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Exosomes, their biogenesis, composition and role

Exosomy, jejich biogeneze, složení a role

Bachelor´s thesis

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, Podpis:

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Abstrakt

Exosomy jsou podtyp extracelulárních váčků, oproti ostatním extracelulárním váčkům se liší svým endozomálním původem a svým typickým tvarem. Tvorba váčků začíná při maturaci časných endozomů a invaginaci váčků dovnitř lumen. Při invaginaci se pomocí proteinových komplexů ESCRT třídí ubiquitinované proteiny dovnitř váčků. Do těchto váčků je také uzavřeno malé množství cytosolu. Po vytvoření se tyto váčky nazývají intraluminální váčky a celé těleso se nazývá multivezikulární tělísko. Multivezikulární tělísko splývá s plazmatickou membránou a intraluminální váčky jsou sekretovány jako exosomy. Exosomy jsou přítomné v mnoha tělesných tekutinách a jsou sekretovány mnoha druhy buněk. V organismu prezentací antigenu na svém povrchu aktivují imunitní systém nebo slouží v buněčné komunikaci přenosem malých RNA molekul.

Klíčová slova:

Exozomy, multivezikulární tělísko, ESCRT, endozomy, RNA, imunita

Abstract

Exosomes are a subtype of extracellular vesicles. Exosomes are distinguishable from other extracellular vesicles by their endosomal origin and their typical cup-shaped morphology. The biogenesis of exosomes begins in the early endosomes by inward budding. The endosomal sorting complex required for transport sorts ubiquitinylated proteins into the vesicles. The small volume of cytosol is also encapsulated during budding. These vesicles are called intraluminal vesicles and the whole body is called multivesicular body. Multivesicular body fuses with the plasma membrane and vesicles are released as exosomes into the extracellular space. Exosomes are present in all bodily fluids and are secreted by a high number of cells. Exosomes present antigens on their surface to trigger immunity or serve in the cellular communication by the transfer of small RNAs.

Keywords:

Exosome, multivesicular body, ESCRT, endosomes, RNA, immunity

Acronyms

A2B1 – hnRNPA2B1

ADAM10 – metalloproteinase domain-containing protein 10

Ag – argentum

APC – antigen presenting cell

ARF6 – GTPase ADP ribosylation factor 6

ASGR – asialoglycoprotein receptor

BAPTA – 1,2-bis(*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid

CDS – coding sequences

CE – cholesteryl ester

Cer – ceramide

CHOL – cholesterol

CLmotif – cellular motif

DAG - diacylglycerol

DC – dendritic cell

DGKα – Diacylglycerol kinase α

Doa4 – Degradation of alpha 4

EGTA – ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

EHD4 – EH domain-containing protein 4

EQ – ExoQuick

ESCRT - Endosomal Sorting Complexes Required for Transport

esRNA – exosomal shuttle RNA

EV – extracellular vesicle

EXOmotif – exosomal motif

GSL – glycosphingolipids

HLA-DM – human leukocyte antigen DM

HLA-DR – human leukocyte antigen - antigen D related

hnRNPA2B1 – heterogenous nuclear ribonucleoprotein A2B1

ICAM-1 – Intercellular adhesion molecule1

IFN – interferon

IL – interleukin

ILV - intraluminal vesicle

Lamp – lysosome-associated membrane protein

LBPA – lysobisphosphatidic acid

LFA-3 – lymphocyte function-associated antigen 3

lncRNA – long non-coding RNA

LPE – lysophosphatidylethanolamine

MC – mast cell

MFG-E8 – milk fat globule epidermal growth factor VIII

MHC – major histocompatibility complex

MIICs – MHC class II-enriched compartments

miRNA – microRNA

MON – Monesin

MVB - multivesicular body

Mvb12 – multivesicular sorting factor

ODG – OptiPrep density gradient centrifugation

PCR – Polymerase chain reaction

PE – phosphatidylglycerol

PG – phosphatidylglycerol

PI – phosphatidylinositol

piRNA – piwi-interacting RNA

PLD2 – phospholipase D2

PM – the plasma membrane

RISC – RNA induced silencing complex

rRNA – ribosomal RNA

S – SUMO

SEC – size-exclusion chromatography

Snf7 – sucrose non-fermenting

snoRNA – small nucleolar RNA

snRNA – small nuclear RNA

TCR – T-cell receptor

TEI – Total Exosome Isolation

Tf – Transferrin

TfR – Transferrin receptor

Tim4 – T-cell immunoglobulin domain and mucin domain-containing protein 4

tRNA – transfer RNA

TSAP6 – Tumor suppressor-activated pathway 6

TSG101 – Tumor susceptibility gene 101

UBC-like – ubiquitin conjugating-like

UC – ultracentrifugation

UTR – untranslated region

Vps - vacuolar sorting protein

Content

1. Introduction

Exosomes are a subtype of extracellular vesicles besides microvesicles and apoptic blebs. Since their discovery, these vesicles have been the subject of intensive studies.

Current research shows that exosomes are involved in cellular communication and modulation of immunity since they carry a number of RNAs and present various antigens on their surface. However, the exosomal pathway is also exploited by viruses for their morphogenesis or for the infection. Exosomes are secreted by cancer cells and circulate in blood stream. Because of this, they could serve as a protentional biomarkers of cancer diseases. Furthermore, they have been shown to be able to cross the brain-blood barrier. Due to these properties, exosomes were proposed to be used as vehicles for drug delivery system. The aim of this thesis is to summarize present knowledge of exosomes, their biogenesis and role in organism. Further, the proposition of exosomes as disease biomarkers, as well as the isolation of exosomes and comparison of the most used isolation methods are presented.

2. Discovery of exosomes, function, possible usage

Exosomes were first observed in the 1980's during maturation of reticulocytes. The gold-labelled transferrin (Tf) was enriched in vesicles, which later fused with the plasma membrane and were released by exocytosis. (Harding, Heuser, & Stahl, 1983) The term "exosomes" for these vesicles was first used in 1987 (R. M. Johnstone, Adam, Hammond, Orr, & Turbide, 1987).

Exosomes are a subtype of extracellular vesicles (EVs). Other types of EVs include apoptic blebs and, microvesicles. They have different sizes and origins. Apoptic blebs are secreted by apoptic cells. These blebs are large vesicles with a diameter of 500-2000 nm, formed on the cell surface. They have been shown to have a role in the regulation of immunity. (Kerr, Wyllie, & Currie, 1972) Microvesicles, also sometimes named ectosomes or prostasomes, have a diameter of 50-1000 nm. They include all structures released directly

from the plasma membrane. (Akers, Gonda, Kim, Carter, & Chen, 2013) It was proposed that the term "exosomes" should be used for vesicles of a specific size that contain the typical exosomal markers. This was proposed because the purification procedures cannot distinguish EVs by their origin, only by their morphology, size and density. (Simons & Raposo, 2009)

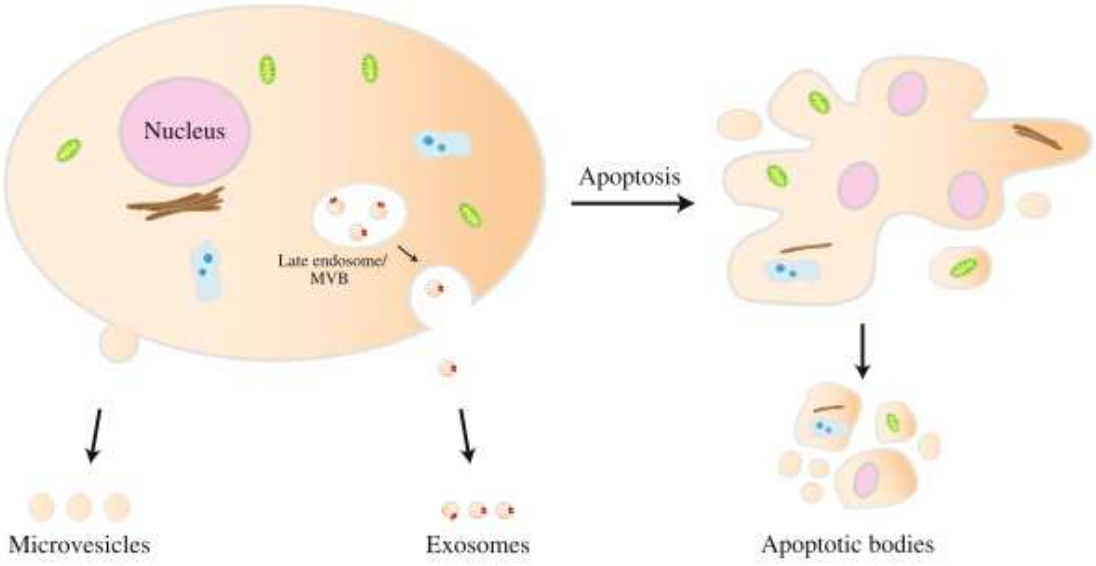

Figure 1: Biogenesis of microvesicles, exosomes and apoptic bodies. Adapted. (Akers et al., 2013)

Exosomes are of a size of about 40-100 nm in diameter with a characteristic cup-shaped morphology. Exosomes float on sucrose gradient to a density between 1,13 to 1,19 g/ml (Théry, Amigorena, Raposo, & Clayton, 2006). They can be characterized by their morphology but more importantly by their specific lipid and protein composition. Exosomes have endosomal origin. They are formed in late compartments named multivesicular bodies (MVBs) and are released from cells by fusion of MVBs with the plasma membrane. As summarised in (Akers et al., 2013). Biogenesis of different EVs is shown in Fig. 1.

