REGULATION OF THE TUMOUR SUPRESSOR PTEN THROUGH EXOSOMES

REGULATION OF THE TUMOUR SUPPRESSOR PTEN THROUGH EXOSOMES

By:

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A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

McMaster University MASTER OF SCIENCE (2013) Hamilton, Ontario (Medical Sciences)

TITLE: Regulation of the tumour suppressor PTEN through exosomes

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SUPERVISOR: Dr. Richard Austin & Dr. Khalid Al-Nedawi

NUMBER OF PAGES: viii, 65

ABSTRACT

PTEN is a potent tumour suppressor protein. Aggressive and metastatic prostate cancer (PC) is associated with a reduction or loss of PTEN expression. PTEN reduction often occurs without gene mutations, and its downregulation is not fully understood. Herein, we show that PTEN is incorporated in the cargo of exosomes derived from cancer cells, and this is an exclusive characteristic of cancer cells; normal cells do not incorporate PTEN in their exosomes. We found that this process is affected by the expression of oncogenes, with activation of oncogenic molecules leading to increased PTEN incorporation into exosomes. PTEN expressed in exosomes can be transferred to other cells that have a reduction or loss of PTEN expression. The transferred PTEN is active, as cells showed a substantial increase in phosphatase activity upon treatment with PTEN-bearing exosomes. PTEN transferred through exosomes is also competent to confer tumour-suppression activity to acceptor cells. After incubation with PTEN-bearing exosomes, recipient cells exhibited decreased AKT phosphorylation, changes in the expression of cell cycle mediators indicating cell cycle arrest, and decreased proliferation. These data suggest that exosomal PTEN may be able to compensate for PTEN loss in cancer cells, by transferring the active protein to cancer cells where it can then perform its role as a tumour suppressor.

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LIST OF ABBREVIATIONS

AR	Androgen receptor
ARF	ADP ribosylation factor
BSA	Bovine serum albumin
CK2	Casein kinase 2
CSF	Cerebrospinal fluid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFRvIII	Epidermal growth factor receptor variant III
EMMPRIN	Extracellular matrix metalloproteinase inducer
ERK	Extracellular-signal-regulated kinase
ESCRT	Endosomal sorting complexes required for transport
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSK3	Glycogen synthase kinase 3
HRPC	Hormone refractory prostate cancer
HSP	Heat shock protein
IF	Immunofluorescent
ILV	Intraluminal vesicle
MAC	Membrane attack complex
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamycin
MVB	Multivesicular body
PBS	Phosphate buffered saline
PC	Prostate cancer

PI	Phosphatidylinositol
PI3K	Phosphatidylinositide-3 kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PS	Phosphatidylserine
PSGL	P-selectin glycoprotein ligand
PTEN	Phosphatase and tensin homolog
SDS	Sodium dodecyl sulfate
STAT	Signal transducer and activator of transcription
TGFα	Transforming growth factor alpha
TSG101	Tumour susceptibility gene 101

CHAPTER 1: INTRODUCTION

1.1 Prostate Cancer

Prostate cancer (PC) is the common cancer (excluding non-melanoma skin cancer) and the third leading cause of cancer-related deaths among Canadian men. In 2012, an estimated 26,500 men were diagnosed with PC and 4,000 will die of it (Canadian Cancer Society, 2012). In Canada, 1 in 7 men will be diagnosed with prostate cancer during their lifetime, with 25% of these men eventually dying of prostate cancer (Fradet et al., 2009).

There are several established risk factors for PC occurrence and likelihood of disease severity. Risk of PC occurrence increases with age, and high risk disease is more likely after the age of 75 (Bechis et al., 2011; Wilt & Ahmed., 2013). Family history of PC is also an established risk factor, with likelihood of disease occurrence increasing with number of affected relatives (taking into account the patient's age) (Brandt et al., 2010). PC risk is also tied to ethnicity, with African American men developing prostate cancer at a significantly higher rate than other ethnicities (Glass et al., 2013). Modifiable lifestyle factors may also play a role, with moderate exercise found to decrease risk of PC (Antonelli et al., 2009), and smoking linked to greater prostate cancer mortality and risk of recurrence (Kenfield et al., 2011).

The typical advancement of PC from local to disseminated disease is well characterized. Early stage prostate tumours are dependent on androgens for growth. Androgens bind to the Androgen Receptor (AR), a nuclear transcription factor that regulates the transcription of pro-growth and pro-survival proteins upon ligand binding

(Saraon et al., 2011). Local PC is treated with radical prostatectomy or radiation. If PC recurs or is diagnosed at a more advanced stage, androgen ablation therapy is used. Androgen ablation is the depletion of androgens through orchiectomy or pharmacological methods (more commonly used). Pharmacological methods include Gonadotropin-releasing hormone (GnRH) super-agonists, which can be paired with AR antagonists for total androgen ablation (Feldman et al., 2001). Although androgen ablation is effective in reducing cancer growth, the cancer will eventually progress to become androgen independent and no longer responsive to hormone therapy (Hormone Refractory Prostate Cancer: HRPC).

Progression to HRPC occurs because cancer cells that lose androgen dependency are selected for through androgen ablation therapy. Loss of androgen dependence occurs through several mechanisms, some involving alterations in AR signalling and others through changes in growth and survival pathways independent of AR (Debes et al., 2004). Common AR signalling alterations include AR gene amplification or mutation and changes in expression of AR corregulators, leading to increased androgen sensitivity or activation of the receptor by binding partners other than androgens. Loss of androgen dependence can also occur through mutations in tumour suppressor genes such as *PTEN* or proto-oncogenes such as *Bcl-2*, allowing cancer cell survival and growth without the need for AR signalling (Debes et al., 2004). Androgen independent HRPC is not curable, and patients typically succumb to the disease within 2 years (Sadar, 2012).

1.2 Microvesicles

Microvesicles are small (40-1000 nm) membrane-bound vesicles shed by a variety of cell types under both physiological and pathological conditions. The term microvesicle has been used to define the heterogeneous population of vesicles secreted by cells, which are formed by at least two major mechanisms (exosomes, through the endosome pathway, and ectosomes, through plasma membrane budding). They carry a cell and cell-state specific cargo, and have been associated with important intercellular signaling functions. They are a unique form of communication with exciting possibilities, because they allow the transfer of insoluble molecules, active proteins, intact membrane-bound receptors, genetic material (mRNA, miRNA), and combinations of molecules which may interact to exert surprising effects (van Doormaal et al., 2009). Microvesicles are found in most bio-fluids, including blood, urine, lymph, and cerebrospinal fluid (CSF), allowing them to conduct messages to distant tissues (Lee et al., 2011).

This important mechanism of intercellular communication has also been implicated in several pathologies, notably cancer. Cancer cells shed a higher volume of microvesicles than normal cells due to oncogenic activation. For example, amplified K-ras and EGFR (or EGFRvIII) activity leads to increased formation and secretion of microvesicles (Al-Nedawi et al., 2008). Microvesicle release is also increased under conditions of UV irradiation, oxidative stress, membrane cholesterol depletion, and intracellular calcium amplification (Pant et al., 2012). Cancer cell derived microvesicles have been implicated in many aspects of tumourigenesis. Microvesicles transfer cancer-promoting mRNA and oncoproteins to a variety of cell types, including indolent cancer

cells, endothelial cells, and immune cells (Castellana et al., 2010). Indolent cells can be transformed to exhibit cancerous characteristics by the transfer of mutated receptors. For example, EGFRvIII bearing microvesicles from cancerous glioma cells can transfer this receptor to the surface of indolent cells (Al-Nedawi et al., 2008). This transfer results in the induction of cancerous changes in the recipient cells, including growth and survival enhancing signaling pathway activation (Al-Nedawi et al., 2008). Microvesicles have also been implicated in the promotion of a pro-tumour microenvironment (Anderson et al., 2010), invasion and angiogenesis (Al-Nedawi et al., 2009), local and systemic cancer-associated hypercoagulability (Al-Nedawi et al., 2005), and immune evasion (Taylor & Gercel-Taylor, 2011).

1.3 Exosome Formation

Exosomes are a subgroup of microvesicles that are formed through the endosome pathway (Figure 1). They are smaller in size (40-100 nm), and characterized by the presence of proteins involved in endocytosis (Rab-5a, Rab-5b, Alix, Tsg 101), lipid raft related proteins (Caveolin-1), heat shock proteins (Hsp70, Hsp90), flotillin-1, and tetraspanins (CD63, CD9, CD81) (Yang & Robbins, 2011). Exosome formation begins with vesicle cargo assembly at clatherin-coated sites of the plasma membrane, which then invaginate, forming early endosomes. This process may also be clatherin independent in some cases. The endosome then matures to form a multivesicular body (MVB) through inward budding of the limiting endosomal membrane to form intraluminal vesicles (ILV).

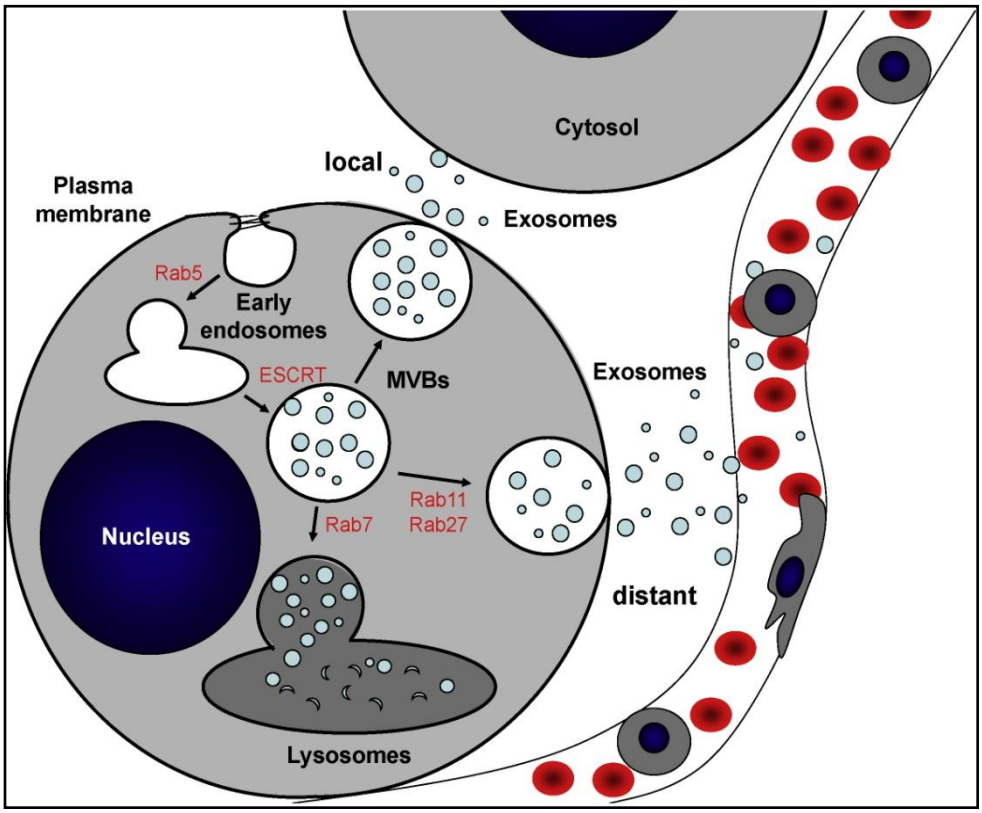

Figure 1. The formation and release of exosomes through the endosome pathway.

Exosome formation begins with endocytosis, forming early endosomes. Early endosomes mature to form multivesicular bodies, which are targeted to the plasma membrane and fuse with the membrane to release exosomes, or are degraded upon fusion with a lysosome. Released exosomes participate in local cell signaling, as well as signaling to distant targets when secreted to the bloodstream. (Ludwig & Giebel, 2012)

This process is facilitated by the ESCRT complexes (endosomal sorting complex required for transport), which recognize and organize ubiquitinated proteins within late endosomes and initiate membrane budding to form ILVs (Wollert & Hurley, 2010). The mature MVBs are then targeted to the cell membrane, fusing with the membrane to release ILVs (exosomes) into the extracellular space (de Gassart et al., 2003). Various Rab-GTPases (Rab 5, Rab 7, Rab 11, and Rab 27a and b) are involved in this process, facilitating maturation of early endosomes to late endosomes, membrane docking, and fusion of MVBs with the cell membrane (Huotari &Helenius, 2011; Ostrowski et al., 2009; Ludwig & Giebel., 2012). Alternately, MVBs fuse with lysosomes, at which point they are degraded. The well-known function of the endosome pathway in which endosomes are targeted for degradation is dependent on ESCRT complexes -I through –II (de Gassart et al., 2003).

ESCRT independent mechanisms of exosome formation and protein sorting have also been identified (Simons & Raposo, 2009). These mechanisms are dependent on ceramide, a sphingolipid that promotes membrane invagination and stabilizes lipid rafts. Ceramide has been found to be crucial for the sorting of proteins and lipids into ILVs destined for release as exosomes rather than degradation, and this process has been found to be ESCRT-independent (Trajkovic et al., 2008). Ceramide-rich domains may also attract tetraspanins, transmembrane proteins that form large webs involving transmembrane and cytosolic proteins. Several tetraspanins are highly represented in exosomes, and have been demonstrated to be involved in protein sorting into exosomes (Rana & Zöller, 2011; Chairoungdua et al., 2010). Lipid rafts are also likely to be

involved in exosome secretion, as exosomes are enriched in raft lipids, and the interface between the lipid raft and surrounding membrane is highly conducive to membrane budding (Simons & Raposo, 2009).

1.4 Ectosome Formation

The other major class of microvesicles is ectosomes, larger vesicles (100-1000 nm) formed through direct outwards budding of the plasma membrane. These vesicles are characterized by the presence of phosphatidylserine (PS) residues on their outer surfaces, raft lipids, flotillin-1, tissue factor, adhesion molecules (PSGL-1, integrin beta-1), matrix metalloproteinases (MMPs), Arf6, and EMMPRIN (Lee et al., 2011). The process of ectosome formation remains somewhat speculative, however some aspects of their biogenesis have been uncovered. Ectosomes are formed preferentially at lipid rafts, plasma membrane regions rich in cholesterol and sphingolipids. Ectosome formation sites are also areas with PS relocation to the outer leaflet of the plasma membrane. This relocation is accomplished by the actions of flippases (Camussi et al., 2011). Deficiencies in these enzymes results in defects in platelet microvesiculation, demonstrating their importance in the formation of ectosomes (Piccin et al., 2007). This flippase-induced lipid asymmetry encourages membrane curvature, promoting the formation of ectosomes. Membrane-associated proteins are also important in ectosome biogenesis, promoting membrane curvature, disrupting membrane interactions with the cytoskeleton, and allowing final release of the ectosome (Muralidharan-Chari et al., 2010).

1.5 PTEN

PTEN (phosphatase and tensin homolog deleted on chromosome 10) is an important tumour suppressor that is deleted or downregulated in a wide array of cancers. PTEN is a lipid/protein phosphatase, whose major biological function involves dephosphorylating the 3-phosphoinositide products of PI3K. These PI3K products are activators of the AKT survival and growth stimulating pathway, so PTEN acts as a negative regulator of AKT through dephosphorylation of PI3K products (Hollander et al., 2011) (Figure 2). PTEN has an N-terminal domain containing the phosphatase active site, and a C2 regulatory domain with no catalytic activity (Lee et al., 1999). The C-terminal domain contains five phosphorylation sites which regulate the protein's stability, and is responsible for binding the plasma membrane (Steelman et al., 2004).

