Intercellular communication via exosomes

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ABSTRACT

Exosomes are small membrane bound vesicles between 30-100 nm in diameter of endocytic origin that are secreted into the extracellular environment by many different cell types. They play a role in intercellular communication by transferring proteins, lipids and RNA to recipient cells. The overall aim of this work has been to further investigate the mechanisms by which cells communicate with each other via exosomes.

In Paper I we hypothesized that exosomes from human cells could be used as vectors to provide cells with therapeutic RNA. Herein, exogenous short interfering RNAs were successfully introduced into various kinds of human exosomes using electroporation. Flow cytometry, confocal microscopy and northern blot confirmed the presence of siRNA inside the exosomes. The results showed that exosomes from blood plasma could deliver the siRNA to human monocytes and lymphocytes. The siRNA delivered to the target cells was shown to be functional causing selective gene silencing of mitogen activated protein kinase 1. Our results imply that exosomes from human cells could be used as vectors for delivery of therapeutic exogenous nucleic acids to cells.

In paper II we investigated if exosomes from activated CD3+ T cells could play a role in an immunological response by conveying signals from their secreting cells to recipient resting T cells in an *in vitro* autologous setting. The role of these exosomes was explored in IL-2 mediated T cell proliferation. The results showed that neither exosomes nor IL-2 alone could stimulate proliferation in resting T cells. However, exosomes from stimulated T cells together with IL-2 were able to induce proliferation. T cell cultures stimulated with exosomes and IL-2 showed a higher proportion of CD8+ T cells than cultures without exosomes. Moreover, a cytokine array showed significant changes in the levels of cytokines and chemokines when exosomes were present. The results indicate that activated CD3+ cells communicate with resting autologous T cells via exosomes.

The main focus in paper III was to study the cellular mechanism by which esRNA is selectively packaged into exosome vesicles during their biosynthesis. Using RNA gel mobility shift assay, we showed the presence of RNA-binding proteins (RBPs) in exosomes. Moreover, we developed a method for the identification of exosomal RBPs able to bind to the esRNA and cellular microRNA. Using this method, we could identify 31 different RBPs in exosomes and 78 in cells. To evaluate the possible role of the identified RBPs in the transfer mechanism of RNA into intraluminal vesicles, five gene transcripts from the identified RBPs were silenced. The results revealed that a selective gene silencing of hnRNPA2B1 caused a reduction of RNA present in the extracellular vesicles. Thus, a novel transport mechanism was suggested for the packaging of esRNA into the exosomes.

In conclusion, the studies presented in this thesis have implications for better understanding the RNA and protein transfer mechanism that occurs between cells via exosomes. The described ability of exosomes to deliver exogenous nucleic acids to cells may be of interest in clinical applications e.g. in gene therapy.

Keywords: exosomes, electroporation, RNA, IL-2, RNA binding proteins

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Populärvetenskaplig sammanfattning på svenska

Kommunikation mellan celler sker kontinuerligt genom utsöndring av olika molekyler och genom direkt kontakt mellan celler. Cellerna utsöndrar även så kallade exosomer som har visat sig vara ytterligare ett sätt för celler att kommunicera. Exosomer är små vesiklar som kan liknas vid membranomslutna bubblor. De är i samma storleksklass som ett virus, ca 30-100 nanometer. Exosomerna bildas genom en specifik mekanism inne i cellen och den slutar med att exosomerna utsöndras från cellen. Olika studier har visat att de flesta celltyper såsom cancerceller och blodceller utsöndrar exosomer. Exosomer har även hittats i olika kroppsvätskor som till exempel urin, blodplasma, saliv och bröstmjölk. Sedan den första upptäckten av exosomer i början av 1980-talet har det påvisats att de har flera funktioner bland annat i kommunikationen mellan cellerna i immunsystemet och även mellan cancerceller. Det finns även studier som påvisar att exosomer har betydelse i normala fysiologiska processer som till exempel vid graviditet för att skydda fostret från att bli bortstött.

Exosomer innehåller flera typer av biomolekyler däribland olika proteiner och genetiskt material i form av RNA och medger därför en mer komplex typ av kommunikation mellan celler. Upptäckten av RNA i exosomer har öppnat upp möjligheten att använda exosomernas RNA som biomarkörer för att kunna upptäcka vissa sjukdomar.

Exosomernas RNA-innehåll ligger till grund för de undersökningar som gjorts i delarbete I i den här avhandlingen. Här var målet att kunna utnyttja exosomernas förmåga att transportera RNA för att i stället transportera syntetiska RNA-molekyler som skulle kunna påverka mottagarceller. Resultaten visade att det gick att föra in syntetiskt RNA i exosomer från blodplasma med hjälp av en metod som kallas elektroporering. Elektroporering innebär att en elektrisk puls öppnar upp exosomens membran för en kort stund så att RNA molekylerna kan komma in. Resultaten visade att de RNA-laddade exosomerna kunde tas upp av blodceller från människa och påverka bildningen av ett visst protein i blodcellerna. Möjligheten att kunna föra in syntetiskt genetiskt material i exosomer innebär att de skulle kunna användas som bärare av genetiskt material för att stänga av den sjukdomsalstrande molekylen i en cell.

Exosomer utsöndras också från olika typer av immunceller och påverkar immunförsvaret. I delarbete II låg fokus på att undersöka hur exosomer från en grupp av immunceller som kallas T-celler påverkar och kommunicerar med andra T-celler. Resultaten visar att T-cells-exosomerna påverkarade andra T-celler främst när de var

tillsammans med en stimulerande molekyl vilket tyder på att de kan vara inblandade i kommunikationen mellan T celler vid ett immunsvar.

För att kunna använda exosomer som bärare av syntetiskt terapeutiskt genetiskt material såsom RNA till människor med olika sjukdomar behöver exosomerna designas så att de endast för över det material som är nödvändigt för just den sjukdomen. Ett steg på vägen är att komma på ett sätt att tömma exosomerna på deras inneboende genetiska material. För att kunna göra det behöver mekanismen för hur RNA-molekyler packas in i exosomerna klargöras. Ett första steg i den riktningen har påbörjats i delarbete III där en metod för att påvisa vilka proteiner i cellen som hjälper till att föra in RNA i exosomerna utvecklades. De här RNA-bindande proteinerna har normalt ansvar för att transportera RNA ut i cytoplasman från cellkärnan. Resultaten i delarbete III visar att flera av de här proteinerna även spelar en roll i transporten av RNA in i exosomerna.

Sammanfattningsvis bidrar den här avhandlingen till att ge ytterligare kunskap i hur celler tar emot och skickar information via exosomer.

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LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Wahlgren J*, Karlson T*, Brisslert M, Sani F, Telemo E, Sunnerhagen P, Valadi H.
Plasma exosomes can deliver exogenous short interfering RNA to monocytes and lymphocytes.
Nucleic Acids Research, 2012 Sep 1; 40 (17).
**These authors contributed equally*

II. Wahlgren J, Karlson T, Glader P, Telemo E, Valadi H.
Activated human T cells secrete exosomes that participate in IL-2 mediated immune response signaling.
PLoS One, 2012; 7 (11).

III. Statello L, Wahlgren J, Ragusa M, Sunnerhagen P, Purello M, Valadi H.
Exosomes contain RNA-binding proteins involved in the transfer mechanism of esRNA
In manuscript.

Content

Abbreviations

CD	Cluster of differentiation
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DLS	dynamic light scattering
EBV	Epstein Barr virus
ESCRT	endosomal sorting complex required for transport
esRNA	Exosomal shuttle RNA
FDC	Follicular dendritic cell
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Hsc	Heat shock cognate
ILVs	Intraluminal vesicles
ISEV	International Society for Extracellular Vesicles
LFA	Lymphocyte function-associated antigen
MAPK	Mitogen activated protein kinase
MHC	Major Histocompatibility complex
MIIC	MHC-class-II enriched compartment
miRNA	microRNA
mRNA	Messenger RNA
MVB	Multivesicular body
NC	Non coding
NK	natural killer
NTA	nanoparticle tracking analysis
PBS	phosphate buffered saline
RANTES	regulated upon activation, normal T cell expressed and secreted
RISC	RNA induced silencing complex
RNAi	RNA interference
rRNA	Ribosomal RNA
SIOS	scanning ion occlusion sensing
siRNA	Short interfering RNA

SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
snRNA	Small nuclear RNA
TCR	T cell receptor
tRNA	Transfer RNA
Tsg101	Tumor susceptibility gene 101 protein
UTR	Untranslated region

Introduction

Intercellular communication occurs through secretion of molecules or by direct contact between cells. In addition, cells release nano-sized membrane enclosed vesicles of different origin. For example, vesicles shed from the plasma membrane referred to as microvesicles or vesicles secreted via the endosomal pathway termed exosomes. These extracellular vesicles are believed to encompass an additional way for intercellular communication and they have been implied to act on cells both at a distance and in the vicinity (1).

Extracellular vesicles such as exosomes allows for a more complex way of intercellular communication compared to single secreted molecules since they are composed of several bioactive molecules such as proteins, lipids and nucleic acids (2). Secretion of exosomal vesicles from late endosomal compartments to the extracellular environment was first described almost 30 years ago (3,4). These studies indicated that the role of exosomes was solely an alternative way to remove unwanted proteins from cells. However, almost a decade later exosomes were shown to have a stimulating role in the immune response (5). This finding inspired a new interest in the field of exosome research and resulted in several reports showing the relevance of exosomes in intercellular communication through their shuttling of bioactive cargo (2,6,7).

Exosomes are known to be secreted by most cell types including tumor cells (8), antigen presenting cells (5,9), T cells (10), stem cells (11) and epithelial cells (12). In addition, exosomes have been purified from most biological fluids such as urine (13), plasma (14), and breast milk (15). Their secretion from most cell types and their presence in most body fluids supports the possible function of exosomes in intercellular communication and consequently a function in normal physiological processes. Moreover, exosomes have been implied to have a role in cancer as well as in the spreading of pathogens such as viruses and prions (16-18).

