# Exosomal Shuttle RNA

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# ABSTRACT

Exosomes are small membrane nanovesicles of endocytic origin that can be released by many different cells to the extracellular environment. Exosomes have been found in a number of body fluids such as blood plasma, breast milk, bronchoalveolar lavage fluid and urine, indicating relevance *in vivo*. Exosomes have been suggested to have a number of different functions and are believed to take part in the communication between cells. Previously, exosomes were believed to consist of a lipid bilayer and proteins, but no nucleic acids. The aim of this thesis was to assess the composition and functions of mast cell exosomes, with focus on the content of nucleic acids and cell to cell communication. We utilized exosomes released from two mast cell lines as well as mouse primary bone marrow derived mast cells. Exosomes were isolated and detected as small 40-80 nm membrane vesicles, which were positive for the tetraspanins CD9, CD63 and CD81 as assessed by electron microscopy and flow cytometry. We showed for the first time that mast cell exosomes contain RNA but no DNA. The exosomal RNA differs from the donor cell RNA. Exosomes contain very little or no ribosomal RNA but a substantial amount of small RNA. We further characterized the RNA using Affymetrix DNA microarray and microRNA array analysis, which revealed that exosomes contain a selection of both microRNA and mRNA. Interestingly, a number of mRNAs were detected in the exosomes but not in their donor cells. Transfer experiments revealed that the exosomal RNA is shuttled to other mast cells and to CD34 positive progenitor cells. Exosomal RNA is functional, as shown by *in vitro* translation and the translation of mouse exosomal RNA to mouse protein after transfer to a human mast cell. In summary, mast cell exosomes contain mRNAs and microRNAs, which can be delivered to another cell. Exosomal RNA shuttle may be a powerful mode of communication between cells, either in the microenvironment or over a distance. We propose that this RNA be called “exosomal shuttle RNA” (esRNA).

# POPULÄRVETENSKAPLIG SAMMANFATTNING

Exosomer är små 30-90 nanometer membranblåsor som bildas inuti celler och sedan kan frisättas till den omgivande miljön. Exosomer identifierades först under 1980-talet. Man trodde då att de fungerar som en soptipp och tar hand om material som cellen inte längre behöver. Sedan dess har de visats ha ett antal funktioner och tros bland annat vara viktiga vid signalering mellan celler. De kan till exempel aktivera immunsystemet eller främja tolerans. Många olika typer av celler kan bilda exosomer och de har olika sammansättning och därmed olika funktion beroende på ifrån vilken cell de frisätts. Några av de celler som kan frisätta exosomer är mastceller, dendritiska celler, epitelceller och cancerceller. Exosomer har också hittats hos människan i ett antal olika kroppsvätskor som blod, urin, lungsköljvätska och bröstmjölk, vilket tyder på att de har en biologisk funktion. Innan den här studien genomfördes var det känt att exosomer består av ett lipidmembran och proteiner, men man trodde att de saknar genetiskt material (RNA eller DNA).

Det huvudsakliga målet med arbetet som presenteras i min avhandling var att undersöka exosomers roll i kommunikationen mellan celler. Mer specifikt var målet att undersöka om exosomer från mastceller innehåller RNA, att karakterisera exosomerna med avseende på RNA- och proteininnehåll samt undersöka om mastceller kan kommunicera med andra celler genom överföring av RNA via exosomer.

För att undersöka detta odlade vi tre olika typer av mastceller, en human- och en musmastcellinje samt primära musmastceller. Exosomerna som frisätts från cellerna ut i cellodlingsmediet isolerades genom upprepad centrifugering och filtrering för att först bli av med celler och cellrester, och koncentrerades sedan genom höghastighetscentrifugering. För att detektera exosomer krävs användning av elektronmikroskopi, eftersom de är för små för att synas i ett vanligt ljusmikroskop. Vi kunde visa att mastcellerna frisätter exosomer genom att titta på dem med hjälp av elektronmikroskop och kunde även kartlägga deras proteininnehåll. Vi fann att exosomerna innehåller RNA, men inget DNA. I celler utgör ribosomalt RNA ca 80% av det totala RNA-innehållet. Det visade sig att exosomerna saknar eller har väldigt lite ribosomalt RNA. Det RNA vi hittade i exosomerna är av typerna mikroRNA och mRNA. MikroRNA är en typ av små regulatoriska RNA som kan reglera genuttrycket och har därför stor reglerande kapacitet. Vid en jämförelse av innehållet av mRNA och mikroRNA i exosomer och deras moderceller visade det sig att exosomerna innehåller ett

urval av RNA. En del RNA är betydligt vanligare i exosomerna än i cellerna medan andra helt saknas i exosomerna. Intressant nog kunde vi hitta något hundratal mRNA i exosomerna som inte alls kunde detekteras i cellerna. Tillsammans tyder den här selektionen av RNA på att det inte är någon slump att RNA finns i exosomerna och att det finns ett specifikt selektions- och packningssystem för RNA till exomerna då de bildas inuti cellen. Genom att märka in exosomernas membran och RNA kunde vi visa att exosomerna kan överföra RNA mellan mastceller och till CD34-positiva celler, som är en typ av stamceller. De fungerar alltså som bärare av genetiskt material mellan celler, kroppens egen genterapi. Vi har också visat att RNA är funktionellt i mottagarcellen. Vi placerade exosomer från musmastceller på humana mastceller. RNA från mus togs upp av de humana cellerna och översattes till musprotein i den humana cellen.

Sammanfattningsvis visar denna avhandling för första gången att exosomer innehåller RNA. Vi har visat att mastceller kan kommunicera med andra mastceller samt med CD34-positiva celler genom exosomal överföring av RNA mellan cellerna. Eftersom exosomer har hittats i blodsystemet skulle en cell kunna sända iväg RNA-innehållande exosomer som antingen kan tas upp av en cell i närmiljön eller likt ett hormon transporteras via systemiska cirkulationen och tas upp och påverka en cell på avstånd. Därigenom skulle man på sikt kunna använda exosomer som bärare av RNA eller DNA vid genterapi vid behandling av sjukdomar som diabetes, cystisk fibros och cancer.

# ORIGINAL PAPERS

This thesis is based on the following papers, which in the text will be referred to by their Roman numerals:

I Ekstrom, K., Valadi, H., Bossios, A., Sjostrand, M., Lee, J. J., and Lotvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9, 654-659.

*Valadi and Ekström have contributed equally to this work.*

II Ekstrom, K., Valadi, H., Sjostrand, M., Bossios, A., Malmhall, C., and Lotvall, J. O. Human mast cell exosomes shuttle RNA between mast cells and to CD34 cells

*In manuscript*

# ABBREVIATIONS

| | |
|---|---|
| BMMC | bone marrow derived mast cells |
| CD | cluster of differentiation |
| cDNA | complementary DNA |
| DC | dendritic cell |
| $dH_2O$ | distilled water |
| DNA | deoxyribonucleic acid |
| dsRNA | double stranded RNA |
| EM | electron microscopy |
| ESCRT | Endosomal Sorting Complex Required for Transport |
| esRNA | exosomal shuttle RNA |
| FACS | fluorescence-activated cell sorter |
| IEC | intestinal epithelial cell |
| IL | interleukin |
| MC | mast cell |
| miRNA | microRNA |
| mRNA | messenger RNA |
| MV | microvesicle |
| MVB | multivesicular body |
| PBMC | peripheral blood mononuclear cell |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| RISC | RNA-induced silencing complex |
| RNA | ribonucleic acid |
| rRNA | ribosomal RNA |
| siRNA | short interfering RNA |
| ssRNA | single stranded RNA |

# CONTENTS

# INTRODUCTION

## *Exosomes*

Exosomes are small membrane vesicles of endocytic origin with a size of between 30 to 90 nm. They correspond to the internal vesicles of multivesicular bodies (MVBs) and are released in the extracellular environment upon fusion of MVBs with the plasma membrane (Thery *et al.*, 2002b). Exosomes can be released by a variety of cells including mast cells (MC) (Raposo *et al.*, 1997), dendritic cells (DC) (Thery *et al.*, 1999), tumour cells (Mears *et al.*, 2004), reticulocytes (Johnstone *et al.*, 1987), epithelial cells (Van Niel *et al.*, 2001), B-cells (Raposo *et al.*, 1996), and neural cells (Faure *et al.*, 2006).

Exosomes were first described by Pan and Johnstone when studying the maturation process of sheep reticulocytes into erythrocytes. They followed the transferrin receptor in the reticulocyte and discovered that it was released on small 50 nm vesicles of endosomal origin upon the fusion of larger vesicles with the plasma membrane. They named the vesicles exosomes (Johnstone, 2005; Johnstone *et al.*, 1987; Pan and Johnstone, 1983; Pan *et al.*, 1985). In the following years it was believed that exosomes only functioned as a way for cells to shed unwanted proteins.

It was not until 1996 that exosomes where shown to have an immunological function. Raposo *et al.* demonstrated that B lymphocytes secrete exosomes and that these exosomes contain MHCII molecules on the surface and are able to present antigen for $CD4^+$ T cells (Raposo *et al.*, 1996). This greatly increased the interest in the field. Today, exosomes have been found in a number of human body fluids, including blood plasma (Caby *et al.*, 2005), urine (Pisitkun *et al.*, 2004), breast milk (Admyre *et al.*, 2007), bronchoalveolar lavage fluid (Admyre *et al.*, 2003), amniotic fluid (Keller *et al.*, 2007) and malign effusions (Andre *et al.*, 2002b) under both healthy and disease conditions.

In this thesis the term exosomes is used for membrane vesicles with endosomal origin and should not be confused with the exosome complex, which is a multi-protein complex capable of degrading RNA.

## Biogenesis

The formation of exosomes starts with the endocytos of proteins at the cell surface. This process may be clathrin dependent (e.g. transferrin receptor) or clathrin-independent (glycosylphosphatidylinositol (GPI)-anchored proteins). The endocytic vesicles are delivered to early endosomes, where house-keeping receptors are uncoupled from their ligands at the mildly acidic pH (pH 6.2), and then recycled back to the plasma membrane or delivered to late endosomes together with other proteins. Late endosomes are acidic (pH 5.0-5.5) and spherical in shape. In late endosomes, proteins are sorted into intraluminal vesicles (ILV) by inward budding of the limiting membrane into the endosomal lumen, forming MVBs. MVBs can either fuse with lysosomes for protein degradation or with the plasma membrane, resulting in the release of the ILVs as exosomes (Van Der Goot and Gruenberg, 2006; Van Niel *et al.*, 2006). Since exosome formation includes two inward budding processes, exosomes maintain the same topological orientation as the cell, with membrane proteins on the outside and some cytosol on the inside.

