# MICROSCOPIC AND FUNCTIONAL STUDIES ON EXTRACELLULAR VESICLES

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Kai Härkönen: Microscopic and functional studies on extracellular vesicles

Master’s thesis 49 pages

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**ABSTRACT:**

Importance of extracellular vesicles as multifunctional tools of intercellular communication has grown during last fifteen years. All cell types secrete these vesicles which conceal, among other things, RNA, DNA and proteins inside a bilipid layer. With the help of these vesicles, cells can send molecules to other cells, even in a distance. The bilipid layer prevents degradation of the contents of the vesicles. The role of extracellular vesicles in the development of cancer has been studied intensively and promising findings have been obtained. Metastasis and invasion of cancer cells require communication between cancer cells and their environment. Especially the use of extracellular vesicles as biomarkers for cancer seem to be possible.

In this study microscopic and functional studies were performed with extracellular vesicles isolated from cultured cells and from human body fluids. Human fibroblasts were used as target cells to study the effect of stable inducible EGFP-HAS3-MV3 melanoma cell -derived vesicles on their migration and invasion speed. Both of these properties are important in cancer metastasis. Secretion of extracellular vesicles were imaged with live cell confocal microscopy. Transmission electron microscopy (TEM) was used to image extracellular vesicles from different sources. Because of their small size, 30-500 nm, examination of extracellular vesicles requires high resolution from research equipment.

The results obtained in this study show that transmission electron microscopy and confocal microscopy are useful methods to study the ultrastructure, diameter and secretion mechanisms of extracellular vesicles as well as their effects on target cell behavior. Effects of extracellular vesicles isolated from melanoma-derived vesicles to the human fibroblast migration are quite low. However, there is an increase in invasion efficiency in those cells which were given melanoma-derived vesicles. After all further studies are needed.

**ABBREVIATIONS**

| | |
|---|---|
| ADP | Adenosine diphosphate |
| DMEM | Dulbecco’s modified eagle medium |
| ERK | Extracellular signal-regulated kinase |
| EMT | Epithelial mesenchymal transition |
| ESCRT | Endosomal sorting complex required for transport |
| EV | Extracellular vesicle |
| FBS | Fetal bovine serum |
| GTP | guanosine-5'-triphosphate |
| HA | Hyaluronic acid |
| HAS | Hyaluronan synthase |
| ILV | Intraluminal vesicle |
| MHC | Major histocompatibility complex |
| MLCK | Myosin light chain kinase |
| MVB | Multivesicular body |
| RAB | Ras related protein in brain |
| SNARE | Soluble NSF attachment protein receptor |
| TAT-5 | Transbilayer amphipathic transporter 5 |
| TEM | Transmission electron microscopy |
| TGN | Trans-golgi –network |
| TSG-101 | Tumor sysceptibility gene 101 |
| VAMP | Vesicle associated membrane protein |
| VSP-27 | Vacuolar protein sorting protein 27 |

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# 1 INTRODUCTION

## 1.1. EXTRACELLULAR VESICLES

### 1.1.1. Extracellular vesicles – Anything but dust

Extracellular vesicles are secreted by the most of cell types and exist all over in our body. They are small vesicles which are surrounded by phospholipid bilayer. There are three main types of extracellular vesicles: exosomes (Trams, et al. 1981, Johnstone, et al. 1987), microvesicles (Holme, et al. 1994) and apoptotic bodies (Kerr, et al. 1972). In this thesis the focus is on exosomes and microvesicles. Exosomes are secreted from cells through the lysosomal machinery. In turn microvesicles are formed by direct budding from the cell surface.

In literature cell-derived vesicles have been suggested to have multiple functions. These functions are for example regulation of angiogenesis (Skog, et al. 2008a), cell signaling, inflammation and immune response (Bhatnagar, et al. 2007), maintaining homeostasis, cell waste management (Sims, et al. 1988, de Gassart, et al. 2003a) and blood coagulation (Chargaff and West. 1946).

Extracellular vesicles were found for the first time with transmission electron microscopy in Peter Wolf’s blood coagulation study in 1967. They found out that small vesicles were detached from cells and called these vesicles as “platelet dust”. These vesicles were thought to be just cell waste. (Wolf. 1967)

Cell-derived vesicles have been found almost all-over in our body after their discovery. Vesicles have been found from semen (Ronquist and Brody. 1985), ascites fluid (Andre, et al. 2002), bronchoalveolar lavage (Admyre, et al. 2003), urine (Pisitkun, et al. 2004), blood (Caby, et al. 2005), synovial fluid (Skriner, et al. 2006), breast milk (Admyre, et al. 2007), amniotic fluid (Keller, et al. 2007), saliva (Ogawa, et al. 2008), cerebrospinal fluid (Harrington, et al. 2009), nasal secretions (Lasser, et al. 2011) and bile (Masyuk, et al. 2010).

### 1.1.2. Nomenclature and classification of extracellular vesicles

Size of extracellular vesicles varies from 30 nm to 2000 nm. Vesicles can be classified in three classes based on their biogenesis i.e. exosomes (Trams, et al. 1981, Johnstone, et al. 1987), microvesicles (Holme, et al. 1994) and apoptotic bodies (Kerr, et al. 1972). Exosomes are smallest

type of vesicles and their size varies between 30 and 120 nm. In turn, size of microvesicles is between 50 and 1000 nm. Apoptotic bodies are largest and their diameter is 500-2000 nm (El Andaloussi, et al. 2013).

In addition to biogenesis, vesicles can also be classified by their origin and biological function. Thus, EVs are named as argosomes (Greco, et al. 2001), tolerosomes (Karlsson, et al. 2001), prostasomes (Brody, et al. 1983), ectosomes (Stein and Luzio. 1991), deteriosomes (Yao, et al. 1993), microparticles (Mackman. 2009), sebosomes (Nagai, et al. 2005), cardiosomes (Waldenstrom, et al. 2012) and oncosomes (Morello, et al. 2013). Abundance of possible names for extracellular vesicles is a reason why a debate for standardizing the nomenclature is currently ongoing. Approved nomenclature will make both cooperation and reading of other groups' papers easier.

### 1.1.3. Biogenesis of exosomes

Exosomes originate via endosomal pathway (**Figure 1**). When an early endosome matures to a late endosome by acidification, intraluminal vesicles (ILVs) form by reverse budding from cytoplasm into the lumen of the endosome. While ILVs bud to late endosome, mRNA, microRNA, DNA, proteins and lipids are packed inside these vesicles. After budding of intraluminal vesicles the late endosome is called as a multivesicular body (MVB) (Johnstone, et al. 1987). Multivesicular body may fuse with a lysosome when it and its cargo will be degradated. Another scenario is that the multivesicular body fuses with the plasma membrane (Futter, et al. 1996). In this case the intraluminal vesicles are released to the extracellular space and after that they are called exosomes (Trams, et al. 1981, Harding, et al. 1983). It is also possible that MVBs act as a storage for some important molecules as in the case of MHC class II in dendritic cells (Kleijmeer, et al. 2001).

What is then a molecular machinery behind sorting of cargo to intraluminal vesicles/exosomes? There is not currently a certain answer to that question, but several possible pathways are described in multiple publications. Sorting of cargo in MVB takes place in two steps. In the first step of the process, specific proteins at the outer membrane of the late endosome are gathered together. In the second step an intraluminal vesicle buds inside the endosome. Best quess is probably a pathway that is regulated by ESCRT machinery (endosomal sorting complexes required for transport) (Katzmann, et al. 2001) . This is a reason why common classification of MVB-formation routes is to divide them to ESCRT-dependent and ESCRT-independent routes.

One observation is that monoubiquitinylation leads to packing of molecules to the multivesicular bodies. However all the cargo of multivesicular bodies are not ubiquitinylated which tells that ubiquitinylation is not necessary and there might be other mechanisms. (Buschow, et al. 2005) Also it has to be remembered that polyubiquitinylation is a signal that leads to degradation of its targets.

With the help of VSP-27, ESCRT -0, -I and –II can identify ubiquitinylated molecules. VSP-27 also provides TSG-101 to help. TSG-101, in turn, recruits AIP/Alix (ALG-2-interacting protein 4), which is responsible for membrane budding. Also VTA1 (vacuolar protein sorting-associated protein 1) is thought to be needed on ESCRT –dependent route. ESCRT complexes are also needed for leading membranes for lysosomes to degradation. (von Schwedler, et al. 2003) Ability of ESCRT-II to bind mRNA advocates the possibility of participitation of ESCRT machinery in sorting of exosome cargo (Irion and St Johnston. 2007).

In the light of current knowledge it is assumed that also ESCRT independent pathway exists. In this pathway a lipid called ceramide is in the biggest role. Ceramide may initiate the secretion of exosomes. Biosynthesis of ceramide is regulated by neutral sphingomyelinase 2 (nSMase2). (Trajkovic, et al. 2008, Kosaka, et al. 2010)

Also in some studies clustering of exosomal cargo seems to play important role in exosome loading. It is called as a luminal domain dependent pathway, a passive mechanism, which is independent from ESCRT, ubiquitination and Hrs. Enrichment of tetraspanins and cholesterol are linked to protein sorting to intraluminal vesicles. (de Gassart, et al. 2003b, Theos, et al. 2006)

