

Exosomes and Exosomal RNA

A Way of Cell-to-Cell Communication

Maria Eldh

Department of Internal Medicine and Clinical Nutrition
Institute of Medicine
Sahlgrenska Academy at the University of Gothenburg



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Cover illustration: Confocal microscopy image showing the uptake of green fluorescent-labelled exosomes by a recipient cell. The image was acquired at the Centre for Cellular Imaging, the Sahlgrenska Academy, at the University of Gothenburg, using a LSM 700 confocal microscope from Carl Zeiss. Photo by Maria Eldh.

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maria.eldh@gu.se

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“Sometimes you wake up. Sometimes the fall kills you. And sometimes, when you fall, you fly.”

— Neil Gaiman, *The Sandman*,
Fables and Reflections

To my beloved family and friends

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ABSTRACT

Exosomes are nano-sized extracellular vesicles of endocytic origin participating in cell-to-cell communication, partly by the transfer of exosomal RNA between cells. These extracellular vesicles are released by most cells and found in many body fluids including plasma and urine. Exosomes differ compared to their donor cells in RNA, protein and lipid composition, and their molecular content has shown prognostic and diagnostic potential. Uveal melanoma is a tumour arising from melanocytes of the eye and despite successful control of the primary tumour, approximately one third of the patients will develop metastases, predominantly liver metastases, with poor prognosis. The overall aim of this thesis was to evaluate the role of exosomes in cell-to-cell communication and the biological role of exosomal RNA.

Exosomal RNA has been extracted by different RNA isolation methods and we identified that the RNA size distribution pattern varied in multiple studies. Therefore, we aimed to determine if this RNA variation was a true variation or merely a consequence of the RNA extraction method used. We evaluated seven different RNA isolation methods using a mouse mast cell line (MC/9) that continuously releases exosomes. The results showed that the exosomal RNA yield and size distribution pattern differed substantially between different RNA isolation methods.

The mRNA content and function of MC/9 cell-derived exosomes was shown to be altered depending on the culture conditions of the cells. Cells exposed to oxidative stress were shown to have the capacity to send a conditioning signal to other cells, resulting in resistance to oxidative stress in the recipient cells. Moreover, this conditioning signal was shown to be eradicated upon UV-C exposure, indicating a possible role for the exosomal RNA in this biological function.

The presence of exosomes in patients with liver metastases from uveal melanoma was established with the isolation, detection and characterisation of exosomes from isolated hepatic perfusion. The results revealed melanoma-specific exosomes, which contained similar microRNA profiles between patients. Furthermore, patients with metastatic uveal melanoma were shown to have a higher concentration of exosomes in their peripheral venous blood compared to healthy controls.

We conclude that exosomes play a role in cell-to-cell communication and their RNA appears to be of biological importance. Furthermore, exosomal RNA may potentially play a role in the diagnosis and prognosis of uveal melanoma.

Keywords: Exosomes, extracellular vesicles, RNA, mRNA, microRNA, RNA isolation, uveal melanoma

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SAMMANFATTNING PÅ SVENSKA

Exosomer är små cellblåsor i nanostorlek som frisätts av kroppens celler och som förekommer i många olika kroppsvätskor, så som blod, urin och bröstmjölk. Exosomer upptäcktes först på 1980-talet och ursprungligen förmodades det att de var ett sätt för röda blodkroppar att bli av med proteiner under sin mognadsprocess. Under 1990-talet och i början av 2000-talet visade det sig dock att dessa små cellblåsor även kan användas av celler i immunsystemet för att aktivera och hämma andra immunsystemceller. Sedan dess har exosomers betydelse visat sig i en lång rad biologiska funktioner. Efter att exosomer frisätts från en cell kan de tas upp av andra närliggande celler eller färdas genom blodcirkulationen och tas upp av celler på långt avstånd. Exosomer består av lipider, proteiner och genetiskt material i form av RNA, vilka kan överföras till andra celler. Specifika cellers förmåga att transportera genetiskt material i kroppen via exosomer har skapat ett stort intresse, då denna förmåga bland annat skulle kunna användas som markör för olika sjukdomar.

I denna avhandling har exosomer från odlade celler, men också från patienter med levermetastaser från uvealt melanom, studerats. Inledningsvis har olika RNA-isoleringsmetoders förmåga att isolera exosomalt RNA undersökts. Därefter har exosomer som frisätts från såväl normala som stressade förhållanden undersökts i syfte att utreda om deras RNA-innehåll förändras samt för att se vad som sker med mottagarcellen. Slutligen har exosomer från patienter med uvealt melanom undersökts i syfte att identifiera nya diagnostiska och prognostiska markörer.