Exosomes contain proteins involved in membrane transport and in biogenesis of multivesicular bodies as well as heat shock proteins, integrins and tetraspanins. Exosomes are also enriched in raft-lipids and glycerophospolipids. (Simons & Raposo, 2009) Some of these

proteins may only reflect the abundance of these proteins in the cell, others may function as exosomal markers.

Exosomes are released by many types of cells and have been found in all bodily fluids including blood, urine, saliva, breast milk, bronchial lavage, amniotic fluid and cerebral spinal fluid as summarised in (Chahar, Bao, & Casola, 2015). Up to this date, they have been shown to originate from many types of cells tested, such as T cells (Blanchard et al., 2002), B cells (G. Raposo, H. W. Nijman, et al., 1996), dendritic cells (Théry et al., 1999), neurons (Fauré et al., 2006), astrocytes (Wang et al., 2012), reticulocytes (R. M. Johnstone et al., 1987), oligodendrocytes (Krämer-Albers et al., 2007), smooth muscle cells (Comelli et al., 2014) and endothelial cells (Walker, Maier, & Pober, 2010). This suggests great importance of exosomes in many cellular processes.

Exosomes can also serve as vesicular carriers for miRNAs, mRNAs, and other small RNAs. These RNAs are protected in the lumen of exosomes from RNases outside the cell. It was also shown that these RNAs are functional and when the exosomes are taken up by the cells, RNAs can be translated into functional proteins. (Valadi et al., 2007)

Exosomes can serve as vehicles for medicaments. The significant advantage is the fact, that exosomes can be derived from patient cells, so they do not induce immune response. Possible usage of exosomes includes medical stimulation of immunity, biomarkers of diseases or in vitro loading of RNAs for silencing of specific genes. (Lai, Yeo, Tan, & Lim, 2013)

3. Biogenesis, sorting, uptake and budding

3.1. Endosomal sorting

It is known that eukaryotic cells use many compartments for uptake, sorting and secretion of cargos. After endocytosis, endocytic vesicles are transported into early endosome. Late endosomes develop from early endosomes by acidification and protein sorting. These two

types of endosomes can be distinguished by their shape and cellular location. Early endosomes have a tubular shape and are located near plasma membrane. Late endosomes are round and are present close to the nucleus. The late endosomes are able to form intraluminal vesicles (ILVs). The ILVs are formed by inward budding which leads to formation of multivesicular bodies (MVBs). The inward budding not only detaches membrane components, but vesicles formed by this process also encapside small volume of cytosol. The fate of the MVBs may vary. Not all MVBs are degraded in lysosomes. MVBs can also fuse with the plasma membrane and ILVs vesicles, which are released into extracellular space are called exosomes. This was summarised in (Scott, Vacca, & Gruenberg, 2014). Figure 2 shows electron microscopy of a cell with multivesicular bodies and release of exosomes after MVB fusion with the plasma membrane. Fig. 3 shows isolated exosomes from neurones. Figure 4 shows schema of exosome biogenesis in the cell.

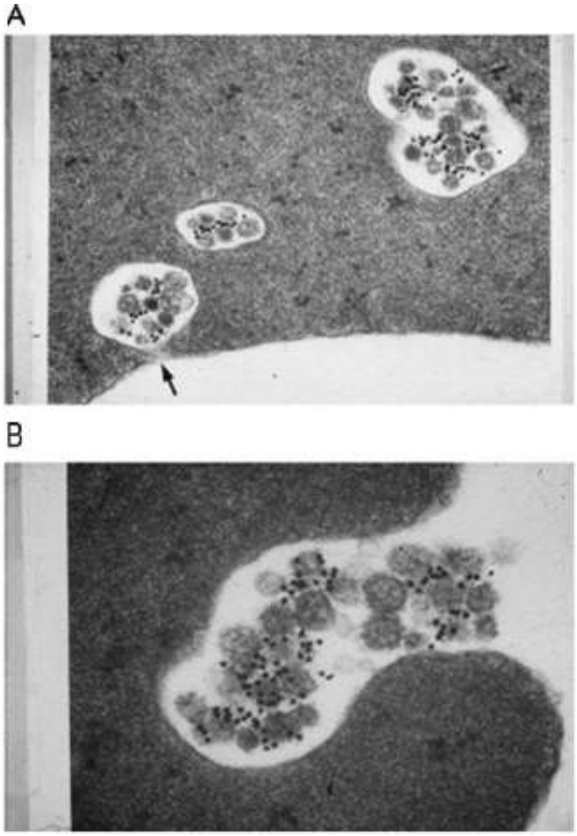

Figure 2: Electron microscopy images of the fusion of multivesicular bodies and release of exosomes in sheep reticulocytes. A: Image taken 18h after incubation with an antibody against

the transferrin receptor B: Image taken 36h after incubation with an antibody against the transferrin receptor Transferring receptor was labelled by immunogold antibody. The arrow shows fusion with the plasma membrane. Copied from (Rose M Johnstone, 2005).

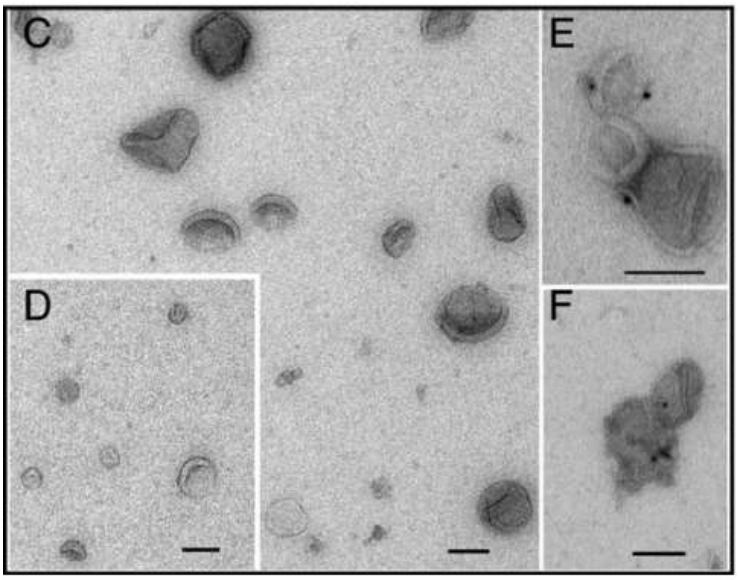

Figure 3: Electron microscopy images of neurone-derived exosomes. C and D show a negative staining. E shows immunogold labelling with anti-GluR2 antibody and F shows immunogold labelling with anti-Alix antibody. Scale bars: 100 nm. Copied from (Fauré et al., 2006).

Apparently, different types of MVBs exist, since one class ends up in lysosomes and is degraded there, while the other class of MVBs fuses with the plasma membrane to release exosomes. However, methods to distinguish these types of MVBs are so far not available. MVBs were tested by labelling of cholesterol by perfringolysin O. It was found that cholesterol negative and positive MVBs coexisted in cells. Interestingly, only cholesterol positive MVBs fused with the plasma membrane and released exosomes. (Möbius et al., 2002)