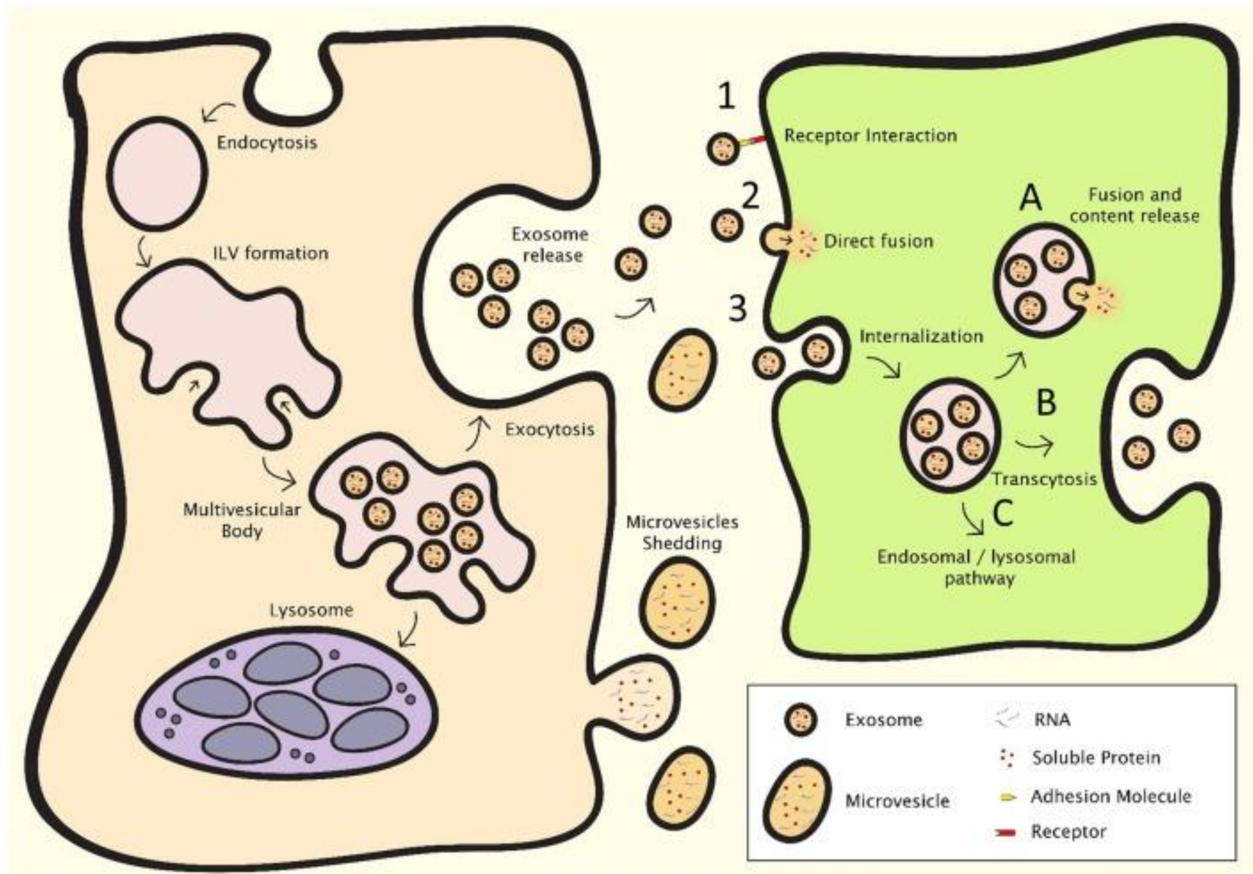


**Figure 1**. Exosomes originate via endosomal pathway. While an early endosome matures to the late endosome, intraluminal vesicles (ILV) bud into the endosome. After budding of intraluminal vesicles, the endosome is called as a multivesicular body (MVB). MVB may end up to lysosomal degradation or fuse with the plasma membrane. In the later phase intraluminal vesicles are released to extracellular space and called as exosomes.

### 1.1.4. Secretion of exosomes

As in the biogenesis of exosomes also molecular mechanisms behind secretion of exosomes remain still unclear but it is ravelling is in the process. It is known that secretion of exosomes is controlled by many factors. Multiple members of RAB (Ras-related protein in brain) – protein family have been found out to be part of the machinery of the exosome secretion. The RAB family members are small GTPase proteins. The role of these small GTPase proteins in exosome secretion is the movement of multivesicular body to the plasma membrane. (Stenmark. 2009)

When the multivesicular body and the plasma membrane are in touch, soluble NSF attachment protein receptor complexes (SNAREs) take over the fusion of MVB with plasma membrane

(Zylbersztejn and Galli. 2011). For fusion at least SNAP-23 (Castle, et al. 2002), VAMP-7 (Hirashima. 2000) and VAMP 8 are needed (Luzio, et al. 2005). Fusion process is also $Ca^{2+}$ regulated in the case of secretory lysosomes. All the MVB secretion is not, however, regulated by SNAREs.

### 1.1.5. Biogenesis of microvesicles

Another type of extracellular vesicles are microvesicles. Microvesicles form via direct budding from cell membrane (Figure 2). Microvesicles are also called as ectosomes (Stein and Luzio. 1991) and microparticles (Mackman. 2009) in literature. Recognition between exosomes and microvesicles is difficult because size ranges of these two type of vesicles partially overlap. In many cases their complete separation is just impossible.

Microvesicles are associated with the cell apoptosis. Budding of microvesicles is a multistep process which starts with certain stimulus like cell stress. This leads to increase of calcium concentration in cytosol followed by enzymatic activity of calpains, gelsolins, scramblases and kinases. The activation of above-mentioned enzymes causes inhibition of translocases and phosphatases. This finally enables formation of microvesicle via alterations in cytoskeleton. The fact that platelets are able to produce microvesicles after inhibition of calpain shows that also other pathways exist for microvesicle formation. (Wiedmer and Sims. 1991, VanWijk, et al. 2003)

Structure of the plasma membrane is closely linked to biogenesis of microvesicles Aminophosphotranslocases regulate the structure of the cell membrane. Function of aminophospotrans-locases is to transfer phospholipids from one side to another of the cell membrane which consists of bilipid layer. Transfer of phosphatidylserine to the outer leaflet of cell membrane promotes budding of microvesicles (Hugel, et al. 2005)

Another factor that affects the structure of the plasma membrane and consequently to the microvesicle formation is distribution of phosphatidylenolamine between inner and outer leaflet of the plasma membrane. It has been found out that the phosphatidylenolamine –translocase (TAT-5) regulates secretion of extracellular vesicles in the study performed using *Caenorhabtidis elegans*. In normal state phosphatidylenolamine is unevenly distributed between outer and inner leaflet of plasma membrane so that only few phosphatidylenolamine exists at outer leaflet. In TAT-5 mutants phosphatidylenolamine is transferred to the outer leaflet which enables attachment of endosomal

sorting complexes to the inner leaflet of the plasma membrane. This makes microvesicle formation possible. (Tuck. 2011)

Contraction of cytoskeleton, which consists of actin and myosin, is the final step that is needed to the microvesicle budding. This is due to that ARF6-GTP (ADP ribosylation factor 6 that binds GTP) activates phospholipase D. After that ERK (extracellular signal-regulated kinase) attaches to plasma membrane. ERK phosphorylates MLCK (Myosin light chain kinase). Phosphorylation activates MLCK, which leads to phosphorylation of the light chain of myosin. (Muralidharan-Chari, et al. 2009)

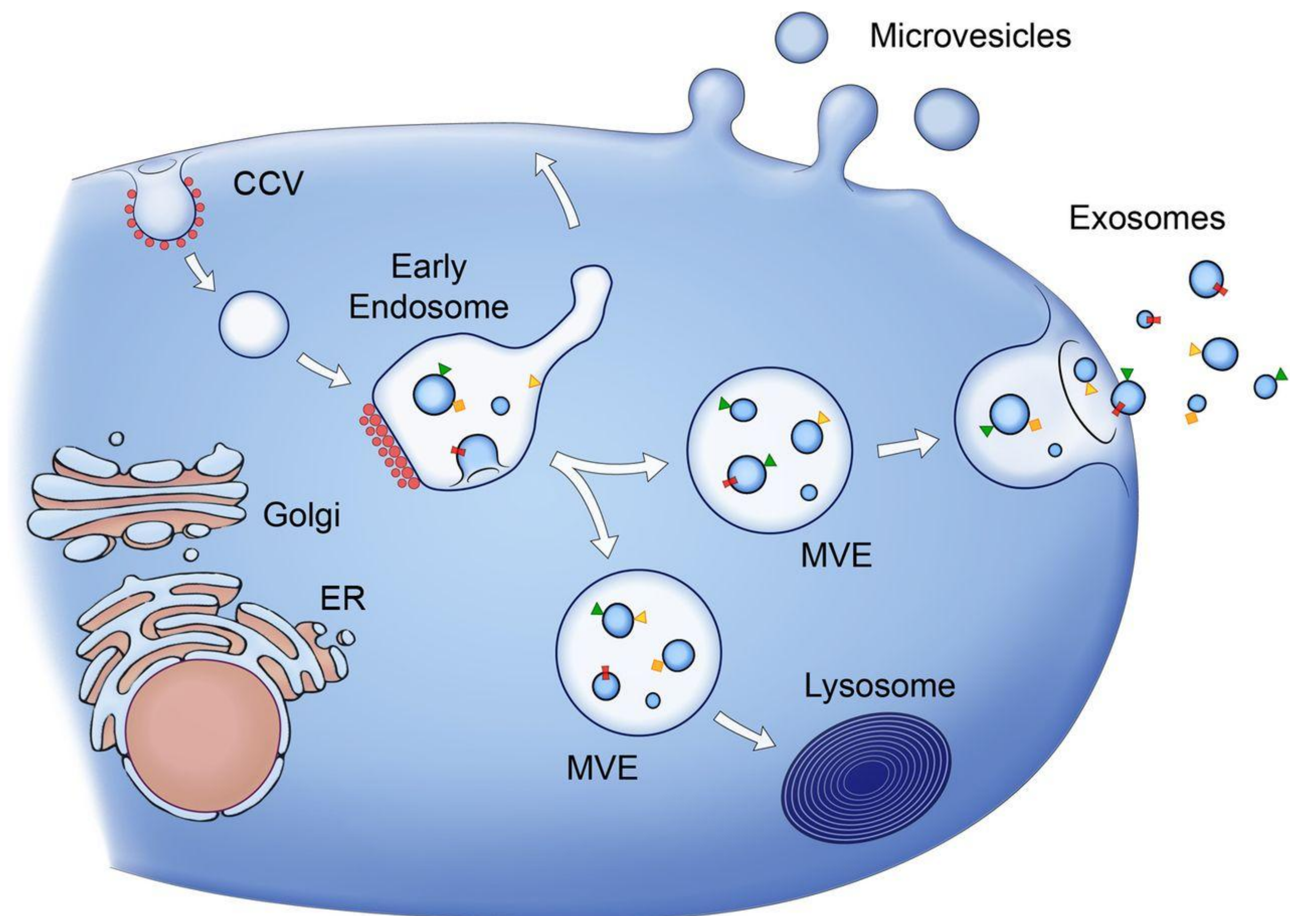


**Figure 2**. Microvesicles are 50 – 1000 nm -sized plasma membrane bubbles which are formed via direct budding from the cell surface.

## 1.2. ROLE OF EXTRACELLULAR VESICLES IN HEALTH AND DISEASE

### 1.2.1. Extracellular vesicles – multitools of cells

Both exosomes and microvesicles have been thought to be part of intercellular communication because they transfer biomolecules between cells. Extracellular vesicles are necessary for the functions of multicellular species. They play a big role in intercellular communication by sending messages to other cells in the shape of transmembrane receptors, mRNAs, miRNAs, proteins and signaling molecules which are carried to target cells wrapped in tiny cell membrane envelopes. This communication is a way of manipulation the extracellular environment.

Recent study points out interestingly that exosomes and microvesicles differ in their ability to transfer information between cells. The study which was performed using transiently transfected cells showed that reported proteins and mRNA were successfully sealed in both types of vesicles but only microvesicles managed to convey reporter function to target cells.(Kanada, et al. 2015) It may still be too early to make too radical conclusion about different abilities of these two types of extracellular vesicles.

Another study shows that exosomes derived from breast cancer carcinoma cell cultures are able to increase cell movement. This study compared the effects of three breast cancer cell lines with different metastatic potential and showed that increase of the cell movement correlated with the metastatic potential of donor cells. Exosomes have been found to have unique protein signatures depending from which cell they originate.(Harris, et al. 2015)

### 1.2.2. Potential of extracellular vesicles as biomarkers

Liquid biopsy is currently being developed alongside traditional biopsy which means taking of piece of tissue from patient. Liquid biopsy, in turn, is much easier to obtain from the patient because it can be taken from body fluids, especially, if tumor exists deep in the body. In short, the principle of liquid biopsy is that biomarkers are observed from the sample. Extracellular vesicles, which are present in every bodily fluid and secreted from every cell type are very potential biomarkers in liquid biopsies. This kind of liquid biopsy could be taken from blood or urine so the invasiveness of sampling is very low if it exists at all. Real-time monitoring is also possible by liquid biopsies.