PTEN activity is regulated through many mechanisms, including phosphorylation, ubiquitination, and differential targeting. C-terminal phosphorylation of PTEN by casein kinase 2 (CK2) and glycogen synthase kinase 3(GSK3) protein kinases results in a "closed" conformation, in which the protein is more stable, but also less active, exhibiting lower phosphatase activity, membrane association, and nuclear localization (Leslie et al., 2008). This increased stability is due in part to the decreased membrane localization associated with phosphorylated PTEN, because membrane-localized PTEN is more susceptible to ubiquitination (Maccario et al., 2010). Ubiquitination is an important control mechanism for PTEN stability and localization. PTEN mono-ubiquitination by Nedd-4 ubiquitin ligase leads to nuclear localization, while poly-ubiquitination leads to cytoplasmic retention and degredation (Trotman et al., 2007). PTEN ubiquitination has

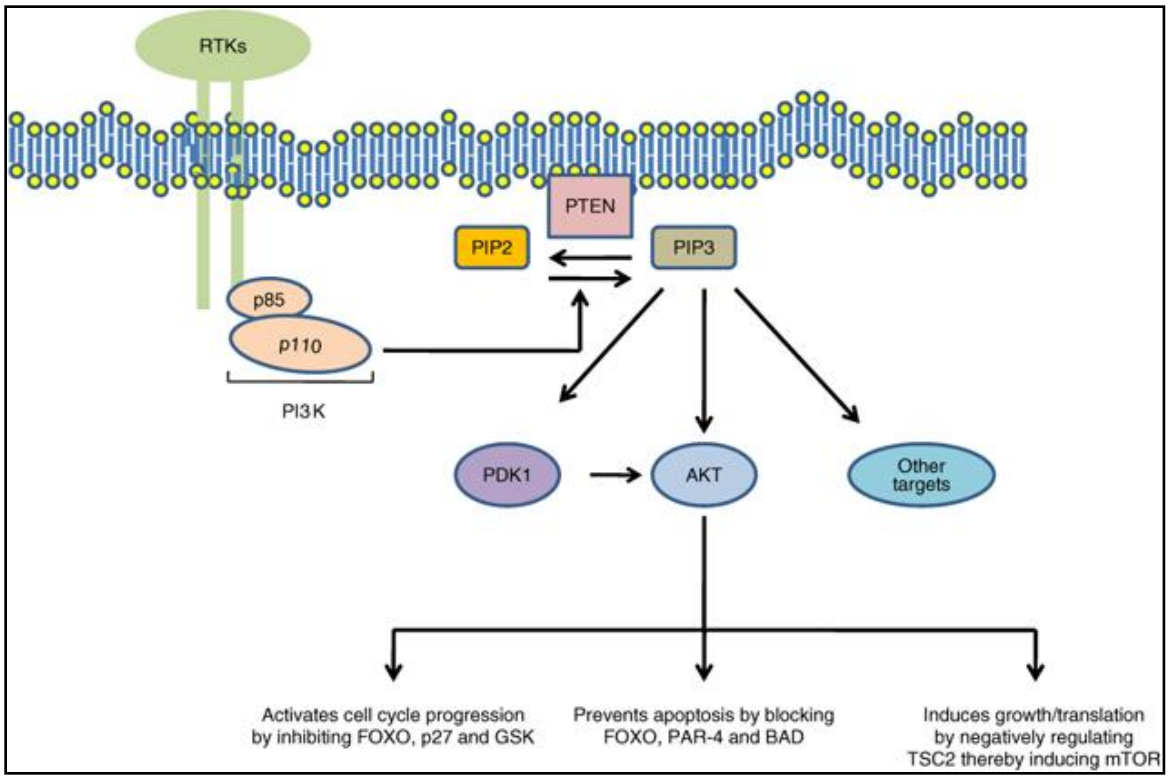

Figure 2. PTEN signaling

The tumour suppressor PTEN is a lipid/protein phosphatase that dephosphorylates PIP3 to PIP2. This antagonizes the Akt pathway, preventing the initiation of downstream pro-growth, proliferation, and survival signaling. (Keniry & Parsons, 2008)

been shown to decrease protein activity by inhibiting its phosphatase activity (Maccario et al., 2010). PTEN localization can also be modified by plasma membrane characteristics. PTEN is recruited to acidic, PS, PI, and PIP3-rich regions of the plasma membrane. PTEN membrane localization can thus be altered by changes in plasma membrane lipid composition, or by the actions of phospholipase C (Leslie et al., 2008). In many cancers, PTEN transcription is downregulated through promoter methylation, and PTEN mRNA is targeted by the overexpression of miRNAs (Hollander et al., 2011).

1.6 PTEN as a tumour suppressor

PTEN acts as a tumour suppressor protein by inhibiting the PI3K-AKT-mTOR pathway. When PTEN function is reduced and this signaling cascade is activated, several different changes occur that contribute to tumourigenesis. Uninhibited signaling through this pathway leads to increased proliferation, promoting rapid tumour growth. PTEN reduction can also lead to the promotion of epithelial-to-mesenchymal transition, leading to increased cancer cell invasiveness and motility (Song et al., 2009). PI3K-AKT-mTOR signaling leads to metabolic reprogramming, promoting glucose uptake and anaerobic glycolysis even in the presence of oxygen (the Warburg effect), which enables rapid cancer cell growth (Eguez et al., 2005; Fang et al., 2010). Reduction of nuclear PTEN leads to increased genomic instability, causing an accumulation of mutations in cancer cells (Puc et al., 2005).

Disruption of PTEN activity is a common feature of many cancer types. Reduced PTEN expression has been documented in glioblastoma, endometrial cancer, non-small

cell lung cancer, breast cancer, ovarian cancer, and colorectal cancer (Soria et al., 2002; Mutter et al., 2000; Perren et al., 1999; Bose et al., 2002; Kurose et al., 2001; McMenamin et al., 1999; Zhou et al., 2002). PTEN is reduced 70-80% in prostate primary tumours, and it is the most mutated gene in metastatic prostate cancer (Whang et al., 1998; Gray et al., 1995; Cairns et al., 1997; Suzuki et al., 1998). PTEN is a haploinsufficient tumour suppressor protein in some tissues, and mutation of PTEN at one allele only is common in prostate cancer (Salmena et al., 2008). Small reductions in PTEN expression or activity can affect its tumour suppressor function, causing increased cancer susceptibility and accelerating tumour progression (Alimonti et al., 2010). Although PTEN gene mutations are frequently seen in cancer, PTEN protein expression is often reduced in the absence of gene mutation (Salmena et al., 2008).

1.7 EGFR

EGFR (epidermal growth factor receptor) is a receptor tyrosine kinase that plays an important role in cell growth and survival (Figure 3). EGFR overexpression or oncogenic mutation is implicated in cancer development and progression (Mitsudomi & Yatabe, 2010). EGFR (also known as ErbB-1), is a member of the ErbB family of receptors, which also includes ErbB-2, ErbB-3, and ErbB-4 (Burgess, 2008). All members of the ErbB family contain four functional domains; an extracellular ligand-binding domain, a transmembrane domain, an intracellular tyrosine kinase domain, and a

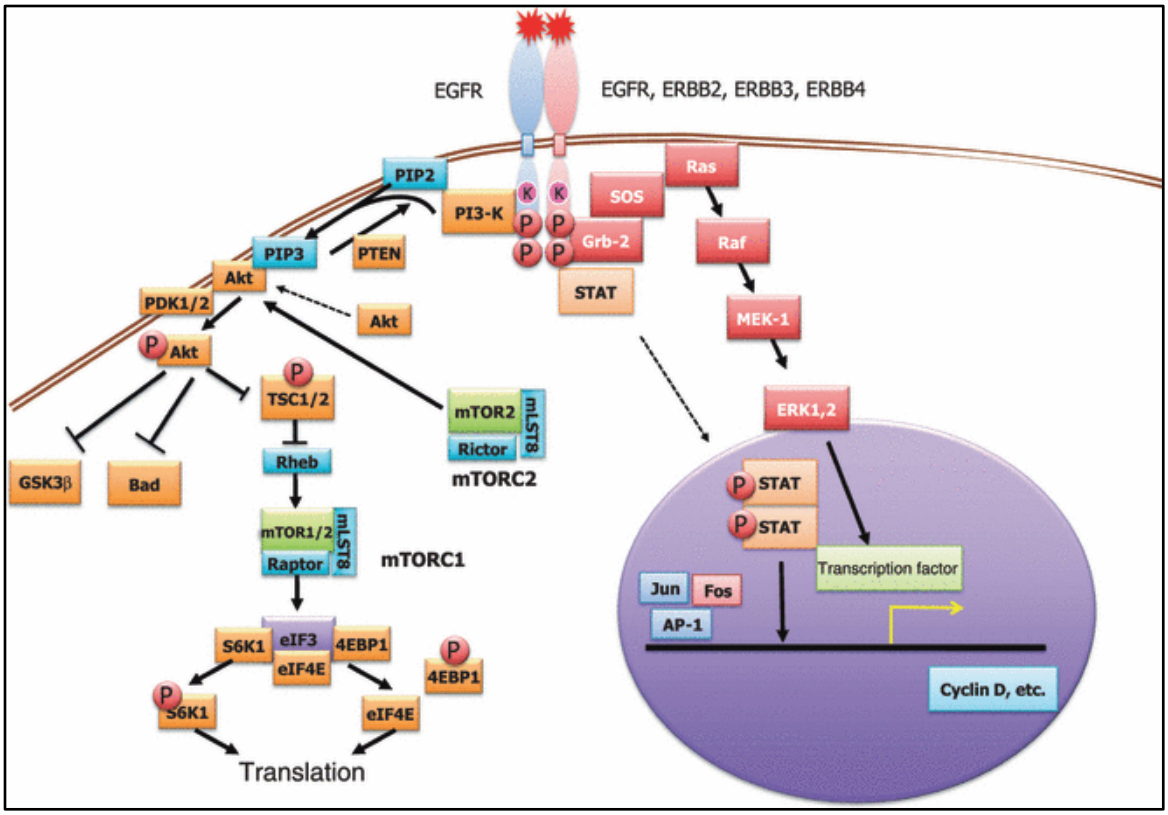

Figure 3. EGFR signaling.

Upon ligand binding, EGFR dimerizes with another ErbB family member, initiating autophosphorylation of the intracellular tyrosine kinase domain. Adaptor proteins bind to the phosphorylated tyrosine residues, allowing signal transducing molecules to bind and initiate signaling cascades. Activated pathways include PI3K/Akt, MAPK/ERK, and STAT, leading to the promotion of cell proliferation, invasion, survival, and metastasis. (Mitsudomi & Yatabe, 2010)

C-terminal regulatory domain (Hynes & MacDonald, 2009). ErbB receptor ligands include EGF (epidermal growth factor), TGF-α (transforming growth factor alpha), epigen, amphiregulin, and others (Riese & Stern, 1998). Ligand binding to the extracellular domain of the receptor initiates the formation of homo- or hetero-dimers. Dimerization stimulates activation of the intracellular tyrosine kinase domain, leading to autophosphorylation of tyrosine residues within the C-terminal domain (Holbro & Hynes, 2004). Adaptor proteins then bind to the phosphorylated tyrosines, anchoring signal transducing molecules which initiate downstream signaling pathways. Signaling pathways initiated by EGFR activation include the MAPK/ERK, PI3K/AKT, and STAT pathways (Normanno et al., 2006). Receptor signaling is inactivated when the receptor-ligand complex is internalized through endocytosis, and then degraded or recycled to the cell surface (Yarden, 2001). EGFR signaling pathways play an important role in cancer development through the promotion of cell proliferation, invasion, survival, and metastasis (Mitsudomi & Yatabe, 2010).

1.8 EGFRvIII

EGFRvIII is a mutated, oncogenic form of EGFR. EGFRvIII contains a deletion of exons 2-7, resulting in the loss of the first two regions of the extracellular ligand-binding domain (Wikstrand et al., 1998). The deletion is an in-frame deletion, and results in the insertion of a new glycine residue at the fusion junction (Figure 4). This mutation is most common in glioblastoma multiforme (20-30% of glioblastoma cases) (Gan et al., 2009), and is frequently accompanied by gene amplification (Voldborg et al., 1997).

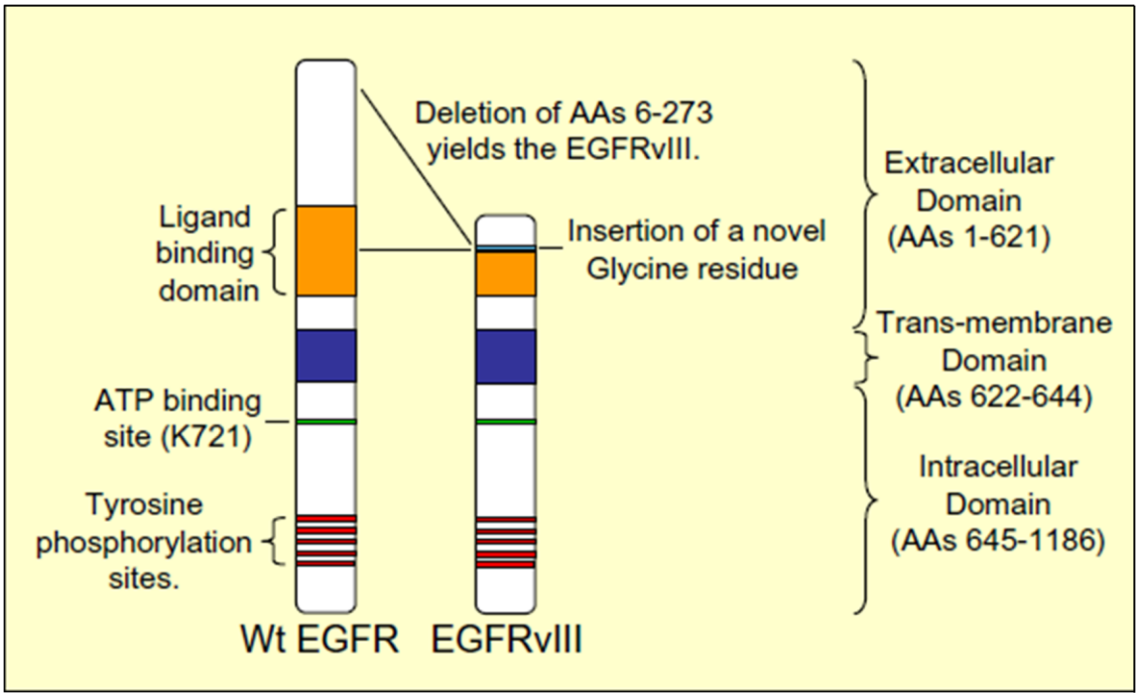

Figure 4. EGFRvIII structure.

EGFRvIII is a mutated, oncogenic variant of EGFR. It contains a deletion of exons 2-7, resulting in the loss of two extracellular domains and the insertion of a novel glycine residue. (Gan et al., 2009)

EGFRvIII has a constitutively activated tyrosine kinase domain, meaning that no ligand binding is required to initiate signaling (Mitsudomi & Yatabe, 2010). EGFRvIII has only approximately 10% of the activity level of wild-type EGFR. However, EGFRvIII is not as easily endocytosed as wild-type EGFR, so it remains at the cell surface longer, compensating for its lower signaling efficiency (Gan et al., 2013). EGFRvIII signaling primarily activates the PI3K/AKT pathway, leading to increased proliferation, enhanced tumorigenesis and resistance to chemotherapy *in vivo* (Gan et al., 2013).

1.9 Hypothesis

Based on the observation that PTEN is found in cancer cell-derived exosomes but is not present in exosomes produced by normal cells, we propose that the inclusion of PTEN in cancer cell exosomal cargo is under the control of oncogenes. We propose that these PTEN-bearing exosomes can transfer PTEN to cells not expressing the protein, and that the transferred PTEN can functionally compensate for PTEN loss in recipient cells.

1.10 Objectives

I. To assess the status of PTEN in cancer cell and normal cell exosomes and the role of oncogenic molecules in PTEN incorporation into exosomes.

The observation that PTEN is included in cancer cell exosomes but not normal cell exosomal cargo suggests that this may be a cancer cell-specific mechanism under the control of oncogenes. We will assess PTEN status in exosomes after EGFR and EGFRvIII transfection into cells, and under conditions of EGFR activation and inhibition.

