Pro gradu

Isolation and Characterization of Extracellular Vesicles (EVs) from Renal Carcinoma Cells

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**Abbreviations**

RCC          Renal cell carcinoma  
DC           Dendritic cell  
EVs          Extracellular Vesicles  
MSCs         Mesenchymal stromal cells  
ABs          Apoptotic bodies  
PS           Phosphatidylserine  
PBS          Phosphate Buffered Saline  
MVs          Microvesicles  
MVBs         Multivesicular bodies  
HSP          Heat shock protein  
MHC          Major Histocompatibility  
HIF          Hypoxia inducible factor  
MM           Metanephric mesenchyme  
YFP          Yellow fluorescent protein  
WT           Wild type  
DMEM         Dulbecco’s Modified Eagle medium  
DMSO         Dimethyl sulfoxide  
HM           Homogenization medium  
WB           working buffer  
SDS          Sodium dodecyl sulfate  
BSA          Bovine serum albumin  
TEM          Transmission electron microscopy  
NTA          Nanoparticle tracking analysis  
LCMS         Liquid chromatography mass spectrometry  
Rpm          Revolutions per minute
1. LITERATURE SECTION

1.1 Introduction

Kidney cancer is one of the major types of cancer among people, being 7th most common cancer among men and 11th among women. This accounts for around 3% among all cancers occurring in Europe alone. In 2012, around 115,000 new cases were reported and more than 49,000 deaths were recorded in 40 European countries because of kidney cancer (Steliarova-Foucher et al. 2014). The mortality rate due to this cancer is higher in Europe and North America in comparison to Asia and South America. Kidney cancer includes multiple tumors that originate in kidney, renal pelvis as well as ureter and other urinary organs (Marcos-Gragera et al. 2015).

Renal cell carcinoma (RCC) is the most common renal tumor, accounting more than 90% renal malignancies (National Comprehensive Cancer Network 2009). The major factor that is responsible for increasing malignancy rate is lifestyle. Major etiological factors include sex, race, age, smoking habit and obesity or overweight. Beside this, genetic factors, nutritional factors and occupations are also involved. Thus RCC still remains a major challenge for the researchers and effective therapeutics able to inhibit growth and propagation of tumor is an urgent need.

Exosomes are the membrane vesicles between size 30-100 nm (Raposo et al. 1996). They are formed by inward budding from multivesicular endosomes fused with plasma membrane. Exosomes are secreted by different cells including RBC, B and T lymphocytes, platelets and dendritic cells (DCs) (Denzer et al. 2000). Exosomes has been purified from different body fluids such as blood, saliva, urine, serum and nasal secretion.

Role of exosome in kidney cancer is not fully understood yet and is currently deriving much interest among researchers. The purpose of our study was to find the general effect of kidney cancer derived exosomes in tumorigenesis. The work includes characterization of these
exosomes using different techniques, study of effects of these exosomes in tumor formation, cell proliferation and cell motility. The study also includes identification of cargo proteins and miRNAs from exosomes.

1.2 Extracellular vesicles

Extracellular Vesicles (EVs) are nano sized vesicles secreted by various cell types including B and T lymphocytes, mast cells, platelets, dendritic cells and mesenchymal stromal cells (MSCs) derived from different tissues, and can be isolated from cell culture supernatant and different biological fluids like urine, blood, semen, amniotic fluid etc. Wide variety of organisms ranging from simple prokaryotes to higher eukaryotes releases EVs. EVs can be categorized as exosomes, microvesicles and apoptotic bodies on the basis of size, morphology, origin and the way they are released in the microenvironment (fig.1) (Nawaz et al. 2014). Exosomes originate from multivesicular bodies, microvesicles are derived from the plasma membrane and apoptotic bodies are produced from cells undergoing apoptosis.
Figure 1: Types of extracellular vesicles (EVs) secreted by cell. Exosomes, microvesicles and apoptotic bodies are the three types of extracellular vesicles secreted by a cell and released in extracellular space. (Kalra et al. 2016)

EVs were first observed as procoagulant platelet-derived particles in 1946 by Chargaff and West (Chargaff & West 1946), and in 1967 Wolf referred these particles as “platelet dust” (Wolf 1967). Thus for a long time these vesicles were considered as a waste product of cells. However, studies were being done and new findings were coming out. In 2006-2007 most exciting finding came into light that EVs contain RNA including microRNA and can serve as mediators of cell to cell communication (Valadi et al. 2007). Since then much interests have been attracted in this field and new results are coming out every day.
The various EVs from body fluids and cell culture can be isolated and characterized by using different protocols. The presence of membrane fragments and all types of vesicles in the starting material can lead to the contamination during specific EVs purification. Therefore, standardization of protocol for effective purification and analysis of EVs is a major challenge at the moment. The problem also arises due to similar or overlapping characteristics among various vesicles and lack of proper biomarkers that can discriminate these vesicles in an effective way (Gould & Raposo 2013).

The major components of EVs include mRNAs, miRNAs, DNA, proteins and lipids. EVs have abilities to transfer these materials to the neighboring cells, thus introducing changes in their phenotype along with the microenvironment (Camussi et al. 2011). So this has been recognized as new effective means of cell to cell communication in both prokaryotic and eukaryotic organisms. The main features of the EVs population have been listed in table 1.