Given that exosomes seem to be secreted by a majority of cell types both during healthy and disease conditions it is crucial that this signaling is tightly regulated in order to maintain homeostasis. Therefore, elucidation of the molecular mechanisms that underlie the secretion and uptake as well as targeting of exosomes to different cell types is fundamental to our understanding of exosome mediated cellular functions in both health and disease. Furthermore, this knowledge would aid in the development of exosomes engineered for use as therapeutic delivery vesicles.

Exosomes and extracellular vesicles

Extracellular vesicles

Apart from exosomes other types of extracellular membrane vesicles can be released from cells. These vesicles are categorized into two main groups: apoptotic bodies and microvesicles. In contrast to exosomes, which are formed in the endosomal pathway, apoptotic bodies and microvesicles are formed by outward budding of the plasma membrane of the cell. Furthermore, both microvesicles and apoptotic bodies have a different molecular composition with regards to membrane proteins and lipids. They also differ in size where apoptotic bodies range between 50 nm to five micrometer and microvesicles have a size range of 100 to 1000 nm (2,19,20). Interestingly, both microvesicles and exosomes have been shown to deliver proteins, microRNA (miRNA) and messenger RNA (mRNA) to cells and to exert a function in recipient cells (21).

Compared to microvesicles the exosomes are generally smaller, between 30-100 nm in diameter. They are formed inside the cell as a part of the endosomal pathway, which results in the formation of multi-vesicular bodies (MVB). The exosomal vesicles are referred to as intraluminal vesicles (ILVs) as long as they reside inside the MVBs. When a MVB fuse with the outer membrane the exosomes are released into the extracellular space (22). The first observations of extracellular vesicles formed via the endosomal pathway were made in the early 1980s by the groups of Johnstone and Stahl during ultra-structural studies of the transferrin receptor (3,4,23).

To enable high quality results in exosome research it is important to be able to separate different types of extracellular vesicles. There are currently no efficient methods to separate microvesicles from exosomes. Although the smaller exosomes will only pellet at high speed centrifugation $\geq$ (100 00 x g) there is evidence that smaller vesicles may bud from the plasma membrane which indicate an overlap in size (24). However, the microvesicles are usually of greater size between 0.1 – 1 µm and are pelleted at lower centrifugation speed (10 000-20 000 x g). Furthermore, microvesicles have a different protein and lipid composition than exosomes since they are formed from different compartments of the cell (2,25,26).

There has been a great increase of research interest in the field of secreted membrane vesicles in recent years. In addition, during the development of the field many different types of vesicles have been described and named with regards to their cell of origin for example ectosomes (shed from neutrophils), cardiosomes (derived from cardiomyocytes) and prostasomes (1,27-29). In addition, when reviewing the literature some studies use the term microvesicles when the method of isolation will

include exosomes and vice versa. The invention of explanatory names in this way may serve a purpose in a specific field but it may as well cause confusion in the general field of extracellular vesicles. Hence there is a need for more commonly defined terms specifying if the vesicles studied are of endosomal or plasma membrane origin. With this in mind it was unfortunate that, during the first meeting of the International Society for Extracellular Vesicles (ISEV) in 2012 the research community could not reach consensus regarding nomenclature. Nevertheless, suggestions were made for investigators to declare the definition of the term and the related methods used for handling and purification of the vesicles (30).

Exosomes

Exosomes are spherical membrane vesicles limited by a lipid bilayer membrane enclosing a content of both proteins and RNA. In general the exosomes contain proteins involved in membrane trafficking such as Rab GTPases and annexins they also comprise proteins involved in MVB formation such as Alix and Tsg101. In addition, exosomes also carry tetraspanins e.g. CD9, CD63 and CD81 commonly found in lipid micro-domains in limiting membranes. This protein content reflects the endosomal origin of the exosomes (31,32). Other proteins commonly found in exosomes from many cell types includes heat shock proteins e.g. Hsp90, cytoskeletal proteins e.g. myosin and proteins associated with metabolism e.g. GAPDH (33). Additionally, the protein composition in exosomes depends on their cell of origin e.g. exosomes from antigen presenting cells are enriched in MHC class I and II (33).

Moreover, exosomes have a characteristic lipid composition which appears to be in some parts dependent on their cell of origin, which was shown when performing lipid analysis of exosomes from several cell types (34,35). A typical lipid feature appears to be an enrichment of lipid raft associated proteins including cholesterol, sphingolipids and ceramide (36,37).

Furthermore, exosomes contain different types of RNA including messenger RNA (mRNA) and micro RNA (miRNA) and compared to their cell of origin the exosomes are highly enriched in small miRNAs (7). The RNA content of exosomes will be described in more detail further down in this thesis.

Biogenesis of exosomes

Until now two routes for protein secretion from eukaryotic cells have been recognized. One involves a specific stimulation triggered release of storage granules and the other encompasses the exocytosis of vesicles constitutively secreted from the cell (38). However, three decades ago several independent experiments confirmed

that the endocytic pathway is another route for secretion of proteins and thereby described a third, now widely accepted, secretion pathway from cells. More specifically it was shown that vesicles confined in endosomal compartments of the cell, termed multivesicular bodies (MVBs), could be released from the cells by fusion of the MVB outer membrane with the plasma membrane (Figure 1). This pathway was first described in reticulocytes in 1983 by Pan et al and was confirmed by others in several cell types in the next decade (5,23,39-42).

The MVBs are formed within the endosomal system, which consists of primary endocytic vesicles and early and late endosomes often fused with lysosomes (43,44). Endocytosis begins with invagination of the plasma membrane where the cell membrane and cytosolic components are sorted into endocytic vesicles (45). This process is thought to be either dependent or independent of clathrin (46). Endocytosed material enters mainly via early sorting endosomes. Inside the cells the early endosomes mature into late endosomes by fusion with deposition tubules or other vesicles e.g. lysosomes. This leads to a maturation process involving change in contents by recycling of proteins, acidification and partial degradation. A process of inward budding of the outer membrane of the late endosome results in the gradual accumulation of intraluminal vesicles (ILV) forming the MVB. Since the MVBs form through inward budding of the limiting membrane of endosomes the resulting ILVs will contain parts of the cytosol (23,47-49). The budding vesicles incorporate lipids and membrane proteins from the MVB membrane and often the sorted proteins are targeted to lysosomes for degradation e.g. for the elimination of growth factors (50-52). However, in exosome biogenesis the MVBs escape degradation and travel to the plasma membrane where they fuse with the membrane and release the ILVs into the extracellular space, which are then referred to as exosomes (53). The late endosomes and MVBs are usually situated close to the nucleus (54-56).

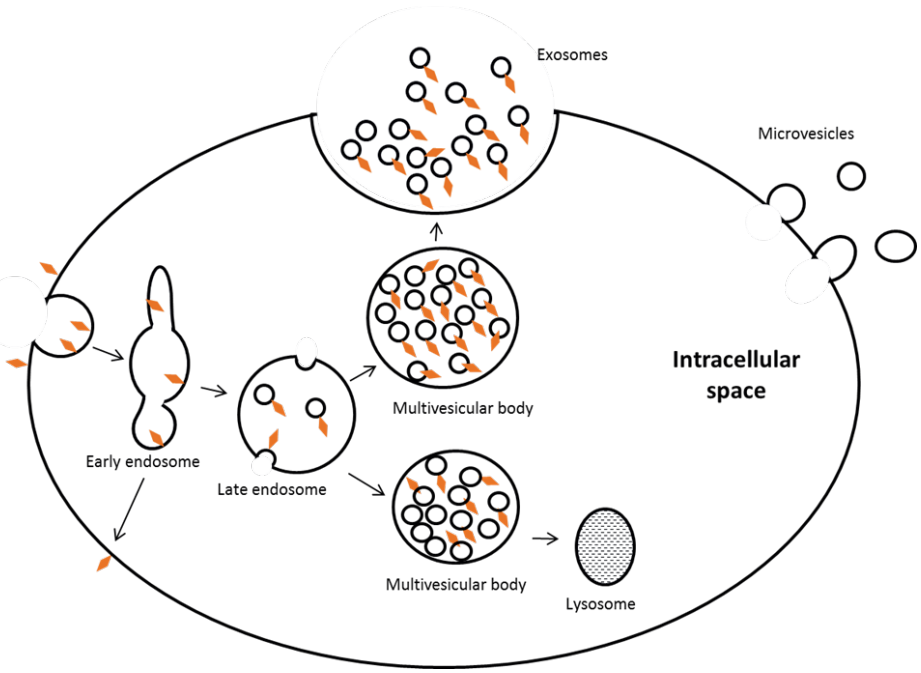

Figure 1. Membrane proteins (shown in orange) are internalized through invagination of the plasma membrane and endocytic vesicles form early endosomes. Early endosomes may recycle the membrane proteins to the plasma membrane or keep them inside the endosomes. From the endosomes intraluminal vesicles may be formed by budding from the limiting membrane into the lumen of endosomes. The vesicle containing endosomes are called multivesicular bodies and they may follow a degradation pathway by fusing with lysosomes or they may fuse with the plasma membrane in which case the internal vesicles are released into the extracellular medium as exosomes. Contrary to exosomes microvesicles are formed through shedding from the plasma membrane of the cell. Image adapted from (22,33,57,58).

At present the exact mechanism of exosome formation in MVBs is not fully understood. However, the sorting of proteins into exosomes may in some cases involve the endosomal sorting complex required for transport (ESCRT) (59). The ESCRT comprises of several protein complexes termed ESCRT- 0 to III and they are thought to recruit cargo to the endosomal membrane for sorting into ILVs and this usually involve ubiquitination of selected proteins. This process begins with adhesion of the ESCRT-0 to proteins bound to the regulatory protein ubiquitin. ESCRT-1 is then recruited through binding of its TSG101 subunit to the ubiquitinated cargo. This, in turn activates ESCRT-II which aids in the formation of the ESCRT-III.