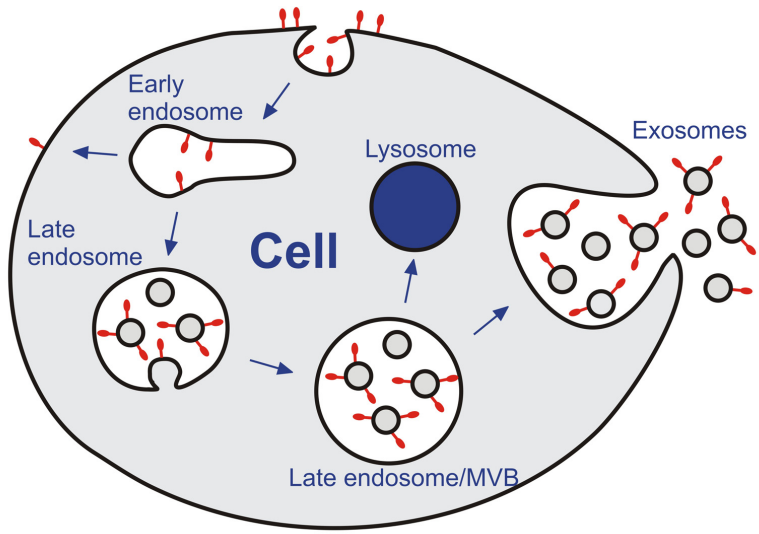


***Figure 1. Exosome biogenesis.*** *Membrane proteins (shown in red) are internalized and delivered to early endosomes. In early endosomes, the proteins can be recycled to the plasma membrane or delivered to late endosomes. Intraluminal vesicles are formed by budding of the endosomal limiting membrane into the*

*endosomal lumen, forming multivesicular bodies (MVB). MVBs can fuse with lysosomes for protein degradation. They can also dock to and fuse with the plasma membrane. This results in the release of the intraluminal vesicles to the extracellular compartment. The vesicles are now called exosomes. Exosomes display the same topology as the plasma membrane, with extracellular domains exposed on the outside and cytoplasm on the inside enclosed by a lipid membrane. Modified from (Fevrier and Raposo, 2004).*

The mechanisms underlying sorting of proteins and lipids into ILVs at the endosomal limiting membrane, and docking and fusion of the MVBs with the plasma membrane are largely unknown, although some potential mechanisms have been suggested. A machinery involved in the sorting and transporting of proteins to the forming ILVs at the endosomal limiting membrane has recently been discovered. This machinery is called Endosomal Sorting Complex Required for Transport (ESCRT), and includes four protein complexes, ESCRT- 0, I, II and III. Ubiquitinated proteins are first recognized by the ESCRT-0 complex, which recruits the ubiquitin binding protein Tsg101 and its complex ESCRT-I. Tsg101 then recruits ESCRT-III via ESCRT-II, and these complexes function together to sequester proteins into the inward budding vesicles of the MVB. ESCRT-III interacts with the AAA-ATPase Vps4, which is required for the recycling of the ESCRT machinery. It has also been suggested that non-ubiquitinated proteins are sorted through the ESCRT complex, probably by directly interfering with ESCRT-II and III (Babst, 2005; De Gassart *et al.*, 2004; Porto-Carreiro *et al.*, 2005; Van Niel *et al.*, 2006). Proteins can also be sorted independently of ESCRT. In a recent publication, it was suggested that not the ESCRT complex but the sphingolipid ceramide is involved in the sorting mechanisms (Trajkovic *et al.*, 2008). The sorting of cytosolic proteins such as heat shock proteins can be explained by the random engulfment of portions of the cytoplasm during vesicle formation. Proteins can also attach to other molecules such as membrane proteins or lipids and be co-sorted into the vesicles (De Gassart *et al.*, 2003; Van Niel *et al.*, 2006).

The release of ILVs as exosomes into the extracellular environment requires the transport and docking of MVBs as well as its fusion with the plasma membrane. Most of these mechanisms are unknown, although many studies have recently been performed with the aim of examining them. A majority of these studies have used the erythroleukemic cell line K562, which produces exosomes in a similar way to reticulocytes. K562 cells can easily be transfected with expression vectors to generate cells that overexpress different proteins, or genes can be knocked out. Thus, erythroleukemic cell line K562 is a suitable experimental system for mechanistic studies (Savina *et al.*, 2005). Using this experimental system, it has been shown that Rab11 is important for the docking and fusion of the

MVB with the plasma membrane in a calcium dependent manner. Rab11 belongs to the GTPase family Rab, whose members are important regulators of membrane trafficking. Rab11 mutants release a lower amount of exosomes (Fader *et al.*, 2005; Savina *et al.*, 2005; Savina *et al.*, 2002).

The transcription factor p53 has been shown to be involved in exosome release. Activation of p53 through irradiation resulted in the release of an increased amount of exosomes (Yu *et al.*, 2006). Moreover, this increase was shown to be due to a p53 gene product, the transmembrane protein TSAP-6. A previous study also reported the involvement of TSAP-6 in exosome release (Amzallag *et al.*, 2004).

## Composition

Exosomes secreted from different cells share some common characteristics such as lipid bilayer composition, size, density and overall protein composition. The exosomal lipid membrane is rigid, enriched in sphingomyelin and exerts rapid flip-flop between the two leaflets (Laulagnier *et al.*, 2004; Wubbolts *et al.*, 2003). The size of exosomes ranges between a diameter of 30 to 90 nm as assessed by electron microscopy, and the density range is between 1.13 and 1.21 g $ml^{-1}$ (Mignot *et al.*, 2006). To date, there is no exosome-specific marker, although proteins enriched in exosomes typically originate from the endosomes, plasma membrane and the cytosol and not from the nucleus, mitochondria, or endoplasmic reticulum, which is consistent with their endosomal origin. Exosomes contain common protein families such as chaperones (Hsc70 and Hsp90), subunits of trimeric G proteins, cytoskeletal proteins (actin, tubulin and moesin), ESCRT proteins (Tsg 101 and Alix), proteins involved in transport and fusion (Rab11, Rab7, Rab2 and Annexines) as well as tetraspanin proteins (CD9, CD63, CD81 and CD82) and have also been shown to contain cell-specific proteins (Escola *et al.*, 1998; Keller *et al.*, 2006). Exosomes from antigen presenting cells (APC) harbour MHCI and MHCII on their surface (Raposo *et al.*, 1996), urinary exosomes harbour aquaporin-2 (Pisitkun *et al.*, 2004), exosomes derived from reticulocytes harbour the transferrin receptor (TfR) (Johnstone *et al.*, 1987), intestinal epithelial cell derived exosomes harbour the A33 antigen (Van Niel *et al.*, 2001), and exosomes from T cells harbour CD3 (Blanchard *et al.*, 2002). The cell-specific proteins may represent their different functions and can additionally be used as a tool to identify exosomes from different sources in, for example, blood plasma.

Interestingly, a single cell line can produce different populations of exosomes. Laulagnier *et al.* showed that the RBL-2H3 mast cell line produces different populations of vesicles, enriched in various phospholipids and proteins. Two main populations were found. MHCII containing exosomes were enriched in phospholipids from the granules, whereas exosomes containing the tetraspanins CD63 and CD81 were enriched in phospholipids from the Golgi. Exosomes containing CD63, MHCII and CD81 accounted for 47%, 32%, and 21%, respectively, of total exosomes (Laulagnier *et al.*, 2005).

## *Isolation methods*

Exosome vesicles can be isolated based on their size, density and biochemical properties. The most common method of purifying exosomes involves a series of centrifugations to remove cells and debris, followed by a final high speed ultracentrifugation to pellet the exosomes (Thery *et al.*, 2002b). Cells and debris can also be removed by a filtration process. The pelleted exosomes may require further purification depending on the downstream application. This can be achieved by another ultracentrifugation step to eliminate contaminating proteins. In addition, exosomes have a specific density and can be purified by floatation into a sucrose density gradient or by sucrosedeuterium oxide ($D_2O$) cushions (Théry *et al.*, 2006). Another purification method is based on exosomes size and utilizes chromatography (Taylor *et al.*, 2006). In addition, exosomes can be isolated based on their membrane properties. Beads coated with an antibody against a protein known to be enriched on the exosome membrane can be added to the supernatant after cell depletion, e.g. anti-MHCII dynabeads (Clayton *et al.*, 2001). One drawback of this isolation method is that unless all the exosomes contain the protein used for the isolation, only a faction of the exosomes will be selected. A GMP approved method has been established for the isolation of exosomes used for clinical applications, which is based on ultrafiltration and diafiltration followed by centrifugation on sucrose cushions, resulting in a highly purified and sterile exosome pellet (Lamparski *et al.*, 2002). This method is best suited for large-scale isolations and requires certain equipment. However, there is a growing need for a fast and reliable method that yields a highly purified exosome fraction.

## Function

The function of exosomes most probably depends on the cells from and conditions under which they are produced, as this provides them with their characteristic composition. When exosomes were first discovered from reticulocytes, they were shown to function as a way to discard proteins, such at the transferrin receptor during the maturation process of reticulocytes into erythrocytes (Johnstone *et al.*, 1987). Reticulocytes lack lysosomes, and it seems as if exosome release is an alternative to lysosome degradation. Another function of exosomes that was later described is as cell free messengers, which can be released from one cell and have an effect on another. This communication with other cells may occur either in the microenvironment, or over a distance (Fevrier and Raposo, 2004). Since exosomes have been found in blood plasma (Caby *et al.*, 2005), they may be transported between organs via the systemic circulation.

How this interaction occurs between exosomes and cells is not fully known. However, several mechanisms describing the interactions of exosomes and cells have been hypothesized. Exosomes can bind to cells through receptor-ligand interactions, similar to cell to cell communication, mediating for example antigen presentation (Admyre *et al.*, 2006; Raposo *et al.*, 1996). Clayton *et al.* showed that B cell exosomes express functional integrines, which are capable of mediating adhesion to extracellular matrix components and activated fibroblasts. This adhesion was strong and resulted in an increase in intracellular calcium (Clayton *et al.*, 2004). Alternatively, exosomes can attach to or fuse with the target cell membrane, thus delivering exosomal surface proteins and perhaps cytoplasm to the recipient cell (Denzer *et al.*, 2000). MHCII positive exosomes have been shown to be attached to follicular DCs. These cells do not express MHCII themselves, and the exosomes provide them with new properties (Denzer *et al.*, 2000). Finally, exosomes may be internalized by the recipient cells due to mechanisms such as endocytosis. Immature DCs have been shown to internalize and process exosomes for antigen presentation to $CD4^+$ T cells (Morelli *et al.*, 2004).