II. To assess the intercellular transfer of PTEN through exosomes to PTEN-negative cells.

We will assess the capacity of PTEN-bearing exosomes to transfer PTEN to other cells. PTEN-reduced and PTEN-negative cells will be incubated with PTEN-bearing exosomes, and PTEN acquisition will be assessed. PTEN mRNA levels in acceptor cells will be

assessed following incubation with PTEN-bearing exosomes to determine if exosomes affect PTEN transcription.

III. To assess the functional consequences of PTEN transfer through exosomes, to determine whether exosomal PTEN can compensate for PTEN loss.

We will assess changes in PTEN-negative cells following incubation with PTEN-bearing exosomes to determine whether transferred PTEN is active, and whether PTEN activity is adequate to compensate for PTEN loss. Characteristics to assess will include proliferation, PTEN phosphatase activity, AKT phosphorylation status, and levels of cell cycle mediators.

CHAPTER TWO: METHODS

2.1 Antibodies

Monoclonal antibodies for PTEN, AKT, Flotilin-1, p27, EGFR, B-actin, and cyclin D1 were purchased from Cell Signaling Technology (Danvers, MA). All the corresponding HRP-conjugated secondary antibodies were purchased from Cell Signaling Technology. Alexa Fluor 488 secondary antibodies were purchased from Molecular Probes (Eugene, OR).

2.2 Cell Lines

DU145 (human prostate cancer cells), PC-3 (human prostate cancer cells), U87 (human glioblastoma astrocytoma), human normal cells [Human aortic endothelial cells (HAOEC), Human aortic smooth muscle cells (HAOSMC) and Human prostate epithelial cells (HPEC)], A549 (human alveolar adenocarcinoma), MDA-MB3 (human breast adenocarcinoma), HCT 116 (human colorectal carcinoma), and BxPC-3 (human pancreatic adenocarcinoma) were purchased from the ATCC (Manassas, VA). DU145 cells with PTEN knockdown (DU145Kd) and DU145 cells transfected with nonspecific siRNA were originally generated in Dr Damu Tang's laboratory at the Hamilton Kidney Research Centre (HKRC), McMaster University. DU145Kd cells were generated by transfection with PTEN siRNA expressed by a retroviral-based H1 promoter–driven shRNA vector (pRIH), as previously detailed (He et al., 2010). DU145vIII cells were generated by stable transfection of DU145 cells to express EGFRvIII. CHO and CHO-EGFR cells were obtained previously as a generous gift from Dr Abhijit Guha's

laboratory at The Hospital for Sick Children, Toronto. All the cell lines used in this study were cultured in microvesicle-depleted FBS (by centrifugation for three hours at 100,000 *g*).

2.3 Isolation of Exosomes

Exosomes from the conditioned media of different cell lines were collected as previously described (Al-Nedawi et al., 2005; Al-Nedawi et al., 2009). Briefly, conditioned medium was collected from cells at approximately 80% confluence, unless indicated otherwise, and this material was subjected to consecutive centrifugations at 300 g for 5 minutes and then at 12,000 g for 20 minutes to eliminate cells and debris. Finally, exosomes were obtained after centrifugation for 2 hours at 100,000 g and then washed twice with a large volume of phosphate buffered saline (PBS). Exosomal proteins recovered were measured using the Bradford assay (Bio-Rad, CA). This centrifugation protocol specifically isolates exosomes.

2.4 PTEN expression profile

Cells (DU145, DU145Kd, and DU145 with control siRNA) and primary cells (HAOEC, HAOSMC and HPEC), along with their corresponding exosomes, were lysed for 10 minutes on ice in a lysis buffer (RIPA lysis buffer) containing: 10 mM Tris (pH 6.8), 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 2% (wt./vol) SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM Na3VO4. Exosomes collected from A549, MDA-MB3, HCT 116, and BxPC-3cells were lysed in the same way. The lysates were resolved by SDS-PAGE and subjected to immunoblotting with rabbit monoclonal

antibodies for PTEN (Cell Signaling Technology, MA). Immunodetection was accomplished using the appropriate HRP-conjugated secondary antibody (Cell Signaling Technology) and chemiluminescence plus kit (ECL kit; Amersham Pharmacia, Buckinghamshire), after which the blots were scanned and protein bands quantitated using the Quantity One software (Bio-Rad).

2.5 Detection of the effects of EGFR stimulation on PTEN incorporation into exosomes

To determine the effects of EGFR on PTEN incorporation into exosomes, CHO and CHO-EGFR cells were grown in F12-K media, then starved in serum free media for 24 hours. Exosomes were collected from the conditioned media as previously described (Al-Nedawi et al., 2009). Cells were collected and lysed in RIPA lysis buffer. Cell and exosome lysates from both cell types were analyzed for PTEN content using immunoblotting. To assess the effect of EGFR stimulation on PTEN incorporation into exosomes, CHO-EGFR cells were plated in 100 mm dishes at a density of 2 x 10^5 cells/mL, grown briefly, and starved for 24 hours (in serum free F12-K media). Cells were treated for 24 hours with 0, 5, 10, and 20 ng/mL of EGF (epidermal growth factor). Media was collected from each treatment, and exosomes were isolated from the media. Exosomes were lysed and analyzed for PTEN content using immunoblotting.

2.6 Detection of the effects of EGFR inhibition on PTEN incorporation into exosomes

To assess the effect of EGFR inhibition on PTEN incorporation into exosomes, DU145 cells were plated in 100 mm dishes at a density of 2 x 10^5 cells/mL, grown briefly, and starved for 24 hours (in serum free DMEM media). Cells were treated for 24 hours with two concentrations (5mM, and 10mM) of CI-1033, an irreversible inhibitor of EGFR. Exosomes were collected from the conditioned media, and PTEN incorporation into exosomes was assessed using immunoblotting.

2.7 Detection of the effects of EGFRvIII on PTEN incorporation into exosomes

The effect of EGFRvIII (a truncated oncogenic form of EGFR that does not require ligand binding to initiate signaling), on PTEN levels in exosomes was assessed using DU145vIII cells (DU145 cells stably transfected to express EGFRvIII). DU145 and DU145vIII cells were grown in DMEM media, then starved in serum free media for 24 hours. Media and cells were collected. Exosomes were isolated from the conditioned media, and cell and exosome lysates were assessed for PTEN levels using immunoblotting.

2.8 Detection of signaling events related to PTEN

To assess the impact of PTEN-bearing exosomes on DU145Kd cells, the latter were plated in 100 mm dishes at a density of 2 x 10^5 cells/ml, grown briefly, and starved for 24 hours (either in DMEM supplemented with 0.5% FBS, or in serum free DMEM). The cultures were then stimulated overnight with different concentrations of exosomes derived from DU145 cells. After exosome treatment, cell lysates were prepared and

analyzed for their signaling effector content using anti-phospho-AKT antibodies (Cell Signaling Technology), according to the supplier's recommendations. Detection of the expression of p27 and cyclin D1 (Cell Signaling Technology) was performed using the same experimental settings used for the detection of pAKT.

2.9 Fluorescent imaging of cells

For *in vitro* analysis of PTEN expression, cells were grown in chamber slides (Nalge Nunc, NY). The cultures were washed with PBS and fixed in preheated (37°C) 4% (wt./vol) paraformaldehyde (PFA) in phosphate buffered saline for 5 minutes. Next, they were washed three times in PBS, and antiquenching was performed in 50 mM NH_4Cl for 10 minutes at room temperature. Cells were then permeabilized by incubation in 0.25% Triton X-100 in PBS for 10 minutes. Subsequently, the cells were washed three times PBS and incubated with BSA [1% (wt./vol) in PBS] for 30 minutes. Incubation with PTEN primary antibody (Cell Signalling Technology) was performed for 1 h, followed by three washes in PBS, and then incubation with Alexa-fluor 488(Molecular Probes) secondary antibody for 30 minutes. After staining, the slides were mounted using Dako fluorescent mounting medium and viewed under a confocal microscope to detect the presence of exosome content (PTEN) in the recipient cells.

2.10 PTEN activity assay

DU145Kd cells were treated with DU145-derived exosomes, lysed, and then subjected to immunoprecipitation using PTEN antibodies (Cell Signaling) and Protein G Sepharose (Sigma-Aldrich, MO). PTEN activity was assessed using the water-soluble substrate

DiC8PtdIns (3, 4, 5) P3 (Echelon Biosciences, UT). The released free phosphates were measured with BIOMOL Green reagent (Enzo Life Sciences, NY) and normalized against a reaction containing only PIP3 substrate.

2.11 Detection of PTEN mRNA

DU145Kd cells were treated with exosomes derived from DU145 cells, followed by extensive washing and the extraction of RNA using Trizol reagent (Invitrogen, NY). RT-PCR analysis was performed using a single-step method (Qiagen, CA) where PTEN was detected using the primer sets: sense 50-ATGACAGCCATCATCAAAGAG-30 and antisense 50-GTGCCACTGGTCTATAATCCAG-30 (He et al., 2010). The reactions were conducted in 50 µL with the initial Taq activation at 95 °C for 30 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, primer annealing at 60 °C for 1 minute, and extension at 72 °C for 30 seconds. The products were resolved on 1% agarose gel and photographed. GAPDH was used as internal control using the primer sets: sense 50-TGATGACATCAAGAAGGTGGTGAAG-30 and antisense 50-TCCTTGGAGGCCATGTGGGCCAT-30.

2.12 Proliferation assay

Proliferation assays were performed using an assay kit (CHEMICON, CA) according to the manufacturer's instructions. Briefly, 0.1 x10^4 DU145Kd cells were plated in a 96-well plate, and after 24 hours the cells were treated with different concentrations of exosomes derived from DU145 cells. Other DU145Kd cells were treated with exosomes collected from DU145Kd cells to show the effect of exosomes with downregulated PTEN on

proliferation. DU145 cells were used as a control. This procedure was also used for U87 and PC-3 cells.

2.13 Statistical Analysis

Experiments were reproduced at least three times with similar results. The quantitative data are presented as the average value of the replicates within the representative experiment ± SEM. Statistical significance was evaluated using a computerized 2-tailed Student's t-test. The differences were considered significant at $P < 0.05$.

CHAPTER THREE: RESULTS

3.1 PTEN status in cancer cell and normal cell exosomes, and the effects of oncogenic activation on PTEN incorporation into exosomes.

I. PTEN status in exosomes from cancer cells and normal cells

We determined the status of PTEN in the following PC cells: DU145, DU145 transfected with PTEN siRNA (DU145Kd), and DU145 transfected with control siRNA (He et al., 2008). Exosomes were collected from the conditioned media of each cell type, and equal protein concentrations from the cells and exosomes were subjected to SDS-PAGE and immunoblotting, and then probed with PTEN antibodies. Both DU145Kd cells (Figure 5A) and exosomes (Figure 5B) showed a downregulation of PTEN expression compared to the wild-type DU145 cells and DU145 cells transfected with control siRNA (Gabriel et al., 2013). We also assessed the phosphorylation status of PTEN in the exosomes derived from these cells. Phosphorylation of PTEN keeps it in a closed state protected from degradation, so that it will later be available to be activated in the cytoplasm, bind the cell membrane upon dephosphorylation, and then initiate cell signaling (Rahder et al, 2009). We found that the PTEN incorporated in exosomes is phosphorylated (Figure 5B, middle panel). Flotilin-1 was used as a loading control for the exosomes (Lespagnol et al., 2008). The proliferation rates of DU145 parental cells and DU145Kd cells (Figure 6) show that the PTEN knockdown cells exhibited a significantly higher proliferation rate.

To determine whether PTEN expression in exosomes is found in other types of cancer cells, we collected exosomes from lung carcinoma cells (A549), breast cancer cells (MDA-MB-231), colon carcinoma cells (HCT 116), and pancreas adenocarcinoma cells (BxPC-3). We performed immunoblotting, and found that PTEN is shed through exosomes in these cancer cell types (Figure 7A). Figure 7B is a scanning electron micrograph showing the shedding of exosomes from a cancer cell.

Human primary cells (normal, non-cancerous cells), such as Human Aortic Endothelial Cells (HAOEC), Human Aortic Smooth Muscle Cells (HAOSMC), and Human Prostate Epithelial Cells (HPEC) express PTEN, but we found that PTEN is not incorporated into the exosomes of these cells (Figure 8). This finding suggests that the incorporation of PTEN in exosomes is a characteristic of cancer cells, but not of normal cells.

II. PTEN incorporation into exosomes is affected by EGFR.

To investigate the mechanism of incorporation of PTEN into exosomal cargo, we have tested the role of the oncogenic receptor Epidermal Growth Factor Receptor (EGFR) in this process. We used the Chinese Hamster Ovary (CHO) cell line, which is spontaneously immortalized (Wurm & Hacker, 2011) and has no EGFR expression. CHO cells stably transfected to express EGFR had similar PTEN levels to parental CHO cells (Figure 9A), and exosomes collected from the transfected cells had similar levels of PTEN as the parental cells (Figure 9B). Upon stimulation of CHO-EGFR cells with

5ng/mL of EGF, there was an increase in the amount of PTEN in exosomes (Figure 10). In addition, we tested the effect of inhibiting EGFR in DU145 cells on PTEN incorporation into exosomes. We treated the cells with the EGFR inhibitor CI-1033 (5, 10 μM). Inhibiting EGFR decreased the incorporation of PTEN into exosomes in a concentration dependent manner (Figure 11). We also assessed the effects of EGFRvIII on exosomal-PTEN levels. DU145 cells transfected to stably express EGFRvIII exhibit increased PTEN levels in exosomes compared with exosomes derived from parental DU145 cells (Figure 12).

3.2 The intercellular transfer of PTEN through exosomes to PTEN-negative cells.

I. PTEN-negative cells acquire PTEN expression following incubation with PTEN-bearing exosomes.

To investigate the intercellular exchange of PTEN, exosomes derived from native DU145 PC cells were collected using the standard procedure of centrifugation. DU145Kd cells were incubated with an exosome preparation derived from parental DU145 cells. The apparent uptake of PTEN by DU145Kd cells was observed using immunocytochemistry (Figure 13). Background subtraction was performed using the untreated cells as control, and the fluorescence was subtracted from both nontreated and treated cells to show the acquisition of PTEN in exosome-treated cells. PTEN acquisition by DU145Kd cells following incubation with DU145 exosomes was also shown with immunoblotting (Figure 14). To determine if PTEN can be transferred from exosomes collected from other cancer cell types, we treated the PTEN-null prostate cancer cell line

PC-3 with exosomes derived from HCT 116 colorectal carcinoma cells. We tested for PTEN acquisition using immunocytochemistry, and found that the PC-3 cells acquired PTEN expression (Figure 15).

II. PTEN expression in cells following incubation with PTEN-bearing exosomes is not due to stimulation of PTEN transcription by exosomes.

To ensure that the increase of PTEN in DU145Kd cells post-incubation with DU145-derived exosomes did not result from the stimulation of PTEN transcription by exosomes, we performed RT-PCR for PTEN in DU145Kd cells treated with different concentrations of DU145-derived exosomes. We observed an inhibitory effect on PTEN transcription (Figure 16). To further ensure that we were observing the intercellular transfer of PTEN protein, as opposed to the stimulation of transcription, we used the glioblastoma cell line U87, a null genotype, which contains a mutation in both PTEN alleles (Wen et al., 2001). When treated with exosomes derived from DU145 cells, U87 cells tested positive for PTEN (Figure 17). We also treated the PTEN-null prostate cancer cell line PC-3 with exosomes derived from DU145 cells, with similar results (Figure 18). These data confirm that the acquired PTEN in DU145Kd, U87, and PC-3 is solely related to the intercellular transfer of PTEN protein through exosomal exchange.

3.3 PTEN transferred through exosomes is active, and leads to functional changes in recipient cells.

I Exosomes transfer active PTEN to recipient cells.

To assess whether the PTEN transferred through exosomes is active, we treated DU145Kd cells with different concentrations of exosomes derived from the DU145 cells. The treated cells were subjected to immunoprecipitation for PTEN. The immunoprecipitates were assessed for PTEN activity using a lipid-phosphatase assay. DU145Kd cells showed a substantial increase in phosphatase activity upon treatment with exosomes derived from DU145 cells (Figure 19).