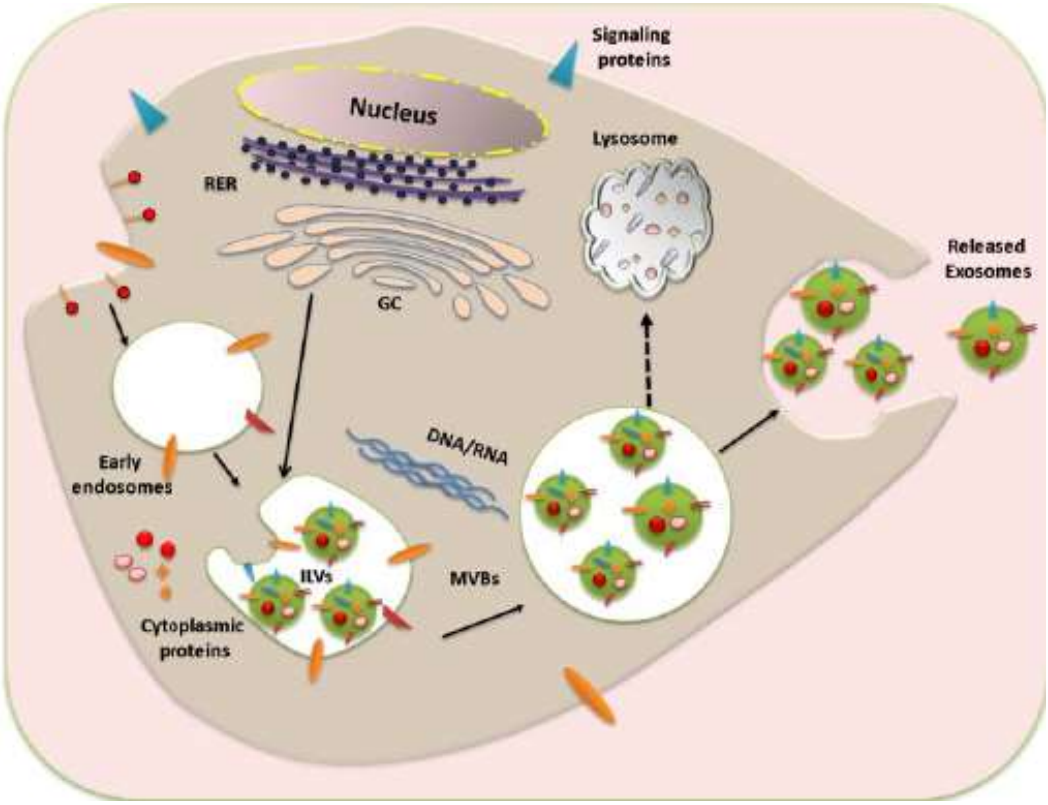

Figure 4: Schema of exosomes biogenesis. The clatrin-coated vesicles are delivered into the early endosomes. The budding of the membrane occurs and proteins and nucleic acids are incorporated into ILVs. Further, there are two possible pathways: One leads to lysosome and to degradation of MVBs, the other one leads to fusion of MVBs with the plasma membrane and to release of exosomes. RER: Rough Endoplasmatic Reticulum, GC: Golgi complex. Copied from (Chahar et al., 2015).

There also exist other domains from which exosomes are released. These domains were deemed by some authors as endosomal-like domains because they retain the endosomal property of inward vesicle budding. The endosomal-like domains were first discovered in Jurkat T cells. It was found that these domains contain endosomal and exosomal proteins such as CD81 and CD63. Furthermore, N-Rh-PE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N- [lissamine rhodamine B sulfonyl]), a lipid secreted in exosomes, was shown to be accumulated in the same domains as CD81. Immunofluorescence analysis showed that these markers are in fact accumulated within these domains. (Booth et al., 2006)

3.2. Sorting of cargo into exosomes

The sorting process and the generation of ILVs require the Endosomal Sorting Complexes Required for Transport (ESCRT) machinery. However, ESCRT independent mechanism was also described. (Chahar et al., 2015)

3.2.1. ECSRT machinery

Poly-ubiquitination of proteins is traditionally associated with degradation in proteasome. However, mono-ubiquitination is associated with other cellular functions, including delivery into MVB pathway. (Buschow et al., 2005) The ESCRT machinery consists of E-vacuolar sorting genes, which are essential for the biogenesis of internal vesicles in the MVB (Odorizzi, Babst, & Emr, 1998). The ESCRT complex is composed of several complexes. These complexes are called ESCRT-I, ESCRT-II and ESCRT-III. ESCRT-I bounds to ubiquitinated cargo and activates ESCRT-II. ESCRT-II then initiates recruitment and assembly of ESCRT-III. This results in the sorting of cargo and the formation of ILVs as summarised in (Hurley, 2009).

ESCRT-I is a 350 kDa protein complex composed of the subunit vacuolar protein 23 (also known as Tumor susceptibility gene 101 (TSG101) in humans), 28, and 37 (Vps23, Vps28, and Vps37). Vps23 has UBC-like (ubiquitin conjugating-like) domain (Babst, Odorizzi, Estepa, & Emr, 2000). This indicates a potential role in ubiquitination, even though it lacks cysteine in the active site. Without this cysteine, Vps23 cannot act as ubiquitin-conjugating enzyme. UBC-like domain has been mutated by PCR and has been screened for a loss of function. A point mutation has been identified, which changed methionine at position 85 to threonine. Even though this Vps23 mutant was inactive, it was still able to assemble itself into the ESCRT-I complex. Because of this, it has been proposed, that even if it is lacking cysteine in the active site, this protein could still interact with ubiquitine. It was

suggested that UBC-like domain of Vps23 is required to bind ubiquitinylated cargo with ESCRT-I and, as a result, this cargo is sorted into the MVB. (Katzmann, Babst, & Emr, 2001)

More recently, another component of ESCRT-I, has been identified. It's a multivesicular sorting factor 12k Da (Mvb12). It was shown, that Mvb12 binds to the Vps23. Mvb12 promotes assembly of ESCRT-I into an oligomeric complex in the cytosol. The loss of Mvb12 results in the formation of stable complex of ESCRT-I and ESCRT-II in the cytosol, so that ESCRT-I cannot function properly. (Chu, Sun, Saksena, & Emr, 2006)

ESCRT-II is approximately 155 kDa soluble protein complex composed of vacuolar protein 22, 25, and 36 (Vps22, Vps25, and Vps36). ESCRT-II is normally found in the soluble pool. However, in the cells with deleted Vps4 gene, the ESCRT-II complex was found in the membrane-bound pellet. This suggests association of ESCRT-II with membranes. The Vps4 ATPase is required for dissociation of this complex from the membrane. To find out how ESCRT-II associates with endosome, a functional Vps36-GFP fusion was constructed to identify the target compartment. Vps36-GFP was accumulated at large perivascular structures, most likely class E compartments. To support endosomal localization, Vps36-GFP cells were stained with antibodies specific for Snf7 (Sucrose Non-Fermentable 7), ESCRT-III subunit, which associates with endosomes. The results showed that Vps36-GFP associates with Snf7 membrane domains. (Babst, Katzmann, Snyder, et al., 2002)

Interaction between ESCRT-II and ESCRT-III was tested as well. It was shown that ESCRT-II binds ESCRT-III via Vps20. ESCRT-II regulates the localization and formation of ESCRT-III. It was also found out that membrane association of ESCRT-II is independent of ESCRT-III, which means that ESCRT-II has the upstream regulatory role. However, ESCRT-I acts upstream of ESCRT-II as overexpression of subunits of ESCRT-II leads to suppression

of the ESCRT-I complex. In contrast, overexpression of ESCRT-I did not suppress ESCRT-II. (Babst, Katzmann, Snyder, et al., 2002)

The ESCRT- III complex promotes ILV detachment. ESCRT-III is capable of cutting membrane necks. For this function, the system does not need any of the upstream ESCRT complexes, cargo, or manipulation of membrane tension. These factors are still important, but they are not essential. (Wollert, Wunder, Lippincott-schwartz, & James, 2009) The ESCRT-III complex consists of vacuolar proteins 20, 7, 24, and 2 (Vps20, Snf7, Vps24, and Vps2) which assemble in the aforementioned order into a 450 kDa protein complex. Vps24 and Snf7 are known to be endosome-associated proteins. (Babst et al., 1998) Vps20 and Vps2 are cytoplasmatic proteins which were found to display high homology to Vps24 (52% similarity) and Snf7 (47% similarity). Endosomal association of Vps2 is dependent on the presence of Vps24 and vice versa. The localization of these four proteins indicates that they assemble into a homo- or heterodimeric complexes. All the four proteins are required for the assembly of ESCRT-III. It was found, that overexpression of one of these proteins does not lead to higher amount of this protein in the ESCRT-III complex suggesting that the stoichiometry of these proteins in ESCRT-III remains the same. (Babst, Katzmann, Estepa-sabal, et al., 2002) However, in a more recent study, it was found, that Snf7 is more abundant that the rest of the protein units and the stoichiometry is not 1:1:1:1 (Teis, Saksena, & Emr, 2008).

Further analysis of the ESCRT-III complex indicated that ESCRT-III is formed by two distinct subcomplexes Vps2-Vps24 and Vps20-Snf7. Vps20-Snf7 subcomplex is responsible for the membrane association with ESCRT-III. Vps2-Vps24 subcomplex binds with ATPase Vps4 via coiled-coil interactions. (Babst, Katzmann, Estepa-sabal, et al., 2002)

It was also indicated that prior to entry of a cargo into the MVB, the ubiquitin is removed from the cargo. Enzyme Doa4 (Degradation of alpha 4) is recruited by Vps24 in order to recycle ubiquitin from ubiquitinilated cargo. (Amerik, Nowak, Swaminathan, &

Hochstrasser, 2000) After deubiquitinilation of the cargo, the AAA-type ATPase Vps4 is recruited by ESCRT-III. Vps4 is a 48 kDa protein, which has one ATPase domain and is localized to the cytosol (Babst, Sato, Banta, & Emr, 1997). This protein is required for disassembly and dissociation of ESCRT-III, specifically the dissociation of Vps24p and Snf7p (Babst et al., 1998). Fig. 5 shows a proposed model of the ESCRT machinery.