Finally, MVB proteins are closed off and ubiquitin is removed by an enzyme before incorporation of the proteins into ILVs (60,61).

Although it is more apparent that the ESCRT system is required in the lysosomal pathway, the role of ESCRT and accompanying ubiquitination in exosome biogenesis is not established. There are reports of both ESCRT-dependent (62,63) and –independent sorting (64) in exosome formation. Of course, this could also indicate several pathways for protein sorting into exosomes as well as different sorting mechanisms in different cell types. One example of an ESCRT independent pathway was implied in a study showing that stimulation of sphingomyelinase resulting in ceramide formation may stimulate the budding of exosomes and their release (35).

Apart from proteins and lipids, different RNAs appear to be loaded into exosomes by, as of yet unknown, sorting mechanisms. This is indicated by the observations that the RNA content in exosomes and their donor cells differ significantly (7). Interestingly, ESCRT-II was early on proposed to be an RNA-binding complex (65) indicating an involvement in the packaging of RNA into the ILVs. Furthermore, it has been shown that miRNA seem to be incorporated into exosomes and then released through ceramide formation (66). There are also indications that RNA interference takes place close to the MVBs (67). RNA and exosomes will be further discussed in a later chapter in this thesis.

Exosome secretion

Exosomes are secreted by fusion of MVBs with the plasma membrane and this was first shown in electron microscopy studies of maturing reticulocytes (3,4). Moreover, studies using fluorescently tagged MHC class II contained in MVBs showed fusion of the MVB with the plasma membrane when visualized with confocal microscopy (68).

The main regulators of specific steps in membrane trafficking are the Rab GTPases that are localized on the cytosolic side of vesicular membranes inside the cell (69). Interestingly, small GTPases pertaining to the Rab protein family seem to play a major role in intracellular trafficking by regulating the docking and fusion of membrane compartments. Consequently, this system is involved in the endosomal pathway that results in exosome release (70). More specifically, studies showed that an increase of calcium together with an overexpression of Rab11 increased exosome secretion (71,72). Moreover, Rab27a has been implicated in exosome secretion since its inhibition led to a decrease in release of exosomes (73), and in one study it was suggested that Rab35 facilitated the secretion of exosomes by involvement in the docking to the plasma membrane (74).

Furthermore, several studies suggest that MVB fate is determined by soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) aiding in the docking and fusion within the transport machinery of the cell (75-77). A combination of SNAREs has been proposed in the fusion of MVBs with the plasma membrane. where vesicular SNAREs found on the MVB membranes form a complex with target SNAREs localized at the plasma membrane (78). The participation of specific SNAREs in MVB fusion has also been reported in an erythroleukemia cell line (79).

In addition, other proteins that have been associated with exosome release include proteins involved in cell stress such as p53, which regulates the transcription of a gene associated with exosome biogenesis (80,81). Finally, cellular stress conditions such as hypoxia and changes in pH has been shown to influence exosome secretion (82,83).

Isolation and characterization of exosomes

Isolation

There are several methods for isolating exosomes however there are still no isolation methods that can guarantee total purity of the yielded vesicles. Exosomes are usually isolated from body fluids and cell culture supernatant by the use of differential centrifugation. Commonly the isolation begins with a low speed centrifugation step to remove cells followed by centrifugation at a higher speed, usually between 10 000 and 20 000 x g, to remove cell debris and other cell components. Finally, an ultracentrifugation step is applied in order to pellet the exosomes, usually at speeds between 100 000 x g and 120 000 x g. Additionally a filtering step, usually through a 0.2 μm filter may be included before the final pelleting to remove any remaining large membrane vesicles or aggregated proteins (7,84). In addition, when bovine serum is used in cell culture medium it is important to remove membrane vesicles including exosomes potentially contained in the serum first. Since serum has a slightly higher viscosity than e.g. growth medium it is recommended to increase the time or speed of the ultracentrifugation (85,86). It is important to be aware that differential centrifugation method will pellet other vesicles of similar sizes that may not be of endosomal origin. Even though this isolation method has the drawback of pelleting other vesicles as well, it has been the most frequently used and hence it can be considered as a benchmark for comparison with other isolation methods (30).

To obtain an even purer exosome isolate it is possible to perform an extra washing step in phosphate buffered saline (PBS) or a density gradient using a sucrose cushion. The density gradient will separate all lipid based vesicles and consequently any protein aggregates that might have formed will be excluded from the isolate (84). In a

recent study it was shown that the washing step did not remove the co-pelleted contaminating proteins. However, the sucrose gradient resulted in the most pure vesicle preparation when compared to ultracentrifugation with less amount of contaminating proteins (87). Exosomes may also be isolated using immunoaffinity capture with antibody coated beads (88). However, since the exosome population is heterogeneous and varies between cell types this method could result in isolation of only a subpopulation of the exosomes (84,88,89). Exosome isolation has also been performed using filtration methods (90) and additionally by the use of a microfluidics device coated with antibodies directed against exosome specific proteins, which could be especially useful for small samples (91). Finally, along with the rapidly increasing interest in the field of exosomes several kits have been launched and are available on the market that promises quick and easy isolation methods. These kits should be used with caution since they purify extra cellular vesicles of all sizes including membrane-free macromolecular aggregates (58). The ability to generate pure exosome isolates is of importance to generate data of high quality. Therefore is it also of great importance to characterize the purity of the isolated exosomes.

In all parts of this work (paper I, II and III) the isolation of vesicles of endosomal origin we refer to as exosomes have been performed with differential centrifugation in three steps both from cell culture medium (paper I, II and III) and plasma diluted 1:1 (paper I). First, one low centrifugation step (300-3 000 x g) is applied depending on cell type and whether the cells were going to be saved for further experiments (300 x g) or not (3000 x g). The second step includes one high speed centrifugation step (16 500 x g) for removal of additional cell compartments such as mitochondria and cell debris. The second step is followed by filtering through a 0.2 μm filter for removal of any additional larger vesicles or cell compartments. It should be noted that exosomes might be lost here if they have formed aggregates larger than the pore size of the filter i.e. 200 nm. Finally, the exosomes are pelleted by ultracentrifugation (120 000 x g). This protocol has been used consistently in all projects (Paper I, II and III) and has been used by other investigators (7,92-94). The exosome pellets herein were dissolved in appropriate buffer depending on downstream application i.e. cytomix buffer (95) when used in electroporation (Paper I) and phosphate buffered saline (PBS) for pulsing cell cultures (Paper II) or for storage and in Trizol for RNA isolation (Paper III). The isolated exosomes were stored at -80 degrees Celsius for long time storage or at -20 for short time storage. It has been shown that the stability of exosomes is not influenced when frozen or by repeated freeze thaw cycles (96).

Characterization

Estimating the purity of the exosome isolate is of essence for downstream experiments in order to make sure that a specific property is associated with exosomes and not to co-purified proteins or larger vesicles. The most common approach for this is to characterize size, morphology and presence of exosome associated membrane proteins. To confirm morphology and size of exosomes electron microscopy may be used which also can reveal presence of other vesicle types in the isolate. The morphology of exosomes visualized by this method was reported to be cup-shaped but this was later described to be due to the experimental preparation and it is not seen in cryo-electron microscopy which reveals round shaped vesicles (2,26,30,97). Furthermore, electron microscopy does not reveal the whole content but only a snap shot of the exosome isolate. In addition, electron microscopy is not suited for everyday use since not all investigators have access to this delicate instrument (87). Exosomes may also be characterized by their density determined by a sucrose gradient. Exosome densities have been described to range between 1.13 and 1.19 g/ml depending on cell of origin (5,31,86,98).

Furthermore, in recent years several alternative methods have emerged to measure the size of exosomes including dynamic light scattering (DLS) (used in paper I, II and III) which measures the diffusion of particles in a suspension based on their Brownian movement which cause different scattering intensities when hit by laser light and with a calculation formula particle size can be analyzed (99). This principle is also employed in nanoparticle tracking analysis (NTA) however, here each particle can be tracked and measured and is therefore preferred for measuring exosome samples known to have a range of vesicle sizes whereas DLS is to prefer for monodisperse samples (87,96,100,101). Recently, scanning ion occlusion sensing (SIOS) have emerged as an alternative or complement to analysis of the size distribution. In SIOS the vesicles are focused through nano-pores and the size measurement is related to the change in the ionic current flow when the vesicle is passing (102). These methods offer a more facile and perhaps more accessible way for measuring the size distribution and in the case of NTA quantification of the whole exosome isolate which cannot be obtained by electron microscopy.

For a complete characterization of extracellular vesicles, the presence of proteins commonly found in exosomes should be determined including tetraspanins e.g. CD9, CD63 and CD81 (paper I, II and III), Tsg101, Alix and/or Hsc70. The protein markers can be measured either with western blot (84) (Paper I), immunoelectron microscopy (5) or by using flow cytometry where the exosomes need to be attached to beads due to the limited detection of small particles in flow cytometers. For flow cytometry different strategies may be employed such as direct adsorption of the exosomes to latex beads or by first coating the latex beads with antibodies against an

exosome specific surface protein and then capture the exosomes by immunoaffinity (84) (Paper I and II). Purity assessment may also be performed using for example calnexin as a negative marker to make sure no contamination of vesicles from the ER is present in the isolate (84,87). Interestingly, it has recently been shown that exosomes can be detected directly without attaching them to beads with a custom made flow cytometer (103).

Finally, exosome purity may also be roughly determined by their RNA profile. By using a gel electrophoresis or a gel on chip system the exosomal RNA profile is characterized by the lack of ribosomal RNA (rRNA) and the presence of large amount of small RNA when compared to the cellular RNA profile which mostly consists of ribosomal RNA (approximately 80%) (7) (Paper III).

Exosome function

Exosomes are released from most cell types and they may either enter biological fluids e.g. blood and carry their content for long distances or they may stay in the vicinity of the secreting cell. They are implicated both in maintenance of homeostasis and disease, where they can influence for example proliferation, inflammation and tumor progression (1).