**Table I**: Characteristics of major extracellular vesicles (Abreu et al. 2016)

<table>
<thead>
<tr>
<th>EVs</th>
<th>Origin</th>
<th>Size</th>
<th>Content</th>
<th>Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exosomes</td>
<td>Multivesicular bodies</td>
<td>30–100 nm</td>
<td>Proteins, lipids, DNA, mRNA, miRNA</td>
<td>CD81, CD63, CD9, HSPs, Tsg101</td>
</tr>
<tr>
<td>Microvesicles</td>
<td>Plasma membrane</td>
<td>100–1000 nm</td>
<td>Proteins, lipids, DNA, RNA, cell organelles</td>
<td>Selectins, Integrins, CD40</td>
</tr>
<tr>
<td>Apoptotic bodies</td>
<td>Membranes of dying cells</td>
<td>50-5000 nm</td>
<td>DNA, noncoding RNAs, cell organelles</td>
<td>Histones, DNA</td>
</tr>
</tbody>
</table>
1.2.1 Apoptotic bodies

Apoptotic bodies (ABs) are the least studied members among extracellular vesicles. These are released from the cells which undergoes apoptosis and the size are measured between 50-5000nm (Simpson & Mathivanan 2012). Apoptosis is important process and helps in removal of damaged, infected and old cells from tissues. So, apoptotic bodies are basically packed structure of cells and cellular debris that needs to be removed. The positive staining for Annexin V (Hristov et al. 2004) and the presence of phosphatidylserine (PS) on external part (Martin et al. 1995) makes it similar to microvesicle (MV) and hence difficult to distinguish from microvesicle.

Since apoptotic bodies were considered as garbage bags for a long time, relatively less is known about the composition of Abs. However, Mallat et al. studied the ABs of human monocytic and lymphocytic from atherosclerotic plaques and found these ABs to contain PS, coagulation factor III and annexin A5 (Mallat et al. 1999). Proteomics studies of ABs from thrombocyte of mice has identified 142 proteins ranging from heat shock protein, histone related, cytosolic, oncogenes and few other proteins that are immunologically important (Turiak et al. 2011).

Further, ABs contains multiple cell organelles that are packed closely along with nuclear fragments which can be seen by electron microscopy (Elmore 2007). Also, ABs containing DNA and RNA separately that is rich in either one of these component have been observed (Halicka et al. 2000). ABs of tumor cells have shown ability to suppress CD8+ cell responses in vivo (Xie et al. 2009) and growth factor VGEF was found to be expressed higher, suggesting possible role of apoptotic bodies in tumor propagation.

1.2.2 Microvesicles

Microvesicles (MVs) represent the extracellular vesicles between 100-1000 nm in size. MVs are released by normal and diseased cells and are often grouped together with exosomes but these vesicles are unique populations with distinct structures from exosomes.
MVIs are formed by budding and fission of plasma membrane and released into extracellular space; process which is dependent in calcium signaling and enzyme activity. The changes in calcium level leads to the activation of calcium dependent enzymes such as scramblase and floppase along with lipid composition of the plasma membrane (Piccin et al. 2007). One of the main features of MVIs is externalization of phosphatidylserine (PS) which is located on the outer membrane of microvesicle shedding site. However, MVIs without secreted PS has also been reported (Barteneva et al. 2013).

The function of microvesicles depends on the cargo they carry. Apart from carrying genetic materials like DNA, mRNA and miRNA, microvesicles also carry transmembrane proteins and cellular receptors on their surface thus reflecting the characteristics of their parent cells. Differentiated cells and tumor cells release microvesicles carrying miRNAs or mRNAs in most of the cases (Mytar et al. 2008).

1.2.3 Exosomes

Exosomes are small vesicles ranging from 30 – 100 nm in size and originate from multivesicular bodies (MVBs). MVBs fuse with the plasma membrane and their intraluminal vesicles are released as exosomes in extracellular space (Harding et al. 1983). The term “exosomes” was given by Trams et al in 1981 (Trams et al. 1981). Exosomes are separated by multiple number of cells including B and T cells, dendritic cells, mesenchymal stem cells, epithelial cells, endothelial cells and cancer cells both at normal as well as pathological condition (Henderson & Azorsa 2012). Apart from this, exosomes are also identified in different types of body fluids such as urine, blood, serum, saliva and nasal secretion. Interestingly, it has been shown that the amount of exosomes secreted by cancer cells is higher than the normal cells (Taylor & Gercel-Taylor 2008; Logozzi et al. 2009).

The major components of exosomes are Proteins, lipids, DNA, miRNA and mRNA. The study carried out in different cell type shows that exosomes contain 4,563 proteins, 194 lipids, 1,639 mRNA and 764 miRNAs (Mathivanan et al. 2012; Mathivanan & Simpson 2009) making exosomes a complex molecule. The major proteins identified are fusion proteins, CD63, CD81, CD9, CD82, heat shock proteins (HSP70, HSP90), signal
transduction proteins, cytoskeletal proteins along with cell type-specific proteins like major histocompatibility complex (MHC) I and II, A33 antigen (Mathivanan & Simpson 2009) and differ according to the cells from which they originate.

Lipids in exosomes are less characterized in compared to other components. Exosomes have their own specific lipid composition differing from parental MVB but still reflect the cells of origin. Their functions are highly contributed by bioactive lipids such as prostaglandins (Subra et al. 2010). DNA and RNA including mRNA and miRNA are other major compositions of exosomes in addition to protein. The presence of RNA makes exosomes as a significant pathway of cell to cell communication. These mRNAs are found to be functional and transferable within target cells where they can be translated into proteins (Ratajczak et al. 2006). Thus mRNA and miRNA both perform specific functions upon their transfer to other cells indicating that exosomes are potential route for signaling between cells.

1.3 Exosomes in cancer

Intercellular communication is an important process by which cells transmit and receive signals between nearby or distant cells. Previously, it was thought that intercellular communication occurs by direct cell-to-cell contact and secreted soluble proteins. Recent studies have shown a new way of communication among cells mediated by extracellular vesicles (Thery et al. 2009). These vesicles are effective for signaling among neighbor cells as well as distant cells.

Metastasis is one of the major causes of deaths in cancer. The mechanism by which the cancers cells escape the site of primary tumor and migrate to distant cells are not yet fully understood (Nguyen et al. 2009). For effective metastasis, cells from primary tumor escape into blood or lymph and grow tumor in a distant cell with favorable microenvironment for tumor propagation. There is growing evidence that exosomes might play an important role in this process (Yang C & Robbins PD 2011). Exosomes are capable of passing proteins, miRNAs as well as anti-cancer drugs from the cells and release regulatory and signaling molecules (Lee et al. 2011). Some factors released from exosomes can enhance stromal
remodeling and transformation of hypoxia-mediated mesenchyme which is important for cancer development (Nieto 2011). By desmoplastic reaction, exosomes can lead to fibroblast proliferation and can cause immune escape by suppressing antigen specific immune responses or by upregulating immunosuppressive cell differentiation (Azmi et al. 2013). This shows that exosomes are critical for tumorigenesis.

