpe, M. Lambros, F. Geyer, M. A. Lopez-Garcia, R. Natrajan, C. Marchio, E. Iorns, A. Mackay, C. Gillett, A. Grigoriadis, A. Tutt, J. S. Reis-Filho, and A. Ashworth. 2010. FGFR1 amplification drives endocrine therapy resistance and is a therapeutic target in breast cancer. *Cancer Res.* 70: 2085-2094.

234. Sharpe, R., A. Pearson, M. T. Herrera-Abreu, D. Johnson, A. Mackay, J. C. Welti, R. Natrajan, A. R. Reynolds, J. S. Reis-Filho, A. Ashworth, and N. C. Turner. 2011. FGFR signaling promotes the growth of triple-negative and basal-like breast cancer cell lines both in vitro and in vivo. *Clin. Cancer Res.* 17: 5275-5286.

235. Chauhan, H., A. Abraham, J. R. Phillips, J. H. Pringle, R. A. Walker, and J. L. Jones. 2003. There is more than one kind of myofibroblast: analysis of CD34 expression in benign, in situ, and invasive breast lesions. *J. Clin. Pathol.* 56: 271-276.

236. Wong, Y. C., and Y. Z. Wang. 2000. Growth factors and epithelial-stromal interactions in prostate cancer development. *Int. Rev. Cytol.* 199: 65-116.

237. Bauer, G. 1996. Elimination of transformed cells by normal cells: a novel concept for the control of carcinogenesis. *Histol. Histopathol.* 11: 237-255.

238. Pavlakis, K., I. Messini, T. Vrekoussis, P. Yiannou, D. Keramopoullos, N. Louvrou, T. Liakakos, and E. N. Stathopoulos. 2008. The assessment of angiogenesis and fibroblastic stromagenesis in hyperplastic and pre-invasive breast lesions. *BMC. Cancer* 8: 88.

239. Fevrier, B., H. Laude, G. Raposo, and D. Vilette. 2005. [Exosomes: carriers of prions?]. *Med. Sci. (Paris)* 21: 132-133.