### Exosomes from mast cells

Mast cell derived exosomes were first characterized in 1997 (Raposo *et al.*, 1997). It was shown that bone marrow derived mast cells (BMMC) release MHCII positive exosomes upon degranulation triggered by IgE-antigen stimulation. MHC class II molecules

synthesized by mast cells accumulate in secretory granules with lysosomal characteristics. MHC class II molecules in these endosomal compartments are associated with 60-80 nm vesicles, which are exocytosed during degranulation triggered exocytosis (Raposo *et al.*, 1997). Mast cell derived exosomes have been shown to have immunological effects. Exosomes released from BMMC cells stimulated with IL-4 have the capacity to induce lymphocyte activation with proliferation as well as IL-2 and IFN-γ production (Skokos *et al.*, 2001a). This induced proliferation was also achieved by exosomes from the mast cell lines MC/9 and P815, although these cells did not require any pre-treatment with IL-4 for the release of active exosomes. Activation of lymphocytes was also shown *in vivo*, by culturing spleen and lymph node cells from mice treated with BMMC exosomes (Skokos *et al.*, 2001a). Moreover, mast cell exosomes have been shown to induce maturation of DCs (Skokos *et al.*, 2003). In addition, Al-Nedawi *et al.* demonstrated that mast cell exosomes can stimulate endothelial cells to secrete Plasminogen Activator Inhibitor Type I (PAI-1), thus leading to pro-coagulant properties (Al-Nedawi *et al.*, 2005).

### *Exosomes from antigen presenting cells*

The first report of the immunological properties of exosomes from antigen presenting cells (APC) was presented by Raposo and co-workers and showed that exosomes secreted by EBV-transformed B cells stimulated human $CD4^+$ T cells in an antigen specific manner (Raposo *et al.*, 1996). Exosomes from APC have been shown to stimulate T cells both *in vitro* (Raposo *et al.*, 1996) and *in vivo* (Zitvogel *et al.*, 1998). How this stimulation of T cells occurs remains unclear and is currently the subject of debate. Some reports indicate that exosomes released from APC directly stimulate T cells (Admyre *et al.*, 2006), while other reports underline the need for a functional APC such as DC for T cell stimulation (Hao *et al.*, 2006; Thery *et al.*, 2002a). Thery *et al.* revealed that DC exosomes stimulate naïve $CD4^+$ T cells *in vivo* by transfer of peptide-MHC complexes to DC. In the absence of DC, the exosomes did not stimulate the $CD4^+$ T cells. This transfer of peptide-MHC complexes between antigen presenting cells may be a mechanism that amplifies the immune response (Montecalvo *et al.*, 2008; Thery *et al.*, 2002a). On the other hand, Admyre *et al.* showed *in vitro* using a sensitive Elispot method that exosomes from DC can directly stimulate $CD8^+$ T cells (Admyre *et al.*, 2006).

### Exosomes from epithelial cells

Intestinal epithelial cells have been shown to release exosomes (Van Niel *et al.*, 2001). Karlsson *et al.* demonstrated that serum isolated from an OVA fed rat could induce antigen specific tolerance in naïve recipient animals. This tolerogenic effect was concentrated in a 70 000 g pellet, identified as exosomes, and believed to originate from intestinal epithelium, since exosomes isolated from *in vitro* pulsed intestinal epithelial cells showed the same characteristics as these tolerogenic serum exosomes. The tolerogenic exosomes were thus named tolerosomes (Karlsson *et al.*, 2001). In contrast, Van Niel *et al.* found that intestinal epithelial cells have an immune stimulatory effect as opposed to a tolerogenic function (Van Niel *et al.*, 2003).

### Exosomes from tumour cells

The mechanism for immune tolerance to tumours is unknown, but since such knowledge may lead to new therapeutic strategies for the treatment of cancer, it is currently under extensive investigation. Tumour exosomes have been widely studied in the last few years, with a diverse range of reported functions such as immune activation (Andre *et al.*, 2002b; Wolfers *et al.*, 2001) and inhibition (Clayton *et al.*, 2007). Biochemical analysis of tumour exosomes has shown that they are enriched in MHC class I molecules, heat shock proteins and tetraspanins. Importantly, they contain tumour antigens such as Mart1 and gp100 (Andre *et al.*, 2004; Wolfers *et al.*, 2001).

Exosomes are produced in malignancies in cancer patients (Andre *et al.*, 2002b). It is believed that tumour exosomes may impair the immune response, since patients with tumour exosomes do not spontaneously obtain a protective tumour immune response. Tumour exosomes may inhibit $CD8^+$ T cells and alter the immune response from cytotoxic toward T regulatory cell responses, which contributes to tumour immune escape (Clayton *et al.*, 2007; Clayton and Tabi, 2005). Moreover, tumour exosomes have also been shown to induce apoptosis of tumour reactive $CD8^+$ T cells through the FasL-Fas pathway (Abusamra *et al.*, 2005).

In contrast, it has been demonstrated that tumour exosomes have a stimulatory function in the immune system, which inhibits tumours. Tumour exosomes harbour tumour antigens, which may be transferred to DCs for T cell stimulation (Wolfers *et al.*, 2001). Andre *et al.*

showed that tumour exosomes isolated from ascite fluid harbour tumour antigens that may be transferred to DCs, which in turn led to differentiation and the expansion of tumour-specific cytotoxic T lymphocytes *in vitro*. However, these T lymphocytes could not be found in cancer patients, indicating that tumour exosomes cannot stimulate T cells *in vivo* (Andre *et al.*, 2004; Andre *et al.*, 2002b).

### *Exosomes in cancer therapy*

The first anti-tumour effect of exosomes was demonstrated by Zitvogel *et al.*, who showed that exosomes produced by mouse DC pulsed with tumour peptides suppressed the growth of established tumours (Zitvogel *et al.*, 1998). Since then, many studies have been performed on the potential use of exosomes in cancer therapy, using either exosomes from DC or tumour cells. Tumour exosomes naturally harbour tumour antigens, while DC exosomes can be loaded with tumour antigens by pulsing the cells with synthetic peptides for loading on MHCII molecules, followed by loading on MHCI by acid elution of the exosome pellet and subsequent incubation with a high concentration of peptide (Hao *et al.*, 2007; Mignot *et al.*, 2006).

Phase I clinical studies using DC exosomes in cancer therapy have been reported. Two of these studies were performed on metastatic melanoma patients (Escudier *et al.*, 2005) and patients with advanced non-small cell lung cancer (Morse *et al.*, 2005), and satisfactory safety and promising results for the future treatment of cancer were reported. Based on these results, a phase II/III study was designed using DC exosomes in patients with non small cell lung cancer (Mignot *et al.*, 2006).

Exosomes from tumour cells have also been evaluated for cancer treatment. Tumour exosomes contain tumour antigens and can trigger MHC class I reactivity *in vitro*. They are believed to function by transferring antigens from donor tumour cells to DCs for antigen presentation. Tumour exosomes can easily be isolated from tumour ascites from cancer patients, and thus provide a valuable source of treatment (Hao *et al.*, 2007). Andre *et al.* showed that tumour exosomes from patients with melanoma can transfer the Mart1 tumour antigen to DCs for cross-presentation to $CD8^+$ T cells. In 7 out of 9 cancer patients, tumour-specific lymphocytes could be efficiently expanded from peripheral blood cells, by pulsing DC with tumour exosomes (Andre *et al.*, 2002a).

Different strategies have been applied to increase the immunogenicity of exosomes, and the addition of an adjuvant such as double stranded RNA or CpG can increase their immunogenicity (Mignot *et al.*, 2006). Another strategy that has been tested is the modulation of the exosome composition. Exosomes from mature DC have been shown to be more immunogenic than those from immature DC (Segura *et al.*, 2005a). In a comparative study between exosomes from DC and tumour cells, it was found that the former induced the best antitumor response both *in vitro* and *in vivo* (Hao *et al.*, 2006). Moreover, as discussed in the tumour exosome section, naturally occurring tumour exosomes are believed to induce tolerance to tumours in cancer patients.

### *Exosomes in pregnancy*

Recent studies have demonstrated the role of exosomes in the immune tolerance to the foetus during pregnancy (Mincheva-Nilsson *et al.*, 2006; Taylor *et al.*, 2006). The exosomes found in the maternal circulation were immunosuppressive and had markers from the placenta, suggesting placental origin. Pregnant women had significantly more exosomes in serum than their non-pregnant counterparts. In addition, there was a difference between women delivering at term and preterm. Women delivering at term had more exosomes than those who delivered preterm. The exosomes were found to suppress the expression of T-cell signalling components, including CD3 and JAK3, which suppression was correlated with FasL on the exosomes. Exosomes from women delivering at term showed a significantly increased expression of FasL and T cell suppression than those from women who delivered preterm (Taylor *et al.*, 2006). In addition, placental exosomes were found to contain MIC, which functions in a tolerogenic way by downregulating the NKG2D receptor and the cytotoxic function of PBMC (Mincheva-Nilsson *et al.*, 2006).

### *Exosomes in inflammation and autoimmunity*

Exosomes have also been found to have anti-inflammatory functions. Exosomes released from DC overexpressing IL-4 or IL-10 suppress delayed-type hypersensitivity (DTH) reactions in an MHCII dependent manner in a mouse model. In addition, the onset and severity of collagen-induced arthritis was suppressed (Kim *et al.*, 2005; Kim *et al.*, 2007a). Moreover, FasL on the exosomes was shown to be important for the suppression of DTH (Kim *et al.*, 2007a). DCs treated with IL-4 and IL-10 have been implicated in the

treatment of inflammatory and autoimmune diseases. Due to their stability, DC derived exosomes may represent a safer and more effective therapeutic approach compared to cells (Andre *et al.*, 2004; Andre *et al.*, 2002a). Plasma exosomes of mice immunized to a specific antigen were shown to have anti-inflammatory functions in the DTH model similar to those of DC exosomes, suggesting relevance *in vivo* (Kim *et al.*, 2007b).

Exosomes may also have a beneficial role in sepsis, through an increased phagocytosis of apoptotic cells (Miksa *et al.*, 2006). Exosomes released from mycobacteria infected macrophages can induce a pro-inflammatory response, which was found to be dependent on TLRs and the exosomes probably contain PAMPs (Bhatnagar and Schorey, 2007; Bhatnagar *et al.*, 2007). Exosomes have also been associated with Alzheimer's disease (Rajendran *et al.*, 2006; Vella *et al.*, 2007).

### *Transfer of infectious agents via exosomes*

In human macrophages, HIV particles have been shown to bud and accumulate within multivesicular compartments enriched in MHCII and CD63, the same types of compartments in which exosomes are formed (Pelchen-Matthews *et al.*, 2004; Raposo *et al.*, 2002). In addition, HIV particles and exosomes from macrophages have a similar protein and lipid composition (Nguyen *et al.*, 2003). These findings formed the background for the "Trojan virus hypothesis", first proposed by Gould *et al.*, which suggests that viruses and prions hi-jack the exosomal pathway for their replication and budding. It states that "retroviruses use the preexisting, nonviral exosome biogenesis pathway for the formation of infectious particles, and the preexisting nonviral pathway of exosome uptake for a receptor-independent, Env-independent mode of infection" (Gould *et al.*, 2003). Released retroviruses could then escape the host defence, since they are released in the form of exosomes.