Delarbete I visar att det kan förefalla som att exosomer innehåller olika slags RNA, beroende på vilken metod man använder för att isolera exosomalt RNA. Det här är en viktigt upptäckt då RNA-profiler från olika exosomer ofta jämförs, dock utan att man har tagit hänsyn till hur detta RNA har isolerats.

Delarbete II visar att exosomer som frisätts från stressade celler kan ge mottagarceller skydd när dessa sedan utsätts för samma typ av stress. Resultaten visar också att dessa exosomers RNA-innehåll skiljer sig från RNA-innehållet hos exosomer som frisätts från celler som odlats under normala förhållanden. Detta tyder på att exosomer som frisätts under olika förhållanden har olika funktioner.

Avslutningsvis visar resultaten av delarbete III att leverperfusat från patienter med levermetastaser från uvealt melanom innehåller melanom-specifika exosomer. Dessa exosomer har en mikroRNA-profil som korrelerar väl mellan olika patienter, men inte med mikroRNA-profiler från cellinjer.

Sammanfattningsvis visar delarbetena att sättet som exosomalt RNA isolerats på påverkar resultaten, samt att en cells nivå av stress påverkar deras exosomers RNA-innehåll, vilket i sin tur påverkar mottagarcellerna. Delarbetena visar även att exosomer potentiellt kan användas som biomarkörer för uvealt melanom.

LIST OF PAPERS

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

- I. **Eldh M**, Lötvall J, Malmhäll C and Ekström K.
Importance of RNA isolation methods for analysis of exosomal RNA: Evaluation of different methods.
Mol Immunol. 2012 Apr;50(4):278-86.

- II. **Eldh M**, Ekström K, Valadi H, Sjöstrand M, Olsson B, Jernås M and Lötvall J.
Exosomes Communicate Protective Messages during Oxidative Stress; Possible Role of Exosomal Shuttle RNA.
PLoS One. 2010 Dec 17;5(12):e15353.

- III. **Eldh M***, Olofsson R*, Lässer C, Svanvik J, Sjöstrand M, Mattsson J, Lindner P, Choi DS, Gho YS and Lötvall J.
MicroRNA in exosomes released from liver metastases in patients with uveal melanoma.
In manuscript. * *These authors contributed equally to this work.*

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Additional publications not included in this thesis:

Lässer C*, **Eldh M*** and Lötval J. Isolation and characterization of RNA-containing exosomes. *J Vis Exp*. 2012 Jan 9;(59):e3037.

* *These authors contributed equally to this work.*

Ekström K, Valadi H, Sjöstrand M, Malmhäll C, Bossios A, **Eldh M** and Lötval J. Characterization of mRNA and microRNA in human mast cell-derived exosomes and their transfer to other mast cells and blood CD34 progenitor cells. *Journal of Extracellular Vesicles*. 2012 Apr 16;1:18389.

Lässer C, Alikhani VS, Ekström K, **Eldh M**, Paredes PT, Bossios A, Sjöstrand M, Gabrielsson S, Lötval J and Valadi H. Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages. *J Transl Med*. 2011 Jan 14;9:9.

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ABBREVIATIONS

Ago	Argonaute
BAL	Bronchoalveolar lavage
Ca ²⁺	Calcium
CD	Cluster of differentiation
cDNA	Complimentary DNA
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ESCRT	Endosomal sorting complex required for transport
FasL	Fas-ligand
FBS	Fetal bovine serum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H ₂ O ₂	Hydrogen peroxide
Hsp	Heat shock protein
ICAM-1	Intercellular Adhesion Molecule 1
IHP	Isolated hepatic perfusion
IL	Interleukin
ILVs	Intraluminal vesicles
Lamp2	Lysosomal associated membrane protein-2
LFA-1	Leukocyte function-associated antigen-1
MHC	Major histocompatibility complex
MIC	MHC class I chain-related protein
miRNA	MicroRNA
mRNA	Messenger RNA
MVB	Multivesicular body
NK cell	Natural killer cell
NKG2D	natural killer group 2 member D
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pre-miRNAs	Precursor miRNAs
pri-miRNAs	Primary miRNAs
RISC	RNA-induced silencing complex

RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RVG	Rabies virus glycoprotein
siRNA	Small interfering RNA
TLRs	Toll-like receptors
TNF- α	Tumor necrosis factor- alpha
Tsg101	Tumor susceptibility gene 101 protein
UTRs	Untranslated regions
UV	Ultraviolet

1 INTRODUCTION

1.1 RNA

The molecular key to the origin of life is ribonucleic acid (RNA), which is a single stranded polymer composed of four different nucleotides; adenine, guanine, cytosine and uracil. RNA can be divided into coding and non coding RNA. Messenger RNA (mRNA) belongs to the coding RNAs and acts as a template for protein synthesis and comprises approximately 1-5% of the total RNA in a cell. The remaining ~95% is made up of noncoding RNA with ribosomal RNA (rRNA) (~80%), transfer RNA (tRNA) and small RNA, such as microRNA (miRNA).

MiRNA are small non-coding RNA molecules, approximately 22 nucleotides in length, that are endogenously expressed and conserved among species [1]. They are gene regulators and act by repressing gene expression at a post-transcriptional level, by binding to complimentary sequences at the 3' untranslated regions (UTRs) of target mRNAs. This group of small gene regulating RNAs was first discovered in the early 90's [1], and over 1000 different miRNA have been identified to date. More than 60% of the human protein-coding genes are predicted to contain miRNA binding sites within their 3'UTRs [2].

The biogenesis of miRNA starts in the nucleus where it is transcribed by RNase polymerase II as long primary miRNAs (pri-miRNAs) [3]. Pri-miRNAs are then processed by the RNase III enzyme Droscha and DGCR8/Pasha creating ~70 nucleotide stem-loop structures called precursor miRNAs (pre-miRNAs) [4-5]. Pre-miRNAs are then transported out of the nucleus to the cytoplasm by Exportin-5, where it is cleaved by the RNase III enzyme Dicer into ~22-nucleotide miRNA duplexes [6-8]. The miRNA duplex is then unwound by a helicase activity, and subsequently one of the mature miRNA strands is assembled into the RNA-induced silencing complex (RISC) which includes Argonaute (Ago) protein, while the passenger strand is degraded. However, in some cases the passenger strand can also become functional. The miRNA-RISC complex then binds to the target mRNA and represses its translation or in some cases degrades it [9-13].

These small gene regulators play a role in a large diversity of biological processes including development, homeostasis, metabolism, cell proliferation, cell differentiation and angiogenesis. Therefore, abnormal

expression of miRNAs can result in changed post-transcriptional regulation of mRNAs, which can lead to a diversity of diseases such as cancer [10, 14-15]. The important role of miRNAs in normal and abnormal biological processes makes them potential therapeutic targets.

1.2 Extracellular Vesicles

Cells release various types of extracellular vesicles, including exosomes, microvesicles, apoptotic bodies, exosome-like vesicles, membrane particles and ectosomes, which can be isolated from cell cultures and body fluids. The different vesicles have different characteristics and properties and are formed in a variety of ways. The various types of vesicles are usually classified according to their intracellular origin, with exosomes derived from multivesicular bodies (MVBs), microvesicles shed from the plasma membrane, and apoptotic bodies produced from cells undergoing apoptosis [16-20].

The nomenclature for extracellular vesicles can be somewhat problematic, and in some studies it can be hard to distinguish if the extracellular vesicles are exosomes or other extracellular vesicles. In some of these studies exosomes are called microvesicles, as this sometimes is used as a collective name for extracellular vesicles. Moreover, microvesicles in some studies can be named exosomes, and as a result create confusion. The term “exosomes”, referring to the nano-sized vesicles of endocytic origin, was not taken until 1987 [21]. Prior to this, the term “exosomes” was proposed by Trams *et al* as early as 1981 for exfoliated membrane vesicles with ecto-enzymatic activity [22]. These exosomes were shown to be released by various cell types and as two populations, one larger (500-1000 nm in diameter) and one smaller (around 40 nm in diameter) population [22]. The term “exosome” is also used to describe a RNA-degrading multiexonuclease complex [23]. However, in this thesis, the term “exosomes” is used for membrane vesicles, below 100 nm in diameter, of endocytic origin.

All extracellular vesicles have been shown to carry both proteins and RNA, with some also containing DNA, which can be transferred from one cell to another, and thus exhibit biological functions [24-27]. Due to the extracellular vesicles unique intracellular origins, different vesicles have different features such as size and furthermore they all exhibit different enrichments in both lipids and proteins. Consequently, they have different densities and sediment at different speeds, although there is some overlap. The key features of the main extracellular vesicle populations are shown in

Table 1. In addition, genetic material has also been found independent of vesicles, as high-density lipoproteins and the RNA binding protein complex Ago2 have been suggested to carry and protect extracellular RNA [28-29].