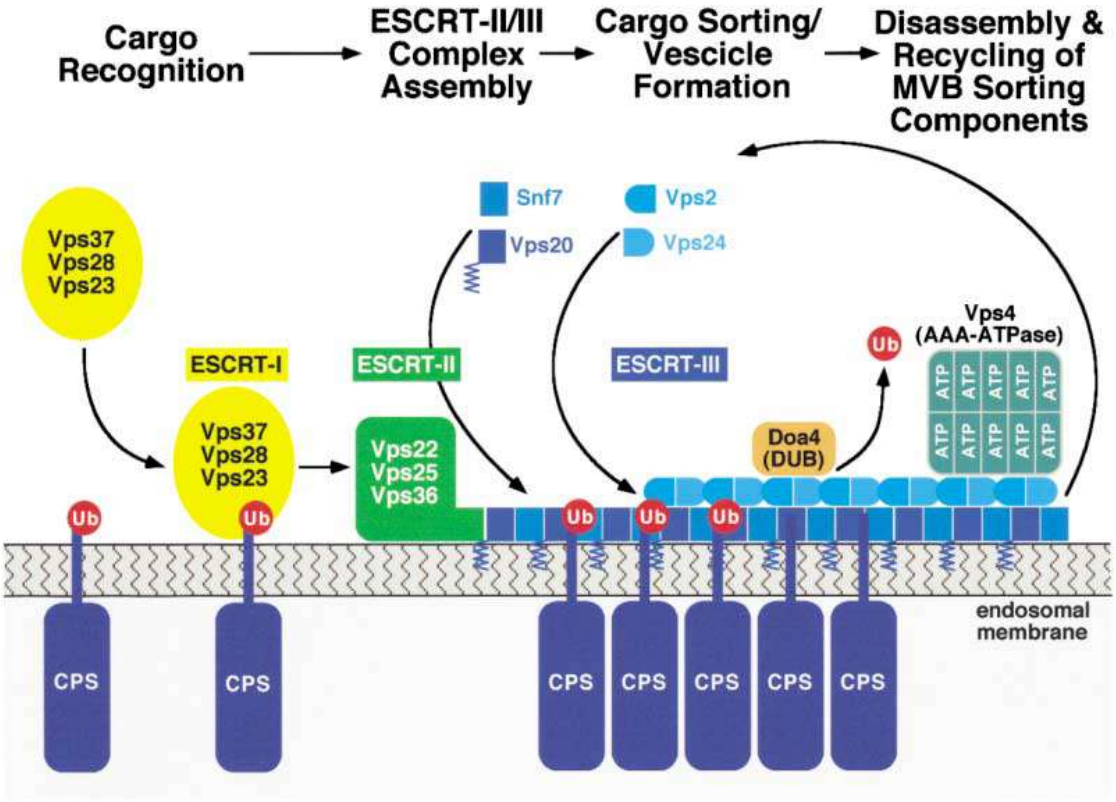

Figure 5: The first proposed model of the ESCRT machinery. Ubiquitinated vacuolar enzyme carboxypeptidase S (CPS) is recognised by ESCRT-I (Vps37, Vps28, Vps23), which activates ESCRT-II (Vps22, Vps25, Vps36). The ESCRT-II complex recruits proteins to form ESCRT-III (Snf7, Vps20, Vps2, Vps24). The ESCRT-III complex sorts CPS and vesicle is formed. The deubiquitination enzyme, Doa4, binds to ESCRT-III and removes ubiquitin from the cargo prior to entry into a vesicle. After MVB sorting, the Vps4 is recruited which results in the dissociation of ESCRT-III from the membrane. (Babst, Katzmann, Estepa-sabal, et al., 2002)

Because of the ATPase Vps4, the MVBs should not contain ubiquitinated proteins. However, it was demonstrated that exosomes do contain poly-ubiquitinated proteins. Western

blot of cell lysate and exosomes was performed with P4D1, an antibody which binds mono- and poly-ubiquitinated proteins. In both cases, Western blot showed smears at the upper part of gel, suggesting for heavily ubiquitinated proteins. The low molecular weight (64-22 kDa) also showed bands, suggesting presence of mono- or oligo ubiquitinated proteins. Significant difference in the composition of ubiquitinated proteins in exosomes was observed when compared to the cell lysate, suggesting that ubiquitinated proteins are incorporated into the MVB pathway. (Buschow et al., 2005)

3.2.2. ECSRT independent pathway - Ceramide and tetraspanins

ECSRT independent sorting pathway also exists. This pathway is mediated by ceramide and tetraspanins. Ceramide is formed by hydrolytic removal of a part of phosphocholine by sphingomyelinases. It was demonstrated that the treatment of exogenous sphingomyelinases induced the formation of vesicles. After treating cells with sphingomyelinase inhibitor, exosome release was reduced. (Trajkovic et al., 2008)

Tetraspanins are used as exosomal markers but their role in sorting proteins and other agents is not clear. Based on interactions of the intracellular tetraspanin-enriched microdomains, interactome was created. This interactome accounted for almost half of the proteins which compose exosomes. This suggests, that tetraspanins-enriched microdomains may play a sorting role that targets specific receptors to exosomes. Tetraspanin-enriched microdomains serve as a platform for placing specific receptors and proteins and this platform aids the sorting of these receptors and proteins into exosomes. There also exists a connection between tetraspanin-enriched microdomain and RNA binding proteins. This connection could have a potential role in incorporation of RNAs into vesicles. This finding could be exploited in gene therapy to sort the wanted agent into vesicles. (Perez-Hernandez et al., 2013) It was also shown that tetraspanin CD63 promotes oligomerization and sorting during melanogenesis (Brousse, Albrechts, & Kiel, 2012).

4. Molecular characterization of exosomes

4.1. Proteomic studies

Protein composition of exosomes depends on the cell type from which they are derived. Exosomes can stimulate T-cells in vitro, because they carry MHC class II-peptide complexes at their surface. However, they are not as efficient in antigen presentation as antigen presenting cells. (G. Raposo, H. W. Nijman et al., 1996) This stimulation requires co-stimulatory molecules. In order to find out, which co-stimulatory molecules are present on the surface of exosomes, the following study has been performed. B-cells were collected by centrifugation, the pellets were lysed, analysed by SDS-PAGE and by Western blotting. Transferrin receptor (TfR) served as a reference protein. MHC class II complex was enriched 8-fold over TfR. Several other proteins of tetraspan family were enriched in exosomes, such as CD86 (9-fold over TfR), CD37 (36-fold over TfR), CD81 (124-fold over TfR), CD82 (41-fold over TfR), and CD53. Western blotting could not detect the presence of CD53, however CD53 was detected on vesicles by immunoelectron microscopy. Other MIIC (MHC class II-enriched compartments) markers, such as Lamp-1, Lamp-2, and HLA-DM are not enriched. Adhesion molecules were also analysed. ICAM-1 (CD54) was enriched 4-fold over TfR. Lymphocyte function-associated antigen 3 (LFA-3 [CD58]) and ICAM-2 were not significantly enriched. This study revealed, that exosomes are highly enriched in proteins that play a significant role in antigen presentation. (Escola et al., 1998)

Several proteomic analyses for cell-type specific exosomes have been performed up to date.

Proteomic analysis of spleen-derived murine dendritic cell (DC) line D1 was performed. This proteomic study identified eight exosomal proteins. Three proteins are transmembrane (Mac-1 A chain, MHC II B chain, and CD9), one associated to membranes (milk fat globule-epidermal growth factor VIII [MFG-E8]), and four proteins are cytosolic

(Gi2A, annexin II, gag from MRV provirus, and Hsc73). It was suggested that Hsc73 and annexin II are present in the lumen of exosomes. Hsc73 is an inducer of antitumor immune responses *in vivo*, which could suggest antitumor effect of exosomes. The most abundant protein, MFG-E8, could target DC-derived exosomes to other antigen presenting cells. CD9 is a co-factor for the interaction of EGF-like growth factor and its receptor. Therefore, CD9 could take part in the interactions between exosomes and antigen presenting cells or T cells in vivo. The cytosolic proteins found in exosomes play a role in membrane budding or fusion of intracellular compartments. (Théry et al., 1999)

Another proteomic analysis of DC-derived exosomes, which identified 21 new exosomal proteins was performed. These proteins were involved in exosome biogenesis and function (cofilin, profilin I, and elongation factor 1A), intracellular membrane transport and signalling (several annexins, Rab7 and 11, Rap1B, and syntenin). A novel category of exosomal proteins related to apoptosis was identified (thioredoxin peroxidase II, Alix, 14-3-3, galectin-3) The presence of these apoptosis proteins suggests relationship between the endocytic pathway and the apoptotic processes. Because of the high number of identified proteins, the first protein map of DC-derived exosomes was established. (Théry et al., 2001)

Mass spectrometry analysis of B cell-derived exosomes revealed the presence of MHC class I and II, Hsc70, Hsp90, integrin A4, CD45 receptor tyrosine phosphatase, moesin, tubulin (A and B), actin GIA2 and other proteins. Other proteins include Na+/K+ ATPase and CD63, which is known as lysosome-associated membrane protein 3 (Lamp 3); it is also commonly used as marker for exosomes. It was also found out, that exosomes are enriched in cholesterol, sphingomyelin, and ganglioside GM3. (Wubbolts et al., 2003)