Some studies have shown the potential of exosomes for cross tumor propagation. Exosomes from renal cells have shown to initiate angiogenesis in lung cancer ascites (Nguyen DX & Massague J 2007) and exosomes from human bone marrow mesenchymal stem cells have shown to stimulate proliferation of proximal tubular cells by transferring hepatocyte growth factor-1 receptor mRNA (Tomasoni et al. 2013). These results suggest that exosomes trigger tumor propagation by horizontal transfer of genetic materials.

There are different mechanisms by which exosomes interact with the recipient cells. One mechanism involves the binding of exosomal membrane proteins with the ligands of the recipient cell membrane such as transferrin receptor, integrins, tumor necrosis factor receptors and tetraspanin proteins like CD9, CD63 and CD81 (Chen et al. 2010). Another mechanism involves the fusion of exosomes with the membrane of the recipient cell thus resulting in the transfer of exosome contents such as proteins, miRNAs, mRNAs and other signaling molecules or endocytosis (Hemler 2003; Tien et al. 2010).

Together with transferring cargo to various cells, exosomes have ability to protect its contents from degradation due to several degradative enzymes such as RNase, trypsin and other chemical components due to the presence of lipid bilayer in exosomes (Koga et al. 2011).
1.4 Hypoxia and exosomes

Adequate oxygen is necessary for normal cellular function and energy metabolism in organisms. Hypoxia refers to the condition of insufficient oxygen supply and in most of the time associated with promoting different diseases such as tumor spreading, pre-eclampsia (PE) and venous thromboembolism (VTE) (Belting & Christianson 2015). Hypoxia inducible factors (HIFs), are the transcription factors associated with the oxygen homeostasis (Kaelin & Ratcliffe 2008). HIFs are formed by two subunits, HIF-α and HIF-β and are responsible for maintenance of different cellular responses (Zhong et al. 1999). HIF dependent hypoxic response depends on intercellular signals, which then plays active role to provide adaptive mechanism for cell survival and tissue remodeling in the hypoxic microenvironment. It was also recently found that hypoxia increased the secretion of tumor enhancing factors which affects the surrounding tissues in the tumor microenvironment (Chaturvedi et al. 2013).

Many studies have shown that hypoxia enhance the exosomes release in various tumors. In breast cancer, hypoxia mediated activation of HIF-1 α have been found to increase release of exosomes which was confirmed by the lower release of exosomes when HIF-1α was knockdown (King et al. 2012). Extracellular vesicles include a diverse range of vesicles and hence are conceivable to believe that different mechanisms play a role in hypoxia-regulated formation and secretion of these vesicles in cell dependent manner.

Hypoxia not only affects during secretion of vesicles but also have great impact on the messages contained within the vesicles. The messages contained and transferred by the vesicles are affected by hypoxia resulting in effects in functions. The study showed that angiogenic pathways and metastatic potential are enhanced by hypoxic microenvironment through secretion of exosomal proteins transpanins and alix while studying A431 carcinoma cell line when subjected to hypoxia and then back to oxygen (Park et al. 2010). These studies show that hypoxia actually promotes the secretion of exosomes inorder to create microenvironment which favors pro-angiogenic environment which results in the development of tumors aggressively.
1.5 Importance of EVs in cancer

1.5.1 As biomarkers

The novel biomarkers play important roles in detection, prognosis and diagnosis of the disease, particularly in cancer and exosomes have potential to act as significant biomarkers (fig.2). Exosomes carries various intercellular molecules, hence may act as a source of unidentified important biomarkers which have potential to predict the disease before it spreads completely and patient goes under formal diagnosis such as MRI, CT or PET scan (Cho et al. 2012; Paidpally et al. 2012). Since exosomes display specific expressions which are most of the time representation of disease state, and also these exosomes can be detected in the body fluids, making these vesicles ideal candidate for biomarkers of the tumor propagation (Tickner et al. 2014). Both exosomal proteins and RNAs serve as potential biomarkers.

In kidney, proteomics studies of urinary exosomes have analyzed proteins from different parts of nephron and several biomarkers have been identified. These proteins are effective for pathological conditions such as acute kidney rejection, acute kidney injury as well as diabetic nephropathy (Ling et al. 2010; Hongjuan et al. 2009). One study suggested that exosomes containing aquaporin-1 protein’s excretion decreased in rats under I/R up to 96 hours but after 480 hours, it returned to normal level. The similar result was later observed in patient with renal allograft transplantation, suggesting that exosomes carrying aquaporin-1 protein is able to detect I/R induced injury of renal cells. This can be used to predict post transplantation failure (Sonoda et al. 2009).

More than 1000 proteins have been identified in humans by analyzing urinary exosomes and among them 34 proteins have been found to be associated with kidney diseases like polycystin-1 protein in autosomal dominant polycystic kidney disease type 1, aquaporin-2 protein in autosomal dominant and recessive nephrogenic diabetes and Na-K-2Cl symport protein in antenatal barter syndrome type 1 (Miranda et al. 2010).
Along with proteins, exosomes also contain mRNA and miRNA which can be used as disease biomarkers. Studies have shown that miRNA of circulating exosomes are useful in diagnosis of cancers including lung and ovarian cancer (Taylor & Gercel-Taylor 2008; Rabinowits et al. 2009). In kidney, various miRNAs (miR-200a, miR-200b and miR-429) were found to be lowered than the normal level in the patient with immunoglobulin A nephropathy and the level of reduction was associated with the disease severity (Wang et al. 2010). It is also found that urinary granzyme A mRNA level can distinguish person between diseases cellular rejection and acute kidney injury (Van Ham et al. 2010). These studies show that the miRNAs and mRNAs are not only associated with the disease biomarkers but also changes in their level in urinary exosomes can help to detect the progression of diseases.
Figure 2: Role of extracellular vesicles as therapeutic agents, biomarkers and in drug delivery. EVs can activate immune response as well as suppress inflammation, can act in blood coagulation process, help in communication for neurons and can repair injured tissues, thus making them ideal for therapeutic agents. Their ability to transfer RNAs to surrounding cells make them suitable candidate for drug delivery. Also proteins present in these vesicles can act as biomarkers (Andaloussi et al. 2013).