Utilization of extracellular vesicles as biomarkers for screening of several diseases have been studied for years. Exosomes and microvesicles have same features as their parent cells such as same surface receptors. RNA and protein content of EVs is also dependent on the original cell type. Possibilities of using extracellular vesicles as diagnostic tools are great. Screening of abundance and content of extracellular vesicles may bring out useful information about diseases which are not yet observed. They can also be used as tools for monitoring progression of diseases which have been already diagnosed.

### 1.2.3. Using of extracellular vesicles as biomarkers for cancer

There is more and more information about importance of interaction between cancer cells and their environment to development of tumors. Cancer cells manipulate surrounding cell types to enable penetration and growth. Extracellular vesicles have been found to play a key role in this interaction. Especially exosomes act as messengers which carry biomolecules between cells. Tumor-derived exosomes has been found out to interact with tumor environment for example by promoting angiogenesis, stimulating the cell movement and evading immune system. (Clayton, et al. 2007, Skog, et al. 2008b, Harris, et al. 2015)

It can be easily thought that these extracellular vesicles transport messages only to cells nearby. Extracellular vesicles carry out also long-distance communication. (Kadiu, et al. 2012) These tiny shuttles have often been observed to contain tumor antigens indicating that they are originated from the tumor. Nowadays, it is generally known that extracellular vesicles can transfer mRNA and proteins in functional form between cells, which advocates their importance to tumor development. Protein p53 is an important regulator of cell cycle, closely associated to cancer development and has been found out to regulate amount of TSAP6 which increases secretion of exosomes. (Yu, et al. 2006)

In light of the considerations set out above it is not surprising that the use of extracellular vesicles as biomarkers for cancer is studied extensively. Various approaches to utilize extracellular vesicles, especially exosomes, as biomarkers have been carried out and promising results have been obtained. Until now possibly applicable biomarkers have been detected related to several common cancers as lung cancer, prostate cancer, breast cancer and ovarian cancer. (Rabinowits, et al. 2009, Li, et al. 2009, Mitchell, et al. 2009, Le, et al. 2014)

In 2008, Taylor et al. measured amount of eight different microRNAs and pointed out that exosomal microRNA can be used to recognize ovarian cancer. (Taylor and Gercel-Taylor. 2008) Next year Li et al. found out that those exosomes in peripheral circulation which contain claudin have a connection to ovarian cancer (Li, et al. 2009).

A few markers have been found from extracellular vesicles linked to prostate cancer. In 2009 Prostate-Specific Antigen (PSA) and Prostate-Specific Membrane Antigen (PSMA) were noticed to act as biomarkers for prostate cancer. (Mitchell, et al. 2009). Also increased levels of a protein called survivin in plasma exosomes were linked to prostate cancer (Khan, et al. 2012)

Additionally, a recent study (Melo, et al. 2015) suggests that glypican-1, a proteoglycan anchored to the membranes of circulating extracellular vesicles, could be utilized as a specific marker for pancreatic cancer.

Levels of exosomal RNA in blood may act as an indicator in screening of lung cancer. Remarkable differences between lung cancer patients and control group have been measured. Also similarity between tumor miRNA and exosomal RNA in blood samples in lung cancer patients were observed. (Rabinowits, et al. 2009)

Levels of microRNA 200 family members are increased in metastatic breast cancer cells. Metastatic potential also may be transferred from metastatic cells to non-metastatic cells via microRNA-200 – containing extracellular vesicles. (Le, et al. 2014)

## 1.3. HYALURONAN

First time hyaluronic acid was found in 1934 when Karl Meyer observed vitreous of bovine eye (Meyer, et al. 1934). This high molecular weight polysaccharide has since been found to exist all over our body in connective tissues. A high capacity to retain water is one of it's specific features. (Kogan, et al. 2007) In 1986 hyaluronic acid was given a name hyaluronan. This new name followed standardization of polysaccharide nomenclature. (Balazs, et al. 1986)

### 1.3.1. Structure and synthesis

In a nutshell, hyaluronan is a mucopolysaccharide which is synthesized by hyaluronan synthases and degraded by hyaluronidases. Hyaluronan is a linear glycosaminoglycan which consists of

disaccharides D-glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc) linked together by alternating β-1,4 and β-1,3 glycosidic bonds. Unlike other glycosaminoglycans, HA does not go through other chemical modification like sulfation or acetylation. (Weissmann and Meyer. ) Synthesis of hyaluronan takes place at plasma membrane. HA is synthesised by three hyaluronan synthases, HAS1-3, in mammals.(Weigel and DeAngelis. 2007)

### 1.3.2. General role of hyaluronan

Otherwise than originally thought hyaluronan is not only the space-filler althought it is the main component of extracellular matrix. Several other roles has been associated with hyaluronan molecule. Maintaining the homeostasis is important role of hyaluronan. It has been found out to be key player in wound healing (Aya and Stern. 2014). Hyaluronan has also multiple ways to regulate inflammation, which is important part in cancer development. It has been also linked to morphogenetic processes which means that HA regulates development. HA has an effect in angiogenesis, skeleton development, chondrogenesis and cell proliferation.(Tammi, et al. 2002) Hyaluronan should not be thought only unambiguous molecule. The molecular weight of hyaluronan seem to be important factor in regulating the effects of hyaluronan.(Cowman, et al. 2015)

### 1.3.3. Hyaluronan, extracellular vesicles and cancer

According to a recent finding, hyaluronan is associated with enhanced microvesicle production in cell cultures. (Rilla, et al. 2013)This suggests that plasma membrane shedding of the vesicles carrying hyaluronan on their surface could enable the horizontal transfer of hyaluronan from tumor cells into the surrounding stroma and enhance tumor-stroma interactions. Additionally, the HA-coated EVs may offer both a potential therapeutic target and a diagnostic tool in cancers and other disease states with excess hyaluronan secretion.(Rilla, et al. 2014) Therefore, it is important to solve the mechanisms that regulate their shedding, to develop novel methods for their identification and clarify their functional effects on target cells. One of the main objectives of this study is to find answers to these questions.

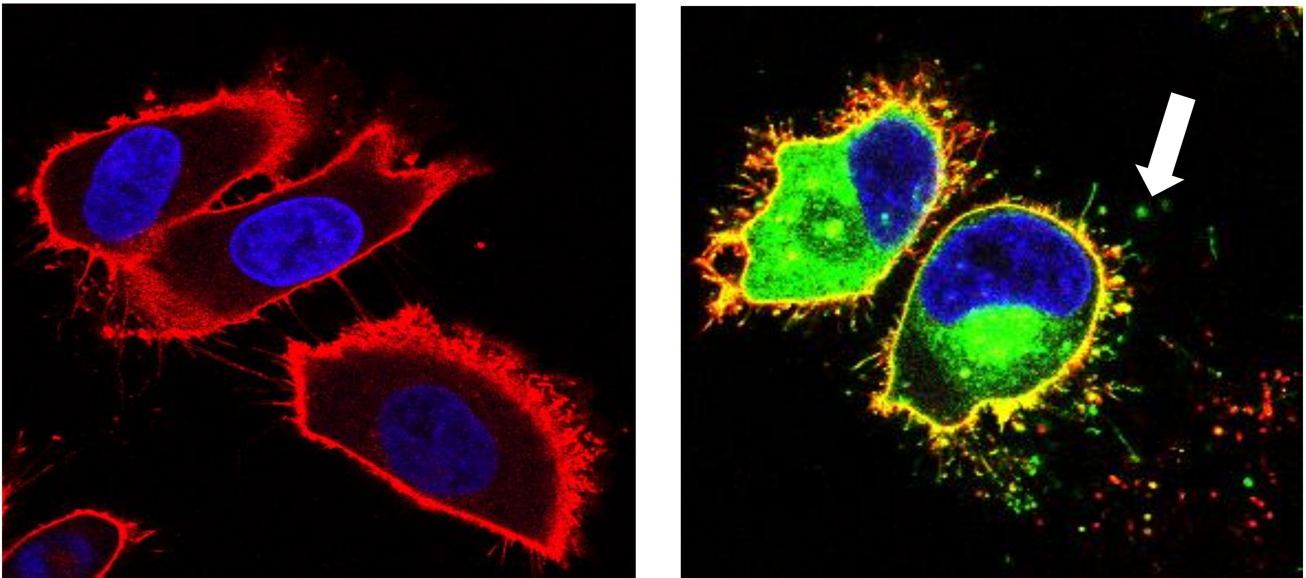

**Figure 3. Human melanoma cells (MV3) with inducible expression of GFP-HAS3.** The left panel shows a group of cells without induction and right panel cells after induction of GFP-HAS3 expression. Note the increased number of EVs in HAS3-overexpressing cells (arrow). Green color shows GFP-HAS signal, red color CD44 immunostaining and blue color indicated nuclei (unpublished data by Kirsi Rilla).

## 1.4. ISOLATION OF EXTRACELLULAR VESICLES

### 1.4.1. Challenges in vesicle isolation

Currently there is not only one proper protocol to isolate extracellular vesicles. One big reason for this is that EVs can be isolated from multiple different sample sources. Also amount of available sample material and other follow-up measurements affect the choice of the protocol.

When purification and isolation procedures are planned for extracellular vesicles, it's important to remember that there is not only one uniform type of vesicles. Each type of extracellular vesicles needs suitable isolation procedure. It may lead to large errors if this is not taken into account.

Once again problems in isolating extracellular vesicles is caused by their small size. List of commonly used isolation methods includes ultracentrifugation with/without a sucrose gradient, ultrafiltration, size exclusion chromatography, affinity capture of magnetic/non-magnetic beads and polymer based precipitation.

In a recent study performed by Jae-Jun Ban et al. attention has been paid to impact of pH to yield of exosome isolation. Interest underlying in the background for this study were the effect of changes of pH in some diseases as cancer and Creutzfeldt-Jakob disease. In cancer low pH has been closely linked in the metastasis and progression of cancer. Exosomes have also been found to have a hand in Creutzfeldt-Jakob disease progression. Differences in exosomal protein and RNA yields turned out to be remarkably high. Levels of total exosomal protein and RNA were measured in medium with three different pHs which were 4, 7 and 11. Acidic medium gave the best result. The second best result was obtained with neutral conditions and the lowest yield, in turn, was due to alkaline pH. In the light of the results is not in vain to draw attention to pH in exosome studies from now. (Ban, et al. 2015)

As mentioned before, the isolation method of extracellular vesicles depends on the type of vesicles to be isolated. A sample type is another major issue that should be noted. Each sample type has its own specific requirements to achieve the desired outcome. Sample types can be roughly divided into two major classes: Extracellular vesicles isolated form cell culture media and extracellular vesicles isolated from body fluids. Both of these fluid types have their own possible sources of error.