Furthermore, exosomes may help cells to communicate in different ways. By direct cell surface interactions exosomes might stimulate other cells and by carrying several ligands they are able to bind to different cell surface receptors at the same time. Additionally, they may load their cargo onto the recipient cell either by binding or fusion which then might confer new surface or cytosol protein molecules (104). Exosomes might also be an alternative to the lysosomal pathway to remove proteins that are resistant to lysosomal degradation (104). There are several different ways for exosomes to interact in a functional way with cells and to participate in the homeostasis of normal physiology. It may be through activation of cell surface receptors, fusion and incorporation of membrane components with the plasma membrane, and also through delivery of effector molecules into recipient cells such as infectious particles, mRNA, miRNA, non-coding RNAs, oncogenes and transcription factors (1,7,16).

Exosomes appear to play a role in the tissue development of *C. elegans*, which was connected to the development controlling protein Hedgehog in the exosomes (105,106). Furthermore, there are indications that exosomes from stem-cells play a role in tissue regeneration e.g. after myocardial ischemia (11). More specifically the

results showed that exosomes isolated from mesenchymal stem cells (MSCs) are able to reduce injury in myocardial ischemia and might be a therapeutic option in treating myocardial infarction (11,107). Interestingly, MSCs have already been set up for large scale production of exosomes which is necessary for use in further development of experimental methods and translation to a clinical setting (108). MSC derived exosomes offer an appealing alternative to overcome problems associated with stem cell transplantation such as deviant differentiation. Moreover, in the field of regenerative medicine there are results indicating that stem cells secrete exosomes that inhibit apoptosis, stimulate proliferation and promote neovascularization and thereby protect the developing cells (107).

Exosomes from tumors might have immunosuppressive effects. One example of this is that melanoma cells can secrete exosomes carrying FasL that induce T cell apoptosis, which could protect the tumor from being attacked (109,110). Exosomes have also been implicated in cancer progression by the induction of tumor specific suppressor cells (111). In addition, exosomes from tumor cells have been shown to inhibit lymphocyte proliferation (112).

Infectious pathogens like viruses can take advantage of exosome properties to infect cells. One study reports how exosomes from immature dendritic cells mediate HIV infection by transferring the virus particles to CD4+ T cells via the endosomal pathway and in this way the virus avoided detection by the innate immune system (113). Moreover, cells infected with Epstein-Barr virus (EBV) have been shown to release functional EBV miRNA in exosomes which is taken up both by cells in the vicinity and cells further away and may increase infection spread (114). Furthermore, exosomes have also been shown to carry prions and release them into recipient cells (18,115). In a similar way exosomes from neuronal cells are implicated in Parkinson´s disease since they have been shown to carry a protein associated with the spreading of the disease (116) (117). In short, exosomes are exploited by many different cell types and pathogens by loading them with their disease framing cargo and thereafter taking advantage of their ability to relatively un-noticed travel between cells.

The discovery of the array of different functions of exosomes has prompted an interest for using them as biomarkers for several diseases. This may be done by harvesting exosomes from easily obtained biological fluids such as plasma and urine. Since exosomes are cell derived it is predicted that they will reflect the disease specific patterns regarding protein and RNA content, which makes them interesting as diagnostic tools (118,119). Exosomes isolated from urine have already been used to diagnose prostate cancer (120).

Exosome function in immunology settings

Exosomes may be used in immunotherapy since they are known to play a natural role in immune function, which has been reported for both cancer and infectious diseases. The initial study by Raposo et al in 1996 elegantly showed that B cell exosomes carrying MHC class II with antigen peptide in place could stimulate T cell responses opened up this avenue (5). The involvement of exosomes in immune response have made them interesting candidate in cancer immunotherapy (121,122), and Zitvogel reported in 1998 that mouse dendritic cell derived exosomes could be used as a vaccine and suppress tumor growth in a T cell dependent manner (42). The anti-tumor effect was mediated by CTLs, but the effect on CTLs was not seen *in vitro* which indicates that there is no direct interaction between exosomes and CTLs. It has been suggested that these exosomes transport antigens from immature dendritic cells in the periphery to recipient dendritic cells and sensitize them for T cell stimulation (38).

B cell exosomes

Almost a decade after the first studies revealing exosome release as a way of removing molecules that are no longer needed for erythrocyte differentiation, a similar mechanism was described for the first time in B cells (5). More specifically it was shown that B cells contain late endosomal compartments known as MHC-class-II enriched compartments (MIICs), resembling MVBs found in other cell types (123-125). The MIICs could fuse with the plasma membrane and release peptide bound MHC class II molecules confined to exosome vesicles. Interestingly, the study revealed that the exosome proteome differed to a great extent from the protein content of the plasma membrane of the B cell. More importantly, the MHC class II positive exosomes stimulated an antigen specific CD4+ T cell response *in vitro* which suggested that these exosomes could have a role in antigen presentation *in vivo*. The authors speculated that these exosomes might aid in the maintenance of long term T cell memory or tolerance by transporting peptide bound MHC class II (5). Worthy of note is that most studies on B cell derived exosomes use EBV transformed lymphoblastic cell lines secreting high yields of exosomes shown to contain EBV proteins, miRNA and integrins for adhesion (17,114,126). In contrast, primary B cell only secrete small amounts of exosomes although the secretion can be stimulated by growing them together with T cells or IL-4 together with a stimulating CD40 antibody (127,128).

Dendritic cell exosomes

The finding that B cells secrete exosomes with antigen presenting properties was soon followed by reports that dendritic cells could secrete antigen presenting exosomes. As mentioned above one of the first reports showed that DC derived exosomes could eradicate tumors in a mouse model (42). Furthermore, it was

reported that these exosomes could directly activate CD8+ T cells (129-131). However, others reported an indirect activation requiring the presence of DCs for initial uptake and subsequent presentation of the antigen to the T cells (132-134). DC derived exosomes have also been shown to stimulate CD4+ T cells but they need to be processed by recipient DCs to activate naïve CD4+ T cells (135,136). Additionally, mature DCs appeared to be more immunostimulatory than immature DCs in these cultures, probably due to the increased presence of co-stimulatory molecules and MHC class I and II molecules on mature DCs (137,138). Moreover, DC derived exosomes have also been shown to have a tolerogenic effects such as reducing inflammation in a mouse arthritis model (139). Finally, it should be noted that so called follicular dendritic cells (FDCs) that lack MHC class II expression do not secrete exosomes but they have exosome like vesicles on their surface. This is probably a mechanism in order for the FDCs to obtain MHC-class II molecules to their plasma membrane, which enable them to activate helper T cells (140,141). These locally activated T cells may help B cells interacting with antigens carried by the FDC and enable isotype switching and formation of memory B cells (38).

T cell exosomes

Exosomes are secreted from most immune cells and initial observations of secreted vesicles from T cells showed that they contained apoptosis inducing ligands including FasL and APO2L/TRAIL, which could play a role in preventing immune damage by spreading the T cell death signals (142-144). One of the first studies that characterized T cell derived exosomes came in 2002 and reported that secretion of exosomes was enhanced upon T cell receptor (TCR) stimulation and that they contained the TCR/CD3/zeta complex (10). These results were corroborated by a co-localization study showing presence of other TCR components in secreted exosomes (145). These reports support the results in Paper II where anti-CD3 simulating TCR engagement, together with co-stimulatory action of anti-CD28 and IL-2 stimulated the secretion of exosomes (Paper II). Furthermore, exosomes from CD4+ T cells have been implicated in atherosclerosis (146). In addition CD4+ T cell derived exosomes have been reported to inhibit CD8+ T cell responses *in vivo* and CD4+ T cell activation *in vitro* by binding to DCs in an antigen specific way (147,148). Moreover, using a very promising new design of high-resolution flow cytometer it was shown that CD4+ T cells regulate the release of different subpopulations of exosomes according to their level of activation and where full activation increased the release of exosomes from the T cells (149). Furthermore, one report showed that T cell derived exosomes transfer microRNA to DCs during antigen/TCR interactions (150). Recent studies have also reported exosome secretion from T regulatory cells contributing to their immunosuppressive function (151-153). The regulatory functions of these different types of T cell derived exosomes on antigen presenting cells was further supported in Paper II. However, in this study the exosomes from CD3+ T cells were given to autologous non-stimulated T cells instead of antigen

presenting cells to investigate if they had a regulatory function on other T cells. This resulted in proliferation of non-stimulated T cells and cytokine production when the exosomes were added together with IL-2 compared to the response in cultures stimulated with either exosomes or IL-2 alone. Moreover, the ratio between CD4+ and CD8+ cells were skewed with a higher proportion of CD8+ cells in cultures treated with exosomes and IL-2. The results indicate that T cell secreted exosomes could have a role in an immune response by regulating neighboring T cells (Paper II).