To investigate whether the transferred PTEN is competent to alter cell signaling in acceptor cells, DU145Kd cells were treated with exosomes derived from DU145 cells for 24 hours. The phosphorylation status of AKT, the main substrate for PTEN, was assessed. In DU145Kd cells, we found a decrease in the phosphorylation of AKT that reached levels comparable to DU145 parental cells (PTEN positive) and DU145 cells transfected with control siRNA (Figure 20). To determine whether the transferred PTEN affects downstream cell signaling pathways associated with AKT, we assessed cyclin-dependent kinase (CDK) inhibitor p27 (KIP1) expression. p27 regulates cell proliferation, cell motility, and apoptosis. A reduction in p27 expression is observed in most lethal epithelial cancers and is associated with poor patient outcomes (Chu et al., 2008). pAKT negatively regulates p27 to support antiapoptotic activity in cancer progression. We found that p27 expression is increased in DU145Kd cells when treated with DU145-derived

exosomes (Figure 21, top panel). It has been reported that G1 cell-cycle arrest is coordinated by PTEN lipid phosphatase activity through the upregulation of p27 and by PTEN protein phosphatase activity through the downregulation of cyclin D1 (Weng et al., 2001). We determined that cyclin D1 is downregulated in the DU145 exosome-treated DU145Kd cells, as anticipated (Figure 21, bottom panel).

II PTEN transferred through exosomes affects biological functions in the acceptor cells.

To assess whether the biochemical changes observed in Figures 19-21 are associated with modification of biological function, we assessed the effect of PTEN-positive exosomes (derived from DU145 cells) on the proliferation of three cell lines that lack PTEN expression. DU145Kd, PC-3, and U87 cells were treated with different concentrations of DU145 exosomes, and proliferation rates were inhibited in all three cell lines in a dose-dependent manner (Figures 22, 23, and 24). Exosomes lacking PTEN expression, derived from DU145Kd, showed little effect on the proliferation rates.

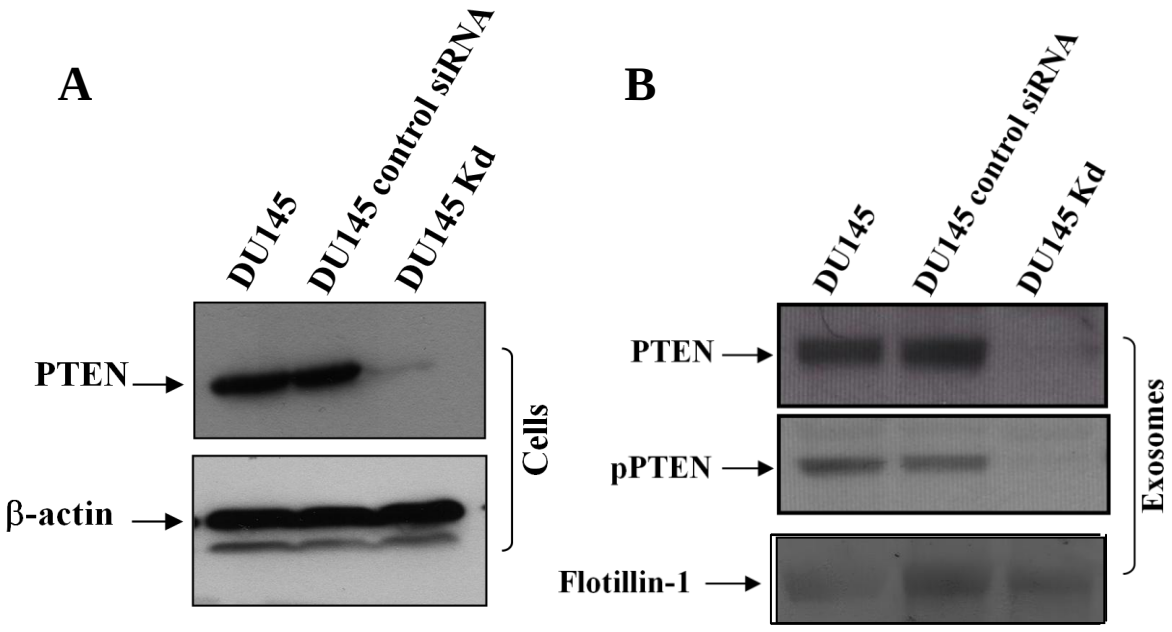

Figure 5. Prostate cancer cells produce exosomes containing PTEN

A. DU145 prostate cancer cells were transfected with the retroviruses for PTEN siRNA and control mismatch siRNA. Cells transfected with PTEN specific siRNA (DU145Kd) showed clear knockdown of PTEN expression, and cells transfected with control mismatch siRNA showed no decrease in PTEN.

B. Exosomes derived from DU145, DU145Kd, and DU145 with control siRNA show the same pattern of PTEN expression as seen in the cells from which exosomes are derived. Middle panel shows that PTEN incorporated into exosomes is phosphorylated, which means it is preserved against degradation and will be activated upon transfer to other cells. Exosomes are positive for Flotillin-1.

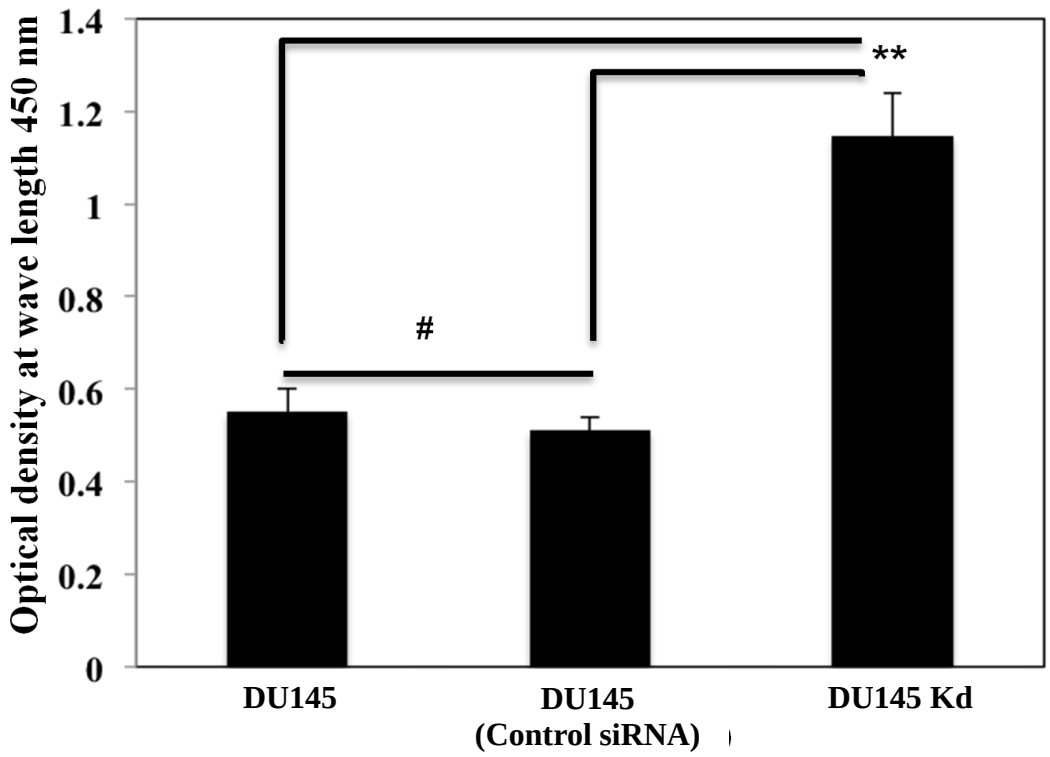

Figure 6. DU145Kd cells exhibit greater proliferation than parental DU145.

Using a proliferation assay, PTEN knockdown cells (DU145Kd), exhibited enhanced proliferation compared to DU145 parental cells and DU145 cells with control siRNA; the results represent the mean of three experiments ±SEM.

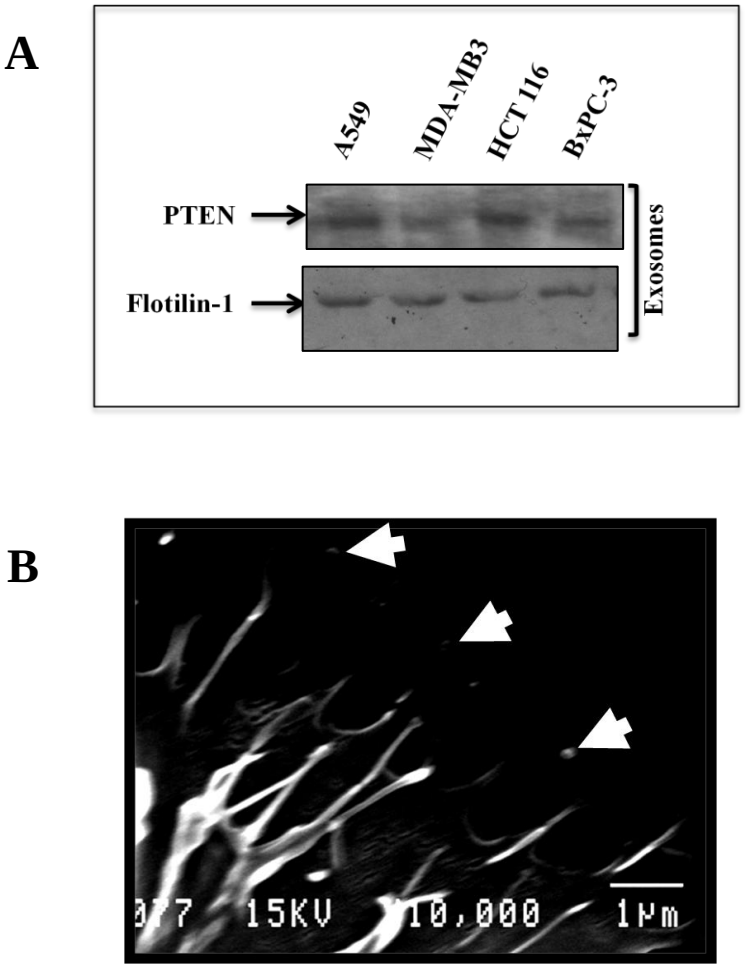

Figure 7. Exosomes from multiple cancer cell types express PTEN.

A. PTEN is expressed on exosomes derived from different types of cancer cells. A549; Alveolar Adenocarcinoma, MDA-MB3; Breast Adenocarcinoma, HCT 116; Colerectal Carcinoma, BxPC-3; Pancreas Adenocarcinoma.

B. Scanning electron micrograph showing shedding of exosomes from a cancer cell.

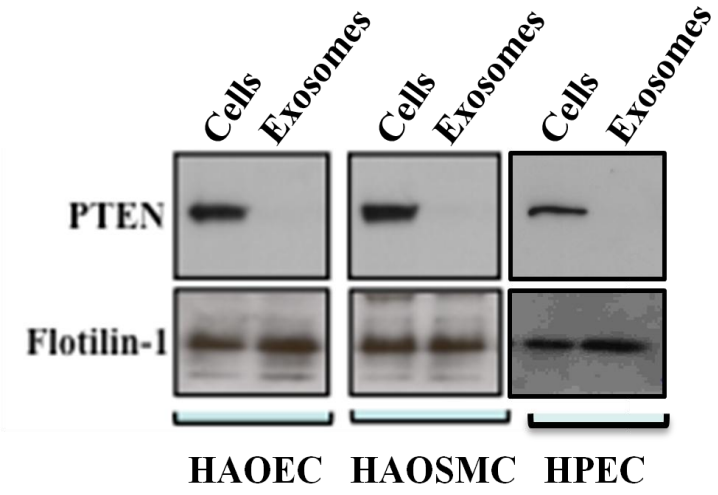

Figure 8. Normal primary cell-derived exosomes have no detectable PTEN

Human primary cells; Human aortic endothelial cells (HAOEC), Human aortic smooth muscle cells (HAOSMC), and Human prostate epithelial cells (HPEC) and their exosomes were profiled for PTEN expression. All three cells have PTEN expression, but no PTEN is detected in their exosomes. The expression for both cells and their exosomes was normalized with Flotilin-1.

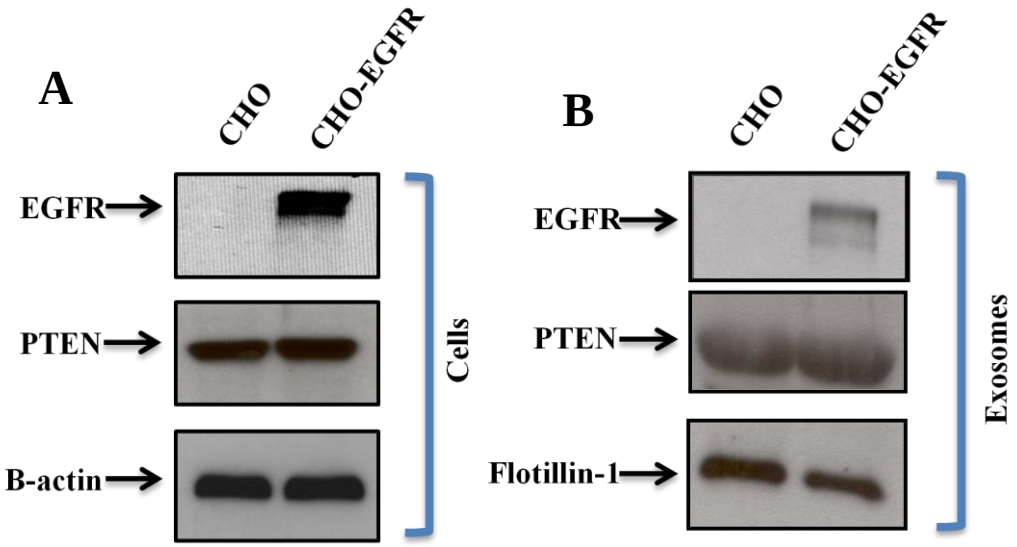

Figure 9. CHO-EGFR cells have similar levels of PTEN in exosomes as wild type CHO cells.

A. Introducing EGFR in CHO cells showed no effect on the expression of PTEN in cells.

B. CHO-EGFR cells have the same PTEN levels in exosomes as parental CHO cells.

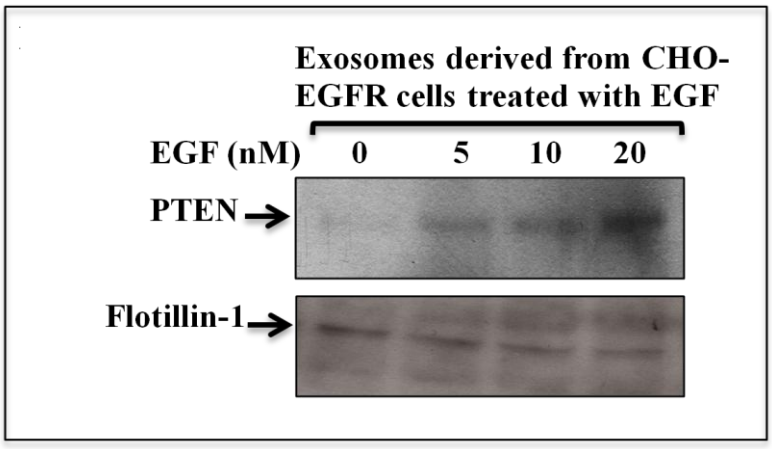

Figure 10. Stimulation of EGFR with EGF increases PTEN incorporation into exosomes.

CHO-EGFR cellswere cultured in 100 mm dishes and treated with the indicated concentrations of EGF (5, 10 and 20 ng/mL) for 24 hours, then exosomes were collected for analysis of PTEN expression by immunoblotting. EGF treatment led to an increase in PTEN incorporation into exosomes. Flotillin-1 was used as a loading control.