Another study of B cell-derived exosomes found out, that there exist different subpopulations of B cell-derived exosomes. The CD81+ exosomes express CD19, CD20, CD24, CD37, and Human Leukocyte Antigen - antigen D Related (HLA-DR). For the CD63+

exosomes, the only detected B cell surface protein was HLA-DR. It was found out, that levels of exosomal markers and B cell markers depend on the host cell origin and there is a large heterogeneity among different B cell lines. (Oksvold et al., 2014)

Mast cell (MC)-derived exosomes were shown to induce blast formation, proliferation, production of IL-2 and IFN-γ but no detectable production of IL-4. A pre-treatment with IL-4 is required for bone mast cells to secrete exosomes, suggesting that this activity is regulated process. It was found that exosomes are enriched in MHC class II, CD86, CD40, CD40L, CD13, annexin VI, actins, CDC25, LFA-1, and ICAM-1. The production of IL-2 and IFN-γ suggest a novel mechanism by which mast cells express their inflammatory and immunoregulatory functions. One of the possible candidates for stimulation of lymphocyte exosomes could be the CDC25 molecule, which is involved in the progression of the cell cycle. The results of this analysis also indicated, that MC-derived exosomes were as potent as MCs in inducing lymphocyte activation. (Skokos, Panse, Villa, Peronet, & David, 2001)

Another study of MC-derived exosomes demonstrated, that MC-exosomes are able to induce phenotypic and functional maturation of DCs *in vitro* and *in vivo*. This is done by inducing immature DCs to up-regulate MHC class II, CD80, CD86, and CD40 molecules and by cross-presentation of Ag to T cells. Two heat shock proteins, Hsp60 and Hsc70 were also identified in exosomes from bone marrow-derived mast cells. However, it was shown, that these two proteins are not present in the exosomes isolated from B cells or macrophages. This could correspond to the fact, that the maturation of DCs is unique to MC-derived exosomes, because exosomes from B cells and macrophages were not able to induce maturation of DCs. (Skokos et al., 2003)

Analysis of exosomes secreted by hepatocytes identified 251 proteins; common proteins as well as several proteins involved in metabolism of lipoproteins, endogenous compounds and xenobiotics. The asialoglycoprotein receptor (ASGR) was detected, which

mediates uptake and intracellular degradation of desialylated glycoproteins and is expressed only in hepatocytes. This suggests that the ASGR receptor could be used as a specific marker for hepatocyte-derived exosomes. (Conde-vancells et al., 2008)

More recent proteomic study revealed that not all the exosomal markers are presented exclusively on exosomes, but they are presented on other types of EVs as well. Among these markers are flotillin-1, actin, HSC70, HSP73, HSP70, and HSP72 or MHC class I and MHC class II. The exclusive exosomal markers are TSG101, metalloproteinase domain-containing protein 10 (ADAM10), and EH domain-containing protein 4 (EHD4). (Kowal et al., 2016).

4.2. Lipidomic studies

Exosomes from rat MCs and human DCs were analysed for their lipid composition. Bilayer organisation of exosomes membrane was established using nuclear magnetic resonance. Exosomes showed enrichment in sphingomyelin (SM). However, cholesterol, as well as, phosphatidylcholine were decreased. (Laulagnier et al., 2004) This decrease of cholesterol is, however, in conflict with the data obtained during studies of exosomes from lymphoid B-cell line (Wubbolts et al., 2003). Exosome membrane rigidity was shown to be increased by increasing pH from 5 to 7. A higher flip-flop of lipids between the two leaflets in comparison with the plasma membrane was demonstrated. The exosomes membrane also exhibited random distribution of phosphatidylethanolamines (PE). These two properties distinguish exosomes from other types of cellular vesicles. In exosomes derived from rat mast cells, neutral lipids were shown to be present in about a half amount, when compared with those in the cells. Interestingly, it was reported that exosomes have a similar phospholipid composition even though they have a different cell type origin, suggesting that the lipid composition is independent of the secretion pathway. (Laulagnier et al., 2004)

More recently, the PC-3 prostate cancer cells and exosomes derived from these cells were analysed for their lipid composition (figure 6). The lipid composition is concurring with

the idea that the membrane of exosomes is highly ordered. A total of 250 and 217 lipids were quantified from cells and exosomes, respectively. It was shown, that the amount of glycosphingolipids, sphingomyelin, cholesterol, and phosphatidylserine is 15 times higher in exosomes than in the cells. It was proposed that glycosphingolipids could serve as a potential biomarker, since glycosphingolipids are present on the outer leaflet. (Llorente et al., 2013)

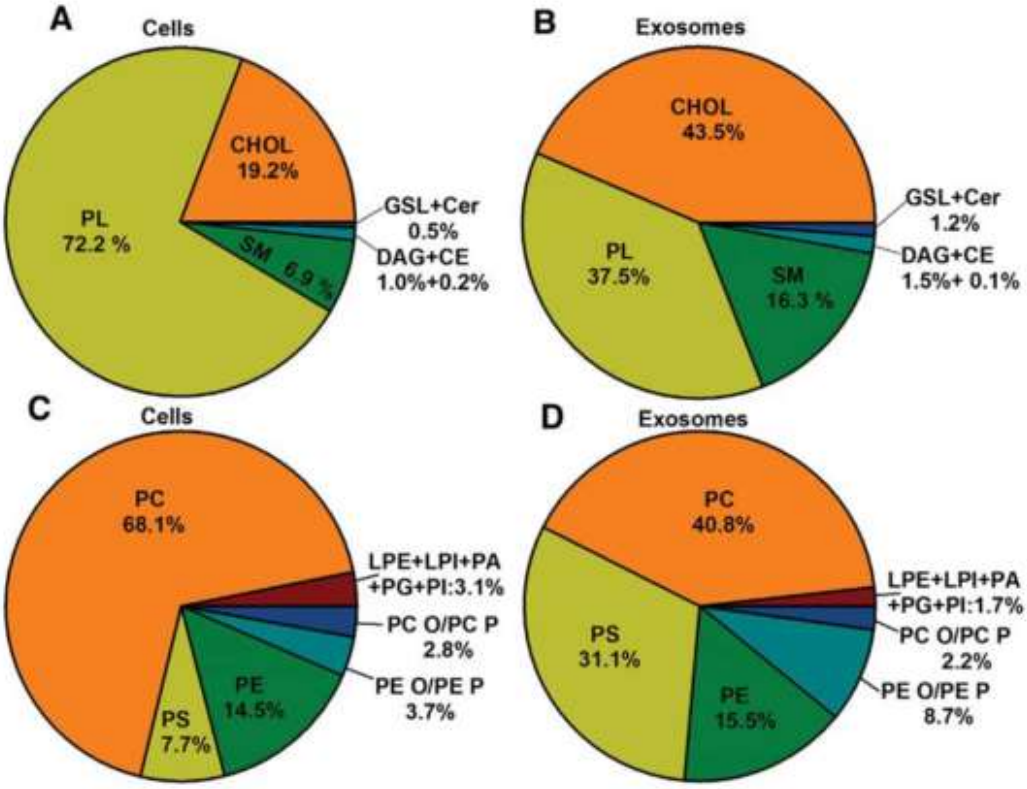

Figure 6: Lipid composition of metastatic prostate cancer cell line, PC-3. Graph A shows the composition of lipids in cells. Graph B shows the composition of lipids in exosomes. Graph C shows composition of PL classes in cells; 100% in graph C corresponds to the 77,2% in graph A. Graph D shows composition of PL classes in exosomes; 100% in graph D corresponds to the 37,5% in the graph B. **Legend:** SM = sphingomyelin, PL = all phospholipids except SM, PC = phosphatidylcholine, PE = phosphatidylethaloamine, PI = phosphatidylinositol, PS = phosphatidylserine, CHOL = cholesterol, Cer = ceramide, DAG = diacylglycerol, LPE = lysophosphatidylethanolamine, PG = phosphatidylglycerol, GSL = glycosphingolipids, CE = cholesteryl ester, PE = phosphatidylglycerol; Taken over and adapted from (Llorente et al., 2013).

4.3. Databases

Because of the amount of proteins, lipids, mRNAs, and miRNAs associated with exosomes and the still ongoing research in this area, there exist three databases, which contain all the data on exosomes. These databases are ExoCarta (http://www.exocarta.org), EVpedia (http://www.evpedia.info), and Vesiclepedia (http://www.microvesicles.org).