Cytotoxic T lymphocyte exosomes

The secretory products of cells from the hematopoietic system are stored in lysosomes (154). The secretory lysosomes of cytotoxic CD8+ T lymphocytes (CTLs) are cytolytic granules reminiscent of MVBs. They contain granzymes and perforin, which has been shown by immunoelectron microscopy and the transport and release of these cytotoxic agents, seems to be closely regulated. Several molecules involved in the binding of CTL to target cells have been identified in the intraluminal vesicles/granules e.g. CD3, CD8 and LFA-1 (155,156). Fusion of the lysosomes with the plasma membrane results in the release of small vesicles and soluble proteins in a regulated timely manner which inhibits the damage on non-target cells (155). Peters et al suggested that the proteins present on the small vesicles e.g. CD8 aid in the targeted transport of the cytotoxic molecules to recipient cells (157). Whether the vesicles released from cytolytic granules from secretory lysosomes in CTLs are exosomes per definition remains elusive. Maybe the vesicles secreted from lysosomes are separate from secretory MVBs that might be present in parallel in the same cell (43). In paper II exosomes from stimulated CD3+ T cells were characterized. However, since the majority of the T cells were CD8 positive we concluded that the majority of exosomes derived from the culture would be of CD8+ T cell (also known as cytotoxic T lymphocytes or CTLs) origin. Interestingly, these exosomes seemed to carry a large amount of CCL5 also known as *regulated upon activation, normal T cell expressed and secreted* (RANTES), since the cytokine was instantly detected after addition of the exosomes to the experimental cultures at 0 h time point (Paper II). RANTES has previously been shown to be present in granules secreted by CTLs and acting as a mitogen upon cell surface aggregation it stimulated release of MIP-1β (158). Interestingly, MIP-1β also named CCL4 was secreted by T cells stimulated with the RANTES containing exosomes (Paper II). In addition CCL3 was secreted (Paper II). Both these cytokines together with RANTES are major suppressive factors secreted by CTLs (159). Furthermore, RANTES has been shown to be released by activated CTLs from a specific storage compartment with exosome features (160). In addition, RANTES containing exosomes from CD8+ cells have been shown to inhibit HIV infection *in vitro*, indicating that exosomes are the means of secretion of this HIV-suppressor factor (161). Considering that we found that the main population in our CD3+ cells to be CD8+ cells it is plausible that the RANTES containing vesicles derive from CD8+ cells (CTLs) (Paper II). Furthermore, this

could indicate that these vesicles originate from another endosomal compartment than the secretory lysosome (38) or that the secretory lysosome is another type of MVB in the CD8+ T cells. Hence these extracellular vesicles could be considered to be CTL derived exosomes. Since the exosomal content varies depending on the cell of origin and the fact that CTLs have a major function in cytotoxic killing events these vesicles could be considered to be CTL exosomes with the function of aiding in the transport of molecules into the extracellular environment and potentially to target cells. Finally, it should be noted that exosomes from CD8+ T cells have also been shown to inhibit antigen-specific immune responses by inducing apoptosis in antigen presenting dendritic cells through Fas/FasL pathway (162). Moreover, in a recent study, exosomes from activated T cells have been shown to induce tumor invasion through the Fas signaling pathway (163).

Exosomes derived from tumor cells

Most cell types secrete exosomes bearing MHC class I molecules and could therefore theoretically activate CD8+ T cells (6). However, we could not detect MHC class I in exosomes from activated human CD3+ T cells (Paper II). Nevertheless, exosomes from tumor cells only appeared to be able to activate CD8 T cells when DCs were present that could process and present tumor antigens (8,164).

In contrast to activating an immune response several studies have shown that tumor derived exosomes contribute to the tumors ability to escape immune recognition. For example, exosomes from leukemic cell lines were shown to carry a ligand (NKG2D) known to suppress T cell and NK (natural killer) cell cytotoxic actions. Interestingly, the secretion of these exosomes was augmented during oxidative and thermal stress and this is of high interest for designing therapeutic methods (165). The results from this study support previous reports showing that tumor exosomes that carry the same ligand, were able to promote tumor growth by inhibiting NK-cell cytotoxicity (166). In addition, the immunosuppressive effects may be due to induction of Fas-ligand mediated apoptosis of T cells (167).

Exosomes derived from other cells

Exosomes derived from cells other than the main immune cells have been associated with immune actions. Similarly to the immunosuppressive actions by tumor derived exosomes, placenta derived exosomes have also been shown to suppress the maternal immune defense by means of NKG2D ligand binding to CD8+ T cells and NK-cells and thereby inhibit their cytotoxicity (168,169). In addition, placenta derived exosomes and exosomes from maternal serum have been shown to bear Fas ligand and suppress T cell activation through the FasL signaling pathway in a similar way to tumor derived exosomes (170,171). Taken together these results supports a role of exosomes in the maternal immune response against the fetus.

Furthermore, exosomes have been shown to play a role in tolerance induction. This has been shown in exosomes from intestinal epithelial cells and from serum derived exosomes from antigen fed rodents. These vesicles were termed tolerosomes since they could induce antigen specific tolerance in recipient animals (12) in a MHC class II dependent manner (172). In addition, tolerance inducing exosomes have also been found in bronchoalveolar fluid (173).

Exosomes and RNA

RNA in brief

Cells synthesize various types of RNA which can be divided into non-coding (nc) RNA and coding RNA. The messenger RNA (mRNA) molecules are protein coding RNAs and consist of transcripts destined for translation into proteins by the ribosomes in the cytoplasm. In an average mammalian cell the mRNA only makes up approximately three to five percent of the total RNA whereas the rest comprises of ncRNA. The major part of ncRNA, approximately 80 percent, is ribosomal RNA (rRNA), which makes up the core structure of the ribosome and catalyzes protein synthesis. Approximately 15 percent of the total RNA is transfer RNA (tRNA) which functions as adaptor molecules in selecting and stabilizing amino acids on the ribosome. The ncRNA also includes small nuclear RNA (snRNA) involved in the splicing of precursor-mRNA into mature mRNA and small nucleolar RNA which have a function in the processing and chemical modifications of rRNA (174,175).

MicroRNA (miRNA) is also a part of the ncRNA and is a category of around 22 nucleotide long transcripts able to regulate gene expression by binding to target mRNAs at the untranslated regions (UTRs) (176). There are indications that more than 60 percent of all protein coding genes are regulated by miRNAs and that a single miRNA species may have the capacity to regulate more than a hundred mRNAs (177,178). The biosynthesis of miRNA starts at transcription by the enzyme RNA polymerase II and results in a hairpin structure called primary (pri-) miRNA. The pri-miRNA is recognized by the RNase enzyme Drosha together with a cofactor called DGCR8 and then cleaved into shorter pre-miRNA which are around 70 nucleotide long structures with a hairpin formation (179,180). The pre-miRNA is then transported from the nucleus to the cytoplasm with the help of the protein Exportin-5 and subsequently cleaved into mature miRNA by the RNase III endonuclease enzyme called Dicer (181). From this step the miRNA can exert RNA interference and in this way regulate translation which is described further down in this thesis.

Since miRNAs are capable of regulating a variety of mRNA transcripts they have been linked to several biological processes such as cell growth, differentiation and apoptosis. Consequently, if the miRNA regulation is disturbed it may result in disease for example cancer (182-184).

RNA in exosomes

It is well known that cells communicate via contact between receptors and ligands as well as through nanotubes and soluble molecules (185). In recent years accumulating evidence indicate that intercellular communication encompass the shuttling of genetic material as well (16). An indication that exosomes might contain RNA was reported in 2004 in hepatitis C studies (186). Worthy of note is that even before reports of exosomes containing genetic material it was reported that cells transfer genetic information via microvesicles from both embryonic stem cells and tumor cells (187,188). However, the presence of genetic material in exosome vesicles was first reported by Valadi et al in 2007. This study reported that exosomes from a human mast cell line contained RNA including microRNA (miRNA) and messenger RNA (mRNA). Furthermore, they showed that the exosome vesicles were capable of shuttling RNA between cells, an activity which served as a novel means of cell-to-cell communication. The RNA present in exosomes was therefore termed *exosomal shuttle RNA* (esRNA) (7). The RNA in exosomes consisted mainly of mRNA and microRNA. Microarray chip analysis revealed that the mRNAs are differentially represented in exosomes compared to their parental cells and only about eight percent of total cellular mRNAs were present in exosomes (7). Furthermore, a microRNA chip analysis showed that the miRNAs in were associated with cellular processes such as exocytosis, tumor formation and angiogenesis. Several reports have supported these results for example in exosomes from ovarian tumor cells (189), urine (120), B cells (114) and dendritic cells (190). However, the differences between cellular and exosomal RNA indicate that a subset of RNA is not randomly packaged into exosomes during their biosynthesis. Rather, a sorting mechanism must exist that provides exosomes with a unique subset of RNA that is the esRNA (Paper III).

Furthermore, in the report by Valadi et al it was stated that the exosomal RNA profile differed compared to their cells of origin. This was true for both the mRNA and miRNA. The pattern of exosomal RNA was shown to almost completely lack ribosomal RNA which is the most enriched RNA species in cells as mentioned above. Furthermore, the exosomes contained a high amount of small RNAs which was shown to include miRNA (7). This exosomal RNA profile have been confirmed in several other studies including exosomes from breast milk, plasma (118,191), urine (120), saliva (192) as well as in supernatant from cultured cells (193). Some

studies have reported the presence of rRNA in some exosome types and this could suggest a presence of other extracellular vesicles that might have a different RNA profile (194-196). However, they may also derive from contaminating cell material where the rRNA is entrapped in aggregates formed during handling or differences in isolation methods.

Moreover, Valadi et al demonstrated that the exosomes could deliver their RNA cargo to recipient cells by isolating exosomes from cells cultured with radioactive uridine. Then, uridine-marked exosomes were added to recipient cells. Next, the transfer of radioactive RNA transferred via exosomes to recipient cells was examined. The results demonstrated that the exosomes were capable of delivering their RNA content to recipient cells. These findings have also been corroborated by others for example in exosomes derived from glioblastoma tumor cells containing an mRNA encoding for a reporter protein (118) and in a similar manner from liver cancer cells (197). Of course, it is not enough to only internalize the exosomes into a receiving cell but the RNA needs to be released into the cytosol to exert its effect. One mechanism of release has been suggested whereby the exosomes release their content by fusion with the plasma membrane and this was shown by adding luciferin loaded exosomes to cells expressing transgenic luciferase as proof of concept (190).