1.5.2 As therapeutic agents

The ability of exosomes to transfer mRNAs and miRNAs between cells of origin and target cells opens a new concept of using exosomes as delivery vehicles in therapeutics (fig.2) (Valadi et al. 2007). These transfers can either increase or decrease in the level of protein expression within the target cells. Angiogenesis in endothelial cells is caused by endothelial progenitor cell-derived exosomes by possible mechanism of transferring mRNA and miRNA molecules in the target
cells (Deregibus et al. 2007). Thus exosomes can influence the gene expression of the target cells.

Exosomes can also serve as important vehicles in the delivery of specific antigens (fig.2). The idea has been useful in vaccination against acute respiratory syndrome where exosomes loaded with acute respiratory syndrome S protein (Kuate et al. 2007) against Toxoplasma gondii where exosomes loaded with specific antigen (Beauvillain et al. 2009) showed positive effects. The antibodies were neutralized to the most extent and in the case of T. gondii the disease level was seen to be reduced in mice.

Since exosomes are secreted by almost every type of cells and are able to transfer their cargos among cells proves their ability to deliver drugs, especially the delivery of therapeutic nucleic acids, fig. 1(b). Thus exosomes can be considered as ideal nanovectors for drug delivery while compared to synthetic lipid vesicles or liposomes (Fais et al. 2013). In 2010, the first publication was done on vesicle mediated transfer of nucleic acid where THP-1 monocyte was transfected with miR-150 and secreted vesicles were found containing miR-150, which was delivered to the recessive, cells (Zhang et al. 2010). The capacity of such vesicles in delivery can be an important success in gene therapy where these vesicles can be engineered to deliver regulatory miRNAs, siRNAs or even tumor suppressors like PTEN (Gabriel et al. 2013).

The possible role of exosomes in the kidney diseases is being studied. Mostly, the exosomes have been found to be associated in the paracrine effects in stem cell therapy. During stem cell therapy of kidney injury, mesenchymal stem cells were able to recover partially through paracrine factors released from exosomes (Gatti et al. 2011). Similarly, in stem cell therapy of glomerulonephritis in rats (anti-Thy 1.1 glomerulonephritis), paracrine factors from injected mesenchymal stem cells were helpful than the factors derived from cells themselves (Kunter et al. 2006). In these paracrine effects, exosome secretion was found to be involved. Thus exosomes can have a significant importance in treatment of different diseases including kidney diseases.
2. AIM OF THE PROJECT

The general aim of this thesis is to study the role of renal tumor cell derived exosomes in kidney.

The specific aims of this thesis are:

- To purify and characterize exosomes derived from mouse kidney cancer cells (Renca).
- To study the effect of exosomes in mouse embryonic kidney (MM) cells
- To see whether Renca derived exosomes have role in tumor growth
- To study proteins and RNA contents of exosomes for biomarker discovery
3. MATERIALS AND METHODS

3.1 Cell culture

The cell lines used in this study were renal carcinoma (Renca WT) cell line (ATCC® CRL-2947™, Manassa, VA) and Renca cells stably expressing enhanced yellow fluorescent protein (Renca YFP), MK3 (early metanephric mesenchyme) and MK4 (later induced metanephric mesenchyme) (Valerius et al. 2002). The Renca cells were cultured in Dulbecco’s modified Eagle medium (Life Technologies, UK): nutrient mixture F-12 (DMEM/F12), MK3 and MK4 cells were cultured in normal DMEM, both supplied with 10% FBS and 100 U penicillin/0.1 mg/ml streptomycin.

During passage of the cells, the cells were washed with PBS one to two times, trypsin was added and plates were incubated for 4 minutes at 37°C in order to detach the cells. After adding media, the cells were splitted in the ratio of 1:3 - 1:5. Cells were cultured in an incubator at 37°C and 5% CO₂ (Panasonic Healthcare, Japan). The cells were frequently checked for contamination.

To maintain cell lines for a long period of time, cells were trypsinized and centrifuged at 1500 rpm for 4 minutes. The pellets were then dissolved in dimethyl sulfoxide (DMSO) 5-10%, put in cryogenic tube and stored at -70°C overnight. The tubes were then finally transferred to liquid nitrogen for long term storage.

3.2 Exosomes isolation

The cells were cultured in 20 big plates until confluency reached up to 80-90%. The previous media was removed and exosome free media was added. Half number of plates were incubated in normal condition (37°C and 5% CO₂) while the remaining plates were incubated in hypoxic condition (37°C and 1% O₂). After 24 hours of incubation, isolation was carried out by ultracentrifugation and density-gradient separation method.
For ultracentrifugation, the cell supernatants were collected and centrifuged at 3000 rpm for 10 minutes to pellet cells and debris. The supernatant was then centrifuged at 2000 rcf and 4°C for 15 minutes inorder to get rid of oncosomes. MVs were pelleted by ultracentrifugation at 20,000 rcf and 4°C for 45 minutes. The supernatant was filtered using 0.22-micron filter and finally ultra-centrifuged at 108,800 rcf and 4°C for overnight or 18-22 hours (Sorvall WX90, Thermo Scientific, US) for exosome isolation. The supernatant was discarded and the pellet was dissolved in 1x filtered PBS.