ExoCarta was the first database designed to specifically contain only the exosomal research data (Mathivanan & Simpson, 2009). ExoCarta contains data on exosomal proteins, RNAs, and lipids. Every study in ExoCarta is annotated with the isolation procedure, so that it is clear, how reliable the data are. (Mathivanan, Fahner, Reid, & Simpson, 2012) ExoCarta features protein-protein interaction networks and biological pathways of exosomal proteins. Currently, ExoCarta contains 41,860 protein, >7,540 RNA and 1,116 lipid entries. (Anand, Zhao, Samuel, Pathan, & Jois, 2016)

Vesiclepedia is a manually organised database that contains molecular data of identified EVs, including ectosomes, exosomes, apoptic bodies, large dense core vesicles, microparticles, and shedding microvesicles. Since only exosomal studies are present in ExoCarta, this database contains the necessary background information to understand more about the molecular composition of all the classes of EVs. Furthermore, with the confusion in the terminology of exosomes it is important not to exclude the other EVs from the database. (Kalra et al., 2012)

EVpedia contains prokaryote, mammalian, and non-mammalian eukaryote data. EVpedia also provides a number of tools and allows a comparison of proteomic data. These tools include browsing of vesicular components, Gene Ontology enrichment analysis, network analysis of vesicular proteins and mRNAs, and a comparison of vesicular datasets by ortholog identification. Furthermore, publications on EVs are listed in the database. EVpedia collects proteomes classified only by high-throughput studies. (Kim et al., 2013)

5. Release of exosomes

Studies of hematopoietic cell line, K562, showed that with the increase of intracellular Ca^{2+}, there is a higher secretion of exosomes. This was confirmed by the fact that exosome release was higher, when the cells were treated with monesin (MON) which induces changes in intracellular Ca^{2+}. It was also demonstrated that MON induces the accumulation of Ca^{2+} in the dilated MVBs. (Savina, Furlán, et.al., 2003) This is in agreement with previous study, which showed that MON induce the dilation of MVBs (Stein, Bensch, & Sussman, 1984). Together, these data indicate, that MON not only induces enlargement of MVBs but also increases the secretion of exosomes (figure 7). The positive effect of calcium was further studied with the help of thapsigargin, which causes inhibition of calcium-ATPase, leading to a rapid increase of intracellular free calcium. Treatment with thapsigargin showed the similar results as the treatment with MON, as well as, treatment with calcium ionophore A23187. Furthermore, the release of exosomes was decreased by the chelators 1,2-bis(*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) or/and ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), which points in the calcium-dependent secretion of exosomes (figure 8). It was also suggested that Tf could stimulate the release of exosomes in a calcium-dependent manner. (Savina, Furlán, et al., 2003) A previous study showed that binding of Tf to TfR increases the intracellular concentration of calcium, suggesting that this could be a physiological stimulus for exosome release (Sainte-marie et al., 1997).

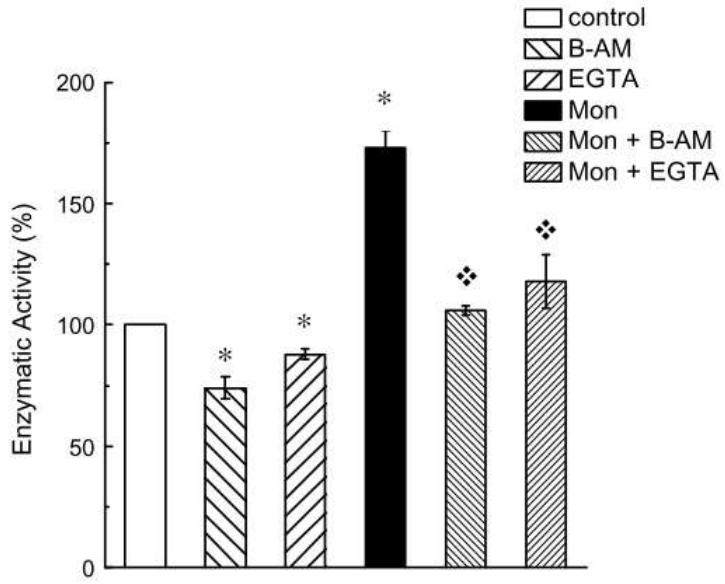

Figure 7: Monesin stimulates exosome release. Cells were incubated for 7 hours with 30 μM BAPTA-AM (B-AM), 1,5 mM EGTA (EGTA), 7 μM monesin (MON), or the combination of 30 μM BAPTA-AM with 7 μM monesin (MON + B-AM) or 1,5 mM EGTA with 7 μM monesin (MON + EGTA). The secreted exosomes were measured for the activity of exosome protein acetylcholinesterase. **Legend:** *Asterisk*, significantly different from the control, $p < 0.05$. *Fragmented diamond*, significantly different from the MON-treated cells, $p < 0.05$. Adapted. (Savina, Furlán et al., 2003)

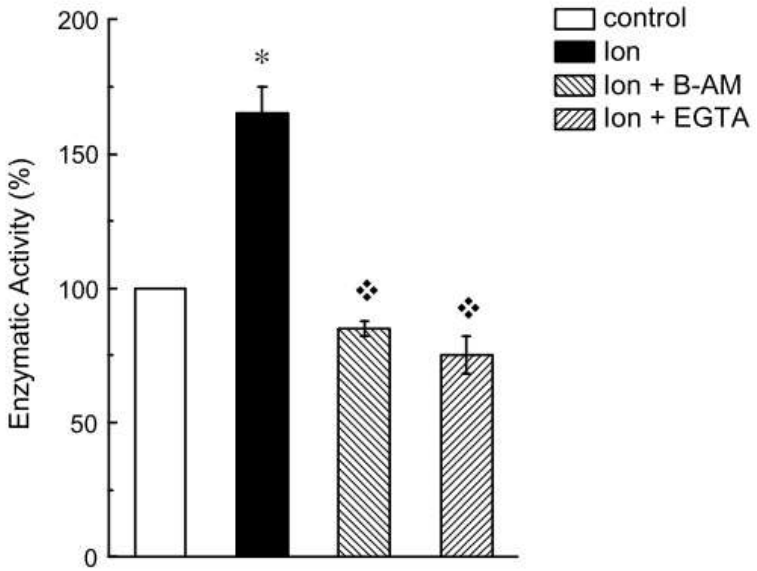

Figure 8: Intracellular calcium rise stimulates exosome release. Cells were incubated for 7 hours with 1 μM A23187 (Ion) or 1μM A23187 and 30 μM BAPTA-AM (Ion + B-AM) or 1 μM A23187 and 1,5 mM EGTA (Ion + EGTA). The secreted exosomes were measured for the activity of exosome

protein acetylcholinesterase. **Legend:** *Asterisk*, significantly different from the control, $p < 0.05$. *Fragmented diamond*, significantly different from the MON-treated cells, $p < 0.05$. Adapted. (Savina, Furlán, et al., 2003)

The influence of Rab GTPases on the exosome pathway has been investigated as well. It was shown that Rab11 modulates exosome pathway. The overexpression of dominant-negative Rab11 mutant inhibits exosome release in K562 cells. A slight increase in exosome release was observed in the cells transfected with Rab11wt, (Savina, Vidal, & Colombo, 2002). It was suggested that Rab11 acts in the tethering/docking of MVBs to promote fusion. However, the calcium is required for the final fusion event. Rab11 was also suggested to drive MVBs to the plasma membrane and not to the lysosome. (Savina, Fader, Damiani, & Colombo, 2005)

GTPase-activating proteins, TBC1D10A-C, also regulate exosome secretion. Rab35 is the target of these proteins and it was demonstrated that the inhibition of Rab35 in oligodendrocytes leads to decrease in exosome secretion, probably by controlling of docking/tethering of endocytic vesicles with the plasma membrane. (Hsu et al., 2010)

6. Exosomes and their influence on immunity

As shown in 4.1., exosomes contain MHC class I and class II complexes and can stimulate T cells in vitro.

It was shown that T lymphocytes release exosomes upon triggering T-cell receptor (TCR). It was proposed that these exosomes present TCR/CD3 on their surface, making them important vehicles to deliver signals to the cells bearing the right peptide-MHC combination. (Blanchard et al., 2002) It was also shown that antigen- or peptide-bearing exosomes induce activation of naïve CD4+ T cells *in vivo* and *in vitro* by the transfer of peptide-MHC complexes to DCs (Théry et al., 2002).

Another study revealed that exosomes secreted by mature DCs are more potent to induce T-cell activation than exosomes from immature DCs. Furthermore, it was shown, that MHC class II and ICAM-1 are necessary for exosomes to prime naïve T cells. The study also revealed that exosomes can not only transfer peptide-MHC complexes, but also T-cell stimulating ability to other antigen presenting cells (APCs), such as B cells. (Segura et al., 2005)

A study about direct exosome stimulation of T cells was performed. It shows that monocyte-derived DC exosomes induce IFN-γ and IFN-α production in CD8+ T cells from peripheral blood without the need of DCs. This induction is more efficient, when using exosomes from mature DCs than from immature ones. This is probably caused by the fact, that mature DC-derived exosomes contain more MHC class I and II molecules, as well as other co-stimulatory molecules. (Admyre, Johansson, Paulie, & Gabrielsson, 2006)

7. Transport of RNA into exosome and from cell to cell

Exosomes were shown to contain nucleic acids, such as mRNAs, miRNAs, and other small RNAs, but no DNA. Treatment of exosomes with RNase did not result in degradation of RNAs, suggesting that RNAs are present in the lumen of exosomes. It was proposed that exosomal RNA should be called exosomal shuttle RNA (esRNA). Furthermore, it was shown that transfer of mouse exosomal mRNA into human cells resulted in the presence of mouse proteins in the human cells, suggesting that exosomal mRNA is functional. The amount of miRNAs found in exosomes was investigated. Exosomes were shown to carry approximately 121 miRNAs. Among these, the most abundant were let-7, miR-1, miR-15, miR-16, miR-181, and miR-375. (Valadi et al., 2007) The presence of more than one type of exosome was suggested. One type may represent exosomes not containing RNA, which might be involved in antigen presentation. The other type, containing RNA could be involved in cellular communication. (Lotvall & Valadi, 2007) Exosomes were shown to transport mRNA

fragments derived from the 3' -ends of mRNAs, which are known to have a regulatory role (Batagov & Kurochkin, 2013).