When extracellular vesicles are isolated from cell cultures it should be kept in mind that supplements which are added to the cell cultures may function as artificial EV sources. Especially fetal bovine serum (FBS) contains vesicles that may cause incorrect observations. Filtering and ultracentrifugation for 18 h at 100 000 x g or greater are used to remove these vesicles. Shorter ultracentrifugation step won't remove all the vesicles.(Shelke, et al. 2014) In addition to used supplements one source of error is culture media itself. Even fresh culture media includes particles of the same size range as the extracellular vesicles. There is different amounts of these particles in media from different manufacturers. Number of these particles alters during storage and those media which are stored in 4 ˚C have less background than those which are stored in room temperature. The cell line used also influences the choice of best protocol. (Jeppesen, et al. 2014)

When extracellular vesicles are isolated from body fluids the complexity of samples is a challenge. Body fluids contain lipoproteins, DNA, RNA, protein aggregates, microbes and platelets which may cause problems in isolation. (Szatanek, et al. 2015)

Platelet-derived extracellular vesicles are the only group of EVs that has accurately determined instructions for storage and isolation. The fresher the better may be a consensus for getting the best

yield of extracellular vesicles. Storing temperature and number of freezing and thawing cycles are critical factores affecting the quality of the samples.

### 1.4.2. Ultracentrifugation

Ultracentrifugation is the most common method to isolate extracellular vesicles. In ultracentrifugation particles are separated based on their size and density so that larger and more dense particles travel farther from a central axis.(Beams, et al. 1933).

Nowadays the most widely used method for extracellular vesicle isolation is differential/ ultracentrifugation. (Witwer, et al. 2013) The main guidelines for this method has been introduced in study performed by Raposo et al. but there is variations depending on different laboratories and studied cell lines. In the study of Raposo et al. exosomes were purified from the conditioned media of transformed human b cell lines. Although used centrifugation speed depends on studied extracellular vesicles and cell lines, mainly the following speeds are used: 300 x g to remove cells, 2000 x g to remove dead cells and apoptotic bodies, 10 000 – 20 000 x g to isolate microvesicles and remove cell debris, 100 000 – 200 000 x g to isolate exosomes. (Raposo, et al. 1996) When microvesicles are wanted to be isolated 10 000 x g -20 000 x g is enough. Too high centrifugal forces will lead to vesicle degradation and fusion. (van der Pol, et al. 2012) Between each centrifugation step supernatant is collected to a new test tube. It is important to validate the centrifugation process according to which size of particles are wanted to be isolated. This will maximize the purity and yield. Common in used procedures is that centrifugation steps are performed in 4˚C except plasma samples. If plasma samples are handled in 4˚C, plateled EVs are formed which are interfering further processing.(Szatanek, et al. 2015)

There is a variation of differential/ultracentrifugation step which uses additional sucrose gradient/cushion step. Reason for using this step is to prevent possibly non-specific proteins to end up to EV samples. In this step the sample is loaded into tube containing Tris/sucrose/$D_2O$ and centrifuged at least 75 minutes at 75 000 x g followed by collection of fraction which density is 1.13 - 1.19 g/ml. This fraction is centrifuged again at 100 000 x g after dilution with PBS. (Escola, et al. 1998, Thery, et al. 2006)

There is multiple problems which should be noted when using ultracentrifugation. Cargo of vesicles affects their density so that it is not possible to make strict size separation. Ultracentrifugation is also time consuming because it is quite slow when the number of samples is

high. Need for high sample volumes causes that it is not able to use with all clinical samples and other samples with small volume. Some vesicles may break down when they are put to ultracentrifuge with high velocity. Also many contaminants may be pelleted with ultracentrifuge. (Peterson, et al. 2015)

### 1.4.3. Size exclusion chromatography

In size exclusion chromatography particles will be separated depending on their size. Particles with different sizes have different abilities to go through stationary phase of size exclusion column i.e. elution speed is different. (Boing, et al. 2014) Normally low-speed centrifugation and filtration is done to samples before chromatography. (Muller, et al. 2014) Centrifugation removes large particles and filtration concentrates the sample. As the other methods also size exclusion chromatography has its own disadvantages. Too high force in filtering EVs may make structural changes to vesicles. Also selection of proper chromatography column should be carried out carefully. (Witwer, et al. 2013)

### 1.4.4. Ultrafiltration (UF)

In ultrafiltration the sample is filtered through a semipermeable membrane. This method is often combined with other isolation methods like ultracentrifugation and size exclusion chromatography (Lamparski, et al. 2002, Nordin, et al. 2015). It can be also used without combination with other methods, resulting in higher purity of the samples as compared to other methods. Main problems associated with ultrafiltration techniques are clogging and shear stress which may lead to unwanted changes in the properties like morphology of the extracellular vesicles.

### 1.4.5. Immuno-affinity purification

Immunoaffinity isolation is a technique which utilizes the information about proteins on the surface of extracellular vesicles. The fact that extracellular vesicles resemble their parent cells is very useful for this technique. By selecting the correct antibodies the desired vesicles can be distinquished from other material. Depending on the approach immunoaffinity isolation can be used to remove unwanted vesicles from the sample or to enrich wanted vesicles. Advantage of immunoaffinity

isolation is that in this method the isolation of extracellular vesicles is based on marker expression on their surface rather than their diameter.

Used antibodies are attached for example to matrix or magnetic/non-magnetic microbeads. These microbeads are coated with specific antibody which is planned to attach to selected markers on the surface of EVs. (Tauro, et al. 2012) Another type of microbeads are surfactant free latex beads, usually made of polystyrene.(Fitzner, et al. 2011)

One method that is based on immunoaffinity uses synthetic peptide called venceremin to isolate extracellular vesicles from cell cultures and biologic fluids. Venceremin has a specific affinity to heat-shock proteins which have been associated to extracellular vesicles. This method needs a pre-purification step to remove cell debris and it is usually performed by centrifugation or filtering with 0,22 µm filter. (Ghosh, et al. 2014).

### 1.4.6. Acoustic purification

One of the newest innovations is an acoustic nanofiltration system. Acoustic purification of extracellular vesicles uses ultrasound waves to separate particles with different sizes from other components of biological sample. Size of wanted particles can be chosen electrically by adjusting acoustic power and flow speed of sample. Mechanical properties like size, density and compressibility of vesicles are the factors that affect how much force they experience. (Lee, et al. 2015)

Advantages of acoustic purification compared to older techniques are that it uses smaller sample volumes and it’s also inexpensive and faster. Either this technique doesn’t require labeling of samples. Also it may have a smaller sample loss than for example ultracentrifugation or ultrafiltration. (Lee, et al. 2015)

### 1.4.7. Microfluidic isolation

Compared to developing microfluidic isolation, traditional techniques like ultracentrifugation are less accurate resulting in weaker purity. It could be said that these older methods are only directed to concentration of extracellular vesicles but not for isolating them. Purity of EV samples is important especially when samples are intended to be used in biomarker studies. Higher purity may

increase the accuracy of testing of diseases. In addition that ultracentrifugation is not so accurate it is also time consuming. Ultracentrifugation takes time from 2- 10 hours. Using of magnetic beads with antibody coating purity is higher compared to ultracentrifugation. Also yield of extracellular vesicles is higher. One problem with magnetic beads is that they don't work to other vesicles than those which contain the protein of interest which leads to lack of detection of other potentially present vesicles. This naturally distort the results.(Liga, et al. 2015)

### 1.4.8. Hydrostatic dialysis

Another new technique, which utilizes hydrostatic dialysis, is developed to isolate extracellular vesicles from urine. Urine samples are centrifuged first at 2000 x g to remove impurities as bacteria and cell debris. After that liquid sample is filtered with 1000 kDa dialysis membrane using hydrostatic pressure. Advantages of this method are that smaller space is required to storage samples and ultracentrifugation is not needed. (Musante, et al. 2014)

### 1.4.9. Commercial isolation kits

Nowadays there are also commercially available exosome isolation kits like Exoquick™ (System Biosciences), Exo-spin (Cell Quidance Systems), Total Exosome Isolation™ (Life Technologies) which are relatively easy and inexpensive to use. Common to these kits are that high-velocity ultracentrifugation is not needed but such as traditional ultracentrifugation, use of these kits is also time consuming because in majority over-night incubation is needed. Disadvantage of commercial kits occurs also in purity of samples which remains below the level of for example OptoPrep density gradient centrifugation.

## 1.5. DETECTION METHODS OF MICROPARTICLES

### 1.5.1. Flow cytometry

Flow cytometry is the most common method for analysis of extracellular vesicles in blood in clinical use. Small size of extracellular vesicles causes problems with accuracy of flow cytometry.

Size of extracellular vesicles compared to human cells varies from 1/200$^{th}$ to 1/10$^{th}$ part. (Inglis, et al. 2015) Lower limit of detection range in most used flow cytometers is 500 nm. (Minciacchi, et al. 2015)

One problem of this method is a difficult detection of false positive events. Main reasons for this are that extracellular vesicles express a relatively low number of antigens, which makes the detection difficult and the background noise may be high because of challenges in washing steps after staining. Some optimization techniques for use of flow cytometry in identification of extracellular vesicles exists. (Inglis, et al. 2015) Study which was performed in 2012 pointed out that nano-sized particles can be quantified precisely. (van der Vlist, et al. 2012) Using of antibody-coated beads that are bigger than exosomes enables counting of small extracellular vesicles. (Lasser, et al. 2012)

### 1.5.2. Dynamic light scattering

Dynamic light scattering, also called photon correlation spectroscopy and quasi elastic light scattering is a technique which utilizes brownian motion to measure the size distribution of particles. Measured particles can be from under 1 nm to 10 µm in diameter.

### 1.5.3. Nanoparticle tracking analysis (NTA)

Nanoparticle tracking analysis is a frequently used method to study extracellular vesicles. In this technique scattered light from the measured particles is captured and computer software calculates absolute size distribution using brownian motion. Mean squared velocity of vesicles brownian motion is determined by tracking and it is used to estimate the diameter of the particle. Size range of detected particles ranges from 50 nm to 1µm in diameter. Though quantitative analysis can't be done to particles larger than 400 nm. (Filipe, et al. 2010)

### 1.5.4. (Tunable) Resistive pulse sensing

In this technique the size distribution of particles in liquid sample is measured by detecting resistance changes when particles go through a small pore. Compared to dynamic light scattering and nanoparticle tracking analysis this technique is more accurate because it gives absolute size distribution. It is so called particle by particle analysis. Size of detectable particles can be from 50

nm to 10 µm. Upper limit is cause of pore size and lower limit is the smallest resistance limit that can be detected. This smallest detectable particle size is about 20% of pore size. One quite high problem with this technique is that particles may clog the pore especially when sample is unknown and if particles clot easily. So careful sample preparation like elimination of big objects by filtering is needed. (Anderson, et al. 2015)

### 1.5.5. Molecular composition

Molecular composition of extracellular vesicles is a widely used method to study the properties of extracellular vesicles. Methods for revealing molecular composition are for example western blotting, peptide mass mapping or immuno-EM.

Different extracellular vesicles can be identified in the basis of their molecular composition. For example exosomes have many lipids and proteins typical specifically for exosomes. At least GTPases, Annexins, flotillins, several tetraspanins (CD9, CD62, CD 63, CD81 and CD82), HSC 70, HSC 90, ALIX, TSG 101, lipid-related proteins, phospholipases and many other markers can be used to detection of exosomes. (Simpson, et al. 2008, Conde-Vancells, et al. 2008, Vlassov, et al. 2012)

## 1.6. MICROSCOPIC METHODS TO STUDY EVS/ IMAGING OF EVS

Because extracellular vesicles are nano-sized particles, their visualization requires the resolution of e.g. electron microscopy or atomic force microscopy. However the largest EVs can be also imaged with confocal microscopy.