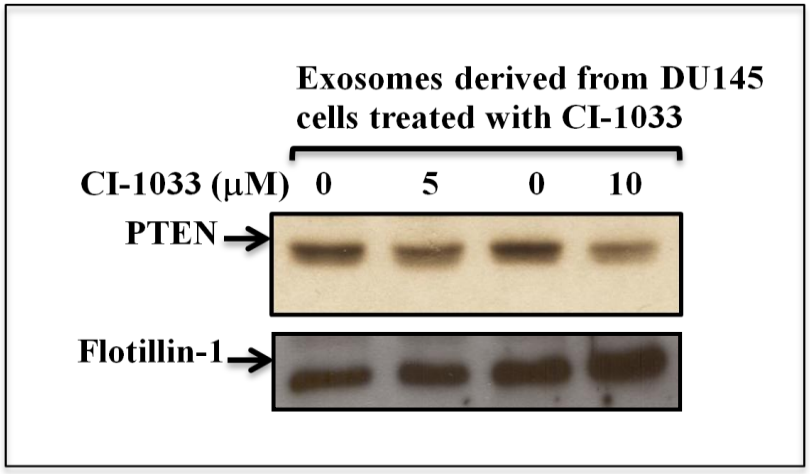

Figure 11. EGFR inhibition decreases PTEN incorporation into exosomes.

DU145 cells were cultured in 100 mm dishes, and treated with the indicated concentrations of the irreversible EGFR inhibitor CI-1033 (5, 10 μm). Exosomes were collected for analysis by immunoblotting for PTEN. CI-1033 treatment results in decreased PTEN incorporation into exosomes. Flotillin-1 was used as a loading control.

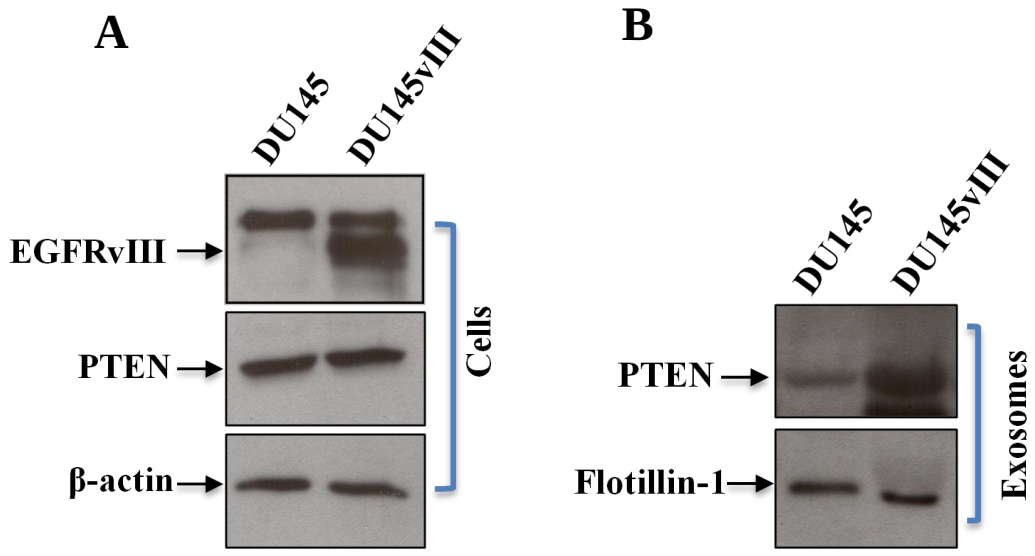

Figure 12. Introduction of EGFRvIII leads to increased incorporation of PTEN into exosomes.

A. DU145 cells were transfected to stably express EGFRvIII (DU145vIII cells). DU145 parental and DU145vIII cells were collected, and immunoblotting for PTEN was performed. DU145vIII cells have similar levels of PTEN as parental DU145 cells.

B. Exosomes were collected from DU145 parental and DU145vIII cells, and analysed for PTEN expression using immunoblotting. DU145vIII-derived exosomes have increased PTEN levels compared with parental DU145-derived exosomes. Flotillin-1 was used as a loading control.

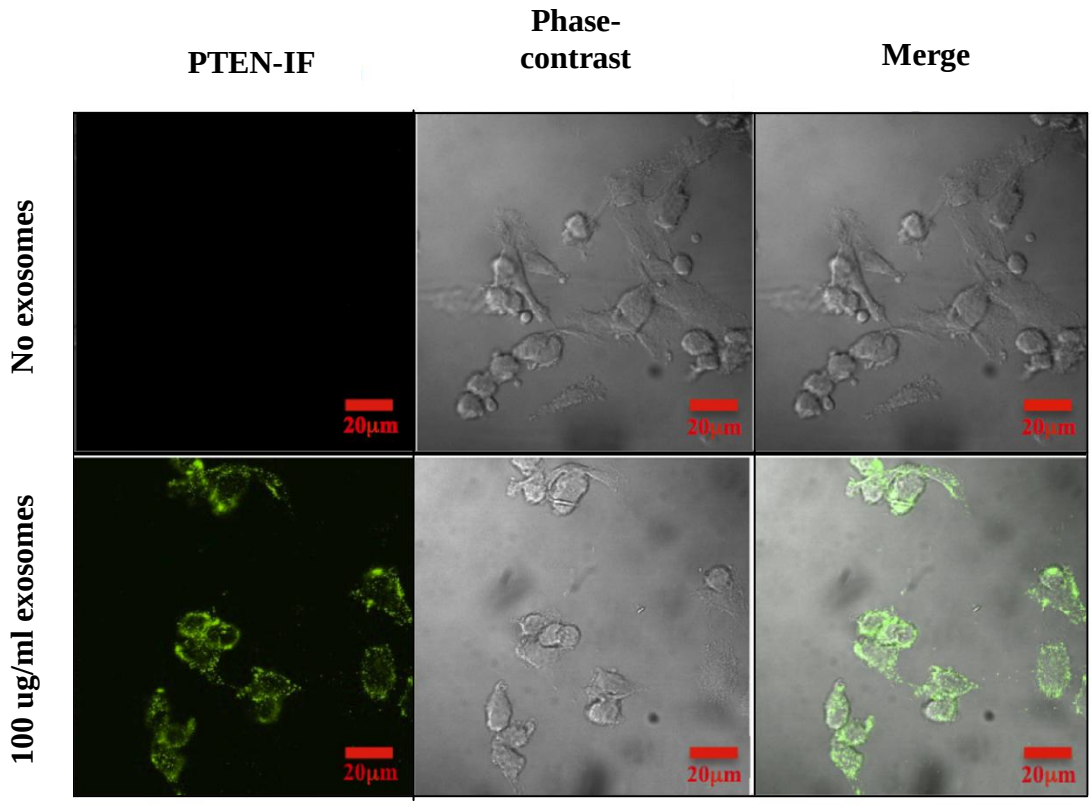

Figure 13. PTEN-bearing exosomes transfer PTEN between prostate cancer cells.

DU145Kd cells were cultured in slide chambers and treated with different concentrations of DU145 exosomes for 24 hours. Cells were then washed twice, fixed, permeabilized, and immunocytochemistry was performed with PTEN antibodies and Alex-fluor 488. The cells were visualised using confocal microscopy (IF; immunofluorescent). DU145Kd cells acquired PTEN (green) with both concentrations of exosomes.

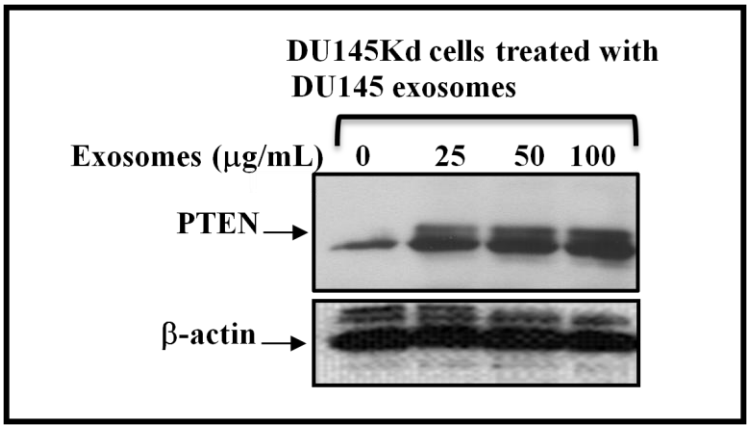

Figure 14. Exosomes transfer PTEN between prostate cancer cells.

DU145Kd cells were cultured in 100 mm dishes and treated with different concentrations of exosomes derived from DU145 cells for 24 hours. Cell lysates were subjected to immunoblotting for PTEN expression. DU145Kd cells acquired PTEN expression.

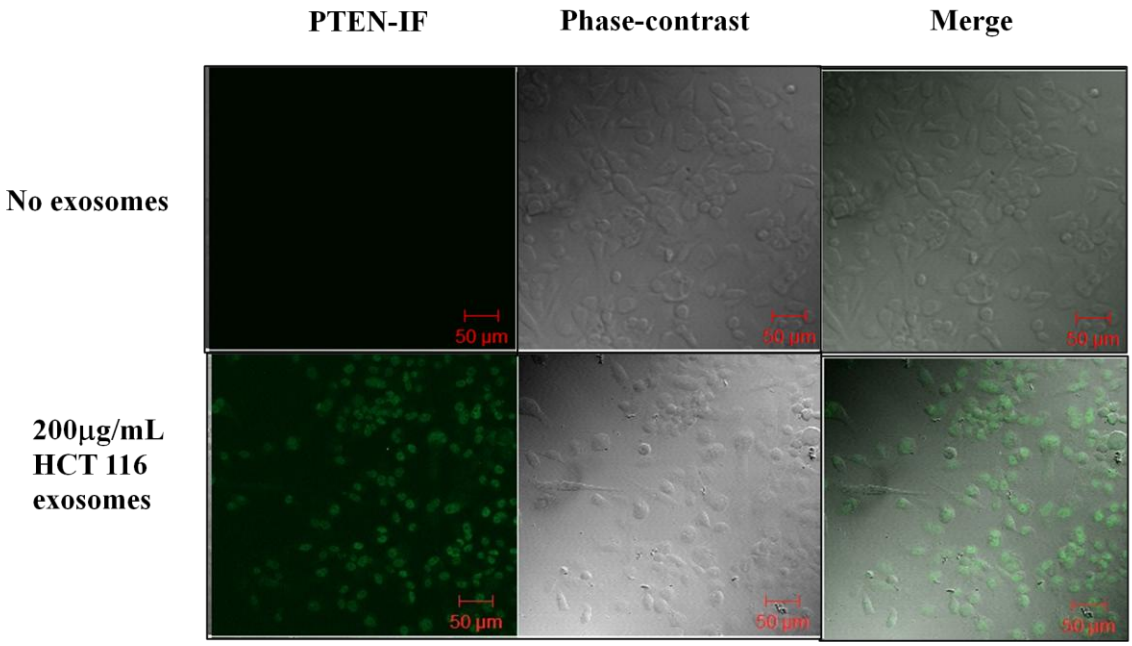

Figure 15. HCT 116 –derived exosomes transfer PTEN to PTEN-null PC-3 cells.

PC-3 cells were cultured in slide chambers and treated with HCT 116-derived exosomes for 24 hours. Cells were then washed twice, fixed, permeabilized, and immunocytochemistry was performed with PTEN antibodies and Alex-fluor 488. The cells were visualised using confocal microscopy (IF; immunofluorescent). PC-3 cells acquired PTEN (green) with both concentrations of exosomes.

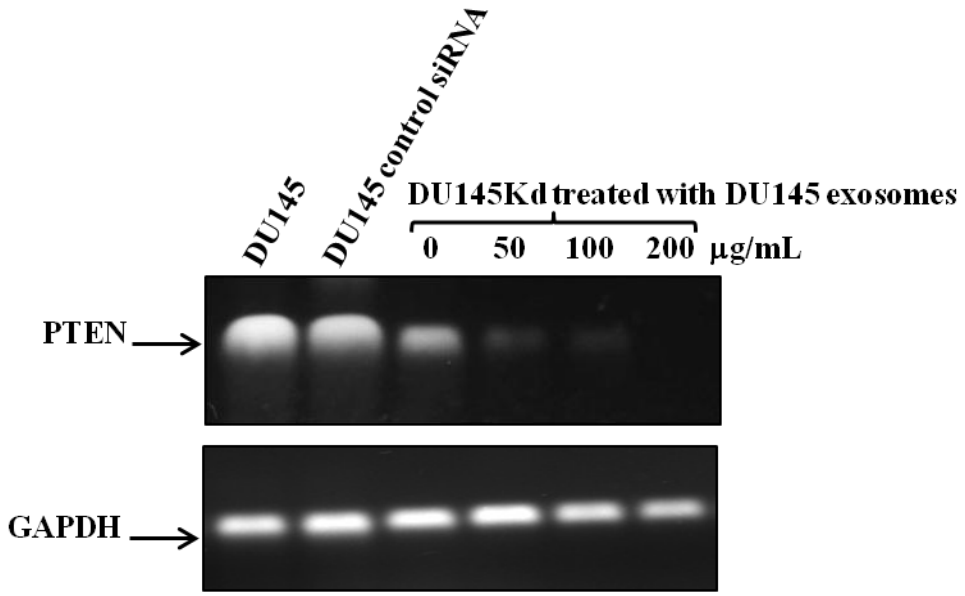

Figure 16. Exosomes have an inhibitory effect on the transcription of PTEN.

DU145Kd cells were treated with different concentrations of exosomes derived from DU145 parental cells. Total RNA was collected and RTPCR was performed using specific primers for PTEN; sense 50-ATGACAGCCATCATCAAAGAG-30 and antisense 50-GTGCCACTGGTCTATAATCCAG-30. GAPDH was used as a control using the primers; sense 50-TGATGACATCAAGAAGGTGGTGAAG-30 and antisense 50-TCCTTGGAGGCCATGTGGGCCAT-30. RTPCR was performed using a one step RTPCR kit (Qiagen). The products were resolved on 1.1% agarose gel. The first three lanes show a comparison between mRNA levels in the three cell lines used in the study (DU145, DU145 with control siRNA, and DU145Kd cells).

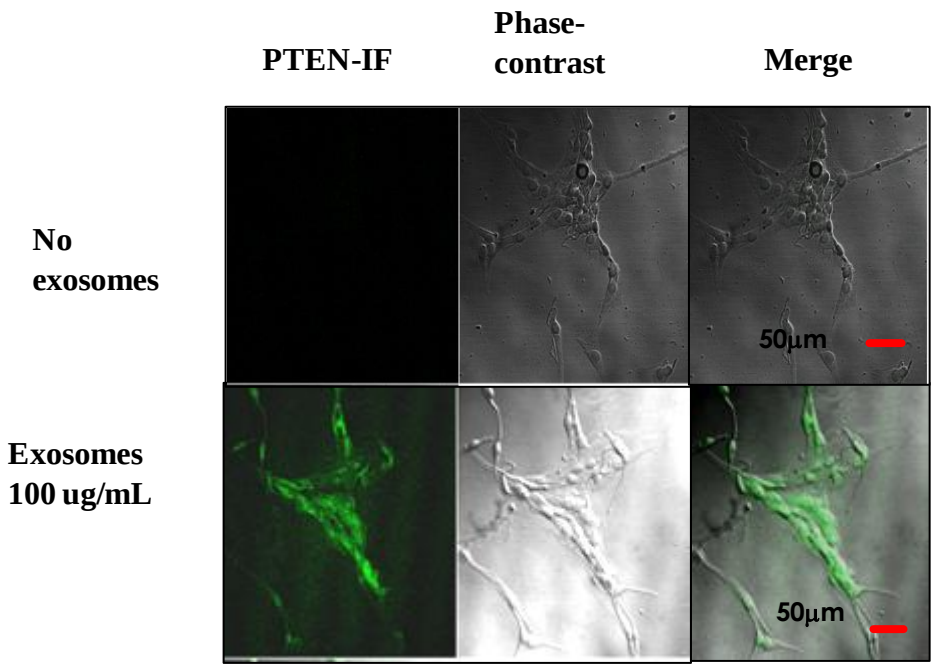

Figure 17. Exosomes transfer PTEN to PTEN$^{-/-}$ U87 cells.