### *Exosomes as biomarkers*

Exosomes have been studied for their potential use as biomarkers in the early detection and classification of disease, choice of therapeutic agents, and assessment of prognosis. Exosomes are accessible, stable, and relatively easy to characterize. They can be collected from blood plasma or urine using non-invasive methods, which favours their use as

biomarkers in the future (Caby *et al.*, 2005; Gonzales *et al.*, 2008; Pisitkun *et al.*, 2006).

### *Microvesicles versus exosomes*

Microvesicles (MV) are circular membrane fragments with a size of 100-1000 nm. They are shed directly from the plasma membrane during activation in most cells and are thus larger than exosomes and derived from the plasma membrane in contrast to the endosomal origin of exosomes. It has recently been found that MV can deliver mRNA between cells and may therefore be involved in cell-cell communication (Bess *et al.*, 1997; Ratajczak *et al.*, 2006a; Ratajczak *et al.*, 2006b).

## *RNA*

RNA is a nucleic acid that serves a number of different functions. It can be subdivided into coding and non-coding RNA. Messenger RNA (mRNA) belongs to coding RNA and serves as a template for protein synthesis and account for about 1-5% of total cellular RNA. Approximately 95% of RNA is non-coding of which ribosomal RNA (rRNA) is most abundant (approximately 80%). The rest of the non-coding RNA includes transfer RNA (tRNA) and small RNA. MicroRNA (miRNA) belongs to the small RNA family and comprises single-stranded molecules of about 22 nucleotides in length. MicroRNAs are regulatory and can downregulate target genes by binding to its complementary mRNA sequence, resulting in either translational inhibition or degradation of the mRNA sequence. Most miRNAs in animals are believed to function by means of the suppression of mRNA translation of target genes through imperfect base pairing with the 3`-untranslated region (3'-UTR) of target mRNAs. In contrast, the binding of miRNA to its target mRNA sequence in plants often leads to mRNA degradation (Alvarez-Garcia and Miska, 2005; Wienholds and Plasterk, 2005).

The miRNA is transcribed in the nucleus to the long RNA precursor pri-miRNA, which is then processed by the RNase III enzyme Drosha/Pasha, which in turn cuts the loop to form pre-miRNA. The latter is exported from the nucleus to the cytoplasm by Exportin-5. In the cytoplasm, another RNase III enzyme, Dicer, cuts the pre-miRNA to generate mature miRNA. The RNA duplex is unwound by a helicase activity and the mature single-stranded miRNA is incorporated into an RNA-induced silencing complex (RISC). Micro-RNA-RISC then binds to the target mRNA sequence and finally leads to

suppression of mRNA translation. MicroRNAs are believed to have an extensive regulatory capacity. It has been suggested that each miRNA can suppress translation of up to 200 mRNA and estimations reveal that 10-90% of all human genes may be targets for miRNA regulation, indicating the great complexity of gene signalling. The functions of many of the miRNAs so far discovered are unknown, although miRNAs have been shown to play a role in a number of biological mechanisms, including hematopoiesis, developmental timing, organ development, cancer, cell differentiation and infectious disease (Carthew, 2006; Lodish *et al.*, 2008; Wienholds and Plasterk, 2005). Moreover, the majority of miRNAs are located in cancer-associated genomic regions. miRNAs are believed to function both as tumour suppressors and oncogenes (Esquela-Kerscher and Slack, 2006).

Short interfering RNAs (siRNAs) are a group of small RNAs that can be used for gene silencing and which have similar mechanisms to miRNAs. siRNA is 21-23 nucleotide dsRNA with two nucleotide 3' overhangs that are processed from longer dsRNA by Dicer. siRNAs occur naturally, are used by bacteria and viruses and can be synthesized for experimental purposes. The introduction of synthetic siRNAs can induce RNA interference in mammalian cells. The RNA interference pathway is often used in experimental biology to study the functions of different genes and can also be used in gene therapy (Rana, 2007).

## *Gene therapy*

Gene therapy is the delivery of genetic material into a specific cell to treat a disease. Basically, the DNA material is carried in a vector and delivered to the target cell where the DNA is inserted in the genome, leading to the production of a functional protein. Alternatively, siRNA can be used to silence a gene that is overexpressed. Since gene therapy can potentially be used to treat genetic disorders such as diabetes, cystic fibrosis and cancer, a great deal of research is being carried out in the field. The main problem has been to find a suitable vector to carry the genetic material. The ideal vector would be non-immunogenic, non-toxic, stable and easily engineered to transfect the cell or organ of interest.

So far, several types of vectors have been investigated to establish their usefulness in gene therapy. They can be subdivided into viral and non-viral, both of which have advantages

and disadvantages. Viruses are naturally occurring vectors for gene therapy, since they introduce their genetic material into the host cell as part of their replication cycle. They have a high transfection rate, but a major disadvantage is their potential toxicity and immunogenicity. Non-viral vectors include liposomes, which are vesicles composed of a lipid bilayer. Hydrophobic chemicals can be dissolved into the membrane and hydrophilic chemicals dissolved on the hydrophilic inside. In addition, DNA can be injected into the liposomes. Liposomes are generally non-immunogenic, but have a much lower transfection rate compared to viruses (Patil *et al.*, 2005).

## Mast cells

Human mast cells (MC) develop from bone marrow myeloid progenitors that express CD34, c-kit and CD13, under the influence of growth factors such as stem cell factor and interleukin 4 (IL-4) (Kirshenbaum *et al.*, 1999). Mature MC do not circulate, but instead acquire their mature phenotype in the tissues in which they reside. Mucosal mast cells are found adjacent to respiratory and intestinal mucosal surfaces while connective tissue mast cells are spread throughout the skin, peritoneal cavity, and musculature. Mast cell functions include immune cell recruitment and activation, epithelial permeability and secretion, vascular permeability, smooth muscle cell constriction resulting in peristalsis and bronchoconstriction, in addition to wound healing. Some of the mediators that lead to these effects are histamine, prostaglandins, leukotriens, heparin, proteases, and cytokines such as IL-5, IL-9, IL-13, TNF and TGF-β. MC may have an important innate immunity function in the defence against bacteria and parasites due to the recruitment of neutrophils, eosinophils and T helper 2 cells to the infection site (Bischoff, 2007). In addition, MC can release exosomes, which is further discussed in the mast cell exosome section (Raposo *et al.*, 1997).

Mast cells have mainly been associated with allergic diseases, but are also involved in other types of human disease, including inflammatory disease, neurological disease and functional disease such as IBS and fibromyalgia (Bischoff, 2007). In allergic disease, cross linking of the high affinity receptor for IgE (FcERI) by the binding of allergen to receptor bound IgE triggers mast cell degranulation, leading to the release of inflammatory mediators and proteases contained in the secretory granules. In addition, MC can be activated by growth factors and cytokines, of which stem cell factor and IL-4

are the most important. Stem cell factor binds to the c-kit receptor, which is expressed on all mast cells. MC can also be activated by bacteria. In addition, binding of FcERI to FcGRII inhibits MC activation (Bischoff, 2007).

A problem in MC research is the difficulty obtaining human MC for *in vitro* cultures and, therefore, the use of cell lines is common. In the studies included in this thesis, we have used two different MC lines; the human mast cell line HMC-1, which is a leukemic mast cell line (kindly provided by Dr Joseph Butterfield, Mayo Clinic, USA), and MC/9, which is a mouse cell line obtained from mouse foetal liver. We have also cultured primary MC from mouse bone marrow cells.

# HYPOTHESIS AND AIMS OF THE THESIS

We hypothesised that exosomes play an important role in cell to cell communication and that this communication occurs as a result of the transfer of genetic material via exosomes.

The overall aim was to assess the composition and function of mast cell derived exosomes, with focus on the content of nucleic acids and cell to cell communication.

The more specific aims were to:

- Detect and characterize the exosomes using electron microscopy, flow cytometry and proteomics.
- Determine whether exosomes contain RNA, and if so:
- Evaluate the RNA content of mast cell derived exosomes, e.g. mRNA and microRNA.
- Evaluate whether exosomes can fuse with other cells, and whether this fusion can result in RNA transfer.
- Evaluate whether any exosomal RNA is functional and if the transfer of mRNA can result in protein production in the recipient cells.

# METHODOLOGY

## Cell culture (papers I and II)

*Paper I)* MC/9 cells (ATCC) were cultured according to manufacturer's recommendations. The human mast cell line HMC-1 (Dr Joseph Butterfield, Mayo Clinic, USA), was cultured in IMDM containing 10% fetal bovine serum (FBS), 100 U $ml^{-1}$ penicillin, 100 μg $ml^{-1}$ streptomycin, 2 mM L-glutamine and 1.2 mM alpha-thioglycerol (all from SIGMA-Aldrich). The cells were passaged every 3-4 days. For release of exosomes, the HMC-1 cells were stimulated with 1 μM calcium ionophore (A23187, SIGMA) for 30 min. Bone marrow derived mast cells (BMMC) were prepared by culturing bone marrow cells from femurs of 7-10 wk old male BALB/c in the presence of 3 ng $ml^{-1}$ IL-3 (R&D systems) as described previously (Razin *et al.*, 1984). After 4 weeks of culture, the cells were harvested and consisted of ≥ 95% pure MCs as analysed by morphology. During the last 48 h before exosome isolation, BMMC were cultured at $3x10^6$ cells $ml^{-1}$ in complete medium supplemented with 10 ng $ml^{-1}$ IL-4 (R&D-systems). For culture of $CD4^+$ T cells, mouse spleens were collected and passed through a 70 μm followed by 30 μm filter. $CD4^+$ T cells were purified by negative selection using the Spincep® mouse $CD4^+$ T cells enrichment cocktail (Stemcell Technologies) according to the manufacturer's instructions. The purity of the $CD4^+$ T cells ranged between 89 and 91%, as analysed by flow cytometry. The cells were cultured in RPMI 1640 containing 10% FBS, 100 U $ml^{-1}$ penicillin and 100 μg $ml^{-1}$ streptomycin at $1x10^6$ cells $ml^{-1}$ in flat bottom 48 well plates.

*Paper II)* HMC-1 cells were cultured as above. Human peripheral blood mononuclear cells (PBMC) were prepared from peripheral blood of healthy subjects by Ficoll-Plaque separation. The CD34 cells were obtained from the PBMC by positive isolation using magnetic separation according to the manufacturer's instruction (Miltenyi Biotec, Germany). The magnetic labelled cells were passed through the magnetic column twice to increase the purity. CD34 cells were cultured in IMDM containing 10% FBS, 100 U $ml^{-1}$ penicillin, 100 μg $ml^{-1}$ streptomycin, 2 mM L-glutamine and 1mM sodium pyruvate. The purity of the separations ranged between 60-85%, as assessed by flow cytometric staining for CD34 combined with 7-AAD for viability.