### 1.6.1. Confocal microscopy

Confocal microscopy is a specialized technique of light microscopy and it has been used to visualize extracellular vesicles for years. Use of confocal microscopy requires staining of the sample with fluorescent markers. It should be remembered that the properties of visible light sets limitations to use of confocal microscopy in extracellular vesicle research. Wavelenght of visible light, properties of sample and restrictions of microscope optics result in the maximum resolution of about 200 nm in confocal microscopy. Resolution means the minimum distance by which two

objects can be detected from each other. (Lodish, et al. 2008) Size of exosomes varies between 40-120 nm. It is therefore natural that the precise structural information about the extracellular vesicles can not be obtained with light microscopy. Using confocal microscopy is however possible to get images of movements of cell surface which is useful when the moment of vesicle budding is wanted to be studied.

### 1.6.2. Transmission electron microscopy

Transmission electron microscopy (TEM) allows better resolution than confocal microscopy. It is important because of the small size of extracellular vesicles. In transmission electron microscopy, electrons are accelelerated with high voltage and electron beam is then shoot through the sample. Advantage of transmission electron microscopy compared to light microscopy is that the wavelength of light is not limiting. Resolution obtained with TEM can easily be less than 1nm. Transmission electron microscopy is for this reason suitable method for studying structural properties of extracellular vesicles(van der Pol, et al. 2010)

# 2. AIMS OF THE STUDY

Existence of extracellular vesicles has been known for decades but the interest in extracellular vesicles has grown exponentially during last fifteen years which can be seen as the increased amount of publications dealing with the topic. Aim of this master's thesis is to gather together up to date information about nomenclature, biogenesis, secretion and properties of extracellular vesicles. Thesis will also shed a light on how these vesicles could be utilized as biomarkers for cancer. Also the role glycosaminoglycan called hyaluronan in the field of extracellular vesicles is discussed in brief. Towards to end of the text existing isolation techniques and the available detection methods for extracellular vesicles will be discussed. Final goal of literature review of this thesis is to make an entity that combines importance of the extracellular vesicles and hyaluronan in cancer environment and development.

The aim of the methodological part of this study was to develop and test microscopic methods for characterization of EVs from different sources by utilizing transmission electron microscopy and live cell confocal microscopy. Another aim is to set up and test the methods to study the effect of melanoma-derived EVs on the migration and invasion rate of target cells. These methods will be further developed and utilized in future studies of hyaluronan-induced EVs, their regulation, shedding and uptake mechanisms and effects on target cell functions.

# 3. MATERIALS AND METHODS

## 3.1. Cell culture

In this thesis following cell lines were used: Stable inducible EGFP-HAS3-MV3 melanoma cell line and human fibroblasts. Culture medium used for both cell lines was Dulbecco's Modified Eagle's Media (DMEM, EuroClone, Pavia, Italy) with high glucose added with 10% Fetal bovine serum, 1% L- glutamine and 1% penicillin. In the case of MV3-HAS-3 (D/S) also hygromycin B was added to the culture medium.

Human mesenchymal stem cells described before in (Qu et al 2014) were cultured in MEM-α (HyClone, South Logan, UT, OSA) supplemented with 10 % FBS, (FBS, fetal bovine serum,

HyClone), 25 µg/ml streptomycin (EuroClone, Milano, Italia) and 25 U/ml penicillin (EuroClone), 10 ng/ml of bFGF (fibroblast growth factor) and 25 µg/ml of vitamin C (Sigma).

### 3.2. Isolation of EVs by ultracentrifugation

Extracellular vesicles were isolated from MV3-HAS-3 melanoma cells. 1,5 million cells were seed to 8 x 10 cm dishes and when cells were attached to bottom, culture medium was replaced with vesicle-free medium. Just after changing of culture medium, doxycyclin (0,25 mg/ml) were added to the half of cell culture dishes and incubated for 48 hours.

Media from culture dishes were collected to the 15 ml falcon tube and centrifuged 1200 x g 20 min in 4˚C. (Eppendorf Centrifuge 5810 Rotor F-34-6-38) Supernatant was then transferred to ultracentrifuge tubes and ultracentrifuged 110 000 x g 90 min. (Sorval Ultracentrifuge, DuPont) OTD Combi, rotor T865-1). After ultracentrifugation supernatants were removed and vesicles were dissolved to 100 µl DMEM high glucose (no additions)/tube. Vesicle samples were stored in eppendorf tubes -80 ˚C.

### 3.3. Transmission electron microscopy (TEM)

EV specimens used for transmission electron microscopy analysis were originally isolated from cultured GFP-HAS3 overexpressing MDCK cells, human mesenchymal stem cells, human ascites fluid and human synovial fluid. The thawed EV preparations were layered onto carbon coated and glow discharged copper grids. Thereafter, specimens were fixed in 2% paraformaldehyde for 10 min. Next the samples were contrasted using 2% neutral uranyl acetate for 10-15 min in dark and embedded in 1,8% methyl cellulose (25 Ctp)/ 0,4% UA. Transmission electron microscopy was performed using JEOL JEM 2100F transmission electron microscope (Jeol Ltd, Tokyo, Japan) operated at 200 kV. Used magnifications varied from 4000-fold to 150 000-fold.

### 3.4. Confocal microscopy

Confocal microscopy was performed with a Zeiss Axio Observer inverted microscope (40 x NA 1.3–objective) equipped with Zeiss LSM 700 confocal module (Carl Zeiss Microimaging GmbH, Jena, Germany). For live cell imaging, Zeiss XL-LSM S1 incubator with temperature and CO2

control was utilized. ZEN 2009 software (Carl Zeiss Microimaging GmbH) was used for image processing, and 3D rendering.

For visualization of hyaluronan coat on live stem cells, a fluorescent group (Alexa Fluor 594) was directly coupled to HA binding complex (HABC) that has been isolated from bovine articular cartilage like described before (Rilla, et al. 2008) Before time lapse confocal imaging, live cell cultures grown on chambered coverglasses (Ibidi GmbH, Martinsried, Germany) were incubated for 30 min at 37°C with fluorescent HABC (5 µg/ml) diluted in culture medium.

For labeling of plasma membrane of live human stem cells, Deep Mask plasma membrane stain (1:4000, Invitrogen, Life Technologies Ltd, Paisley, UK) was used. The imaging was initiated immediately after addition of the stain.

### 3.5. Migration assay

Migration was studied by traditional scratch wound healing assay. For this assay 70 000 – 80 000 fibroblasts were seeded in 12 wells in 24-well plates (see table below). After 48 hours incubation in 37 ˚C wounds were scraped using a 200 µl pipette tip. After wounding the wells were washed once with 1 x PBS to remove cell debris.

Treatments to migration assay:

| Wells 1-4: | Wells 5-8 | Wells 9-12 |
|---|---|---|
| 300 µl | 300 µl | 300 µl |
| 1:4 DMEM high glucose (no additions) to DMEM(1% FBS, 1% P/S, 1% L-glutamine) | 1:4 Vesicles from DOX- MVs in DMEM high glucose (no additions) to (1% FBS, 1% P/S, 1% L-glutamine) | 1:4 Vesicles from DOX+ MVs in DMEM high glucose (no additions) to (1% FBS, 1% P/S, 1% L-glutamine) |

The wells were imaged using Nikon DS-L1-5M attached to light microscope in time points 0, 6, 16 and 24 hours.

### 3.6. Invasion assay

For invasion assay 55 000 cells were seeded onto 6 wells of a 8-well chamber slide (Ibidi GmbH, Martinsried, Germany). After 24 hours when wells were confluent the medium was removed and 150µl of DMEM high glucose (1% FBS, 1% P/S, 1% L-glutamine) was pipetted to all six wells. For wells 1 and 2, 50 µl of DMEM high glucose (no additions) was added for control, for wells 3 and 4 50 µl of vesicle suspension from MV3-HAS3 dox - cells and for wells 5 and 6 50 µl of vesicle suspension from MV3-HAS3 dox + cells was added. After 6 hour incubation, a layer of basement membrane extract gel (Cultrex, Trevigen Inc., Gaithensburg, MD) was added on the top of the monolayer.

After 24 hour incubation, the cell cultures were fixed the actin cytoskeleton of the cells were stained with AlexaFluor 594-labeled phalloidin (Molecular Probes, Eugene, OR, USA) to visualize the invaded cells in the gel. Next the cell cultures were imaged with confocal microscope. Side views created from stacks of optical sections were used to estimate the invasion area covered by the cells.

## 4. RESULTS

### 4.1. Transmission electron microscopy

Relatively low amounts of extracellular vesicles were found in all TEM samples. Most of the vesicles were individual, but also some clustered vesicles were found. Also variable morphology of vesicles was seen in all samples. Some of the vesicles were electron dense, while some vesicles appeared very light. Many vesicles had a typical concave shaped morphology, some appeared rounded. The size distribution also varied. In cell culture samples the size range was from 30 nm to 500 nm (**Figure 4**), and in human fluids from 50 nm to 1000 nm **(Figure 5).** We used glow discharge treated grids to strengthen the vesicle attachment to the grids, which improved the results (**Figure 4D**), but also increased the amount of impurities on the grids.