For density gradient separation, the cell supernatants were collected in centricon plus-70 filter device (Merck Millipore, Ireland) and concentrated by centrifugation at 3500 rpm and 4°C for 40 minutes. The concentrated sample was recovered by short spin at 1000 rpm for 2 minutes. Homogenization media (HM) and working solution buffer (WB) were prepared. The working solution (WS) was prepared by adding 1 volume of WB to 5 volumes of optiprep solution (Sigma). The gradient solution was prepared in a following way:

<table>
<thead>
<tr>
<th>Percentage (%)</th>
<th>WS (ml)</th>
<th>HM (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>15</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>20</td>
<td>8</td>
<td>2</td>
</tr>
</tbody>
</table>

Each gradient solution was sequentially layered on the top of each other in polyallomer ultracentrifuge tube. The concentrated sample was finally added on the top of the gradient. The gradients were ultracentrifuged at 24,200 rcf, 4°C for 18 hours (overnight). 1 ml fractions were collected and diluted in 1x PBS and centrifuged at 24,200 rcf, 4°C for another 3 hours. The supernatant was discarded and pellet was resuspended in 1x PBS.
Gradient solutions were prepared as follows:

**Homogenization medium (HM)**
- 0.25 M sucrose
- 1 mM EDTA
- 10 mM Tris-HCl
- pH 7.4

**Working solution buffer (WB)**
- 0.25 M sucrose
- 6 mM EDTA
- 60 mM Tris-HCl
- pH 7.4

### 3.3 Exosomes labeling

10x Exo-Red was added to isolated exosomes in a 1.5 ml eppendorf tube and mixed gently without vortex. The solution was incubated in 37°C for 10 minutes. The labeling reaction was stopped by adding ExoQuick-TC reagent (System Biosciences, CA) to the labeled exosomes samples and mixed by inverting. The tube was incubated on ice for 30 minutes. The sample was centrifuged at 14,000 rpm for 3 minutes. The supernatant was removed and the pellet was suspended in 1x PBS. The exosomes were labeled and ready to be monitored for tracking.

### 3.4 SDS-PAGE

Before characterization of exosomes by western blot, proteins were first separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). 10% resolving gel was first prepared and after its polymerization, 4% stacking gel was layered on the top of resolving gel. Protein samples were mixed with 1x laemmlie buffer and heated at 95°C for 5 minutes. The 2-mercaptoethanol reduced disulfide bonds and SDS denatures the proteins and provide
negative charge which will now separate on the basis of molecular weight. The samples were loaded on each well and run for 90 minutes at 15 mA current. Protein ladder was used inorder to determine the protein size separated in the gel.

10% resolving gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>5.22ml</td>
</tr>
<tr>
<td>40% acrylamide</td>
<td>2.6ml</td>
</tr>
<tr>
<td>1.85M Tris, pH 8.8</td>
<td>2ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>50 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

4% stacking gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>3.92ml</td>
</tr>
<tr>
<td>40% acrylamide</td>
<td>500µl</td>
</tr>
<tr>
<td>1.85M Tris, pH 6.8</td>
<td>500µl</td>
</tr>
<tr>
<td>10% SDS</td>
<td>50µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>31µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>6.3µl</td>
</tr>
</tbody>
</table>

3.5 Western blot

After completing SDS-PAGE, the proteins were transferred to nitrocellulose membrane. The nitrocellulose membrane was placed on the top of gel and sandwiched between Whatman papers on both sides. The blotting was done for 70 minutes at 150V using ice inorder to avoid excessive heating. Once the transfer was complete, the membrane was blocked in a 5% blocking buffer for 1 hour. Primary antibody CD81 (Santa Cruz Biotechnology, US) was added and the membrane was left in a cold room in a shaker overnight. The membrane was washed with 1xPBST three times 5 minutes each and secondary antibody anti-mouse (Dako, Denmark) was added and incubated in a shaker for 1 hour. This was followed by another wash with 1xPBST three times 5 minutes each. Detection reagent was added to the membrane and incubated for 1 minute and finally imaging was done on the imager.
Blocking buffer
2.5-gram casein milk was dissolved in 50 ml 1x PBST.

3.6 Cell migration assay

Approximately 30000 cells per well were cultured in a 96 well image lock plate. The plate was incubated overnight and next day, the wells were more than 80 percent confluent to carry out experiment. The wells were then treated with mitomycin (1:10 dilution) and incubated for 3 hours at +37°C.

Scratching on the cell plate was done carefully by using wound maker (Essen Bioscience). The media was removed and the cells were washed with 1xPBS once. After adding new media, the cells were treated with hypoxia and normoxia exosomes (1µl per well) and incubated in +37°C incubator. The imaging was done by using incucyte zoom system (Essen Bioscience, US) for the next 24 hours.

3.7 Cell proliferation assay

Approximately 3000 cells were cultured in a 96 well plate. The cells were 80-90% confluent after growing for 24 hours. Next day, the old media was removed and new media was added. The cells were then treated with hypoxia and normoxia exosomes. Sytox Green (1:20 dilution) was added in all wells and incubated at +37°C. The imaging was done by incucyte zoom (Essen Bioscience US) for next 24 hours.
3.8 Mass spectrometry

The concentration of protein sample was measured by BCA assay. Specific amount of protein solution was taken and precipitated by adding 3 aliquots of cold acetone and mixed by vortexing. The mixture was incubated at -20°C overnight.

The sample was centrifuged at 12,000g for 20 minutes at 4°C. The supernatant was discarded and the pellet was air dried for 3 minutes. A double volume of Tris buffer (50mM, pH 8.5) with trypsin at an enzyme: substrate ratio of 1:30 (w/w) was added, vortexed and incubated at 37°C for 3 hours. The sample was then reduced with 25mM DTT at 95°C for 5 minutes and then alkylated with 100mM iodoacetamide at 37°C for 30 minutes in the dark place. This was followed by addition of second aliquot of trypsin at an enzyme: substrate ratio of 1:25 (w/w) and incubated at 37°C overnight. This allowed for the complete digestion of the protein sample.