In addition, the functionality of the exosomal RNA has to be confirmed i.e. that the RNA exert a biological effect in recipient cells. To show that the mRNA was functional an in vitro translation assay was used in the study by Valadi et al. (7). Thereafter, the functionality of transferred exosomal mRNA and miRNA has been shown by others using a luciferase reporter gene assay (190) (197). In one study the cells were transfected with a vector that codes for a luciferase reporter gene in conjunction with a sequence complementary to the target miRNA found in their exosomes and as an indication of a successful transfer and function a decrease of luciferase activity in the cell was detected (190). Another study observed the functionality by adding exosomes from transfected cells stably expressing luciferase mRNA to non-transfected cells (197). Moreover, the functionality of delivered exosomal miRNA has been shown in exosomes isolated from Epstein Barr virus (EBV) transformed B lymphocytes. These exosomes were shown to contain EBV miRNAs that subsequently were taken up by recipient cells (114). Lastly, exosomal miRNA has also been shown to exert function in cells transfected with lentiviral vector short hairpin RNA (shRNA). This resulted in down-regulation of the surface expression of CD81 in the recipient cells pulsed with exosomes that were isolated from cells transfected with lentiviral vector shRNA against CD81 (198).

The presence of RNA in exosomes has been reported numerous times and from a variety of sources since the account by Valadi et al in 2007. Since there is still no consensus regarding the nomenclature of exosomes it is still possible to find

publications where microvesicles are called exosomes as well as the other way around. It is therefore important to consider publications using both names in order to not miss key findings in the field. Although most extracellular vesicles appear to carry RNA there are probably differences in their RNA profiles since they are isolated in different ways and are formed through different mechanisms (199). It is of course of high interest to have some kind of consensus regarding nomenclature in order to evaluate the reported experimental results.

Although little is known about exosomes *in vivo* they are likely to have an important role in maintaining normal physiological processes in homeostasis. For instance, it has been shown *in vitro* that human mast cell derived exosomes transfer esRNA to CD34+ progenitor cells, and thus convey signals for their maturation (200). As mentioned above, miRNA has been shown to down-regulate large numbers of target mRNAs and one miRNA is thought to be able to regulate over 100 different mRNAs (178,201). Since miRNA has this power of influence and in combination with the potential of mRNA transcription the exosomal RNA is likely to play an influential role in many physiological processes. Accordingly, it has been shown that exosomes released from cell cultures affected by oxidative stress may have a protective effect on the recipient cells making them more tolerant to oxidative stress. This effect was abolished when the RNA was damaged by exposing the exosomes to UV-light, indicating that the effect was due to the exosomal RNA. In addition, this most likely specifies that different conditions in the cell environment influence the mRNA content of exosomes (92).

As mentioned previously there are also indications of a one way transfer of miRNA by T cell derived exosomes to antigen presenting cells i.e. dendritic cells and B cells occurring only during antigen recognition and resulting in altered gene expression in the antigen presenting cell (150). Moreover, involvement of exosomal RNA in innate immunity has been suggested for exosomes from airway epithelial cells (202). Investigations of breast milk exosomes have revealed that they also contain miRNAs related to development and immunity. This could imply that exosomal RNA in breast milk exert RNA interference and inhibit transcription of certain genes in the intestine of the nursing infant (203,204). Although their role in the fetal/maternal immune interaction remains elusive, exosomal RNA also appear to be secreted in pregnancy. This was suggested in an *in vitro* model where placental cells were shown to secrete exosome associated miRNA and this suggests an involvement in the communication between the mother and fetus (205).

Moreover, exosomal RNA may regulate neural function when it is transported over synapses in the nervous system and hence travel over long distances to deliver their genetic material (206,207). Additionally, exosomes from brain tumors have been

shown to deliver functional mRNA and miRNA that promote tumor growth by modulation of tumor stroma (118).

Several studies have shown an increase in the production of extracellular vesicles in a diseased compared to a healthy state, which make them possible candidates for use as biomarkers as well as targets to prevent dissemination of diseases including cancer. For example, exosomes from colorectal cancer cells were shown to contain mRNAs involved in proliferation of endothelial cells which indicate a role in tumor growth and metastasis (196). Furthermore, one study found that exosomes from serum of patients with glioblastoma contained less transcripts of certain RNAs, but a higher overall yield of exosomal RNA compared to healthy subjects (208). In addition to transfer of proteins it is possible that transfer of miRNAs could contribute to disease spreading e.g. by influencing cells to make a favorable environment for tumor growth and invasion (209,210). Hence, the identification of specific miRNA and mRNA in exosomes in different types of cancer adds an additional prospect of using RNAs as biomarkers (118,196) in addition to their protein content (211). The exosomal RNA from cancers may be detected and used as biomarkers when isolated from different body fluids (212) for example in peripheral blood (213) and saliva (214).

Moreover, the exosomes may also contribute to the protection of miRNA as it has been shown that miRNA in serum can withstand conditions that otherwise would degrade most RNAs (215). In addition, it has been shown that ovarian cancer exosomes exist in peripheral blood and the miRNA pattern correlated with primary tumor miRNA which was not seen in healthy controls (189). Similarly, exosomes from the circulation where shown to have a similar miRNA signature as the originating tumor cells in lung adenocarcinoma (216). Furthermore, it has been shown that some tumor cells might exploit exosomes and microvesicles to remove miRNA that have the ability to inhibit the progression of tumors. More specifically, this has been shown in case of let-7 miRNA family which suppress tumors by targeting oncogenes. It was shown that exosomes secreted from a gastric cancer cell line contained let-7 miRNAs and they were hypothesized to play a role in continuing oncogenesis in the secreting cells by transferring the miRNA out of the cell (217). Moreover, let-7 miRNA has been identified in plasma from lung cancer patients and were proposed to be a potential biomarker associated with survival (218).

In addition to analyzing the exosomal mRNA and miRNA profiles using array hybridization methods such as microarrays, recent publications have shown a more detailed profile of small RNA species by using deep sequencing. Studies using exosomes derived from DCs interacting with T cells (219), prion infected neuronal cells (220), human saliva (221) and human plasma (222) all showed presence of small non-coding RNA including for example transfer RNA (tRNA), piwi interacting

RNA (piRNA), small nuclear RNA and small nucleolar RNA. The characterization of the exosomal RNA profiles in human body fluids is important in the search for finding suitable biomarkers for detection of disease.

In conclusion, exosomes are an important part in intercellular communication by transferring genetic information between cells (198). This communication between cells through exosomal RNA includes both maintenance of normal physiological processes and aiding in disease mechanisms. This makes exosomes interesting as candidates for use as vectors for delivering exogenous therapeutic genetic material. One process involved in this mechanism of transfer is RNA interference (RNAi) which has the potential to be exploited in a therapeutic manner.

RNA interference and exosome delivery

RNA interference

RNA interference (RNAi) was discovered in 1998 and contributed to the knowledge of gene regulation by showing that small double stranded RNAs could mediate gene silencing in *C. elegans* (223). The target RNA molecule is between 21 and 23 nucleotides long, double stranded and can interfere with transcription of mRNA and therefore it was termed short interfering RNA (siRNA) (224). The general process of RNAi from the duplex RNA molecule starts with the enzyme Dicer. This enzyme cleaves and process the double-stranded (ds) RNA before it is loaded onto a protein complex named RNA induced silencing complex (RISC), consisting of Dicer, RNA-binding proteins and a catalytic protein called Argonaute (Ago)-2 (225,226). When loaded onto the RISC, one of the RNA strands are selected whilst the other one is discarded (227). Lastly, the RISC scans the cell and binds to the target mRNA complementary to the siRNA sequence leading to cleavage or inhibition of translation (sometimes followed by degradation) of the mRNA (Figure 2). The process of RNAi occurs for all dsRNA in the cytoplasm i.e. both endogenous miRNA formed in the nucleus and exogenous siRNAs for example viral siRNAs (228-230). Furthermore, it is also possible to exploit this process by the use exogenous synthetic siRNA. The first report regarding RNAi using synthetic siRNA for transcriptional gene silencing of human cells came in 2001 (231). This was then followed by a rapid expansion of the RNAi field with an array of commercially available siRNA as well as expression systems generating siRNA molecules (232).

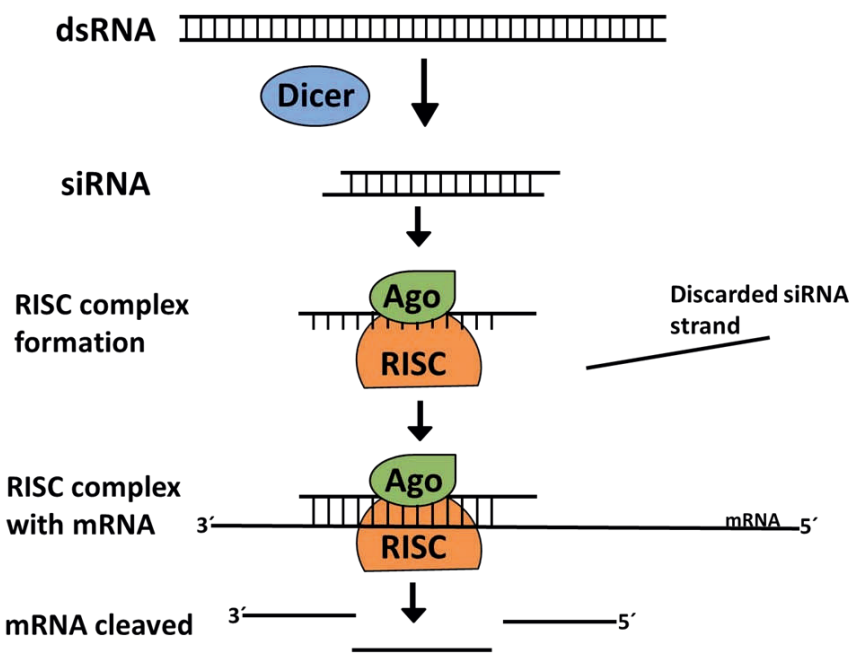

Figure 2. The molecular mechanism of RNA interference. The RNAi pathway initiates with the enzyme dicer which cleaves double stranded RNA (dsRNA) into 20-25 bp fragments usually referred to as short interfering RNA (siRNA) when they are artificially introduced and microRNA (miRNA) when they are produced endogenously. The RNA-induced silencing complex (RISC) is then formed by complementary base pairing between mRNA and one of the two strands of each new fragment. The formation of the RISC complex is followed by degradation of the complementary mRNA by the enzyme Argonaute (Ago). RNA interference has become a valuable research tool since it allows the prevention of translation of specific gene transcripts by introducing siRNA complementary to the mRNA one wishes to suppress. Adapted from (21,228).