Table 1. The key features of the main extracellular vesicle populations.

Feature	Exosomes	Microvesicles	Apoptotic bodies
Size range	30-100 nm	100-1000 nm	0.50-5000 nm
Flotation density	1.10-1.21 g/ml	Undetermined	1.16-1.28 g/ml
Sedimentation	100 000-200 000 × g	10 000-20 000 × g	1 200 × g, 10 000 × g or 100 000 × g
Lipid composition	Cholesterol-, sphingomyelin- and ceramide-rich lipid rafts, low phosphatidylserine exposure	High phosphatidylserine exposure, cholesterol	Undetermined
Main markers	CD63, CD9, CD81, Alix, Tsg101	Selectins, Integrins, CD40	Histones, DNA
Intracellular origin	MVBs	Plasma membrane	Undetermined
Mechanism of release	Exocytosis of MVBs	Budding from plasma membrane	Cell shrinkage and plasma membrane blebbing during cell death

Adapted from [16, 20, 30-31]

1.3 Exosomes

Exosomes are small nano-sized membrane vesicles, which were first discovered in the early 80's [32-34] and termed "exosomes" soon after [21]. Exosomes were first found in the process of eliminating transferrin receptors during reticulocyte maturation, and thought to function as cells "garbage bins", thus received little attention. This garbage bin hypothesis was challenged in the 90's, when exosomes were shown to have an immunoregulatory effect, with the demonstration that B cell-derived exosomes could stimulate T cells [35]. Subsequently, the immunological roles of exosomes have been studied extensively and exosomes have been shown to have many functions in immune modulation, spread of neurodegenerative disease and in tumourigenesis [35-38].

Exosomes are released by virtually all cells such as mast cells [39], dendritic cells (DCs) [40-41], T cells [42], B cells [35], epithelial cells [43] and tumour cells [44-45]. In 2002, exosomes were also found in malignant effusions, suggesting a role *in vivo* [45]. Since then, exosomes have been shown to be present in many human body fluids including blood plasma [46], urine [47], bronchoalveolar lavage (BAL) fluid [48], breast milk [49] and saliva [50].

1.3.1 Biogenesis of Exosomes

The endocytosis of proteins at the cell surface is the start of exosome biogenesis (Figure 1). This process leads to the formation of early endosomes, which can be processed in an either clathrin-dependent (e.g. transferrin receptor) or clathrin-independent manner, such as lipid-raft-mediated endocytosis (e.g. glycosylphosphatidylinositol (GPI)-anchored proteins) [51-53]. In the early endosomes, due to the mildly acidic pH, the membrane receptors and their ligands are separated. The proteins can then either be recycled back to the cell surface or continue in the endosomal pathway, where the early endosomes mature into late endosomes [54-55].

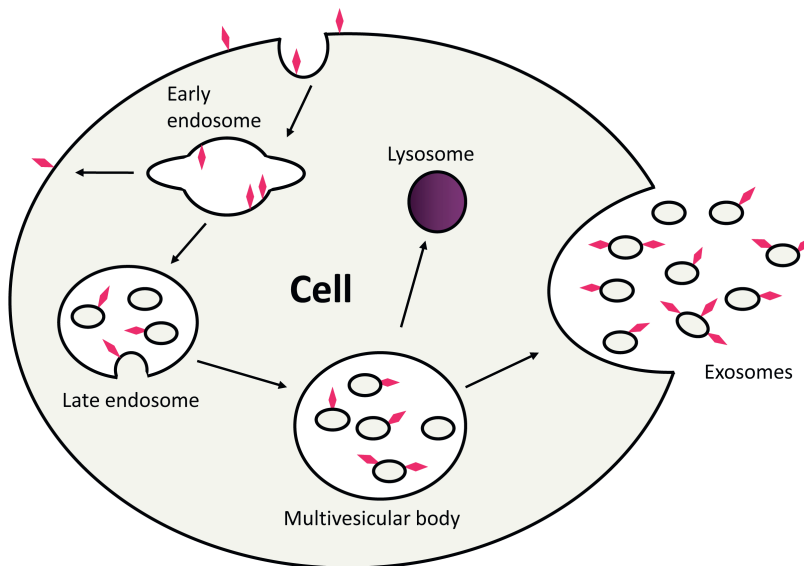


Figure 1. Schematic representation of the biogenesis of intra luminal vesicles (ILVs) and the release of exosomes through the endosomal pathway. Image adapted from [56-59].