U87 cells were cultured in slide chambers and treated with exosomes derived from DU145 cells. The cells were washed twice, fixed, permeabilized, and stained with PTEN antibodies and Alexa-fluor 488 secondary antibodies, and visualised using confocal microscopy (IF; for immunofluorescent). Cells became positive for PTEN after incubation with the DU145 exosomes.

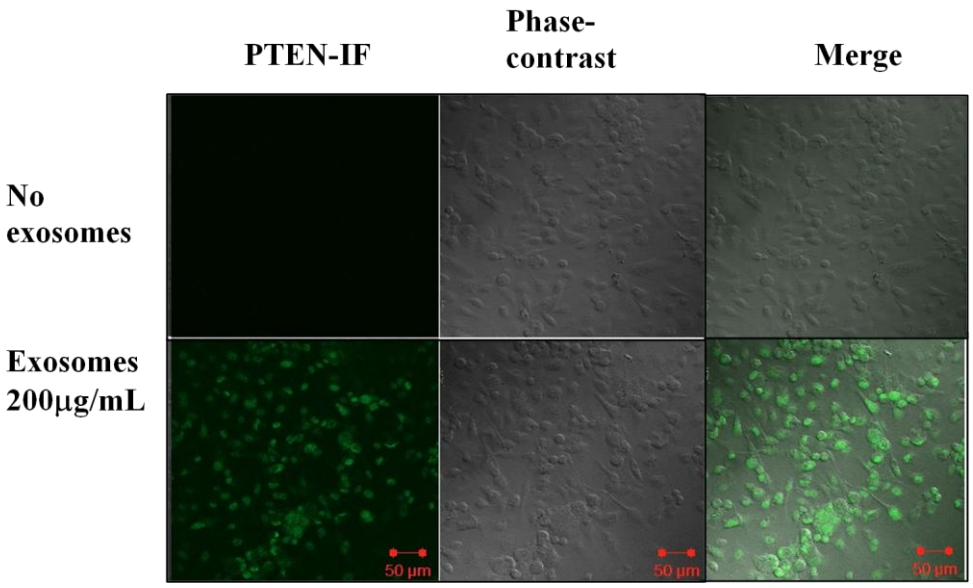

Figure 18. Exosomes transfer PTEN to PC-3 PTEN$^{-/-}$ prostate cancer cells.

PC-3 cells were cultured in slide chambers and treated with exosomes derived from DU145 cells. The cells were washed twice, fixed, permeabilized, then stained with PTEN antibodies and Alexa-fluor 488 secondary antibodies. Slides were visualised using confocal microscopy (IF; for immunofluorescent).

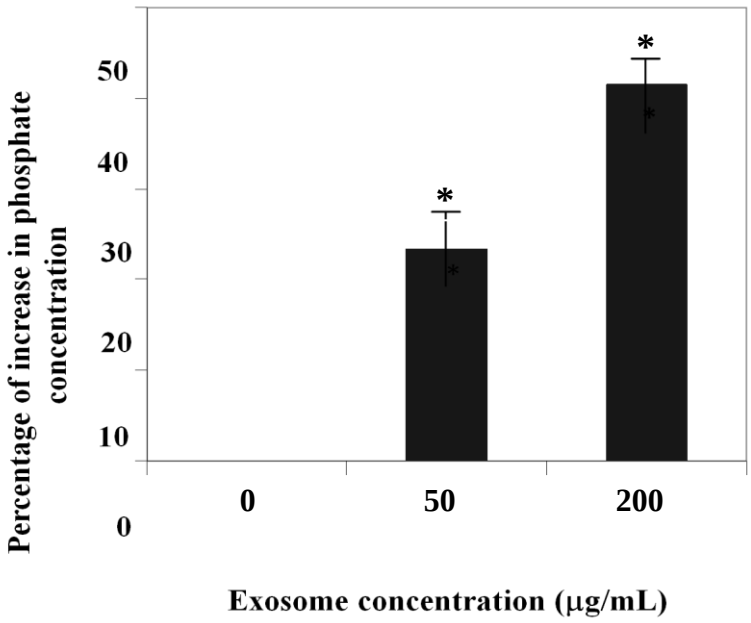

Figure 19. Exosomes transfer active PTEN to acceptor cells.

DU145Kd cells were treated with exosomes derived from DU145 cells. PTEN was immunoprecipitated with PTEN antibodies, and PTEN phosphatase activity assessed using the water soluble substrate DiC8PtdIns (3,4,5) P3 (Echelon). Released free phosphates were measured with BIOMOL Green reagent and normalised against a reaction containing PIP3 substrate only. The result represents the mean of three experiments ± SD; the results were significant $P<0.01$.

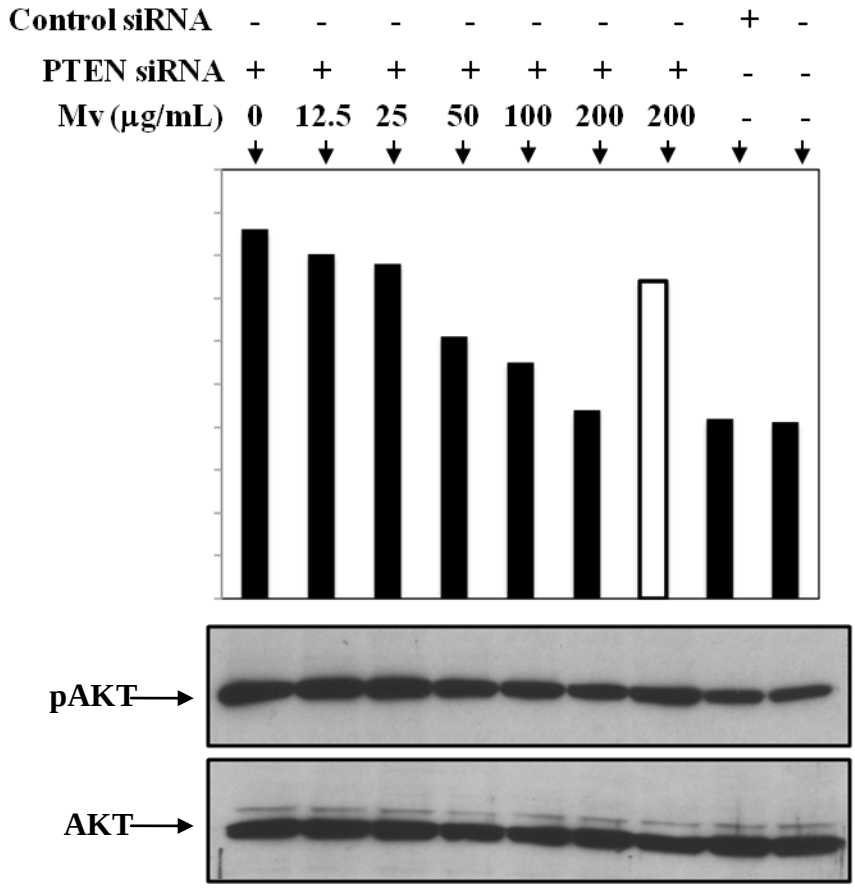

Figure 20. PTEN-positive exosomes decrease AKT phosphorylation in acceptor cells.

AKT phosphorylation in DU145Kd cells incubated with different concentrations of exosomes derived from DU145. AKT phosphorylation decreased to be comparable to DU145 with control siRNA, and the parental counterpart DU145 (last two lanes on the right). The histogram represents the optical densities for the band in the blot; the optical densities were obtained using Image quantity one.

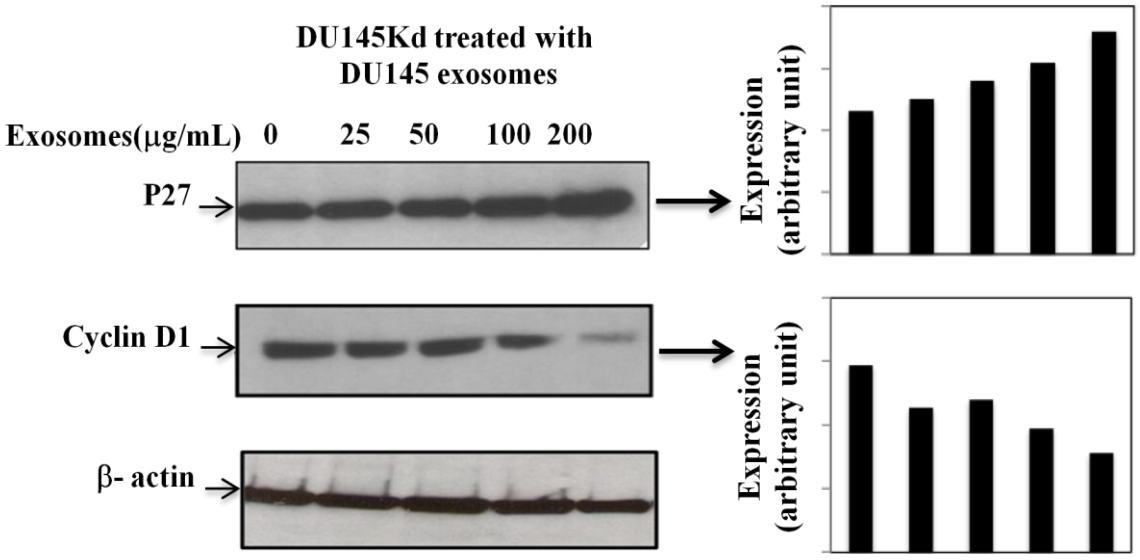

Figure 21. PTEN transferred through exosomes modulates the cell cycle arrest pathway of the acceptor cells.

DU145Kd cells were plated in 100mm cell culture dishes and treated with different concentrations of exosomes derived from DU145 cells for 24 hours. The cells were lysed and subjected to immunoblotting with p27 and cyclin D1 antibodies. PTEN induced the expression of p27 (upper panel), and reduced the expression of cyclinD1 (middle panel). These two events led to the cells entering into cell cycle arrest. B-actin used as a loading control protein.

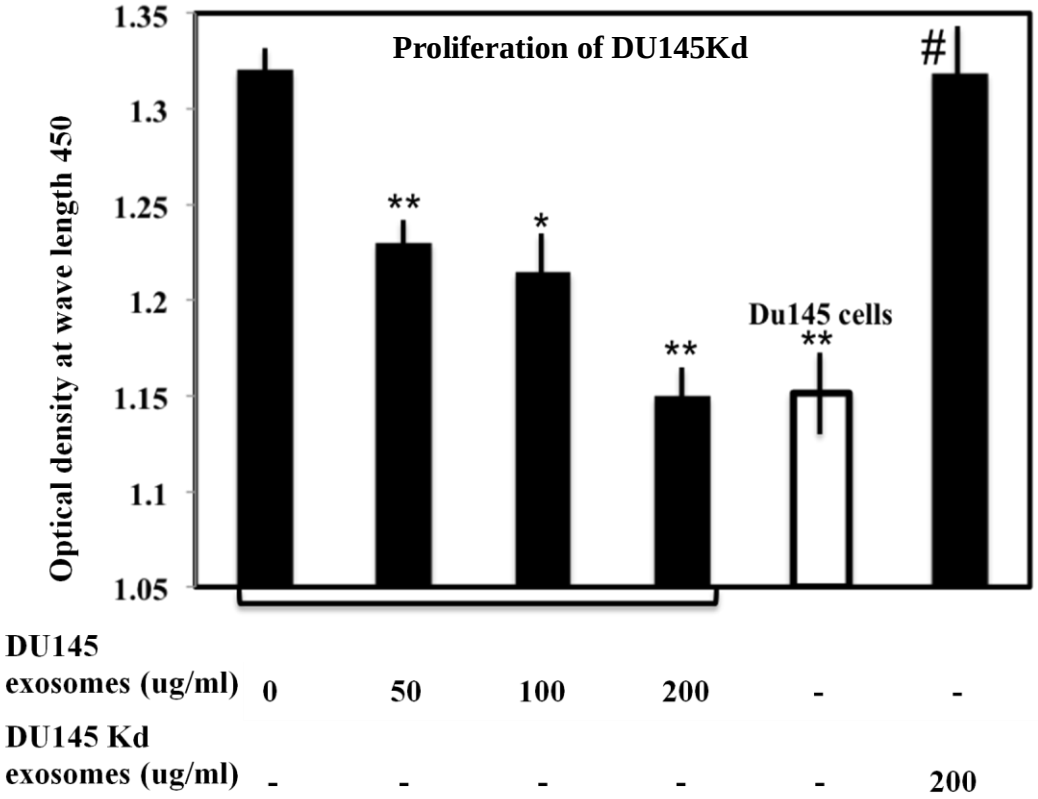

Figure 22. Modulation of cell proliferation in DU145Kd cells by transferred exosomal-PTEN derived from DU145 cells.

DU14Kd cells were treated with different concentrations of DU145 exosomes. A proliferation assay was performed using an assay kit (Millipore), and according to manufacturer instructions. Proliferation rates were inhibited in a concentration dependent manner. DU145Kd cell proliferation rates reached levels comparable to DU145 cells, meaning that exosomes compensated for PTEN loss. Exosomes derived from DU145Kd (lacking PTEN) showed no effect on cell proliferation. Results shown as the mean of three experiments ± SD. Differences from the control (0 exosomes) are shown as (significant * $P<0.05$, ** $P<0.01$ and # not significant).

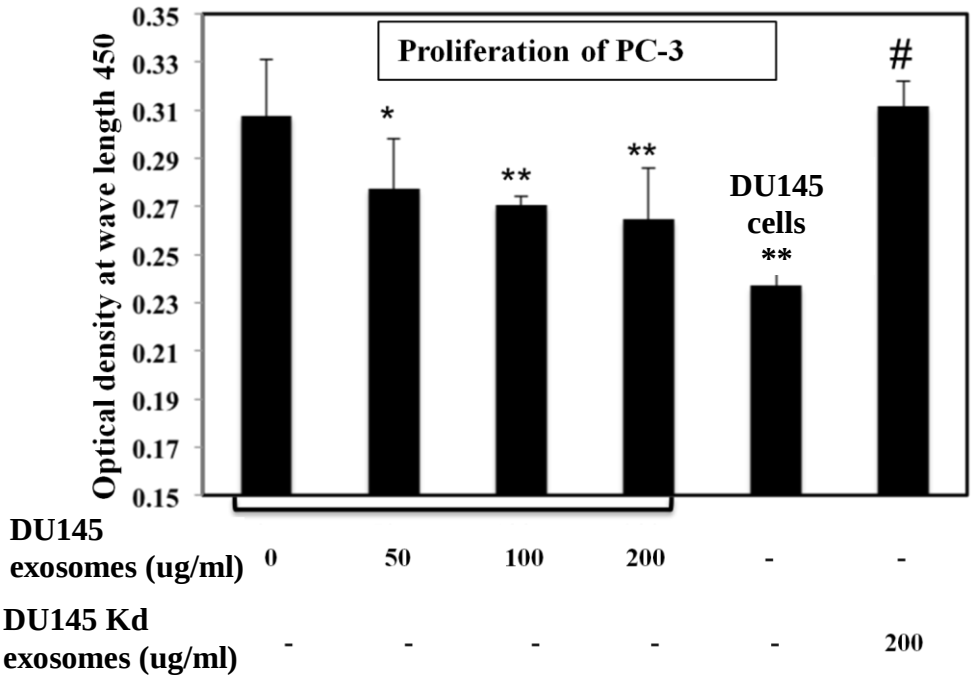

Figure 23. Modulation of cell proliferation in PTEN-null PC-3 cells by transferred exosomal-PTEN derived from DU145 cells.

PC-3 cells were treated with different concentrations of DU145 exosomes. A proliferation assay was performed using an assay kit (Millipore), and according to manufacturer instructions. Proliferation rates were inhibited in a concentration dependent manner. Exosomes derived from DU145Kd (lacking PTEN) showed no effect on cell proliferation. Results shown as the mean of three experiments ± SD. Differences from the control (0 exosomes) are shown as (significant * $P<0.05$, ** $P<0.01$ and # not significant).