All cells were incubated at 37 °C in a humidified incubator in an atmosphere containing 5% $CO_2$. FBS and Rat T-stim (BD Biosciences) used for all cell cultures for exosomes isolations were ultracentrifuged at 120 000 g for 90 min using a Ti70 rotor to eliminate exosomes present in the serum.

## *Exosome isolation (papers I and II)*

Exosomes were prepared from the supernatant of MC/9, BMMC and HMC-1 cells by differential centrifugation and filtration steps. Cells were harvested, centrifuged at 500 g for 10 min to eliminate cells and at 16 500 g for 20 min, followed by filtration through 0.22 μm filter to remove cell debris. Exosomes were pelleted by ultracentrifugation (Beckman Ti70 rotor) at 120 000 g for 70 min.

*Paper I)* For mass spectrometry and electron microscopy, exosomes were washed once in a large volume of PBS.

*Paper II)* For transfer experiments with PKH67 labelling, exosomes were washed once in a large volume of PBS. For electron microscopy, exosomes were diluted in PBS, filtered through 0.1μm filters and pelleted by ultracentrifugation.

When indicated, exosomes were measured for their protein content after protein extraction using BCA™ Protein Assay Kit (Pierce).

### *Sucrose gradient (paper I)*

Exosome vesicles harbour a specific density ranging between 1.13 and 1.21 g $ml^{-1}$ (Mignot *et al.*, 2006). This can be utilized for the purification of exosome vesicles. For the sucrose density gradient experiment, the 120 000 g exosome pellet was floated in a sucrose gradient (0.25-2 M sucrose, 20 mM Hepes/NaOH, pH 7.2). The exosomes were dissolved in 2.5 M sucrose and the gradient was layered on top of the exosome suspension. The gradient was centrifuged at 100 000 g for 15 h. Gradient fractions (10 x 3.8 ml) were collected from the bottom of the tube, diluted with 10 ml PBS and ultracentrifuged for 2 h at 150 000 g. The different fractions were examined for their content of RNA, DNA and CD63 protein as a marker of exosomes. For Western blot, total proteins from the 10 fractions were extracted and run on polyacrylamide gels before

transfer to nitrocellulose membranes (BioRad Laboratories). Membranes were blocked in TBS containing 0.5% skim milk, incubated with the CD63 antibody (Santa Cruz) followed by horseradish peroxidase-coupled secondary antibody (Harlan Sera-lab), and subjected to enhanced chemiluminescence (Amersham Biosciences, Inc.).

## *Flow cytometry analysis (papers I and II)*

### *Flow cytometry analysis of exosomes (papers I and II)*

Due to the small size of exosomes, they need to be conjugated to larger particles before FACS analysis.

*Paper I)* For FACS analysis, exosomes from MC/9 and BMMC cells were adsorbed onto 4-µm aldehyde/sulphate latex beads (Interfacial Dynamics) for 15 min in 30 µl PBS followed by 3 h with agitation in 200 µl PBS at RT (for MC/9; 25 µg exosomes per $1.5x10^5$ beads and for BMMC; exosomes from $10^7$ cells per $1.5x10^5$ beads). The reaction was stopped by incubation in 100 mM glycine for 30 min. Exosome-coated beads were washed three times, incubated with CD63 antibody (Santa Cruz), washed twice, incubated with PE-conjugated secondary antibody (Santa Cruz), washed twice and analysed on a FACSScan (Becton Dickinson, San Diego CA).

*Papers I and II)* For immunoisolation, 30 µg of HMC-1 exosomes were incubated with $1.5 \times 10^5$ anti-CD63 or mouse IgG1 beads in 30 µl PBS at RT for 15 min, the volume was made up to 400 µl and the beads were incubated at 4 °C overnight under gentle agitation. The reaction was stopped by incubation in 100 mM glycine for 30 min. Exosome-coated beads were washed twice, incubated in 1% human serum at 4 °C for 15 min, washed twice and incubated with PE-labelled CD9, CD63 or CD81 antibody (BD), or the corresponding isotype control for 40 min, washed and analysed on a FACSScan (Becton Dickinson, San Diego CA).

### *Flow cytometry analysis of cells (papers I and II)*

Cells were harvested and treated with 50 µg human IgG (human cells, SIGMA) or 1% mouse serum (mouse cells, DAKO) for 15 min at 4°C to prevent non-specific binding. The cells were then immunostained with the following antibodies; PE anti-CD34 (BD

Biosciences), PE anti-CD4 (BD Biosciences) or appropriate isotype control for 30 min at 4°C. 7-AAD was used for viability. The cells were washed twice, fixed in 1% FA and stored in dark at 4 °C until analysis using a FACSScan or FACSAria.

## Electron microscopy (papers I and II)

The exosome pellets from MC/9 cells and HMC-1 cells resuspended in PBS were loaded onto formwar carbon coated grids. Exosomes were fixed in 2% paraformaldehyde and washed. The exosomes were post-fixed in 2.5% glutaraldehyde, washed, contrasted in 2% uranyl acetate, embedded in a mixture of uranyl acetate (0.8%) and methyl cellulose (0.13%), and examined in a LEO912AB Omega electron microscope (Carl Zeiss NTS, Germany).

*Paper I)* HMC-1 exosomes were immunogold labelled with anti-CD63 (BD) antibody followed by 10 nm gold labelled secondary antibody (Sigma Aldrich) after the first fixation step.

## Proteomics (paper I)

MC/9 exosomal proteins were extracted and collected in the stacking part of a 10% SDS gel. Total proteins were cut from the gel, trypsinated and analysed using the LC-MS/MS by Core facilities (http://www.proteomics.cf.gu.se/). All the tandem mass spectra were searched by MASCOT (Matrix Science, London, UK) program to identify proteins. The analysis was repeated three times. The proteins were compared with proteins detected in exososomes from other cellular sources.

## RNA isolation (papers I and II)

RNA, DNA and proteins were isolated using Trizol® (Invitrogen) or RNeasy® mini kit (Qiagen) according to the manufacturer's protocol. For co-purification of miRNA and total RNA, the RNA was extracted using Trizol, followed by the RNeasy® mini kit. Cells and exosomes were disrupted and homogenized in Buffer RLT (Qiagen) and 3.5 volumes of 100% ethanol were added to the samples prior use of the RNeasy mini spin column. The rest of the procedure was performed according to the manufacturer's protocol.

## RNA detection (papers I and II)

### Bioanalyzer (papers I and II)

Detection of RNA from MC/9 and HMC-1 exosomes and their donor cells was performed using Agilent 2100 Bioanalyzer® by Exiqon (www.exiqon.com).

### Agarose gel electrophoresis (papers I and II)

RNA from HMC-1 and MC/9 exosomes and cells was separated on agarose gel followed by Etidiumbromide (EtBr) staining. To confirm that the RNA is confined inside the exosomes, which have a robust and stable lipid membrane, and not merely attached on the outside, MC/9 exosomes were treated with 0.4 μg $\mu l^{-1}$ RNase (Fermentas) for 10 min at 37 °C. As a control, 5 μg cellular RNA was added to the exosomes before the RNase treatment. RNA was extracted and separated on an agarose gel followed by EtBr staining.

### cDNA synthesis (paper I)

For detection of mRNA in both BMMC and MC/9 exosomes, cDNA was synthesised using reverse transcriptase (Fermentas) in the presence of radioactive [α-32P]-CTP, 10 mCi $ml^{-1}$ (Amersham) according to manufacturer's recommendations. Total cDNA was run on a 0.8% agarose gel, dried over night, and visualized using phosphoimager. As control, samples were RNase treated before cDNA synthesis.

## MicroRNA analysis (papers I and II)

Identification of microRNA was performed by Exiqon (www.exiqon.com). The quality of the total RNA was verified by an Agilent 2100 Bioanalyzer. Total RNA from the exosome and the cell samples (MC/9 paper I, HMC-1 paper II) were labelled with Hy3™ and Hy5™ fluorescent stain, respectively. The Hy3™-labelled exosome samples and Hy5™-labelled mast cells were mixed pair-wise and hybridised to the miRCURY™ LNA array (v8.0 paper I, v9.2 paper II). The quantified signals were normalised using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm. The experiment was performed in triplicate samples, and the signal was calculated as

logMeanRatio Hy3/Hy5.

## mRNA analysis (papers I and II)

The microarray experiments were performed by SweGene (www.swegene.org/) according to Affymetrix microarray DNA chip analysis. Four exosomal samples and four cellular samples were analysed (MC/9 paper I, HMC-1 paper II). The expression level signals were scaled in GCOS 1.2 to give a median array intensity of 100. This was done to enable the comparison between different arrays. The program Spotfire DecisionSite 8.2 (www.spotfire.com) was used for gene-profiling analysis. For the network analysis to identify biological mechanisms, the program Ingenuity was used (www.ingenuity.com).

## PKH67 transfer experiments (paper II)

Exosomes were labelled with PKH67 (Molecular Probes, Invitrogen), which is a green fluorescent dye that labels the lipid membrane, and thus make it possible to track the exosomes using flow cytometry or fluorescence microscopy. Exosomes were incubated with 2 μM PKH67 for 5 min, washed four times using 300 kDa filter (Vivaspin 6, Sartorius ) to remove excess dye and mixed with HMC-1 cells or CD34 cells in culture. Cells were harvested after different time points (HMC-1: 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 24, 48, 72 h, CD34: 0, 20 h), washed three times and analysed on a FACSAria. CD34 cells were stained with PE labelled anti-CD34 antibody and 7-AAD before acquisition. As control for non-specific labelling of the cells, PBS was PKH67 stained, washed and added to cells in parallel experiments.

## RNA transfer experiments (papers I and II)

To label exosomal RNA, cells were cultured in complete medium supplemented with $^{3}$H-uridine. Exosomes were isolated according to the isolation protocol and washed by ultra-filtration (10 kDa, Millipore) to remove free nucleotides.

*Paper I)* MC/9 exosomes were added to MC/9, $CD4^{+}$, and HMC-1 cells and incubated for 24 h.

*Paper II)* HMC-1 exosomes were added to HMC-1 and CD34 cells and incubated for 20 h.

Cells were harvested and washed twice. RNA was isolated by RNeasy® mini kit and the signal of radioactive RNA was measured using scintillation. Medium supplemented with 1 µl $ml^{-1}$ $^{3}$H-uridine absent from donor cells was treated equally and used as negative control.

## *In vitro translation (paper I)*

Total exosomal RNA was purified using RNeasy® mini kit and 0.5 µg was used for the translation. The *in vitro* rabbit lysate translation kit (Promega Corporation) was used according to manufacturer's recommendation to translate exosomal mRNA to proteins. A sample without exosomal RNA was treated equally and used as negative control. After the translation procedure was accomplished, total proteins were precipitated using acetone and determined using RC DC protein assay (BioRad). The protein content of the samples (presence and absence of the exosomal RNA) was compared using 2D-PAGE. The 2D-gels were visualised using SyproRuby (BioRad) and digitalised using phosphoimager. Protein spots of the samples were compared and a selection of the newly produced proteins was cut, trypsinated, and identified using LC-MS/MS followed by MASCOT program search. The newly produced proteins of mouse origin were compared to the genes identified from the DNA microarray analysis.