The tubes were collected from incubator and centrifuged at 10,000g for 5 minutes. The supernatant was collected in new tube and equal volume of 100% acetonitrile was added. The mixture was transferred to glass vials avoiding bubbles at the bottom and dried using speedvac (Thermo Scientific, US). The sample was then processed for mass spectrometry.

3.9 Immunohistochemistry

The section slides were taken out from -70°C freezer and thaw them at room temperature for 15 minutes. The samples were blocked by adding 500µl blocking solution at room temperature for 1 hour. The samples were then incubated by primary antibody; anti-GFP (abcam ab 290) at 4°C overnight.

Next day, the samples were taken out from 4°C and allowed to stay at room temperature for 1 hour. Samples were washed with 1x PBS for 6 times 15 minutes each. Secondary antibody; AF goat-anti-rabbit 546 along with Hoechst was added to the samples and incubated for 1 hour at room temperature. Since secondary antibody is light sensitive, this step was carried
out in dark place. The samples were then washed with 1x PBS for 3 times for 15 minutes and finally washed with water.

**Blocking solution**

- 5% gat serum: 2.5ml
- 5% FBS: 2.5ml
- 1% BSA: 0.5ml
- 0.1% Triton-X: 50µl

Dissolved in 0.3M glycine PBS

**Primary antibody (1:500)**

3µl anti-GFP dissolved in 1500 µl 1x PBS.

**Secondary antibody (1:1000)**

2.5µl AF goat-anti-rabbit 546 and 1.25µl Hoechst (1:2000) dissolved in 2.5ml 1x PBS.

### 3.10 RNA extraction

TRIZOL was added in RNaseA treated exosome and mixed. Chloroform was then added to the sample, mixed by inverting 30 times. The mixture was left for 5 minutes at room temperature and span for 15 minutes at 10,000 rpm and 4°C. The aqueous phase was transferred to the tube containing isopropanol, mixed by vortexing and kept at -80°C for 2 hours. The sample was thaw span for 15 minutes at 13000 rpm 4°C, supernatant was removed and 1ml 75% ethanol was added to the pellet. After brief vortexing, the sample was span for 5 minutes at 1000 rpm 4°C, supernatant was discarded and the tube was span for a few minutes at 12000 rpm at room temperature. The rest of the liquid was removed carefully and tube was left to dry for few minutes and RNase free H₂O was added and vortexed. After 5 minutes incubation on ice, concentration was measured. DNAsel buffer and DNAsel was added to the sample and incubated for 10 minutes at room temperature. Trizol and chloroform were immediately added and mixed by inverting. The mixture was left at room temperature for 5 minutes and then centrifuged for 15 minutes at 10,000 rpm 4°C. The
aqueous phase was transferred to the tube with isopropanol, mixed by vortexing and incubated overnight at -80°C.

Next day, the sample was centrifuged for 15 minutes at 13000 rpm. The supernatant was removed and 75% ethanol was added, span for 5 minutes at 10000 rpm and supernatant was removed. The sample was further span for 2 minutes at 12000 rpm at room temperature and rest of the supernatant was removed. The sample was left to dry for few minutes and RNase free water was added, vortexed and put in ice. After 10 minutes concentration of RNA was measured and precipitated again with RNase free glycogen, RNase free sodium acetate and 100% ethanol. The mixture was vortexed, incubated at -80°C for 1 hour. After spinning at 13000 rpm for 15 minutes, the sample was washed with 75% ethanol.

The samples were processed by Next Generation Sequencing (NGS), Ion Torrent in Biocenter Oulu Sequencing Center and results were analyzed.

3.11 Electron microscopy

Former coated copper grids (400 mesh) were glow-discharged before samples were applied. Sample solution was mixed gently and 3µl droplet was used for each grid and incubated for 20 minutes. The grid was washed 2 times for 1 minute each with a drop of PBS and for 10 minutes in 1% BSA in PBS. The grid was incubated on a drop of primary antibody for 20 minutes, washed 3 times for 1 minute each in a drop of 1% BSA in PBS and incubated on another drop of secondary antibody for 20 minutes. The grid was washed 3 times for 1 minute each in a drop of 1% BSA in PBS, incubated on a drop of gold-conjugated protein A for 20 minutes and washed 3 times for 1 minute in a drop of PBS. Sample was fixed by incubating grid on a drop of 1% glutaraldehyde in PBS for 5 minutes, washed 8 times 1 minute each in a drop of water and then stained with 2% UA in H₂O for 5 minutes inside the fume hood. Finally, the grid was coated with 2% methylcellulose-UA (0.4%) solution for 10 minutes and dried at room temperature.
3.12 Injection of chicken embryos: *ex ovo* culture

Fertilized Hy-line chicken eggs were obtained from Haaviston farm (Finland). The eggs were incubated in a rotary thermostat at 37.5°C for 2 days. At day 3, egg shell was cut carefully using sterile scissors and the entire egg content was transferred to petri dish with water covered with plastic.

Renca YFP cells were trypsinized and centrifuged at low speed for 5 minutes. The pellet was dissolved in 1x PBS and around 1 million cells were injected into embryo. The injections were intracardiac and were done in two different conditions; some embryos were injected with only renca YFP cells and others were injected with renca YFP cells with exosomes. The embryos were then incubated in incubator at +37°C. They were allowed to grow for 7-10 days and images were taken.
4. RESULTS

The aim of this thesis was to study the function of kidney cancer derived exosomes. Therefore, the project includes characterization of these exosomes using multiple methods, their effects on normal mesenchyme cells and role in tumor enhancing. In the last part of the thesis, we analysed proteins and RNA contents of the exosomes.

4.1 Characterization of EVs

4.1.1 Electron Microscope analysis

We first investigated the purity of isolated exosomes by transmission electron microscopy (TEM). The exosomes were immunogold labelled with CD63 antibody and negative stained with uranyl acetate. Images were taken with Tencai, TEM. We found that majority of vesicles were labeled with CD63 (fig. 3).
Figure 3: TEM of renca exosomes. Exosomes characterized for known exosome markers CD63. Normoxia exosomes (A) and hypoxia exosomes (B). Both exosomes express CD63; however, cells in hypoxic condition release more exosomes and also express CD63 in higher amount than in normoxic condition.