Moreover, the RNAi mechanism holds a great potential for therapeutic exploitation. The use of siRNA as a therapeutic reagent was first described in 2003 where the researchers directly injected siRNA against Fas into the blood stream in a mouse model of autoimmune hepatitis which then induced protection from apoptosis (233). These results have been corroborated by others mostly using permeable hepatocytes or tumor cells as recipients for nano-vectors delivering the siRNA (234-237). The most efficient delivery vectors of RNAi to date have been reported to consist of lipid based material including cholesterol (238,239). Still, there are not many synthetic vectors able to carry therapeutic compounds over more complex barriers e.g. the blood brain barrier (21). In this context exosomes with their innate properties for carrying cargo between cells arise as an interesting alternative.

Delivery vectors

There are three common types of delivery vectors that have successfully been used in RNAi gene silencing including synthetic liposomes and polycationic polyethylenimine (PEI) nanoparticles as well as viruses. However, there are still efficiency problems with regards to targeting and silencing along with toxicity and immunogenic concerns (240).

The PEI nanoparticles as well as the liposomes may be engineered with targeting molecules and readily protect the siRNA cargo from clearing. Nevertheless, siRNA containing PEI particles have been difficult to target to tissues other than kidney, liver, lung and spleen (241). In addition, after endocytosis the particles (both PEI nanoparticles and liposomes) they cause a rupture of the endosome resulting in activation of inflammatory responses (241). Moreover, liposomes have been shown to be very efficient at delivering siRNA to the liver however toxicity issues remains. In addition, liposomes may activate complement due to opsonin adsorption when internalized thereby leading to an innate immune response (242). In contrast, exosomes have been shown to contain factors that actively inhibit complement activation (243).

Viruses can be modified to target a specific tissue and cause efficient gene silencing. However, they have several disadvantages such as activation of immune responses or being cleared from circulation by antibodies before reaching their target cell. Inflammatory responses caused by the viral vector may be especially detrimental in the therapy for certain diseases and may result in the death of a patient (244,245). In addition, repeated injections may not be possible due to the formation of neutralizing antibodies. Moreover, viral vectors have the potential risk of causing mutations which could result in dysfunctional gene expression and cancer in recipient cells (246).

Taken together these vector types are functional but not optimal. Hence, a safe and efficient vector is needed in gene therapy to introduce therapeutic RNA/DNA molecules into target cells in vivo. Since exosomes are capable of delivering RNA between cells and since they can be isolated from the patient's own cells, they are interesting candidates for use as delivery vectors for both siRNA and other therapeutic molecules (7).

Exosomes as delivery vectors

The innate properties of exosomes, including their composition, size and their natural ability to cross biological membranes enables them to deliver their cargo to target

cells (247). These features of exosomes make them promising candidates for use as vectors of therapeutic material.

Exosomes have been shown to integrate several different molecules with the aim to show their therapeutic potential including RNA molecules (248)(Paper I), proteins (249) and small molecule drugs (250). When using exosomes for delivery of proteins the proteins have to be incorporated into the exosomes. This can be achieved by fusing the gene encoding the protein of interest with a specific exosomal protein and this has been shown for protein antigens e.g. ovalbumin fused with the exosome protein lactadherin (involved in cell adhesion) (251,252) but also other fusion combination have been demonstrated (248,253).

The first proof-of-concept for using exosomes as delivery vectors of siRNA came in 2011 by Alvarez-Erviti et al (248). In this study exosomes were isolated from mouse immature dendritic cells (previously shown to be non-immunogenic (254)). These cells were transfected to express the exosomal protein Lamp2 together with rabies virus glycoprotein (RVG) known to specifically bind to receptors on neuronal cells. Furthermore, siRNA against the transcript of GAPDH was electroporated into the isolated exosomes before they were injected into mice. The results proved successful for knocking down GAPDH expression in the mouse brain. Subsequently, the delivery model was successfully used for delivering a siRNA against BACE-1, an enzyme significant to target in treatment of Alzheimer´s disease. Importantly, the study reported little or no immunogenicity or toxicity after injecting the exosomes repeated times which underlines their usefulness as delivery vectors (248). The low toxicity interpreted from the low accumulation of exosomes in the liver was further corroborated by another study where exosomes were administered intranasally (255).

The report by Alvarez et al was supported by our results in Paper I where siRNAs against MAPK-1 mRNA were shown to be successfully introduced into exosomes isolated from human peripheral blood plasma. This was also done by electroporation and the results could be detected with flow cytometry by using a fluorescently tagged siRNA in addition to sequence specific detection of the siRNA by using northern blot (Paper I). This was the first report where exogenous synthetic siRNA was introduced into exosomes derived from human peripheral blood plasma as well as into exosomes from two human cell lines i.e. HeLa and the lung cancer cell line HTB-177. In addition, the exosomes from blood plasma appeared to be able to deliver the MAPK-1 siRNA to recipient cells including primary human monocytes and lymphocytes (Paper I). This was confirmed by using northern blot (detecting the siRNA fragments inside the cells) as well as confocal microscopy and flow cytometry which indicated a better uptake in the monocytes compared to the lymphocytes. This procedure could also be of interest for transfecting lymphocytes in different experimental settings since they are known to be difficult to transfect using regular methods (57). More

importantly, the exosome derived siRNA caused post-transcriptional gene silencing in the recipient cells apparently without affecting their viability to a higher extent (Paper I). Together the report by Alvarez et al (248) and in paper I show the potential use of exosomes as delivery vectors for siRNA. The targeted vector in the report by Alvarez et al also indicates that exosomes may function as delivery vectors of other therapeutic agent to specific cell types containing a specific targeted protein (248).

In addition, chemical transfection was also tested as an alternative method for introducing siRNA into exosomes (paper I). However, the lipid micelles formed with this method could not be distinguished from the exosomes themselves rendering them unsuitable for this purpose (Paper I). Thus, from these results the electroporation method seemed to be the most suitable method for introducing siRNA into exosomes from several different cells of origin for subsequent delivery to a variety of recipient cells. However, a recent study suggest that the electroporation method have a drawback based on the observations of a high degree of siRNA aggregate formation, which could be hiding the true loading efficiency into the exosomes (256). This could be an additional explanation to the fluctuating efficiencies we observed between different donors when electroporating siRNA into plasma exosomes (Paper I). Furthermore, the electroporation method has also been tested for introducing miRNA into exosomes secreted by HEK-293 cells (253). However, this proved unsuccessful indicating that some RNA species (or exosome types) are less suitable for this method, perhaps due to their structure (253,257). Taken together these reports show the potential of exosomes as therapeutic vectors as well as the need to continue the development of reliable and effective methods for introducing therapeutic RNA (and DNA) molecules into the exosomes.

The mechanism by which exosomes are crossing biological membranes to enter cells is currently not fully understood. However, one hypothesis is that exosomes move through several cells via the endosomal system before reaching their target and this could explain how exosomes cross thick tissues like the blood brain barrier (258). It is speculated that the unique composition of the exosomal membrane compared to liposomes with regards to cholesterol and phosphatidylserine holds one aspect to their ability to cross membranes. However, experiments with vesicles synthesized with similar lipid content as exosomes were unable to enter cells which suggests that the protein content is important as well (36).

As well as exogenous siRNA it has been reported that exosomes can deliver exogenous miRNA to other cells. In this model the exosomes where loaded with miRNA by transfecting the cells of origin to overexpress the RNA of interest (259). This is a less specific way for introducing RNAi molecules into exosomes compared to electroporation since it cannot be guaranteed that this certain mRNA is part of the exosomal RNA content. However, the isolated exosomes from the transfected

monocyte cell line were shown to be enriched in the miRNA of interest and able to transfer it to recipient cells with preserved function (259). This has been corroborated by others in mammary tumor cells (253), monocytes/macrophages (260), hepatic cells (198) and MSCs (261). More specifically, Ohno et al reported that exosomes modified to have EGF on their membrane could transfer miRNA to an EGFR expressing human mammary tumor in a mouse model (253). In yet another report, genetically engineered exosomes derived from cells transfected with an apoptosis inducing mRNA/protein combination could inhibit nerve sheath tumor growth in mice (262). This study also showed that overexpression of the mRNA in the secreting cell resulted in increased transcription of the corresponding protein and accumulation of the same protein into the exosomes (262).

The mechanism of RNA packaging into exosomes

A key mechanism that is needed to be uncovered in order to engineer an exosome vector is how a subset of RNA, that is esRNA, is sorted and packaged into the exosomes. Exosomes, as well as microvesicles, aside from RNA, also shuttle cytoplasmic proteins and membrane proteins between cells (104,263). Some of these proteins found in these extracellular vesicles have been shown to be associated with the transport and stabilization of RNA (264). In addition, proteins involved in translation and transcription have also been identified in these vesicles for example Argonaute-2 which is the catalytic part of the RNA induced silencing complex (RISC) (265).

The fact that exosomes contain specific RNA, different in comparison to their cells of origin, indicates a specific sorting mechanism. This mechanism has not been elucidated yet but there are studies reporting an association between MVBs and the RISC associated proteins Argonaute-2 and GW182 suggesting a possible site for packaging the esRNA into exosomes (67,265). One suggestion has been reported that the loading of RNA into exosomes is part of the endosomal sorting complex for transport (ESCRT). Since it is involved in MVB formation, budding of endosomal membrane and implicated in the sorting of ubiquitinated proteins into exosomes, the ESCRT system may serve as a mean of entry (60). Moreover, the MVB/ESCRT associated protein Alix has been implied to play a role in the sorting of RNA into exosomes, however it did not influence the secretion of miRNA when inhibited (66). In contrast, the secretion of miRNA could be inhibited by inhibition of sphingomyelinase. This result, that miRNA remained unaffected but was not secreted, indicated a functional role of the sphingomyelinase in loading of RNA into exosomes (66). In short these reports underline the significance of MVBs and their proteins together with sphingomyelinase in the mechanism of loading and selecting specific miRNA.