## *In vivo translation (paper I)*

MC/9 exosomes (1000 µg) were added to HMC-1 cells ($8 \times 10^6$) at three different time points (0, 3, 6 h) and the cells were incubated for approximately 24 h. The HMC-1 cells were harvested, washed, and the total proteins of the cells were separated by 2D-PAGE according to Core facility (www.proteomics.cf.gu.se). A sample without exosomes was treated equally and used as negative control. The newly produced proteins were detected using PDQUEST and 96 spots were cut and identified using MALDI-tof followed by MASCOT program search. The newly produced proteins of mouse origin were compared to the genes identified from the DNA microarray analysis.

## Data analysis and statistics

Data are expressed as mean ± SEM. Statistical analysis was carried out using a non-parametric test of variance (Kruskal-Wallis test) to determine the variance among more then two groups, followed by Mann-Whitney's test to determine significance between two groups or within one group. A P value $<0.05$ was considered statistically significant.

Statistics for Affymetrix microarrays was performed according to Affymetrix guidelines.

Statistics for miRNA array analysis was performed according to Exiqon. Each signal was normalised against the background and with global Lowess regression algorithm. Data is expressed as mean values ±SD.

# RESULTS AND DISCUSSION

In this section I will summarize and discuss the results obtained in papers I and II. For more detailed information concerning the results, please see the respective paper and supplementary information. Supplementary information for paper I is assessable on the Nature Cell Biology web site (http://www.nature.com/ncb/index.html) and supplementary information regarding paper II is available upon request.

Before we performed the studies in papers I and II, we spent about a year studying exosomes in relation to allergic inflammation in a mouse model. We hypothesised that exosomes play a role in allergic inflammation and may be a key messenger between airways and bone marrow in such conditions. To test this hypothesis we utilized a mouse model. Exosomes were isolated from the serum of allergen sensitized and exposed mice, after which they were transferred to sensitized mice which were then exposed to the allergen. The results were contradictory, in some experiments exosome transfer resulted in an increase in the allergic inflammation, while in others the inflammation decreased. The results were not reproducible. We had to decide whether to stop the exosome research or to continue it, using a simplified and well-controlled system. We opted to continue the research and went on to study exosomes released from mast cells *in vitro*, which resulted in papers I and II.

## *Detection and characterization of mast cell exosomes (papers I and II)*

In papers I and II, we studied exosomes released from a mouse mast cell line (MC/9), a human mast cell line (HMC-1) and mouse primary bone marrow derived mast cells (BMMC). These cells have previously been shown to release exosomes, but the exosomes have not been described in detail (Caby *et al.*, 2005; Raposo *et al.*, 1997; Skokos *et al.*, 2003; Skokos *et al.*, 2001b). The aim of the first part of this study was to detect and characterize the mast cell derived exosomes. BMMC were generated by culture mouse bone marrow cells for 4-5 weeks under stimulation with IL-3. Over this period, the culture of 100 million BM cells from the femurs of five Balb/c mice generated between 20 and 280 million MCs in different experiments. When assessed by morphology, the

purity of the BMMC cells at harvest was ≥ 95%, see Fig. 2.

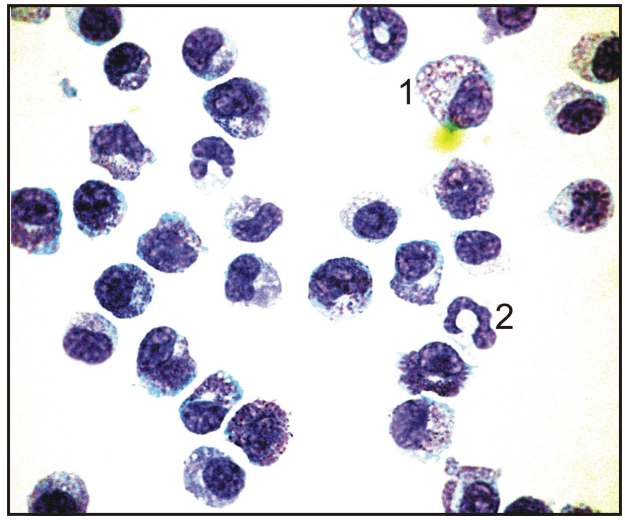


***Figure 2. Photomicrograph of BMMC cells.*** *Bone marrow cells cultured for three weeks under stimulation with interleukin-3 and stained with May-Grünewald-Giemsa. 1) mast cell and 2) non-mast cell.*

The exosomes were isolated by a method based on repeated centrifugation and filtration steps to remove cells and cell debris, followed by high-speed ultracentrifugation to pellet the exosomes. Since exosomes are too small to easily visualize and as there are no exosome specific markers, detection is often based on a combination of methods. We used electron microscopy, flow cytometry (FACS) and proteomics for the detection and characterization of mast cell derived exosomes. In the electron microscope, exosomes were observed as small 40-80 nm vesicles that were CD63 tetraspanin positive as visualized by immunogold labelling (Fig. 3, paper I Fig. 1 and paper II Fig. 1). The exosomes are similar but not identical to previously identified exosomes (Caby *et al.*, 2005; Raposo *et al.*, 1996; Segura *et al.*, 2005a; Skokos *et al.*, 2001a). Differences between electron microscopy pictures may be due to technical variations such as the method of isolation and the electron microscopy preparations. It is therefore difficult to compare different pictures of exosomes.

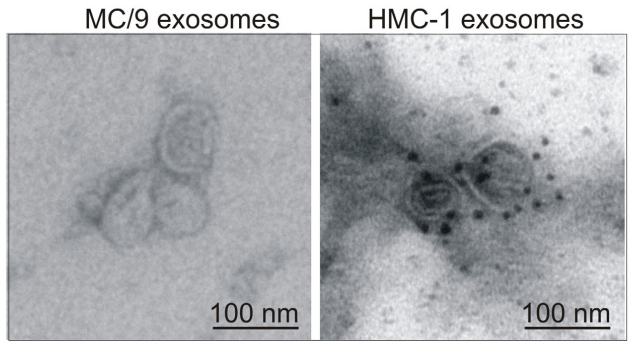


***Figure 3. Electron microscopy picture of MC/9 and HMC-1 exosomes.*** *The exosomes were placed on grids and HMC-1 exosomes were immunogold labelled for CD63 (black 10 nm dots). They were fixed, contrasted, embedded in a mixture of methylcellulose and uranylacetate and examined in an electron microscope.*

We tried to establish a method for directly analysing exosomes by means of FACS, but due to their small size it was impossible to distinguish them from the background. Instead, we conjugated the exosomes to larger particles (4 μm latex beads) prior to the FACS analysis in accordance with a previously described method (Caby *et al.*, 2005). The human exosomes were added to anti-CD63 coated beads, while the mouse exosomes were added directly to non-coated beads due to the lack of a suitable mouse antibody. The exosome-bead complexes were immunostained against CD9 (HMC-1), CD63 (MC/9, BMMC, HMC-1) and CD81 (HMC-1) tetraspanins. These tetraspanins are proteins that are often enriched on the exosome membrane and can be utilized for exosome detection. FACS analysis revealed that all exosomes were CD63 positive and, in addition, that the HMC-1 exosomes were CD9 and CD81 positive, which agrees with previous studies (Caby *et al.*, 2005) (paper I Fig. 1 and paper II Fig. 2).

Finally, we performed an extensive protein analysis of the MC/9 exosomes, using LC-MS/MS technology (paper I). A total of 271 proteins were identified from three preparations of the isolated vesicles (paper I Supplementary Information, Table S1.). The 47 proteins that appeared in all three samples are shown in Table 1. We compared the identified proteins with those identified in exosomes from other sources (i.e., intestinal epithelial cells, urine, dendritic cells, microglia, melanoma, T-cells and B-cells). A large number of the proteins found in our preparations have been previously identified in

exosomes produced by other cells. These are shown in bold in Table 1. It should be noted that due to data handling limitations, our results were compared with a small but representative sample of proteomic publications, which resulted in underestimation of the true values. Later publications have not been included in the comparison.

**Table 1. Proteins identified in MC/9 exosomes.** *Three experiments were performed and the 47 proteins identified in all three experiments are shown in the table. Bold type indicates proteins that were also identified in exosomes secreted by other cells in line with the references provided below.*

| | |
|---|---|
| Adhesion | CD43, **CD63,** CD97, Integrin α |
| Chaperones | **Hsc70,** Hsp75, **Hsp84,** Stress-induced-phosphoprotein, **TCP-1 δ**, γ |
| Cytoskeleton | **Actin β, Ezrin, Moesin, Ras GTPase-activating-like protein IQGAP1, Tubulin beta**-4, **5** |
| Enzymes | ADP-ribosylation factor 1, **ATPase NA+/K+ transporting, ATP-citrate lyase, Carboxypeptidase A, Enolase α, Pyruvate kinase M2, Splicing Factor 2,3** |
| Membrane Fusion | **Annexins (3, 5, 6, 11)** |
| Other | **Histone H2A, H2B, H3.1, H4, Serum albumin, Transferrin receptor** |
| Oxygen transport | **Hemoglobin α, γ** |
| Protein synthesis | Heterogeneous nuclear ribonucleoprotein H, **Elongation factor 1-α 1,** Eukaryotic translation initiation factor 3 subunit 7, **Ribosomal proteins (**S3, **S18),** Transitional endoplasmic reticulum ATPase |
| Signalling | **Gi2α,** RAP-1A |
| T cell stimulation | H2D1, H2K1 |
| Transcription | Activated RNA polymerase II transcriptional coactivator p15 precursor |

*References: Intestinal epithelial cells: (Van Niel et al., 2001), Urine: (Pisitkun et al., 2004), Dendritic cells: (Thery et al., 2001), (Segura et al., 2005b), Microglia-derived exosomes: (Potolicchio et al., 2005), T-cells: (Blanchard et al., 2002), B-cells: (Wubbolts et al., 2003), Melanoma-derived exosomes (Mears et al., 2004).*

In conclusion, electron microscopy, FACS and detailed protein analyses provide extensive evidence to support the identification of isolated vesicles as exosomes. Moreover, we detected a number of proteins involved in protein synthesis, such as

ribosomal proteins, mainly in the first sample analysed. This detection of ribosomal proteins led us to hypothesise that exosomes contain RNA.