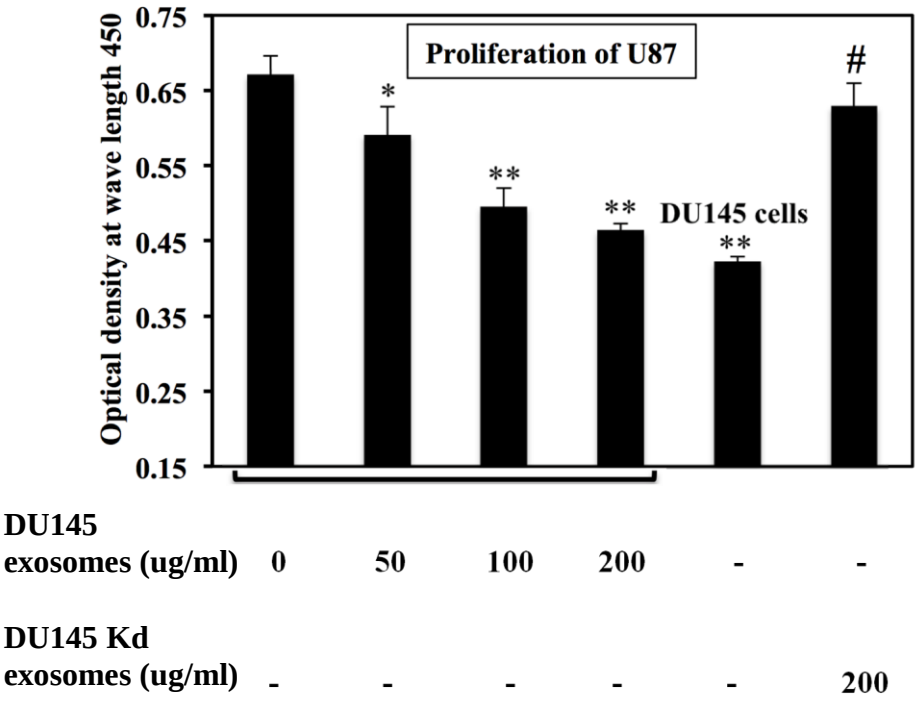

Figure 24. Modulation of cell proliferation in PTEN-null U87 cells by transferred exosomal-PTEN derived from DU145 cells.

U87 cells were treated with different concentrations of DU145 exosomes. A proliferation assay was performed using an assay kit (Millipore), and according to manufacturer instructions. Proliferation rates were inhibited in a concentration dependent manner. Exosomes derived from DU145Kd (lacking PTEN) showed no effect on cell proliferation. Results shown as the mean of three experiments ± SD. Differences from the control (0 exosomes) are shown as (significant * $P<0.05$, ** $P<0.01$ and # not significant).

CHAPTER FOUR: DISCUSSION

The cancer-promoting effects of tumour-derived exosomes have been well established in the past few years. Cancer cell derived exosomes have been found to facilitate cancer growth through the horizontal transfer of mutant oncogenic proteins (Al-Nedawi et al., 2008) and pro-growth mRNA (Skog et al., 2008). Exosomes have also been implicated in tumour immune evasion (Valenti et al., 2007), and remodeling of the tumour microenvironment to promote cancer growth (Sidhu et al., 2004). However, a subject that has received little attention is the potential tumour-suppressive characteristics of exosomes.

An important observation made during the present study is that the tumour suppressor PTEN is expressed in exosomes derived from cancer cells (Figure 5), and it is not expressed in exosomes from normal cells, although the normal cells themselves express PTEN (Figure 8). Thus, the incorporation of PTEN in exosomes is an exclusive characteristic of cancer cells. This finding may suggest an exclusionary mechanism used by cancer cells to downregulate PTEN. The sequestering of anti-growth and pro-death molecules into exosomes by cancer cells has been noted in previous studies. K562 erythroleukemia cells have been found to avoid complement-induced cell death by shedding accumulated membrane attack complex (MAC) from the cell membrane through exosomes (Pilzer et al., 2005). Cancer cells have also been shown to eliminate chemotherapeutic drugs by shedding them through exosomes. For example, ovarian carcinoma cells avoid doxorubicin-mediated death by packaging it into exosomes which

are shed from the cell (Shedden et al., 2003). The finding that cancer cells incorporate PTEN into exosomes may represent another cancer cell adaptation to allow uninhibited growth by removing the tumour suppressor from cancer cells through exosomes.

This new mechanism requires further investigation to characterize the molecules responsible for directing PTEN to exosomes. Because this mechanism is exclusive to cancer cells, we hypothesize that it might be under the control of oncogenes. Therefore, we studied the effect of introducing EGFR in CHO cells, which do not have any expression of EGFR. Introducing EGFR in these cells showed no effect on exosomal PTEN levels (Figure 9). However, when the introduced EGFR was activated by EGF treatment there was an increase in PTEN expression in exosomes (Figure 10). We also tested the effects of the irreversible EGFR inhibitor CI-1033 on the incorporation of PTEN in exosomes, and found that DU145 cells treated with the inhibitor decreased exosomal PTEN in a concentration dependent manner (Figure 11). We assessed the effects of introducing EGFRvIII, the truncated, constitutively active EGFR mutant, on PTEN incorporation into exosomes. We found that DU145 cells transfected to stably express EGFRvIII produced exosomes with higher levels of PTEN (Figure 12). Recently, it has been reported that Ndfip1, an adaptor protein for members of the Nedd4 family of E3 ubiquitin ligases, has a role in directing PTEN to exosomes (Putz et al., 2012). EGFR activation through EGF binding has been shown to activate Nedd4 ubiquitin ligase activity by increasing intracellular calcium (Wang et al., 2010). Nedd4 activation may result in increased PTEN incorporation into exosomes. This mechanism could explain the role of EGFR in the incorporation of PTEN into exosomes, but further study is needed to

confirm this pathway. Determining the mechanism of PTEN direction to exosomes could provide a new, cancer-exclusive pathway to target in the development of cancer therapeutics. Blocking this mechanism could lead to PTEN accumulation within cancer cells, causing cancer cell death or inhibition of growth.

This study demonstrates the ability of PTEN-bearing exosomes to transfer PTEN to cells with no expression of the protein. After incubation with PTEN-positive DU145-derived exosomes, PTEN knockdown DU145Kd cells (Figure 13) and PTEN null U87 (Figure 17) and PC-3 cells (Figure 18) became positive for the protein. Other types of cancer cell exosomes were also found to be positive for PTEN (Figure 7A), and exosomes collected from the colorectal carcinoma cell line HCT 116 also transferred PTEN to PC-3 cells (Figure 15). Our study suggests that the source of the PTEN observed in DU145Kd, PC-3, and U87 cells is related to the transfer of PTEN from PTEN-positive cells via exosomes. This is evidenced by the fact that exosomes have an inhibitory effect on the transcription of PTEN, as demonstrated by RT-PCR (Figure 16). This inhibitory effect may be explained by the expression of miRNA 21 in exosomes, which negatively regulates transcription of PTEN in acceptor cells (Collino et al., 2010). In the transfer experiments using U87 cells, PTEN was acquired from transferred exosomal PTEN, as U87 cells have mutations in both PTEN alleles, resulting in a null genotype (Wen et al., 2001). Both approaches indicate that the presence of PTEN is solely related to PTEN transfer via exosomes.

PTEN transferred through exosomes is active. Upon uptake of PTEN-enriched exosomes, acceptor cells acquired significantly higher PTEN activity, demonstrated by a PTEN-activity assay (Figure 19). In addition, PTEN activity is evidenced by decreased phosphorylation of AKT in acceptor cells following incubation with PTEN-enriched exosomes, which is a reflection of the dephosphorylation of PIP3 to PIP2 by PTEN (Figure 20) (Di Cristofano et al., 1998; Stambolic et al., 1998; Wu et al., 1998). Furthermore, it is reported that PTEN coordinates cell-cycle arrest in G1 by downregulating cyclin D1 via PTEN protein phosphatase activity, and upregulating p27 via PTEN lipid phosphatase activity (Weng et al., 2001). We found that p27 is upregulated by exosomal PTEN transfer, and that cyclin D1 is downregulated (Figure 21). After incubation with DU145-derived PTEN positive exosomes, DU145 Kd, PC-3, and U87 cells exhibited inceased proliferation (Figures 22, 23, and 24). Taken together, these findings imply that exosomal PTEN may be able to compensate for PTEN loss in cancer cells, by transferring the active protein to cancer cells where it can then perform its role as a tumour suppressor.

There are some questions that arise from the observation that cancer cells remove PTEN though exosomes, a presumably pro-growth phenomenon, but also take up PTEN-positive exosomes, resulting in decreased proliferation. Because of the novelty of the results, we do not yet understand the role of PTEN-exosomes in tumour pathology. The pro-growth effect of PTEN excretion and the anti-growth effect of PTEN uptake through exosomes may both play a role in tumour development, with the overall effect on tumour growth dependent on a range of other factors, including PTEN level and concurrent

mutations. Tumour-derived exosomes are found in the peripheral blood in high volumes, so PTEN-exosomes may also be flushed away into the bloodstream and have little effect on the tumour which produced them. Many questions remain surrounding the role of exosomal PTEN in cancer development and its potential as a cancer therapeutic, which should be addressed in future studies.

CHAPTER FIVE: CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, the present results suggest that the incorporation of PTEN into exosomes is a cancer-specific phenomenon not present in normal primary cells. This process may be regulated by oncogenic molecules, as we found that EGFR activation resulted in increased incorporation of PTEN into cancer cell exosomes. Cancer cells transfected to express EGFRvIII, the mutated, constitutively active form of EGFR, have greater levels of PTEN in exosomes. These findings suggest that the incorporation of PTEN into exosomes may be a cancer cell-specific mechanism which allows cancer cells to remove the tumour suppressor from the cell, preventing its anti- growth and survival activity. Another major finding of this study is that exosomal PTEN can be transferred to cells with reduced or absent levels of PTEN. PTEN transferred retains phosphatase activity, and is biologically active in recipient cells. PTEN transfer through exosomes resulted in decreased AKT phosphorylation, changes in levels of cell cycle mediators, and decreased proliferation in recipient cells.

The results of this project raise many questions which merit attention in further studies. One such result is the observation that PTEN is incorporated in cancer cell exosomes but not normal cell exosomes, and that this process may be under the control of oncogenic molecules. This indicates that the incorporation of PTEN into exosomes may be a cancer-specific mechanism, which may allow cancer cells to accelerate growth by ejecting the tumour suppressor from cells. If this is indeed a cancer-specific pathway, it

represents a promising new target for anti-cancer therapeutics, as shutting down this pathway would presumably not affect normal cells. If the molecules responsible for directing PTEN into exosomes could be determined, this pathway could potentially be blocked, causing the accumulation of PTEN in cancer cells and leading to cell cycle arrest or cell death.

Another therapeutic option that could potentially stem from this project is the use of PTEN-exosomes against PTEN-reduced or negative prostate cancers. This possibility stems from the observation that PTEN can be transferred through exosomes to cancer cells. Once transferred, PTEN performs its activity as a tumour suppressor in the recipient cell, resulting in decreased AKT phosphorylation, cell cycle arrest, and reduced proliferation. If exosomes could be loaded with PTEN and given intravascularly to prostate cancer patients, the exosomes could potentially serve as a delivery system to re-introduce PTEN to PTEN-reduced or null tumours. However, many questions remain surrounding the half-life of exosomes in the bloodstream, as well as the specificity of their interactions with cells. The effectiveness of PTEN-exosomes as a delivery mechanism should be assessed using an *in vivo* model. PTEN-positive exosomes will be introduced through the tail vein in mice harbouring PTEN-reduced or negative tumours. Tumour growth rate and PTEN status will be compared between these mice and control mice injected with PTEN-negative exosomes. This will allow us to determine the ability of PTEN-exosomes injected into the bloodstream to reach the tumour and affect its growth.

REFERENCES

Al-Nedawi, K., Meehan, B., Kerbel, R. S., Allison, A. C., & Rak, J. (2009). Endothelial expression of autocrine VEGF upon the uptake of tumour-derived microvesicles containing oncogenic EGFR. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(10), 3794-9.

Al-Nedawi, K., Meehan, B., Micallef, J., Lhotak, V., May, L., Guha, A., & Rak, J. (2008). Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nature cell biology*, *10*(5), 619-24.

Al-Nedawi, K., Szemraj, J., & Cierniewski, C. S. (2005). Mast cell-derived exosomes activate endothelial cells to secrete plasminogen activator inhibitor type 1. *Arteriosclerosis, thrombosis, and vascular biology*, *25*(8), 1744-9.

Alimonti, A., Carracedo, A., Clohessy, J. G., Trotman, L. C., Nardella, C., Egia, A., ... & Pandolfi, P. P. (2010). Subtle variations in Pten dose determine cancer susceptibility. *Nature genetics*, *42*(5), 454-458.

Anderson, H. C., Mulhall, D., & Garimella, R. (2010). Role of extracellular membrane vesicles in the pathogenesis of various diseases, including cancer, renal diseases, atherosclerosis, and arthritis. *Laboratory investigation; a journal of technical methods and pathology*, *90*(11), 1549-57.

Antonelli, J. A., Jones, L. W., Bañez, L. L., Thomas, J. A., Anderson, K., Taylor, L. A., ... & Freedland, S. J. (2009). Exercise and prostate cancer risk in a cohort of veterans undergoing prostate needle biopsy. *The Journal of urology*, *182*(5), 2226-2231.

Bechis, S. K., Carroll, P. R., & Cooperberg, M. R. (2011). Impact of age at diagnosis on prostate cancer treatment and survival. *Journal of Clinical Oncology*, *29*(2), 235-241.

Bose, S., Crane, A., Hibshoosh, H., Mansukhani, M., Sandweis, L., & Parsons, R. (2002). Reduced expression of PTEN correlates with breast cancer progression. *Human pathology*, *33*(4), 405-409.

Brandt, A., Bermejo, J. L., Sundquist, J., & Hemminki, K. (2010). Age-specific risk of incident prostate cancer and risk of death from prostate cancer defined by the number of affected family members. *European urology*, *58*(2), 275-280.

Burgess, A. W. (2008). EGFR Family: Structure, physiology, signalling and therapeutic targets. *Growth Factors, 26*(5), 263-274.

Cairns, P., Okami, K., Halachmi, S., Halachmi, N., Esteller, M., Herman, J. G., ... & Sidransky, D. (1997). Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. *Cancer research, 57*(22), 4997-5000.

Camussi, G., Deregibus, M.-chiara, Bruno, S., Grange, C., & Fonsato, V. (2011). Exosome/microvesicle-mediated epigenetic reprogramming of cells. *Cancer Research, 1*(1), 98-110.

Castellana, D., Toti, F., & Freyssinet, J. M. (2010). Membrane microvesicles: macromessengers in cancer disease and progression. *Thrombosis research, 125 Suppl* , S84-8.

Chairoungdua, A., Smith, D. L., Pochard, P., Hull, M., & Caplan, M. J. (2010). Exosome release of β-catenin: a novel mechanism that antagonizes Wnt signaling. *The Journal of cell biology, 190*(6), 1079-91.

Chu, I. M., Hengst, L., Slingerland, J. M. (2008). The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy. *Nature reviews Cancer* 8**,** 253-267

Collino, F., Deregibus, M. C., Bruno, S., Sterpone, L., Aghemo, G., Viltono, L., ... & Camussi, G. (2010). Microvesicles derived from adult human bone marrow and tissue specific mesenchymal stem cells shuttle selected pattern of miRNAs. *PLoS One, 5*(7), e11803.

Debes, J. D., & Tindall, D. J. (2004). Mechanisms of androgen-refractory prostate cancer. *New England Journal of Medicine, 351*(15), 1488-1490.

de Gassart, A., Geminard, C., Fevrier, B., Raposo, G., & Vidal, M. (2003). Lipid raft-associated protein sorting in exosomes. *Blood, 102*(13), 4336-44.