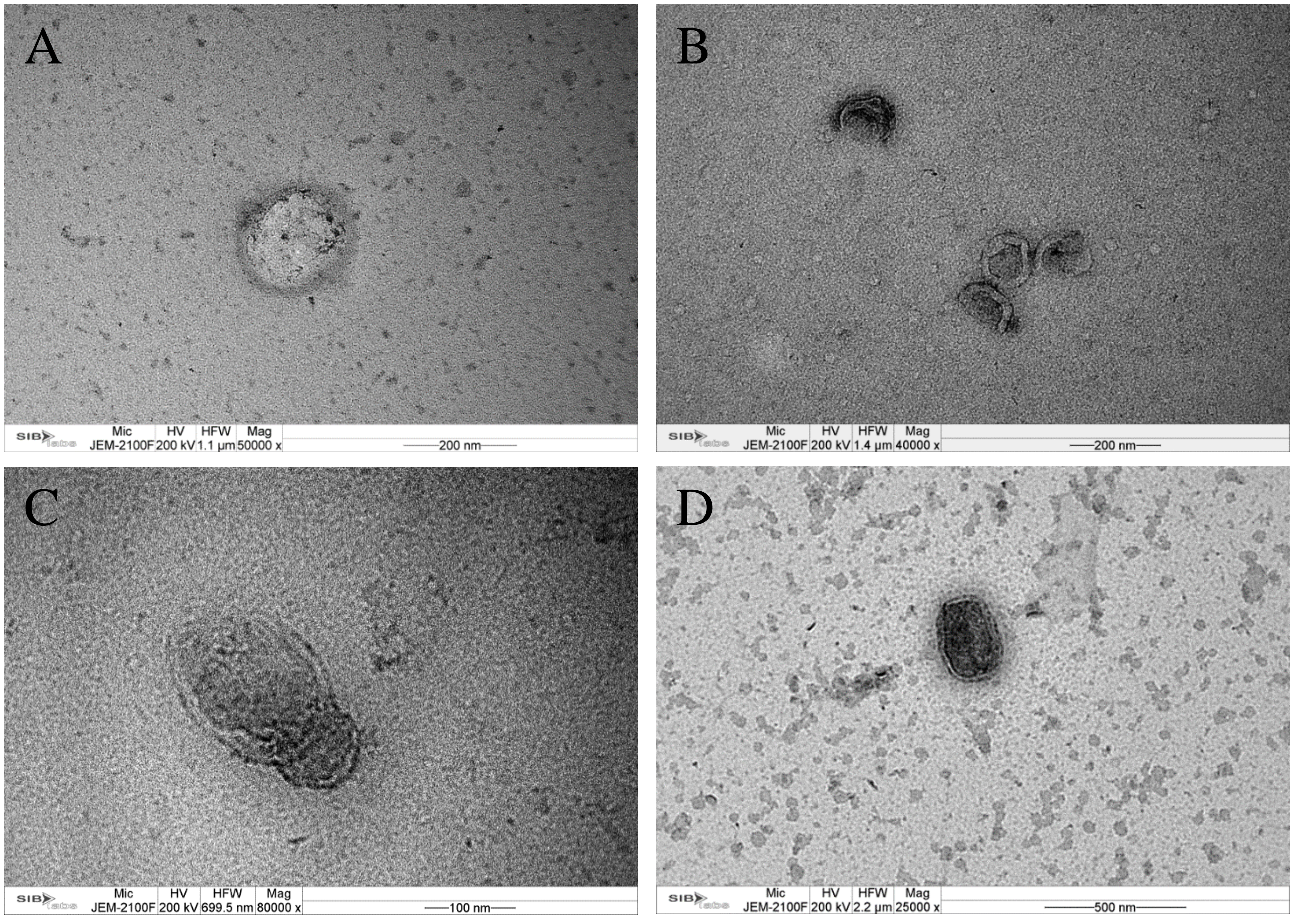


**Figure 4. Transmission electron microscopy of EV preparations purified from different cell cultures.** A) GFP-HAS3 overexpressing MDCK cells, B) GFP-HAS3 overexpressing MV3 cells C) Human mesenchymal stem cells D) Human mesenchymal stem cells with Glow Discharged grid

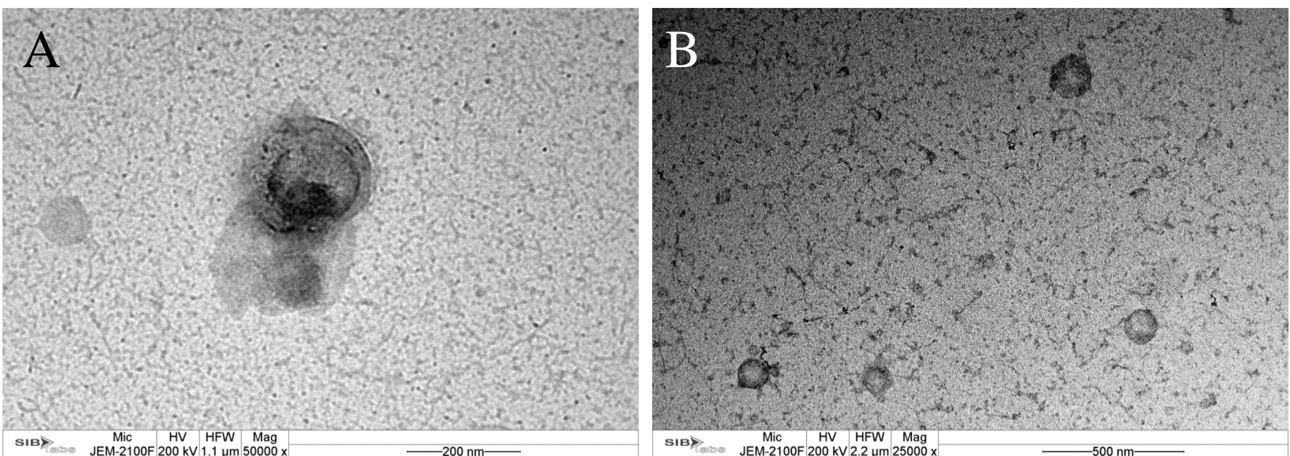


**Figure 5. Transmission electron microscopy of EV preparations purified from human biological fluids.** A) human synovial fluid and B) human ascites fluid.

### 4.2. Live cell confocal microscopy

Live cell imaging was utilized to visualize the shedding of EVs in cultured live human mesenchymal stem cells. Cell Mask plasma membrane marker visualized the plasma membrane clearly, and time lapse series of images (**Figure 6**) showed shedding of vesicles of different size derived from the plasma membrane of cells (**arrows in Figure 6**).

To detect the hyaluronan coat and possible shedding of hyaluronan-coated vesicles from hMSCs, a fluorescently labeled hyaluronan binding probe that detects specifically hyaluronan in live cells was utilized. hMSCs were surrounded by a thick and intensive hyaluronan coat as an indication of active hyaluronan secretion. Additionally, plenty of hyaluronan-positive vesicles were detected around the cells (**arrows in Figure 7**). Many of the vesicles were attached to the plastic substratum, and were immobile (**white arrows in Figure 7**), while some vesicle movements were detected (**yellow arrows in Figure 7**), which suggests active secretion of EVs that carry hyaluronan coat on their membranes.

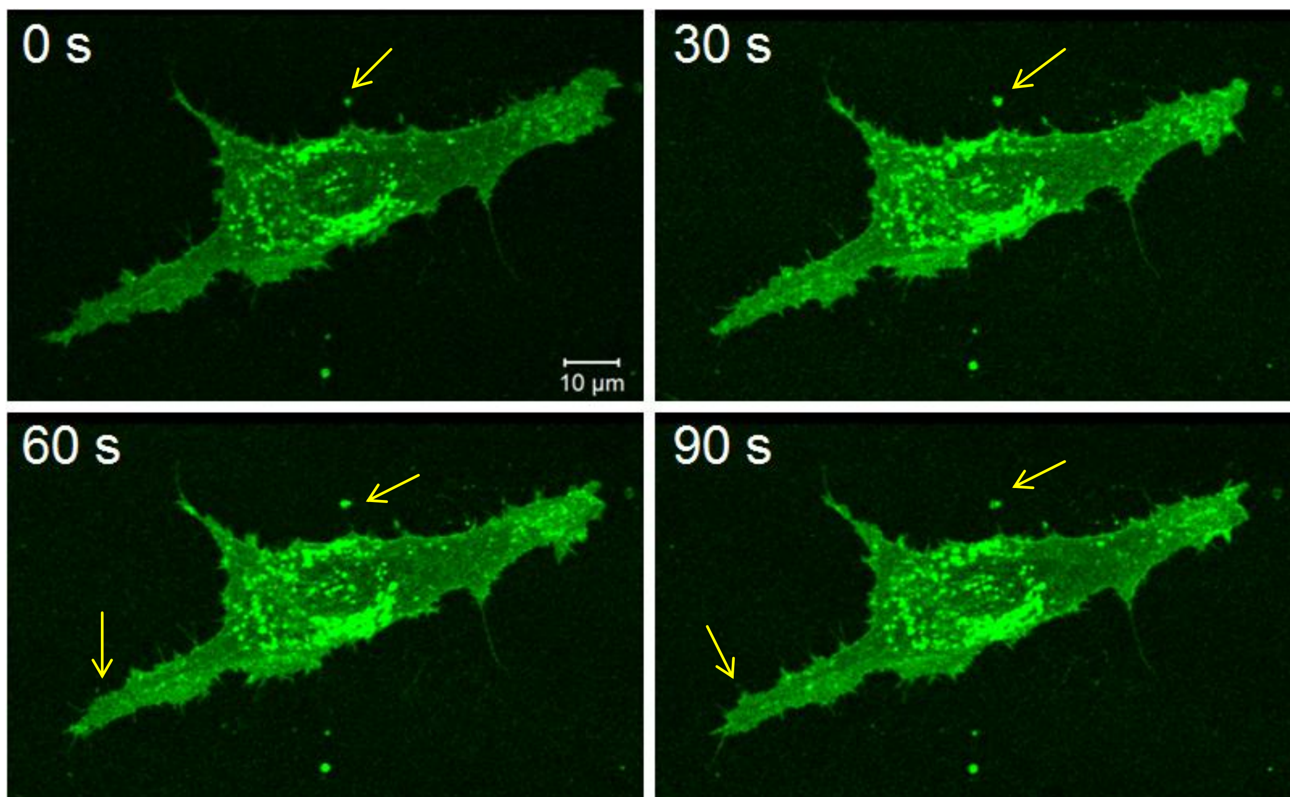


**Figure 6. Time lapse images of a hMSC cell labelled with CellMask plasma membrane marker (green).** Horizontal 3D projections created from a stack of optical sections are shown at each time point.

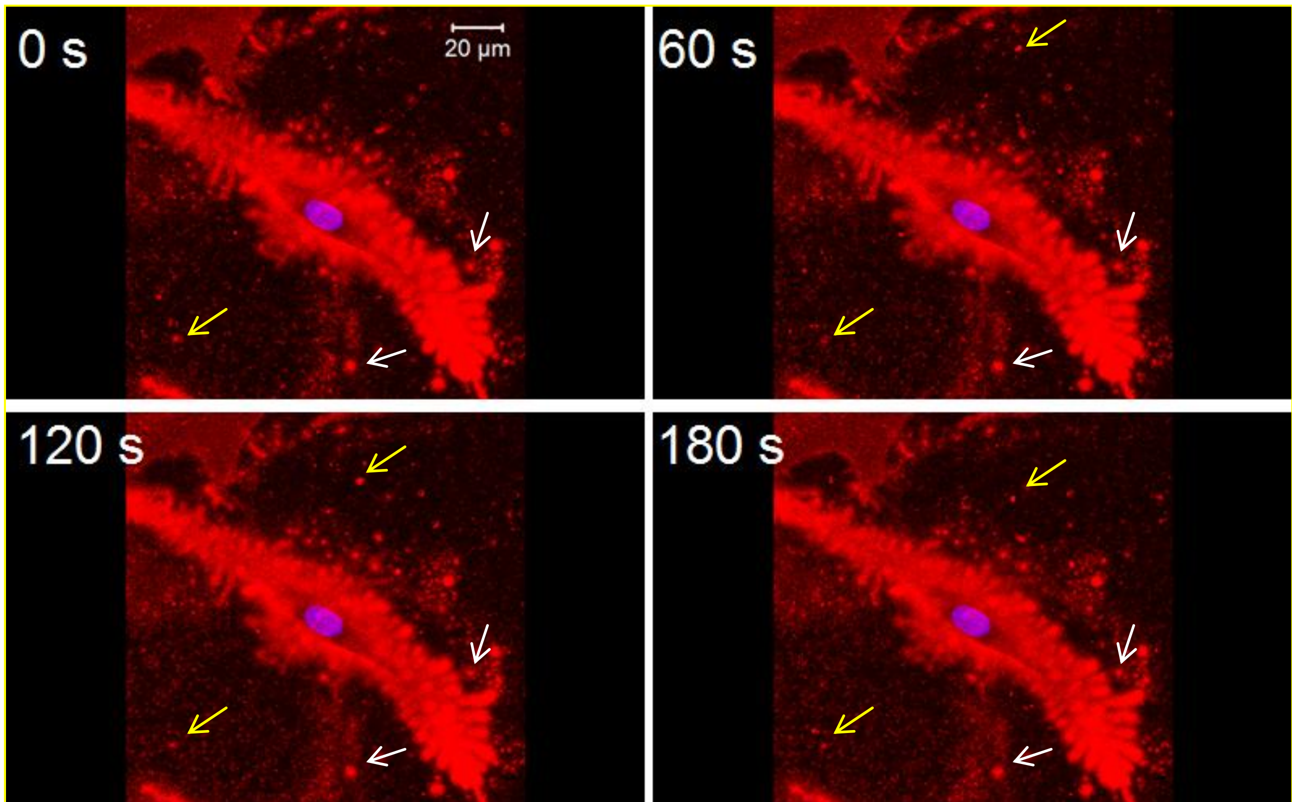


**Figure 7. Hyaluronan coat and hyaluronan-positive EVs visualized by fluorescently labeled hyaluronan-binding probe (fHABC, red)**. Nucleus is stained with DNA-binding label DRAQ5 (blue). Horizontal 3D projections created from a stack of optical sections are shown at each time point.