## *Mast cell exosomes contain RNA (papers I and II)*

At the time of the present study, exosomes were believed to contain lipids and proteins, but no nucleic acids (Fevrier and Raposo, 2004). The discovery of RNA related proteins in the exosomes from the proteomics experiments caused us to hypothesise that exosomes contain RNA and that cells shuttle RNA to other cells via exosome transfer.

To examine this aspect we examined the presence of nucleic acids in the exosomes and their donor cells, which revealed that mast cell exosomes contain RNA, but no DNA (Fig. 4, paper I Fig. 2 and paper II Fig. 3). Interestingly, the RNA pattern differs between exosomes and their donor cells. Exosomes contain little or no ribosomal RNA compared to the donor cells, as visualized by agarose gels and the Bionanalyzer (18s, 28s, Fig. 4). In contrast, they seem to contain a large amount of small RNA, which may indicate a mechanism for packing the RNA into the exosomes. If the RNA were incorporated into the exosomes by chance or just as contamination, they could be assumed to contain a representative sample of RNA from the cells.

For the BMMC exosomes, we had to amplify the signal in order to detect the RNA. This was carried out by cDNA synthesis using radioactive nucleotides (paper I Fig. 2). Since we used the oligodT primer binding to the mRNA polyA tail for cDNA synthesis, the results also reveal that the exosomes contain mRNA. The difficulties in detecting the RNA in BMMC exosomes may be caused by a limited cell number for exosome release, due to problems generating a large number of primary mast cells. It may also be that the BMMC cells release less exosomes or that their exosomes contain less RNA.

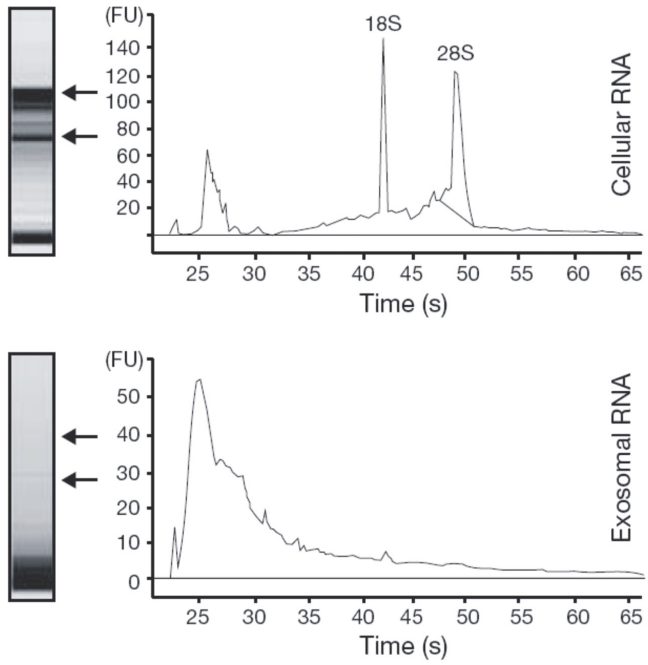


***Figure 4. RNA detection in MC/9 mast cell exosomes and cells using a Bioanalyzer.*** *The exosomes contain a substantial amount of small RNA, but little or no ribosomal RNA compared to the donor cells. Similar results were obtained on exosomes from HMC-1 cells.*

To date, exosomes have only been known to contain lipids and proteins and were believed to have no genetic material. However, microvesicles (MV), which are vesicles with a size of 100-1000 nm, have been reported to contain RNA (Ratajczak *et al.*, 2006a). In our exosome preparations, we used filtration through 100 nm filters in control experiments and observed no difference in the RNA pattern or CD63 as detected by Western blot compared to the standard 200 nm filtration, indicating that our preparations are devoid of MVs (data not shown).

Exosomes have a specific density that can be utilized for the purification procedure. To examine the purity of the exosomes and confirm that they, and not other vesicles, contain RNA, the isolated exosomes were floated in a sucrose gradient. RNA detection on an agarose gel and detection of CD63 protein as an exosome marker by Western blot analysis, as well as DNA detection, were performed on the different fractions.

Interestingly, the results revealed that RNA and CD63 protein are co-localised at the characteristic density of the exosomes. RNA and CD63 protein were also found at higher densities (1.27-1.30 g $ml^{-1}$), probably due to aggregated material. Moreover, no DNA could be detected in any of these fractions (Fig. 5). This implies that RNA is contained within the exosomes and not in other vesicles in the sample.

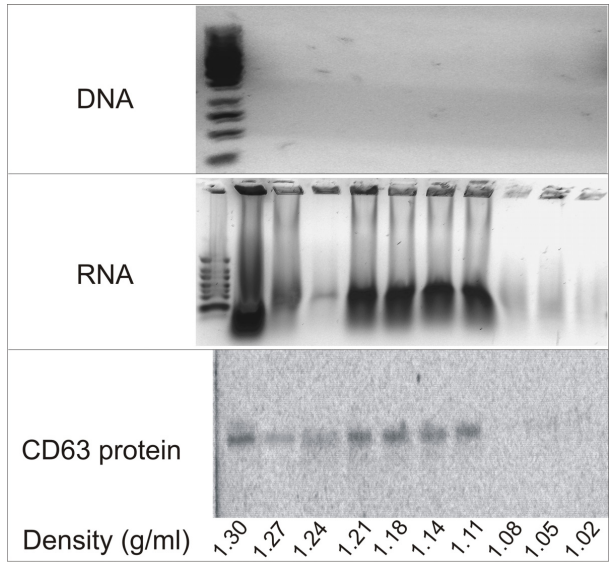


***Figure 5. Sucrose density gradient of MC/9 exosomes.*** *The exosome pellet was floated in a sucrose gradient and 10 different fractions were collected and examined for DNA, RNA and CD63 protein content. No DNA was detected in any of the fractions. The RNA and CD63 proteins were co-localized at the characteristic densities of the exosomes. RNA and CD63 proteins were also detected at higher densities (1.27-1.30 g $ml^{-1}$), probably because of aggregated exosomes.*

In order to confirm that RNA is confined within the exosomes, we performed a control experiment where we treated the exosomes with RNase (paper I Fig. 2). The exosomal RNA resisted RNase treatment, indicating that the RNA is confined within as opposed to outside the exosomes.

## *MicroRNA analysis of mast cell exosomes (papers I and II)*

The presence of large amounts of small RNA in exosomes suggested that the latter may contain microRNA (miRNA, ~22 nucleotides). MicroRNAs are small regulatory RNAs

that can suppress translation by binding to their target mRNA sequence. miRCURY™ LNA Array (www.exiqon.com) was employed to identify the miRNA in MC/9 (paper I) and HMC-1 (paper II) exosomes and cells and revealed the presence of 121 miRNAs in MC/9 and approximately 500 miRNAs in HMC-1 exosomes (paper I Fig. 4 and Supplementary Information Table S5. and paper II Fig. 3 and Supplementary Information Table S1 respectively). Some of the miRNAs were expressed at higher levels in exosomes than cells, again implying that some miRNA may be uniquely packed in the exosomes. It should, however, be noted that the platform used for the HMC-1 exosomes was updated with more miRNAs subsequent to the MC/9 analysis, which was performed at an earlier date. Therefore, the MC/9 exosomes may contain more miRNAs than we detected at that time. The miRNA field is growing; it is not known how many miRNAs exist, and, in addition, the function of most of them remains unclear.

The finding that exosomes contain miRNA suggests that they have regulatory functions. Indeed, some of the miRNAs we detected have been shown to be involved in cancer (miR-17, miR-96) (Alvarez-Garcia and Miska, 2005; Bandrés *et al.*, 2006), hematopoiesis (miR-451, miR-181, miR-185) (Alvarez-Garcia and Miska, 2005; Choong *et al.*, 2007; Masaki *et al.*, 2007) and apoptosis (miR-184) (Chen and Stallings, 2007). This indicates that mast cell exosomal miRNA has multiple biological functions. Whether exosomal miRNA has such functions needs to be investigated in future experiments.

## *mRNA characterization of mast cell exosomes (papers I and II)*

Affymetrix DNA microarray analysis was applied to detect the various mRNAs in MC/9 (paper I Fig. 3 and Supplementary Information, Table S2-3) and HMC-1 (paper II Fig. 4 and Supplementary Information, Table S2-4) exosomes and cells. This analysis revealed the presence of approximately 1300 different gene transcripts in MC/9 exosomes and 2400 transcripts in HMC-1 exosomes. The number of mRNAs identified in the exosomes represented approximately 10% of the mRNAs detected in their donor cells (16 000 mRNAs in MC/9 cells and 21 400 mRNAs in HMC-1 cells). Interestingly, the most abundant mRNAs differed between the exosomes and their donor cells. Furthermore, we detected a number of genes by means of Affymetrix DNA chip analysis that were expressed as mRNAs in the exosomes (270 in MC/9, 79 in HMC-1) but undetectable in

their donor cells. This again implies some kind of mRNA selection and packing mechanism, knowledge of which would be of immense value. Such knowledge would putatively make it possible to engineer the exosomes and regulate what genetic material they contain. In addition, it would be possible to generate exosomes lacking RNA. Perhaps the exosomal RNA contains a consensus sequence that destines them to the exosomes by binding them to RNA binding and transport proteins that carry the RNA from the nucleus to the late endosomes and into the ILVs as they are forming.

Interestingly, a few years ago a publication showed that 50 nm DNA containing vesicles are released in the peyer´s patch of sheep. The authors also found some RNA associated with the vesicles, although it was believed to be a contaminant on the outside of the vesicles in contrast to the DNA, which was shown to be enclosed by a stable vesicle membrane. The authors also hypothesised about nucleic acid communication between cells, but never actually demonstrated such transfer (Collas *et al.*, 2002).

A network-based analysis (Ingenuity® software; www.ingenuity.com) of MC/9 exosomal mRNAs suggested that a key function of their transcripts is "cellular development, protein synthesis, RNA post-transcriptional modification" (paper I, Fig. 3). This network involves 47 gene products, all encoded by mRNAs present in exosomes. Interestingly, the best fitting network for the HMC-1 exosomes was "protein synthesis, gene expression and RNA trafficking" and involved 35 mRNAs, where all except NF-κB are exosomal mRNAs (paper II, Fig. 5). The network suggests that the exosomal RNA transfer results in an indirect activation of NF-κB in the recipient. If exosomal shuttle RNA activates NF-κB, it may lead to an inflammatory response in the recipient cell. Whether the exosomes have this kind of functions needs to be further tested.

To confirm that the exosomal mRNA was functional we performed *in vitro* translation of the MC/9 exosomal mRNA utilizing a rabbit lysate *in vitro* translation assay. In brief, the total mRNA was isolated, translated to proteins and separated by 2D-PAGE. Seven new protein spots were excised and analysed by LC-MS/MS, showing the presence of Cox5b, Hspa8, Shmt1, Ldh1, Zfp125, Gpi1 and Rad23b mouse proteins. These results suggest that the exosomes carry intact and functional mRNA that can give rise to proteins in the presence of functional protein machinery. We hypothesized that exosomes have a protein translation machinery, but incubation of mast cell exosomes in a medium supplemented with $^{35}$S-methionine demonstrated that the exosomes themselves do not have the

components necessary for autonomous protein synthesis.