In addition, it has been reported that mRNA enrichment in exosomes might be dependent on a zip-code like sequence (266). Zip-codes are regulatory sequences involved in mRNA localization and post-transcriptional regulation found in 3´-untranslated regions of mRNA transcripts. Moreover, they mediate binding of mRNA to a RNP complex which transports the mRNA through the cytoskeleton (266,267).

RNA-binding proteins (RBPs) and more specifically ribonucleoproteins (RNPs) involved in transport of RNA as well as components of the RISC have been associated with exosomes (264,265)(Paper III). It has been shown that exosomes and microvesicles contain RNPs such as Staufen homolog (STAU)1, STAU2 and Argonaute-2 together with trinucleotide repeat containing gene 6A protein, also called GW182, suggesting a role of these RNPs in packaging the exosomes with RNA (259,265). Moreover, a recent deep sequencing study also implies the involvement of signal recognition particle RNA (SRP-RNA), vault RNA and Y-RNA in the sorting of RNA into the exosomes (219). All of these RNAs are a part of RNP complexes but only SRP-RNA, which is a part of the SRP-RNP complex, is known to be involved in trafficking and secretion of proteins in the cell (268).

RBPs have been implied to be involved in regulation of RNA biogenesis, maturation, transport as well as the post-transcriptional processing of RNA (269,270). Certain RBPs can form complexes with the RNA and change its conformation and are then referred to as RNP complexes. The RNP complexes where RBPs have associated with miRNA are able to regulate translation by their binding to mRNA and consequently regulate post-transcriptional expression (271). The results from these reports are supported by the findings in Paper III where several RBPs were identified in exosomes, including various heterogeneous nuclear RNPs (hnRNPs) such as hnRNPH1, hnRNPK, hnRNPE1, hnRNPM, and hnRNPA2B1. Indeed, hnRNPs are a family of RBPs primarily known to suppress mRNA splicing but are also involved in other functions such as RNA maturation, transport and localization (272). Moreover, hnRNPs are mainly localized in the nucleus but some of them travel continually between the nucleus and the cytoplasm and this includes hnRNPA2B1 which was identified in Paper III (272,273). The protein hnRNPA2B1 is known to be involved in the packaging of nascent mRNA, alternative splicing, and cytoplasmic RNA trafficking. Nevertheless, in Paper III we succeeded to identify 31 RBPs in exosomes and 78 in cells. Twenty-one of the RBPs identified in exosomes interacted with esRNA, while 10 with miRNA. Furthermore, post transcriptional gene silencing of hnRNPA2B1 showed a detectable decrease of the total RNA in the secreted extracellular vesicles. However, no detectable silencing was detected in four other selected transcripts (Paper III). The observations made in in Paper III suggest the involvement of RBPs, including hnRNPA2B1 in the transport and incorporation of RNA into exosomes during their biosynthesis. In the light of the results in paper III

we have described a plausible mechanism for the transfer/packaging of esRNA. We suggest that a number of hnRNP granules (RNA and their associated proteins), in the cytosol utilize a budding mechanism to cross the membrane of multivesicular endosomes and thereby end up inside the intraluminal vesicles subsequently released as exosomes (Paper III).

Therapeutic potential of exosomes

Since exosomes are important players in the diverse process of intercellular communication they are interesting candidates for use in therapy. A markedly increased research activity in the field has contributed to a better understanding of the biological role of exosomes. However, probably due to differences in isolation, characterization and cell environmental conditions contradictory results have been reported, for example that MSC derived exosomes are capable of both inhibiting and promoting tumor growth (274,275). Moreover, cellular growth conditions have been observed to be important for both the protein and the RNA content of the exosomes (276). In addition, the induction of temperature and oxidative stress has been shown to increase exosomal production and release (92,165).

For translation into a clinical setting it is of importance to develop standardized protocols for isolation and characterization. An alternative to the time consuming differential centrifugation protocol would be of essence, and liquid chromatography based purification is one method currently tested (57). For the characterization of exosomes for clinical therapy it is suggested to use a combination of all available methods i.e. sucrose density gradient, electron microscopy as well as RNA and protein profile to ensure quality and safety (57).

The growing evidence that exosomes could act as vehicles for transfer of genetic material in normal physiological settings as well as in disease makes them desirable study objects in the field of therapeutics. Nevertheless there are several hurdles that remain to be overcome. First, the mechanism of exosome biogenesis and uptake need to be elucidated further in order to develop reliable methods for exosome loading and large scale production. Second the function of exosomes in vivo need to be unraveled and would further the understanding of why exosomes shuttle RNA between cells. There is currently no consensus regarding exosome isolation although there have been attempts to reach at least an understanding of how to describe the methods used (277). However, further insight in intercellular genetic communication via exosomes through optimal isolation and characterization techniques is needed. In this way the exosomes could be separated from other vesicles that most likely will be present in the isolate and lead to higher quality experiments. Moreover, it is of interest to characterize all subpopulations of extracellular vesicles since they all have the

potential to be harvested and utilized in therapy. Reliable isolation methods will also give a more true picture of the genetic content of exosomes from different cell types and this will aid in the use of this content as biomarkers (278). There is still no method available where all characteristics of an exosome can be validated at the same time.

In order to comprehend the function that exosomes have in the shuttling of RNA, the packaging of RNA into exosomes and targeting of exosomes to specific cells need to be further elucidated. One suggestion has been made that short nucleic patterns associated with RNA binding proteins might have a function in the transfer of RNA into the exosomes (279). Evidently the uptake and processing of the exosomes in recipient cells is still not clarified. There are studies suggesting uptake of exosomes by phagocytosis as well as through fusion with the plasma membrane (280-282). There are also indications of selective uptake by means of exosomal membrane proteins (263).

Even though exosomes can be engineered to be targeted to brain cells the technology needs to be developed further (21). It would be of interest to try out additional targeting proteins for targeting to tissues other than the brain. Furthermore an alternative to cloning and expression systems for generating targeted exosomes would be desirable, including direct attachment of ligands to the exosomal membrane (21). In addition, a comparison between exosomes where siRNA has been inserted through electroporation and exosomes generated from cells transfected with siRNA would be of interest for determination of optimal dosage (21). Naturally, it would be of interest to try out exosome vectors for carriage of other therapeutic nucleic acids for example miRNA, miRNA inhibitors and plasmids for expression of shRNA (allowing long term expression in target cells) (21). Moreover, a stable and consistent production of homogenous exosomes engineered and proven to be optimal for therapeutic purposes as well as being produced in large amounts, would make screening for new targeting peptides easier since there would be a large supply of testing material. Large scale production would also be of use in future possible clinical settings (283). It would also be of interest to engineer exosomes lacking their innate RNA content. In this way there would be no other RNA delivered except for the exogenous RNA introduced for therapeutic purposes. One step towards the engineering of exosomes empty of endogenous RNA could be to identify and inhibit proteins involved in the transport of RNA to exosomes. Obviously these proteins first need to be identified and a first step could be proteomic studies of exosomal compartments.

Since exosomes have been implicated in diseases like cancer, where a remarked increase in release has been observed, it is important to investigate if a therapeutic intervention can be done at a vesicular level (57,211). Therapeutic interference may

be done at three levels. First, inhibition of exosome formation is one alternative to decrease disease spreading dependent on increased exosome secretion. It has been shown that inhibition of the formation of ceramide can decrease exosome secretion. Moreover, ceramide was previously shown to play a role in the invagination events forming intraluminal vesicles in the multivesicular bodies (35). Inhibiting exosome formation may be tested by inhibiting other molecules known to be involved in exosome biogenesis in order to find a safe therapeutic strategy. Second, therapeutic intervention could encompass hindrance of exosome secretion. The process of exosome secretion is still not completely known. Nevertheless, several proteins have been associated with exosome release and more specifically RAB27A in certain cancer cells. Therefore RAB27A proved to be a potential target in therapeutic inhibition of exosome release since it led to reduced tumor growth and metastasis in mice (73). This approach might also be valid for other proteins involved in exosomal release (72,74). Third, spreading of disease might be prevented by inhibiting uptake of exosomes by recipient cells. The sorting and targeting of exosomes for uptake by specific cell types has not yet been completely elucidated. It has been reported that blocking the cell adhesion molecule phosphatidylserine in exosomes resulted in reduced tumor growth. However, this approach might interfere with normal physiological actions (284). In addition, if specific targeting molecules on the exosome can be identified they could be inhibited and serve as a strategy for therapy (163). Importantly, interference and inhibition of the different processes in exosome formation and trafficking usually involves proteins that play important parts in other biological mechanisms and needs to be evaluated thoroughly (57). In addition it is plausible that exosomes from different cell types play different roles and therefore it would not be wise to block exosomes in general but to find a way to inhibit the pathogenic vesicles specifically (57).

Conclusions

Exosomes play an important part in intercellular communication through the trafficking and delivery of functional proteins, lipids and RNA able to alter the state of the recipient cells. Their role in homeostasis and disease mechanisms makes exosomes important study objects to understand and to target for therapeutic purposes. Therapeutic approaches could include using their natural ability to deliver RNA between cells over long distances and through biological barriers for delivering therapeutic RNA and also other drug molecules. In order to ensure the safety of an exosomal vector careful engineering is needed where specific targeting and abolishment of potentially interfering endogenous cargo such as miRNA. Finally, the understanding of RNA and protein transfer mechanisms in exosomes together with their ability to carry genetic material between cells underlines their potential in

clinical applications. However more generalized methods for isolating exosomes and introducing the genetic material is needed.

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