Di Cristofano, A., Pesce, B., Cordon-Cardo, C., Pandolfi, P. P. (1998) Pten is essential for embryonic development and tumour suppression. *Nature genetics,* 19**,** 348-355.

Eguez, L., Lee, A., Chavez, J. A., Miinea, C. P., Kane, S., Lienhard, G. E., & McGraw, T. E. (2005). Full intracellular retention of GLUT4 requires AS160 Rab GTPase activating protein. *Cell metabolism, 2*(4), 263-272.

Fang, M., Shen, Z., Huang, S., Zhao, L., Chen, S., Mak, T. W., & Wang, X. (2010). The ER UDPase ENTPD5 promotes protein N-glycosylation, the Warburg effect, and proliferation in the PTEN pathway. *Cell, 143*(5), 711-724.

Feldman, B. J., & Feldman, D. (2001). The development of androgen-independent prostate cancer. *Nature Reviews Cancer*, *1*(1), 34-45.

Fradet, Y., Klotz, L., Trachtenberg, J., & Zlotta, A. (2009). The burden of prostate cancer in Canada. *Canadian Urological Association Journal*, *3*(3 Suppl 2), S92-S100.

Gabriel, K., Ingram, A., Austin, R., Kapoor, A., Tang, D., Majeed, F., Qureshi, T., & Al-Nedawi, K. (2013). Regulation of the Tumor Suppressor PTEN through Exosomes: A Diagnostic Potential for Prostate Cancer. *PLOS ONE*, *8*(7), e70047.

Gan, H. K., Cvrljevic, A. N., & Johns, T. G. (2013). The Epidermal Growth Factor Receptor Variant III (EGFRvIII): Where Wild Things Are Altered. *FEBS Journal*.

Gan, H. K., Kaye, A. H., & Luwor, R. B. (2009). The EGFRvIII variant in glioblastoma multiforme. *Journal of Clinical Neuroscience*, *16*(6), 748-754.

Glass, A. S., Cary, K. C., & Cooperberg, M. R. (2013). Risk-Based Prostate Cancer Screening: Who and How?. *Current urology reports*, 1-7.

Gray, I. C., Phillips, S. M., Lee, S. J., Neoptolemos, J. P., Weissenbach, J., & Spurr, N. K. (1995). Loss of the chromosomal region 10q23–25 in prostate cancer. *Cancer research*, *55*(21), 4800-4803.

He, L., Ingram, A., Rybak, A. P., Tang, D. (2010) Shank-interacting protein-like 1 promotes tumourigenesis via PTEN inhibition in human tumour cells. *The Journal of clinical investigation*, 120**,** 2094-2108.

Holbro, T., & Hynes, N. E. (2004). ErbB receptors: directing key signaling networks throughout life. *Annu. Rev. Pharmacol. Toxicol.*, *44*, 195-217.

Hollander, M. C., Blumenthal, G. M., & Dennis, P. A. (2011). PTEN loss in the continuum of common cancers, rare syndromes and mouse models. *Nature reviews Cancer*, *11*(4), 289-301.

Huotari, J., & Helenius, A. (2011). Endosome maturation. *The EMBO Journal*,*30*(17), 3481-3500.

Hynes, N. E., & MacDonald, G. (2009). ErbB receptors and signaling pathways in cancer. *Current opinion in cell biology*, *21*(2), 177-184.

Kenfield, S. A., Stampfer, M. J., Chan, J. M., & Giovannucci, E. (2011). Smoking and prostate cancer survival and recurrence. *JAMA: the journal of the American Medical Association*, *305*(24), 2548.

Keniry, M., & Parsons, R. (2008). The role of PTEN signaling perturbations in cancer and in targeted therapy. *Oncogene*, *27*(41), 5477-5485.

Kurose, K., Zhou, X. P., Araki, T., Cannistra, S. A., Maher, E. R., & Eng, C. (2001). Frequent loss of PTEN expression is linked to elevated phosphorylated Akt levels, but not associated with p27 and cyclin D1 expression, in primary epithelial ovarian carcinomas. *The American journal of pathology*, *158*(6), 2097-2106.

Lee, J. O., Yang, H., Georgescu, M. M., Di Cristofano, A., Maehama, T., Shi, Y., ... & Pavletich, N. P. (1999). Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. *Cell*, *99*(3), 323-334.

Lee, T. H., D'Asti, E., Magnus, N., Al-Nedawi, K., Meehan, B., & Rak, J. (2011). Microvesicles as mediators of intercellular communication in cancer-the emerging science of cellular "debris." *Seminars in immunopathology*, *33*(5), 455-467.

Leslie, N. R., Batty, I. H., Maccario, H., Davidson, L., & Downes, C. P. (2008). Understanding PTEN regulation: PIP2, polarity and protein stability. *Oncogene*, *27*(41), 5464-76.

Lespagnol, A., Duflaut, D., Beekman, C., Blanc, L., Fiucci, G., Marine, J.C. ... & Telerman, A. (2008) Exosome secretion, including the DNA damage-induced p53-dependent secretory pathway, is severely compromised in TSAP6/Steap3-null mice. *Cell death and differentiation*, 15**,** 1723-1733.

Ludwig, A. K., & Giebel, B. (2012). Exosomes: Small vesicles participating in intercellular communication. *The international journal of biochemistry & cell biology*, *44*(1), 11-15.

Maccario, H., Perera, N. M., Gray, A., Downes, C. P., & Leslie, N. R. (2010). Ubiquitination of PTEN (phosphatase and tensin homolog) inhibits phosphatase activity and is enhanced by membrane targeting and hyperosmotic stress. *Journal of Biological Chemistry*, *285*(17), 12620-12628.

McMenamin, M. E., Soung, P., Perera, S., Kaplan, I., Loda, M., & Sellers, W. R. (1999). Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage. *Cancer research*, *59*(17), 4291-4296.

Mitsudomi, T., & Yatabe, Y. (2010). Epidermal growth factor receptor in relation to tumor development: EGFR gene and cancer. *FEBS Journal*, *277*(2), 301-308.

Muralidharan-Chari, V., Clancy, J. W., Sedgwick, A., & D'Souza-Schorey, C. (2010). Microvesicles: mediators of extracellular communication during cancer progression. *Journal of cell science*, *123*(10), 1603-1611.

Mutter, G. L., Lin, M. C., Fitzgerald, J. T., Kum, J. B., Baak, J. P., Lees, J. A., ... & Eng, C. (2000). Altered PTEN expression as a diagnostic marker for the earliest endometrial precancers. *Journal of the National Cancer Institute*,*92*(11), 924-930.

Normanno, N., De Luca, A., Bianco, C., Strizzi, L., Mancino, M., Maiello, M. R., ... & Salomon, D. S. (2006). Epidermal growth factor receptor (EGFR) signaling in cancer. *Gene*, *366*(1), 2-16.

Ostrowski, M., Carmo, N. B., Krumeich, S., Fanget, I., Raposo, G., Savina, A., ... & Thery, C. (2009). Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nature cell biology*, *12*(1), 19-30.

Pant, S., Hilton, H., & Burczynski, M. (2012). The multifaceted exosome: Biogenesis, role in normal and abberant cellular function, and frontiers for pharmacological and biomarker opportunities. *Biochemical Pharmacology*, *37* (12), 1-11.

Perren, A., Weng, L. P., Boag, A. H., Ziebold, U., Thakore, K., Dahia, P. L., ... & Eng, C. (1999). Immunohistochemical evidence of loss of PTEN expression in primary ductal adenocarcinomas of the breast. *The American journal of pathology*, *155*(4), 1253-1260.

Piccin, A., Murphy, W. G., & Smith, O. P. (2007). Circulating microparticles: pathophysiology and clinical implications. *Blood reviews*, *21*(3), 157-71.

Pilzer, D., & Fishelson, Z. (2005) Mortalin/GRP75 promotes release of membrane vesicles from immune attacked cells and protection from complement-mediated lysis. *International Immunology*, *17*(9), 1239–1248.

Puc, J., Keniry, M., Li, H. S., Pandita, T. K., Choudhury, A. D., Memeo, L., ... & Parsons, R. (2005). Lack of PTEN sequesters CHK1 and initiates genetic instability. *Cancer cell*, *7*(2), 193-204.

Putz, U., Howitt, J., Doan, A., Goh, C. P., Low, L. H., Silke, J., & Tan, S. S. (2012). The tumour suppressor PTEN is exported in exosomes and has phosphatase activity in recipient cells. *Science Signaling*, *5*(243), ra70.

Rana, S., & Zöller, M. (2011). Exosome target cell selection and the importance of exosomal tetraspanins: a hypothesis. *Biochemical Society transactions*, *39*(2), 559-62.

Rahdar, M., Inoue, T., Meyer, T., Zhang, J., Vazquez, F., & Devreotes, P. N. (2009). A phosphorylation-dependent intramolecular interaction regulates the membrane association and activity of the tumour suppressor PTEN. *Proceedings of the National Academy of Sciences of the United States of America,* 106**,** 480-485.

Riese, D. J., & Stern, D. F. (1998). Specificity within the EGF family/ErbB receptor family signaling network. *Bioessays*, *20*(1), 41-48.

Sadar, M. D. (2012). Advances in small molecule inhibitors of androgen receptor for the treatment of advanced prostate cancer. *World journal of urology*, *30*(3), 311-318.

Salmena, L., Carracedo, A., & Pandolfi, P. P. (2008). Tenets of PTEN tumour suppression. *Cell*, *133*(3), 403-414.

Saraon, P., Jarvi, K., & Diamandis, E. P. (2011). Molecular alterations during progression of prostate cancer to androgen independence. *Clinical chemistry*, *57*(10), 1366-1375.

Shedden, K., Xie, X. T., Chandaroy, P., Chang, Y. T., & Rosania, G. R. (2003). Expulsion of small molecules in vesicles shed by cancer cells association with gene expression and chemosensitivity profiles. *Cancer research*, *63*(15), 4331-4337.

Sidhu, S. S., Mengistab, A. T., Tauscher, A. N., LaVail, J., & Basbaum, C. (2004). The microvesicle as a vehicle for EMMPRIN in tumour–stromal interactions. *Oncogene*, *23*(4), 956-963.

Simons, M., & Raposo, G. (2009). Exosomes--vesicular carriers for intercellular communication. *Current opinion in cell biology*, *21*(4), 575-81.

Skog, J., Würdinger, T., van Rijn, S., Meijer, D. H., Gainche, L., Curry, W. T., ... & Breakefield, X. O. (2008). Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nature cell biology*, *10*(12), 1470-1476.

Song, L. B., Li, J., Liao, W. T., Feng, Y., Yu, C. P., Hu, L. J., ... & Zeng, M. S. (2009). The polycomb group protein Bmi-1 represses the tumour suppressor PTEN and induces epithelial-mesenchymal transition in human nasopharyngeal epithelial cells. *The Journal of clinical investigation*, *119*(12), 3626.

Soria, J. C., Lee, H. Y., Lee, J. I., Wang, L., Issa, J. P., Kemp, B. L., ... & Khuri, F. R. (2002). Lack of PTEN expression in non-small cell lung cancer could be related to promoter methylation. *Clinical Cancer Research*, *8*(5), 1178-1184.

Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M, Mirtsos, C., Sasaki, T., ... & Mak, T. W. (1998). Negative regulation of PKB/Akt-dependent cell survival by the tumour suppressor PTEN. *Cell*, 95**,** 29-39.

Steelman, L. S., Bertrand, F. E., & McCubrey, J. A. (2004). The complexity of PTEN: mutation, marker and potential target for therapeutic intervention. *Expert opinion on therapeutic targets*, *8*(6), 537-550.

Suzuki, H., Freije, D., Nusskern, D. R., Okami, K., Cairns, P., Sidransky, D., ... & Bova, G. S. (1998). Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues. *Cancer research*, *58*(2), 204-209.

Taylor, D. D., & Gercel-Taylor, C. (2011). Exosomes/microvesicles: mediators of cancer-associated immunosuppressive microenvironments. *Seminars in immunopathology*, *33*(5), 441-454.

Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L., Wenzel, D., Wieland, F., ... & Simons, M. (2008). Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science*, *319*(5867), 1244-1247.

Trotman, L. C., Wang, X., Alimonti, A., Chen, Z., Teruya-Feldstein, J., Yang, H., ... & Pandolfi, P. P. (2007). Ubiquitination regulates PTEN nuclear import and tumour suppression. *Cell*, *128*(1), 141-156.

Valenti, R., Huber, V., Iero, M., Filipazzi, P., Parmiani, G., & Rivoltini, L. (2007). Tumour-released microvesicles as vehicles of immunosuppression.*Cancer research*, *67*(7), 2912-2915.

Van Doormaal, F. F., Kleinjan, A., Di Nisio, M., Büller, H. R., & Nieuwland, R. (2009). Cell-derived microvesicles and cancer. The *Netherlands Journal of Medicine*, *67*(7), 266-273.

Voldborg, B. R., Damstrup, L., Spang-Thomsen, M., & Poulsen, H. S. (1997). Epidermal growth factor receptor (EGFR) and EGFR mutations, function and possible role in clinical trials. *Annals of Oncology*, *8*(12), 1197-1206.

Wang, J., Peng, Q., Lin, Q., Childress, C., Carey, D., & Yang, W. (2010). Calcium activates Nedd4 E3 ubiquitin ligases by releasing the C2 domain-mediated auto-inhibition. *Journal of Biological Chemistry*, *285*(16), 12279-12288.

Wen, S., Stolarov, J., Myers, M. P., Su, J. D., Wigler, M. H., Tonks, N. K., & Durden, D. L. (2001). PTEN controls tumour-induced angiogenesis. *Proceedings of the National Academy of Sciences*, *98*(8), 4622-4627.

Weng, L. P., Brown, J. L., & Eng, C. (2001). PTEN coordinates G1 arrest by down-regulating cyclin D1 via its protein phosphatase activity and up-regulating p27 via its lipid phosphatase activity in a breast cancer model. *Human molecular genetics*, *10*(6), 599-604.

Whang, Y. E., Wu, X., Suzuki, H., Reiter, R. E., Tran, C., Vessella, R. L., ... & Sawyers, C. L. (1998). Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression. *Proceedings of the National Academy of Sciences*, *95*(9), 5246-5250.

Wikstrand, C. J., Reist, C. J., Archer, G. E., Zalutsky, M. R., & Bigner, D. D. (1998). The class III variant of the epidermal growth factor receptor (EGFRvIII): characterization and utilization as an immunotherapeutic target. *Journal of neurovirology*, *4*(2), 148-158.

Wilt, T. J., & Ahmed, H. U. (2013). Prostate cancer screening and the management of clinically localized disease. *BMJ: British Medical Journal*, *346*.

Wollert, T., & Hurley, J. H. (2010). Molecular mechanism of multivesicular body biogenesis by ESCRT complexes. *Nature*, *464*(7290), 864-869.

Wu, X., Senechal, K., Neshat, M. S., Whang, Y. E., & Sawyers, C. L. (1998). The PTEN/MMAC1 tumour suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway. *Proceedings of the National Academy of Sciences*, *95*(26), 15587-15591.

Wurm, F. M., & Hacker, D. (2011). First CHO genome. *Nature biotechnology*,*29*(8), 718-720.

Yang, C., & Robbins, P. D. (2011). The roles of tumour-derived exosomes in cancer pathogenesis. *Clinical & developmental immunology*, *2011*, 1-11.

Yarden, Y. (2001). The EGFR family and its ligands in human cancer: signalling mechanisms and therapeutic opportunities. *European journal of cancer*, *37*, 3-8.

Zhou, X. P., Loukola, A., Salovaara, R., Nystrom-Lahti, M., Peltomäki, P., de la Chapelle, A., ... & Eng, C. (2002). PTEN Mutational Spectra, Expression Levels, and Subcellular Localization in Microsatellite Stable and Unstable Colorectal Cancers. *The American journal of pathology*, *161*(2), 439-447.