## Mast cell exosomes can be transferred to other cells (paper II)

We hypothesised that mast cells communicate with other cells by sending exosomes containing RNA. This communication may take place with adjacent mast cells and/or to other, more distant cells. Since mast cell exosomal RNA may have an extensive regulatory capacity and the identified miRNA could perhaps regulate hematopoiesis, we hypothesised that exosomes can shuttle RNA not only to other mast cells, but also to CD34 positive hematopoietic progenitor cells. This would be an intricate way to regulate cell differentiation and/or activation. More specifically, we investigated whether the human mast cell exosomes were transferred to other human mast cells and to human CD34 progenitor cells in culture by labelling the exosome membrane with PKH67 green fluorescent dye and co-cultured the green exosomes with mast and CD34 cells. The cells and exosomes were incubated for different lengths of time and investigated by FACS, which revealed that both HMC-1 and CD34 cells take up PKH67-labelled (green) exosomes (paper II, Fig. 6). An interesting issue not definitively determined here is whether the exosomes fuse with the cell membrane or whether they are internalized by the cell. Both mechanisms have been suggested for different exosomes and cell systems (Denzer *et al.*, 2000; Morelli *et al.*, 2004).

## Mast cell exosomes shuttle RNA to other cells (papers I and II)

We then investigated whether the fusion of exosomes with mast and CD34 cells results in exosomal RNA shuttling. We labelled the MC/9 (paper I) and HMC-1 (paper II) exosomal RNA, by culturing the donor cells in a medium supplemented with $^{3}$H-uridine. Exosomes were isolated, washed and incubated with MC/9, HMC-1, CD34 or $CD4^{+}$ T cells for 20-24 h. The cells were harvested, washed, RNA extracted, and the signal measured by scintillation counts. This revealed that MC/9 exosomes shuttle RNA to other MC/9 and HMC-1 cells, but not to $CD4^{+}$ T cells under these experimental conditions (paper I Fig. 3). In addition, HMC-1 exosomes shuttle RNA to both HMC-1 and CD34 cells (paper II Fig. 6). CD34 cells are hematopoietic progenitor cells that are produced in

the bone marrow and can be found in asthmatic airways. Exosomes from several cellular and body fluid sources contain RNA (our unpublished data), and hypothetically this RNA may be shuttled to a number of different cell types. The shuttle of exosomal RNA may represent a powerful mode of communication between cells in addition to previously known signalling pathways.

## *Exosomal shuttle RNA is translated to proteins (paper I)*

The capacity of exosomes to shuttle RNA between cells raises the next question, whether transferred mRNA translates to proteins in the recipient cell. To test this, human mast cells were incubated with mouse MC/9 exosomes, proteins extracted, separated by 2D-PAGE, the gels matched and new spots identified by MALDI-tof. After incubation of the human cells with mouse MC/9 exosomes for 24 hours, 96 new or enhanced protein spots were identified. Interestingly, three distinct mouse proteins that are not present in MC/9 exosomes were identified in the human cells. These proteins were mouse CDC6 (O89033), mouse Zinc finger protein 271 (P15620) and mouse CX7A2 (P48771). The mRNA of the first two proteins was present in two of the microarray experiments, and the third was present in all four microarrays performed, thus suggesting that mRNA delivered by exosomes to a recipient cell can be translated to proteins.

In conclusion, this study shows that exosomes carry miRNAs and mRNAs and that the RNA can be transferred to other cells. This transfer of genetic material between cells may be an intricate way for cell communication and regulation.

# CONCLUSIONS

Mast cells release exosomes which can be visualized in an electron microscope as small 40-80 nm vesicles. Flow cytometry indicated that the exosomes are positive for the tetraspanins CD9, CD63 and CD81. Moreover, proteomics revealed that they are positive for a number of proteins commonly found on exosomes.

The exosomes contain an extensive amount of RNA, but no DNA. We propose that the exosomal RNA be called “exosomal shuttle RNA” (esRNA).

Exosomal shuttle RNA differs from cellular RNA. Exosomes contain little or no ribosomal RNA but a large amount of small RNA.

Exosomes contain a range of mRNAs and microRNAs. 1300 mRNAs and 121 microRNAs were detected in mouse mast cell exosomes, whereas 2400 mRNAs and 500 microRNAs were detected in human mast cell exosomes. The RNA pattern differs between exosomes and their donor cells. In general, the most abundant mRNAs in exosomes differed from the most abundant mRNAs in the donor cells. Additionally, some mRNAs were only found in the exosomes and not in their donor cells, suggesting a specific RNA selection and packing mechanism.

Mast cell exosomes can fuse with other mast cells as well as CD34 cells, thus delivering the RNA.

Assessment of *in vitro* translation and protein production after the transfer of RNA to other mast cells indicated that the mRNA is functional, thus resulting in protein production in the presence of a functional translation machinery.

# GENERAL DISCUSSION AND FUTURE PERSPECTIVES

In summary, the studies in my thesis show for the first time that exosomes contain RNA, which can be shuttled to other mast cells as well as to CD34 progenitor cells. This genetic material transfer between cells may represent a powerful mode of cell to cell communication. We have resolved many questions and presented new data. However, the results generate even more questions and there are several points I would like to discuss and examine further.

Mast cell exosomes contain a selection of RNA. We found a number of mRNAs in the exosomes that were not detected in the cells, showing that exosomal RNA is a selection and not merely a representation of the cellular RNA content. In addition, preliminary results obtained in our laboratory reveal that the RNA content in exosomes is altered upon altered condition for the producing cells, which strongly suggests that the RNA is selected and packed into the exosomes as opposed to being obtained by chance. Knowledge of these mechanisms could hypothetically make it possible to engineer exosomes to contain specific RNAs or produce exosomes lacking RNA. A potential system for this selection and packing of RNA could be through the binding of RNA binding and transport proteins to the exosomal RNA followed by the transport and delivery of the RNA from the nucleus to the forming ILVs in the late endosomal compartments, similar to the delivery of proteins by the ESCRT machinery. It is intriguing to speculate that if such a system exists, exosomal RNA may contain a consensus sequence that destines it to the forming ILVs in the endosomal compartments by binding to transport proteins. It would be interesting to perform a data analysis of exosomal shuttle RNA sequences to identify any consensus sequences that might destine it to the exosomes.

Whether all exosomes contain RNA can only be speculated upon. A single cell line can release different exosome populations, with differing protein and lipid compositions (Laulagnier *et al.*, 2005). In addition, as previously discussed, the packing of RNA into the exosomes seems to be highly regulated. In view of these results, it is tempting to speculate that one cell may release both exosomes that contain and lack RNA. Whether or not the exosomes contain RNA may depend on the condition of the exosome producing cell and the signalling it needs to send out. Moreover, it could also be that some cells

release RNA containing exosomes, while others release exosomes lacking in RNA. We have some very preliminary data in our laboratory indicating that not all exosomes contain RNA. However, as RNA is highly unstable and may be present in a very low amount, it will be necessary to perform extensive studies in order to state that exosomes do not contain RNA. If these exosome species exist, it would be interesting to evaluate how they are formed and their production regulated.

One interesting topic is the cells with which mast cells communicate through exosomal RNA transfer. We have shown the transfer of exosomal RNA to other mast cells and CD34 progenitor cells, but not to T cells. Thus, RNA transfer seems to be selective and to occur both in the microenvironment and over a distance. However, previous publications show that mast cell exosomes have an effect on T cells (Skokos *et al.*, 2001a; Skokos *et al.*, 2001b), although these studies did not investigate whether the exosomes fuse with the cells, or merely bind through receptor-ligand interactions, resulting in a signalling cascade and cell activation. Thus, the effect of the exosomes on the T cells may therefore be due to receptor-ligand interaction rather than RNA transfer, which would explain the different results obtained. It will be necessary to perform extensive screening experiments in order to ascertain the cells with which the exosomes interact and to which they shuttle RNA.

Another exciting subject not included in this thesis is how the exosomal RNA transfer occurs; whether the exosomes fuse with the cellular plasma membrane, are internalized by the cell or merely attach to the membrane and inject the RNA in a similar way to a virus. This question could be explored by means of either electron or confocal microscopy. We have planned to follow green PKH67 labelled exosomes added to mast cells in a confocal microscope. Morelli and co-workers have followed the internalizing of exosomes by immature DC in a confocal microscope, showing how the exosomes are internalized and sorted in the cell followed by presentation on MHCII to $CD4^+$ T cells (Morelli *et al.*, 2004). These methods would make it possible to follow the exosomes inside the cell, as would a successful non-radioactive RNA labelling method.

One important and extensive issue that needs to be examined is the function of the exosomal RNA shuttle. We have shown that the mast cell exosomal RNA is shuttled to other mast cells as well as to CD34 cells and that the mRNA is translatable to proteins after transfer. However, the functional response to exosomal RNA is unfortunately not resolved in this thesis. I have performed some preliminary studies where we hypothesised

that exosomal RNA signalling to CD34 cells results in differentiation of the CD34 progenitors, potentially to mast cells. We looked for transcription factor activation, differentiation markers, cell viability and proliferation, but did not succeed in finding anything. Since there is a great deal to investigate and we chose a couple of endpoints, these studies were inconclusive. In addition, the timing, *in vitro* environment (e.g. cytokines and growth factors) and the amount of exosomes need to be well established in order to register a response. Our network analysis of the HMC-1 exosomal RNA suggested that the human mast cell exosomal RNA is involved in a network that includes the indirect activation of NF-κB through inactivation of the inhibitor protein IκB. Preliminary studies to examine this did not reveal any NF-κB activation. However, the timing is very important, and we might have missed the signal. The continuation of these functional experiments would be to repeat the NF-κB experiments with more time points. In addition, a screening experiment might be suitable for examining all changes to exosomal RNA at the RNA and protein level. If a difference were to be found, it might indicate what to look for next.

Today, there is a great deal of research on the identification of suitable vectors for gene therapy. A vector should be non-immunogenic, non-hazardous, easy to load with the nucleic acids and possible to target to the right cell. Our data show that exosomes are naturally occurring vectors for gene transfer in the body and would probably be perfect vectors, as they fulfil the above-mentioned criteria. They have already been shown to be well tolerated in clinical phase I cancer studies (Escudier *et al.*, 2005; Morse *et al.*, 2005). Furthermore, it would be possible to collect cells from one patient, produce the exosomes *in vitro* and transfer them back to the same patient. In the *in vitro* system, the exosomes could be loaded with specific genes. In addition, it has been shown that it is possible to engineer the exosomes with specific proteins, thus making possible specific targeting *in vivo* (Delcayre *et al.*, 2005).

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