### 4.3. Migration assay

Human fibroblasts spread evenly through the wound during 24 hour observation time (**Figure 8**), resulting in the closure of the wounded area. However, there were no detectable differences between control group and EV treatments in migration efficiency of human fibroblasts (**Figure 9**). Mean migration speed of human fibroblasts was about 25 µm per hour.

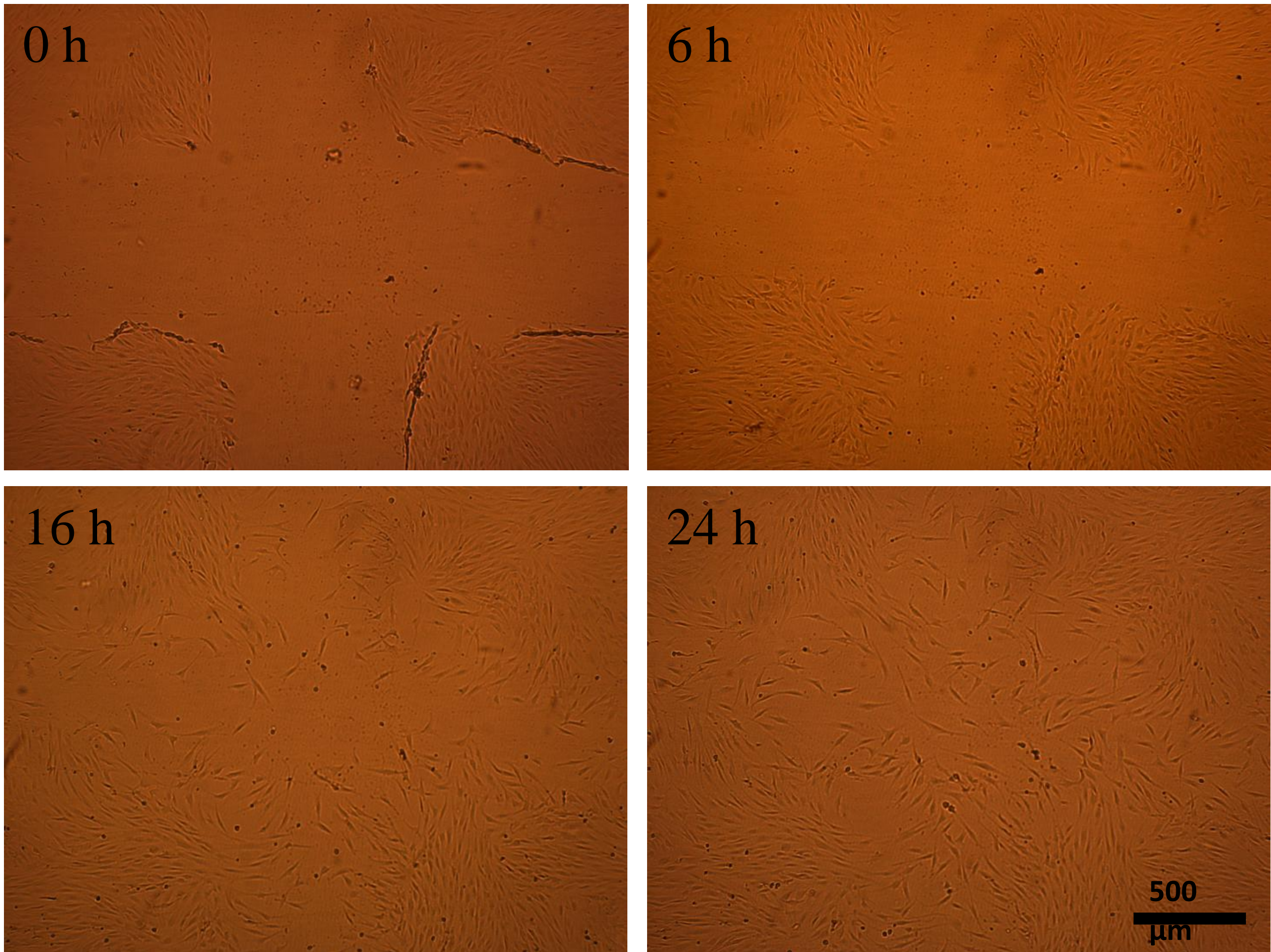


**Figure 8. Phase contrast microscopic images of migration assays with human fibroblasts.** Panels show examples of control cells in different time points after scratch wounding of the cultures (0, 6, 16 and 24 hours). Magnification bar 500 µm.

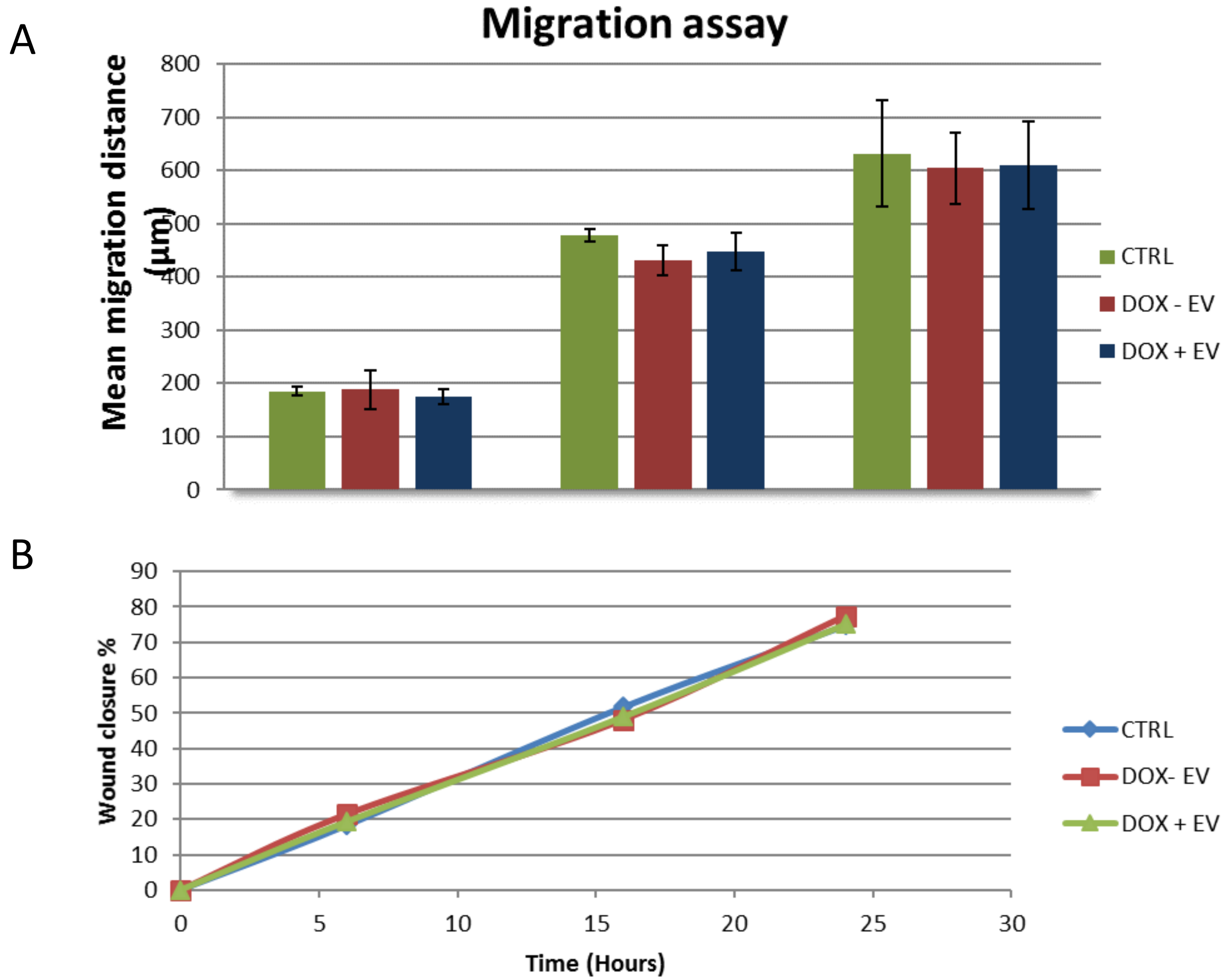


**Figure 9. Migration assay.** A) Effect of EVs derived from MV3 cells expressing GFP-HAS3 on the migration speed of human fibroblasts. Error bars indicate standard deviation (SD) of three independent experiments with three parallel samples in each experiment. B) Effect of EVs derived from MV3 cells expressing GFP-HAS3 on the speed (%) of wound closure of human fibroblasts.

### 4.4. Invasion assay

Invasion of fibroblasts in 3D gel was imaged with confocal microscope (**Figure 10**). Invasion efficiency was higher in fibroblasts treated with extracellular vesicles isolated from MV3-HAS3–cells than the control group (**Figure 11**). No difference between extracellular vesicles isolated from doxycycline induced MV3-HAS3–cells and non-induced MV3-HAS3 (D/S) –cells were found.