4.1.2 Western Blot

We then investigated exosomes purified from differential centrifugation and sucrose gradient fractions with western blot using CD 81 marker. The result shows the strong exosome signals on hypoxia derived exosomes than the normoxia derived exosomes as shown in figure 4. This shows hypoxia increases the exosomes secretion by the cells.
Figure 4: Western blot analysis of tumor derived exosomes. A) Vesicles from differential centrifugation were tested with CD81 marker. The signal was seen in exosome fractions but no signals were seen in oncosomes and microvesicles fractions. B) Exosomes from hypoxic condition show strong signal when treated with CD81 marker. This signal is higher than the normoxia derived exosomes. C) Normoxia derived exosomes have signals when treated with CD81 marker. The signal shows less secretion of exosomes than hypoxic condition.

4.1.3 Nanoparticle tracking analysis (NTA)

The size and concentration of exosomes was measured using nanoparticle tracking device (Malvern Instruments, Malvern, UK). No significant difference in average values was seen between hypoxia and normoxia exosomes particle sizes. The size distribution in hypoxia sample is wide that shows the presence of particles with the size above 100 nm. The NTA result also shows the amount of exosomes is higher in hypoxia than the normoxia condition (fig. 5).
Figure 5: Exosomes analysis by NTA. A) Normoxia exosomes have particle distribution between 50-300nm with highest concentration at 77nm. B) Hypoxia exosomes have wide size distribution between 50-500nm. Higher amount is seen is 82 and 123 nm, with possibly some other bigger particles than exosomes between 300-500nm. C) Graph shows the concentration difference between hypoxia and normoxia exosomes. Exosomes in hypoxia conditions are released in higher amount than normoxia condition (p-value <0.05).
4.2 Effects of exosomes

4.2.1 Exosome labeling

We first wanted to study whether exosomes can be taken by kidney cells. Exosomes from hypoxia condition were labeled using far red CellVue Claret dye and images were taken in confocal microscope. The result shows exosomes moving towards the nucleus of the surrounding cells (fig. 6). This is the indication that the exosomes can work as a cargo and transfer genetic materials like DNA, RNA or proteins into the surrounding cells.

![Image of exosomes labeled with far red dye](image)

**Figure 6: Exosomes labeled with far red dye.** Unstained Renca YFP cells appear green and blue represents the nucleus of the cells. When these cells were treated with labeled exosomes (red), exosomes seem to move towards nucleus. This means cells can uptake exosomes regularly and transfer of nucleic acids via exosomes is possible.
4.2.2 Cell proliferation and motility assay

Next we studied exosomes function in vitro. Cell proliferation and motility assay was performed using IncuCyte zoom (Essen Bioscience). MM cells were grown in 96 well plates overnight, treated with hypoxia and normoxia derived exosomes and images were taken every 2 hours for the next 24 hours. The result shows that exosomes can enhance cell proliferation. The activity was found to be higher in hypoxia exosomes than normoxia exosomes when compared to control (figure 7; A).

The motility assay was done in order to observe the effect of exosomes in cell migration. The result shows that normoxia exosomes have higher activity but hypoxia exosome somehow did not show significant effect (figure 7; B).

Figure 7: Effect of exosomes in cell proliferation and cell motility. A) Hypoxia exosomes has higher activity in cell proliferation than normoxia. When compared to control, cell proliferation increases after cells were treated with exosomes. B) Normoxia exosomes increases the cell migration after treatment with the MM cells. The effect is quite high when compared to hypoxia exosomes.
4.2.3 Injection of chicken embryos

Chicken embryos were used as an *in vivo* model. Renca YFP cells were injected in chicken embryo. The intracardiac injection was done in embryos which were at early stage of development; day 3, ex ovo. The embryos were injected in two different conditions; some embryos were injected with renca YFP cells only while rest of the embryos were injected with mixture of renca YFP cells and exosomes derived from hypoxia conditions. The embryos were observed for another 7-10 days and the preliminary result showed that when renca YFP cells are treated with exosomes then they form bigger tumor colonies (fig. 8).

![Figure 8: Injection of chicken embryos with renca YFP cells and exosomes.](image)

A) Embryo was injected with renca YFP cells alone led to the formation of green colonies. B) Tumors grow after the renca YFP cells were treated with exosomes and injected in embryo. Exosomes seem to enhance tumor growth in the latter case.
4.3 Protein analysis by mass spectrometry

Exosomal proteins were studied after acetone precipitation method and analyzed by using liquid chromatography mass spectrometry (LCMS). The study was carried out on both hypoxia and normoxia derived exosomes and results were analyzed. The result shows that hypoxia induces more proteins secretion in compared to normoxia condition. Several different classes of proteins were observed in the mass spectrometry result in both hypoxia as well as normoxia derived exosomes. The mass spectrometry results are given in table II and III.

Table II: Exosomal proteins expressed under hypoxic condition. Higher number of proteins was detected in exosomes treated under hypoxia condition. Several of these proteins are expressed under extreme conditions and hence can serve as important biomarkers most of the time.

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Table III: Exosomal proteins expressed under normoxic condition. Fewer proteins were detected in exosomes under normoxia conditions. Some proteins are unique and cells express them under normal conditions, thus making them good candidate for important biomarkers.

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4.4 RNA analysis

The exosomes were analyzed for their miRNAs presence, since miRNAs are important contents with important functions. Both hypoxia and normoxia derived exosomes were studied and several miRNAs were found to be present with different expression levels. Most miRNAs were found to be expressed higher in hypoxia derived exosomes than normoxia exosomes (fig. 9).