It was shown that some RNAs are selectively enriched in exosomes, pointing to the controlled sorting of specific RNAs to exosomes. For example, the miRNA let-7 family is enriched in exosomes from metastatic gastric cancer cells. The role of this miRNA is anti-tumorigenic and it was proposed that the role of these exosomes is to maintain the tumorigenic and metastatic properties of these cells. (Ohshima, Inoue, Fujiwara, Hatakeyama, & Kanto, 2010)

The mechanism of RNA sorting into exosomes is still unknown. However, it was shown that RNA induced silencing complex (RISC) is associated with MVB. (Cassidy et al., 2009) It was also shown that endosomes or MVBs are co-localized with miRNA-loaded RISC (Gibbings, Ciaudo, Erhardt, & Voinnet, 2009).

The release of exosomes containing miRNAs was shown to be regulated by ceramide-dependent pathway, specifically by neutral sphingomyelinase 2. Furthermore, the ESCRT-dependent pathway was not shown to be essential for the release of miRNAs. (Kosaka et al., 2010)

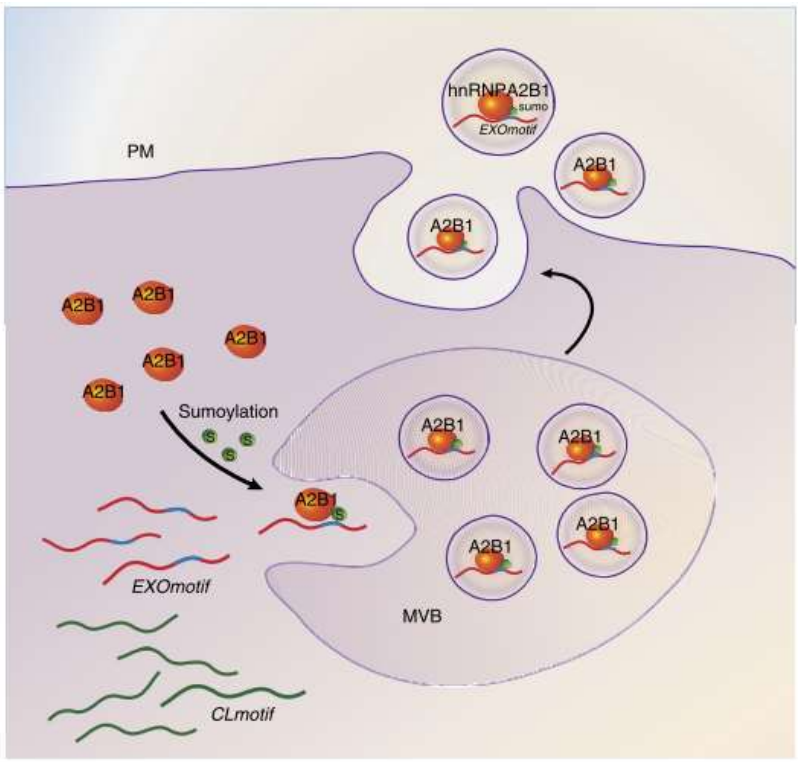

Figure 9: Proposed mechanism of the sorting of miRNA into exosomes. The hnRNPA2B1 after sumoylation, on binds to RNAs with EXOmotif and sorts them into MVB. Legend: A2B1 = hnRNPA2B1, S = SUMO, MVB = multivesicular bodies, PM = plasma membrane, EXOmotif = motif, which binds miRNAs with A2B1, CLmotif = cellular motif. Copied. (Villarroya-Beltri et al., 2013)

The exosomal RNAs are enriched in specific motifs, which were proposed to serve in targeting of RNAs into exosomes. These motifs however negatively correlate with the life time of RNAs, resulting in twice shorter life time than intracellular RNAs. (Batagov, Kuznetsov, & Kurochkin, 2011) Another study also described specific motifs and showed that heterogenous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) that interacts with nascent pre-mRNAs, packaging them into hnRNP particles, binds RNAs through these motifs and controls their loading into exosomes. Furthermore, sumoylation controls hnRNPA2B1-miRNA binding. (Villarroya-Beltri et al., 2013) The proposed mechanism of miRNA sorting into exosomes is illustrated in figure 9.

Next-generation sequencing revealed small non-coding RNA species and other small RNAs, such as Y-RNA, vault RNA, and fragments of tRNA from immune cell-derived

vesicles. The length of these RNAs is in the range of 20-200 nt. (Nolte'T Hoen et al., 2012) Another characterization of RNAs by sequencing revealed the length of these RNAs to be 18-28 nt. The identified RNAs can be found in figure 10. The most abundant were miRNAs, however the second were rRNAs, which were not found in other studies in such a high abundance. These discrepancies could be accounted for a different cell lines or different isolation protocols. (Huang et al., 2013) The characterization of human semen-derived exosomes also showed high abundance of rRNA (Vojtech et al., 2014).

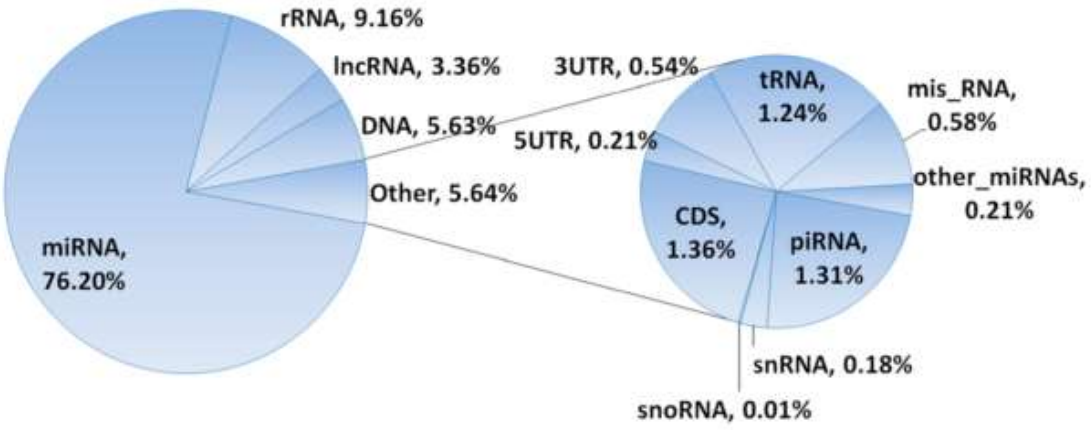

Figure 10: The summary of RNAs identified in plasma-derived exosomes by sequencing. Misc RNAs are the RNA sequences that mapped to the human genome but not in any of the categories listed. The DNA category represents transcripts that have no annotation in the human RNA database. Legend: miRNA = microRNA, rRNA = ribosomal RNA, lncRNA = long non-coding RNA, UTR = untranslated region, tRNA = transfer RNA, piRNA = piwi-interacting RNA, snRNA = small nuclear RNA, snoRNA = small nucleolar RNA, CDS = coding sequences. Copied from (Huang et al., 2013)

The stoichiometric analysis by nanoparticle tracking of exosomal miRNAs was performed. The exosomes were isolated from five different sources. The analysis showed that even the most plentiful miRNAs are present in concentration less than one molecule per exosome, suggesting that individual exosomes do not transfer biologically significant numbers of miRNAs. (Chevillet et al., 2014)

8. Exosomes as disease biomarkers

Since exosomes may contain RNAs, it was proposed, that these exosomes could serve as diagnostic biomarkers.

Exosomes containing mRNA and miRNA characteristic of gliomas were found in the serum of glioblastoma patients, suggesting potential therapeutic usage (Skog et al., 2008). Tumour-derived exosomes containing miRNA were also found in ovarian cancer. Furthermore, the malign and benign disease was proposed to be distinguishable by the levels of 8 specific miRNAs, (Taylor & Gercel-Taylor, 2008) Non-small cell lung cancer was shown to be associated with levels of specific miRNAs, such as let-7f, miR-20b, and miR-30e-3p. These specific miRNAs could serve as circulating tumour biomarkers. (Provencio et al., 2011)

In another study, exosomes from peripheral blood were used as gene delivery vector to transport exogenous siRNA to monocytes and lymphocytes (Wahlgren, Karlson, Brisslert, & Sani, 2012). Exosomes isolated from dendritic cells and loaded with siRNA *in vitro,* were also shown to deliver siRNA to the mouse brain and silence of a wanted gene, suggesting that exosomes could be used as the treatment of brain diseases, since the transport through the blood-brain barrier is complicated (Alvarez-Erviti et al., 2011).