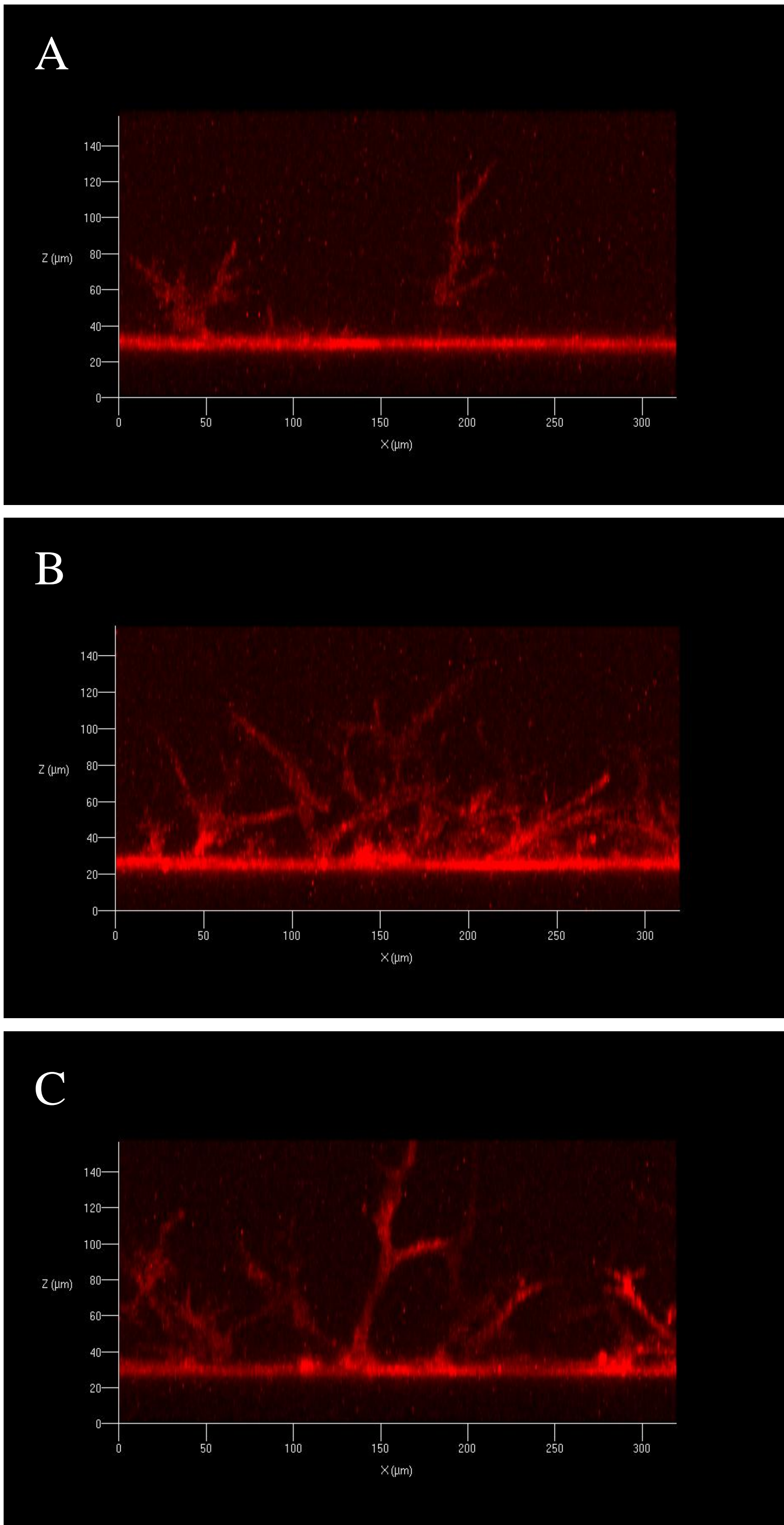


**Figure 10. Invasion assay.** Fibroblast cultures grown under a Cultrex 3D gel. Panels show side views of 3D projections, created from stacks of confocal optical sections. A) Control cells without additions, B) cells after addition of EVs originating from GFP-HAS3-MV3 cells without doxycycline induction and C) after doxycycline induction.

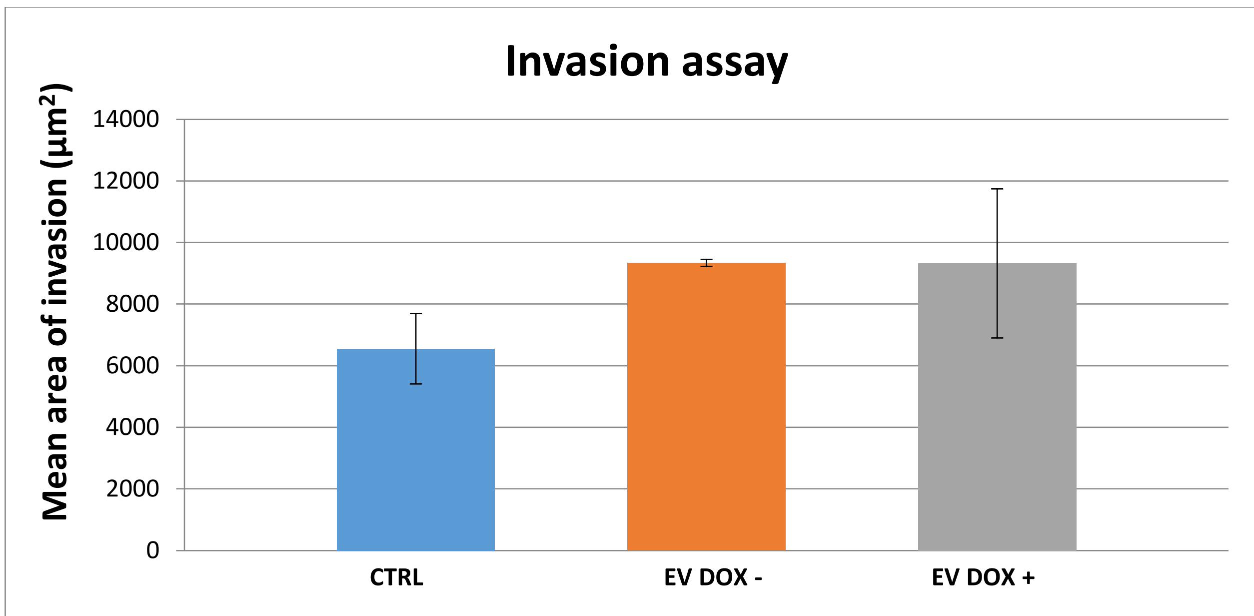


**Figure 11. Effect of EVs on invasion of human fibroblasts.** Invasion efficiency of fibroblasts in cultrex 3D gel was determined from three groups. Control group was given DMEM. EV DOX- group was given extracellular vesicles isolated from not induced MV3-HAS3 (D/S) cells and EV DOX + was given extracellular vesicles isolated from doxycycline induced MV3-HAS3 (D/S) cells. Error bars indicate range of duplicate samples.

# 4. DISCUSSION

Extracellular vesicles seem to be everywhere and to be associated with almost all or all processes in the human body. The research field of extracellular vesicles is therefore exciting and full of possibilities. Research of extracellular vesicles is also full of pitfalls. It may sound exaggerative but almost everything could be done more precisely when studying extracellular vesicles. Isolation and analyzing procedures need to be standardized, terms could be more consistent, research equipment are still in its infancy and also researchers should be more patient when they are doing conclusions based on obtained results. Otherwise in the beginning of new type of research it could be useful to test all the possible ways to study the topic and then determine which way is the best.

It is somehow interesting that when you think that you have learned something about for example exosome biogenesis that “fact” is invalidated in the next sentence. In turn, this all uncertainty inspires to questionate everything and keeps researchers alert when they analyze results from their own and other researchers’ studies. Being too sceptical may slow the research too much so it’s quite important to found a suitable compromise.

The explosive growth of the discipline has brougth a multiple new techniques with it. The most of these new equipment like particle size analyzators and isolation kits for EVs need still more development before they are working absolutely reliably. In some cases the problem is in the inexperienced user and, in turn, in the equipment. For example it is quite hard to separate extracellular vesicles from other same sized particles in the sample with particle size analyzators.

Somehow it may be difficult to understand that different types of EVs – exosomes and microvesicles – may look similar at first glance but they represent totally different entireties. Different type of biogenesis and cargo advocates the view that these vesicles should be separated when research is performed. Partly overlapping size distribution and otherwise similar characteristics of exosomes and microvesicles make this task hard to fulfill. Although idea of separating these two types of EVs to entirely separated entities may sound rational, it should be remembered that these two types of vesicles are actually at the same time and place. Maybe they will work together in order to achieve the desired result?

International society of extracellular vesicles (ISEV) has done much to promote these topics. One example is the setting of basic quidelines for isolation and analysis of extracellular vesicles(Lötvall, et al. 2014).

In the future the extracellular vesicles will pretty sure be powerful tools to research, analytics and treatment of multiple diseases. When the EVs has been harnessed to the use of disease screening it will be possible to recognize pathological conditions much earlier than nowadays. For example in cancer analytics it may be critical if we think survival rate. It's a fascinating thought that development of liquid biopsy may allow easy way to screen development of diseases which cannot be detected with traditional techniques at early stage. The threshold for taking liquid biopsy is also lower because it does not require such invasive operation as traditional biopsy. For example in some cases urine sample is enough and it's also easier to give blood sample than biopsy which needs surgical procedure.

In the practical part of this master's thesis the aim was to study properties of extracellular vesicles using microscopic methods and invasion and migration assays.

Although it may seem pointless to focus to the culturing of cells, culturing conditions of cells are certainly a key factor in the case of secretion of extracellular vesicles. It is easy to think that culturing conditions are equal if the protocol is followed but there is a number of "microchanges" which should be taken into account like the temperature and age of used media and additions. Also pH of cell culture may be good to be monitored.

Vesicle isolation using ultracentrifugation is quite challenging technique from the perspective that the pellet of vesicles is not visible. For more accurate studies the presence of wanted extracellular vesicles should be determined from every isolation batch. This determination could be done for example with transmission electron microscopy. Also the counting of the numbers of existing extracellular vesicles is important for further analyses. Without these measurements the result of these studies are rather preliminary results than accurate results. Different variations of centrifugation /ultracentrifugation speeds should be tested systematically to be sure which results in best outcome. Especially if for example the effects of only microvesicles or exosomes are wanted to be tested. Also this will help to reach the best yield of extracellular vesicles overall.

Live cell imaging with the confocal microscopy was performed to visualize the moment of budding of extracellular vesicle. Imaging of extracellular vesicles with confocal microscopy was done by staining of cell membrane with plasma membrane marker and hyaluronan binding probe. Small size of extracellular vesicles sets limits to the required resolution. In addition to the small size also relatively fast movement of EVs makes imaging difficult. Best resolution of z-stack image would be

achieved with high number of pictures per stack and low imaging speed. However this is not possible to do because of movement of EVs. In the future developing imaging equipment will result in higher resolution and imaging speed will be higher. Slow imaging speed is mainly due to mechanical properties of confocal microscope.

Ultrastructural studies of extracellular vesicles were done succesfully. The bilipid layer and typical cup-shaped form of extracellular vesicles were visible in many cases. Also the size range of vesicles in images were as assumed. Although there were in some cases thick layer of surfactant especially at the border area of grids, imaging could be done without bigger problems. Also ripping of surface of grids made imaging more difficult in several cases. Using of Glow-Discharge treatment of grids increased the amount of captured vesicles but there were also high amount of other "trash". Anyway, in the future experiments, more concentrated EV preparations are required to catch a sufficient number of vesicles for reliable analysis.

In the invasion assay the major shortcoming may be the number of repeats which was only one. This was because of a tight deadline of assay. In the future, more repetition would be done for better reliability. Another shortcoming may be possible practical problems as light drying of cells in used Ibidi chamber slides were observed. This may has changed result especially in the case that the drying were not uniform in all wells. In the future comparing of different cell lines and culturing conditions may lead to better accuracy of results.

In the migration assay there were no significant changes between the control group and treatments. The growth rate of used fibroblast was so high in every group that possible differences between groups may not be detected because of that. On source of error is actually the use of fibroblast because they spread rapidly through the wound. They do not grow as a steadily moving wall. Thus the use of epithelial cells may give more accurate results in future experiments.

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