![Figure 9: Selected miRNA in renca cell exosomes.](image)

miRNAs were expressed in both hypoxia and normoxia exosomes with different expression level. Almost all miRNAs were expressing in both conditions except miRNA 5112 which expresses only in normoxia condition but was absent in hypoxia condition.
5. DISCUSSIONS

Exosomes are extracellular vesicles secreted by almost all type of cells. They are enriched in biological molecules such as DNA, mRNA, miRNA, proteins and lipids. Hence, the cells use these vesicles as a means of communication and transfer biological information from parent cells to the target cells. Such properties make them important players in the field of diseases including cancer. In this study, tumor derived exosomes from renal carcinoma cells were purified, characterized and studied. The proteins and RNAs were analyzed, and role of exosomes in tumor formation was studied.

Currently, there is a lack of standardized purification methods for extracellular vesicles that can distinguish exosomes from other vesicles. Here, we tried to purify exosomes by two methods: sequential ultracentrifugation and OptiPrep™ density gradient ultracentrifugation methods (Van Deun et al. 2014). Western blot analysis with CD81 marker showed stronger signal in density gradient purification (fig. 4) than simple ultracentrifugation method (fig. 4 A). However, nanoparticle tracking analysis (NTA) confirmed the presence of vesicles larger than exosomes (>100 nm) (fig. 5). Thus getting 100% pure exosomes or any other vesicles with the existing purification methods is still a big challenge.

To study the effect of exosomes in cell proliferation and migration in recipient cells, MM cells were either treated with exosomes or with diluent. A significant increase in cell proliferation and migration was observed after the cells were treated with exosomes (fig. 7). Many studies support the role of exosomes in tissue repair and positive effects have been seen in cell migration and proliferation (Zhao et al. 2015). It is assumed that the ability of exosomes to exert such affect is because of the RNA and protein contents of the exosomes. These biological components are released into the cells by exosomes resulting in a promotion of cell migration and proliferation.

The in vitro tracking of the labeled exosomes showed that exosomes actually enter into the cells. The labeled exosomes were uptaken by the cells (fig. 6). This result supports the work of Ramesh et al. where they treated cells with exosomes of tumor origin and found the exosomes entering into the cell cytoplasm (Ramesh et al. 2015). Our result shows that cells uptake exosomes and exosomes release their active biological substances into the cells, promoting cellular activities such as proliferation, migration and even tumor formation.
The role of exosomes in intercellular communication has been studied widely. The ability of exosomes to transfer genetic materials like DNA, RNA and proteins has been found to be associated with tumor propagation (Minciachhi et al. 2015). As an in vivo experiment, the chicken embryos were injected with YFP labelled cancer cells either treated with exosomes or Phosphate Buffered Saline (PBS). Significant tumor colonies growth was observed when the embryos were injected with tumor derived exosomes (fig. 8). This provides evidence that exosomes are key player in the tumor propagation. This result supports the work done by Du et al. who investigated the effect of exosomes in renal carcinoma cells. The human Wharton’s jelly mesenchyme stem cells derived exosomes stimulated tumorigenesis as well as growth in the tumor size (Du et al. 2014).

Exosomes are rich in protein contents and in many cellular processes, exosomes can transfer these protein cargos to target cells thus affecting cellular environment. The transfer of these protein cargos via exosomes shows the potential role of exosomes in diseases including cancer (Boukouris & Mathivanan 2015). In this study, we analyzed exosomal proteins from tumor derived cells treated under hypoxia and normoxia conditions. Diverse range of proteins was found in exosomes derived from both conditions; however, more proteins were detected in hypoxia derived exosomes (table II) than in the normal condition (table III). The result is comparable with the previous findings as many of these proteins have been reported in ExoCarta protein database. However, many ribosomal proteins are also found in this study. This may be due to the presence of apoptotic bodies in significant amount. During purification, apoptotic bodies might have mixed with exosomes.

miRNAs refer to the small non-coding RNAs which play important roles in different biological processes. One of the aims of our study was to investigate the miRNAs present in Renca exosomes. Hypoxia and normoxia exosomes were analyzed and result showed the presence of different miRNAs (fig. 9). Many of these miRNA have been detected in exosomes previously and our result supports them. One of the miRNA we found in our study is let7b, which has been described as one of the first miRNAs discovered in mammals and involves in the regulation of differentiation and proliferation (Peter 2009). MiR-210 is expressed higher in hypoxia exosomes which is relevant as miR-210 is known to be associated with HIF molecules and hypoxia (McCormick et al. 2013). We found that miR-5112 is expressed in normoxia condition but is
completely unexpressed in hypoxia condition. The cells might loss expression of this miRNA under stressed conditions like hypoxia. This can act as RNA biomarker and can be used in diagnostic and prognostic process.
6. CONCLUSIONS

We investigated the role of exosomes in kidney in this study. Exosomes are released constantly by cells and can be extracted from different body fluids and cell culture supernatants. The lack of standard purification methods and suitable biomarkers that can separate exosomes from other extracellular vesicles is still a problem and challenge for the researchers in this field.

The in vitro study showed that exosomes have effects in kidney cells. Metanephric mesenchyme (MM) cells when treated with exosomes enhanced cell proliferation and motility activity when compared to control. Hypoxia increases the release of exosomes as compared to the normal condition.

The in vivo study of chicken model suggested the ability of exosomes to enhance tumor growth. This suggests the role of exosomes in intercellular communication. The exosomes can transfer their biological materials into the recipient cells and change microenvironment. This proves exosomes can enhance tumor growth.

The proteins and RNA analysis of exosomes show the presence of many important proteins and miRNAs. These exosomal proteins and miRNAs might have capacity to alter biological functions.

In summary, we were able to purify and characterize exosomes from kidney cancer cell line, show effect of exosomes in kidney both in vitro and in vivo.
7. REFERENCES


McCormick RI, Blick C, Ragoussis J, Schoedel J, Mole DR & Young AC (2013) miR-210 is a target of hypoxia-inducible factors 1 and 2 in renal caner, regulates ISCU and correlates with good prognosis. Br J Cancer 108; 1133-1142.