The summary of possible utilisation of exosomes as cancer vaccine is reviewed in (Tan, Peña, & Seifalian, 2010).

9. Isolation of exosomes

Exosomes have been isolated from cell culture medium and bodily fluids. For the isolation of exosomes from the cell culture medium, it is important for the cells to be healthy. Otherwise there could be present apoptic bodies and other cellular contaminants. (Théry et al., 2006) Isolation from plasma is more complicated. Exosomes from plasma are not produced by one cell type, unlike exosomes from medium of cultured cells. Because of this, other

factors must be considered as well. Firstly, plasma-derived exosomes can be of different size, because exosomes could fuse with each other after their release. Secondly, plasma-derived exosomes are surrounded by a high number of proteins in the plasma. Thirdly, biological activity is desired, because exosomes can serve as potential biomarkers of human diseases. (Hong, Funk, Muller, Boyiadzis, & Whiteside, 2016) Despite of this, a precise protocol to how isolate exosomes, in the most effective way, has not yet been described. Several isolation methods have been proposed.

The most common method to isolate exosomes is by differential centrifugation, followed by ultracentrifugation. This centrifugation procedure can be modified. First centrifugation step can be replaced by filtration step. (G Raposo, H W Nijman, W Stoorvogel, R Liejendekker, C V Harding, C J Melief, 1996) However, because the isolation by this method takes a long time and the resulting exosomes can be aggregated and not biologically active, another methods were proposed (Arraud, Linares, Tan, & Brisson, 2015).

An alternative to ultracentrifugation are ultrafiltration devices, which are much faster, and provide higher particle yield. Furthermore, when coupled with size-exclusion chromatography (SEC), this method is comparable to a density gradient purification. It was shown that the best concentrating device is centrifuge-based, when dealing with volumes of 50-200 mL of media. Pressure-driven devices are appropriate when working with volumes of more than 400 ml. (Lobb et al., 2015)

Another isolation method involves trapping exosomes on magnetic beads, which are bearing anti-MHC Class II antibodies. By using this method, B cell marker, CD20, on B cell exosomes was discovered. This method however does not isolate MHC Class II deficient exosomes. (Clayton et al., 2001)

Size-exclusion chromatography is another method used to isolate exosomes. When compared to centrifugation method, SEC method is not lengthy and there is no aggregation of vesicles. Furthermore, biological activity remains intact. The efficiency of this method was examined, as well as separation from high-density lipoprotein, a possible contaminant during ultracentrifugation. It was shown that exosomes with size more than 75 nm can be isolated from human platelet-free medium, as well as from human plasma, by sepharose CL-2B single-step SEC. (Böing et al., 2014)

Comparison of different methods of exosome isolation was performed. Four exosome isolation protocols have been compared including OptiPrep density gradient centrifugation (ODG), ExoQuick (EQ), Total Exosome Isolation (TEI) and ultracentrifugation (UC). Results of these methods were evaluated for yield, size, morphology, and protein and RNA content of isolated exosomes. ODG obtained the purest exosomes. It was shown that ODG method eliminated even albumin contamination. Comparison between ODG and sucrose-based density gradients was not tested. However, it was proposed that the results would be similar. Pros and cons of these four methods are listed in Table I. (Van Deun et al., 2014)

The stability of exosomes after isolation from plasma was tested. The results surprisingly showed that after 90 days, all the samples stored at 4, -20, and -80 °C contained exosomal marker TSG101, indicating stability of exosomes. However, the most stable exosomes were those stored at -80 °C. Biological activity was tested with exosomes stored at -20 °C for 30 days. It was shown that these exosomes were internalised by cancer cells, suggesting that they are indeed active. (Kalra et al., 2013)

Technique	Purity	Exosome yield	Protein yield	RNA yield	Ease-of-use	Turn-around time (h)	Hands-on-time (h)	Cost (€)
UC	••	•••	••	•••	••	4	<1	5
ODG	••••	••••	•	•	•	20	1	15
EQ	•	•	•••	•••	•••	13	<0.5	15
TEI	•	•	••••	••	•••	13	<0.5	5

Table I: Comparison of 4 methods based on different factors. Legend: • = low; •• = moderate; ••• = high; •••• = very high. (Van Deun et al., 2014) UC = ultracentrifugation, ODG = OptiPrep density gradient centrifugation, EQ = ExoQuick, TEI = Total Exosome Isolation.

10. Conclusion

In the present work, the biogenesis and the role of exosomes in organism was summarised. The exosomes were introduced as extracellular vesicles with a typical cup-shaped morphology and endosomal origin.

The biogenesis of exosomes relates to the sorting of cargo into endosomes. The Endosomal sorting complex required for transport pathway and ceramide pathway was shown. These two pathways sort cargo into endosomes. The ESCRT pathway sorts ubiquitin-marked proteins into endosomes and compounds of three complexes, each one with slightly different function. ESCT-I sorts ubiquitinylated cargo into endosomes. ESCRT-II is associated with membrane and promotes the assembly of ESCRT-III, which arranges membrane scission between intraluminal vesicles and membrane of multivesicular body. The function of ceramide pathway is not clear.

The composition of exosomes was also showed in this work. It was showed that exosomes contain a high number of proteins, depending on the cell from which they are derived. Some of these proteins are associated with the biogenesis of exosomes, others are cell specific. The lipidomic studies also revealed difference between the distribution of exosomal lipids and cellular lipids. Exosomes are highly enriched in cholesterol and sphingomyelin. The high

abundance of proteins, lipids and nucleic acids associated with exosomes led to the creation of online databases, where all the data on exosomes are present.

Release of exosomes was demonstrated to depend on the level of intracellular Ca^{2+}. The higher levels promote secretion of exosomes; this was induced by the ionophore monesin or A23187. The Ca^{2+} chelators were shown to induce decrease of secretion of exosomes. The interesting fact was that binding of transferrin to its receptor increases levels of Ca^{2+}, suggesting physiological regulation of secretion of exosomes.

The mechanism of RNA loading has not yet been fully understood. However, the role of hnRNPA2B1 in sorting RNAs into exosomes was proposed. The high abundance of some RNAs and not others point out to the selective sorting of RNAs into exosomes. Exosomes can contain a different RNAs, among the most common are miRNAs and mRNAs, but Y-RNA or fragments of tRNA were also present. The presence of rRNA differs in sequencing studies. The presence of non-coding RNAs was also showed, including pervasive transcripts, RNA cleavage products overlapping with protein coding regions and repeating sequence transcripts.

Because of the fact, that exosomes circulate in the blood, its advantageous to use them as diagnostic markers since exosomes contain different RNAs and these RNAs correspond to the cells from which they were produced. Exosomes could also serve as a drug delivery system. They do not induce inflammation responses because they could be derived from the patient's cells. It was also shown that exosomes can passage through blood-brain barrier, indicating that they could be efficiently used in the treatment of brain diseases.

Concerning exosome isolation, universal protocol is not yet available. The most used isolation technique is ultracentrifugation. Since the isolation greatly influences the specificity of exosomes and their RNA content, it is imperative to introduce new isolation methods and create a universal protocol for isolation of exosomal RNAs.

Evidence is growing that exosomes play important roles in viral infections. Since biogenesis of exosomes and viruses is closely connected, the viral exploitation of this pathway is at hand. Particularly, retroviruses exploit this pathway and that is why the Trojan exosome hypothesis has been proposed. This hypothesis proposes the exploitation of cellular vesicle trafficking and exosome exchange by retroviruses for the biogenesis of their retroviral particles and as tool for infection. (Gould, Booth, & Hildreth, 2003) It was shown that exosomes secreted by infected cells contain many regulatory factors, including viral RNAs and proteins. For example, genome of HCV is secreted within exosomes. HAV exploit endocytic pathway by acquiring a host-derived membrane for the transmission between cells. These coated capsids are less susceptible to antibody neutralisation. As summarised in (Anderson, Kashanchi, & Jacobson, 2016). Other example includes Epstein-Barr virus, which loads miRNAs into exosomes to suppress EBV targeting genes (Pegtel et al., 2010).

It is clear, that exosomes are used by variety of viruses and that this usage is constantly exanimated.

In my opinion, the biggest problem of specific EVs, such as exosomes, from the high abundancy of different EVs is the isolation. There is a clear need of unified protocol for isolation of exosomes. Different isolation protocols lead to discrepancies in some results of above-mentioned chapters. Different studies present different sizes of exosomes which vary significantly. Size discrepancies are in a tight relation with exosomal markers. Some of these markers are in high probability not only exosomal but of other EVs as well. Optimal isolation protocol is also needed for the isolation of exosomal RNAs. For example, studies about whether exosomes contain ribosomal RNA or not vary. However, the majority of studies present that exosomes do not contain any ribosomal RNA. The unified and optimized isolation protocol could remove at least some of the confusion.

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