The late endosomes are, through inward budding of the limiting membrane, formed into MVBs containing intraluminal vesicles (ILVs). These ILVs will have the same topological orientation as the cell due to the two invaginations, i.e. membrane proteins on the outside and cytosol on its inside. Furthermore, direct transport of proteins from the Golgi complex to MVBs, where they can be inserted in the MVBs limiting membrane, can lead to the formation of ILVs. The MVBs, containing ILVs, can either fuse with the lysosome, leading to the degradation of the content, or they can fuse with the plasma membrane, resulting in the release of ILVs into the extracellular environment as exosomes [57, 60].

Formation and Packaging of ILVs

The mechanisms behind the formation of ILVs and the sorting of proteins and lipids into these vesicles are not fully understood, although the endosomal sorting complex required for transport (ESCRT) have been shown to be involved in this process. The ESCRT machinery comprises of a series of protein complexes, including five distinct protein complexes, ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and Vps4, which direct ubiquitin tagged cargo to endosomes where they are sorted into ILVs [61-63]. The process is initiated by ESCRT-0 that binds to ubiquitinated proteins. ESCRT-0 then binds to the ubiquitin binding protein Tsg101, which is a subunit of ESCRT-I. ESCRT-I then interacts with ESCRT-II which, with the help of ESCRT-adaptor protein Alix, leads to the recruitment of ESCRT-III. The final step of ILVs formation also requires Vps4, which binds to ESCRT-III and also induces the ESCRT machinery's release from the endosomal membrane, thus enabling it to be recycled [61-62, 64-65]. Furthermore, the ESCRT machinery have also been suggested to be involved in sorting of non-ubiquitinated proteins [38, 60, 66-67]. In the absence of the ESCRT machinery, cells may employ different mechanisms for the formation and protein sorting of MVBs, and consequently ILVs.

These ESCRT-independent pathways seem to be driven by cholesterol, tetraspanins and lipids, such as sphingomyelin, that form specialised microdomains in the endosomal limiting membrane, called lipid rafts, which can bend inwards and consequently form ILVs [68-70]. Proteins can also be co-sorted into ILVs, by binding to membrane proteins or lipids [70]. Additionally, engulfment of the cells cytoplasm during the vesicle formation can lead to random packaging of cytosolic proteins.

Proteins and lipids are not the only content that is sorted into ILVs, as exosomes have been shown to also contain different RNA species [71]. The sorting of RNA into ILVs is an even a greater enigma, however the poor correlation between the mRNA content of exosomes and their donor cells [71-72] suggests that the RNA is specifically packed into the vesicles, rather than randomly engulfed from the cells cytoplasm [71, 73-74]. Some studies have suggested that MVBs are the location of the assembly and loading of RISC and therefore miRNA [75-76].

Secretion of Exosomes

In addition to an incomplete understanding of biogenesis and sorting, the mechanisms underlying the secretion of exosomes is also poorly understood. Secretion involves the docking and fusion of MVBs with the plasma membrane, with several different factors involved in the regulation of this process. Several members of the GTPase family Rab, regulators of membrane trafficking, have been shown to be involved in the docking and fusion of MVBs with the plasma membrane, and thus the release of exosomes. One member of the family, Rab11, has been shown to be involved in this process in a calcium (Ca^{2+})-dependent manner [77-78]. It was first suggested that an intracellular increase of Ca^{2+} stimulate exosome secretion [77]. Later, it was shown that Rab11, together with Ca^{2+} , are involved in the secretion of exosomes. An overexpression of Rab11, together with an increased intracellular Ca^{2+} concentration, promotes MVBs docking and fusion with the plasma membrane, and thus increases the secretion of exosomes [78]. Members of the Rab family, particularly Rab27a and Rab27b, also control intracellular trafficking of lysosome-related organelles and their release [79-80], which has also been shown to be required for efficient release of exosomes [81-83]. The inhibition of Rab27a results in decreased or inhibited secretion of exosomes [36, 81-82]. Furthermore, Bobrie *et al* showed that by inhibiting Rab27a they could inhibit a subpopulation of exosomes, suggesting that cells release subpopulations of extracellular vesicles, released by different intracellular mechanisms [82]. An additional Rab family member, Rab35, has also been implicated in the regulation of exosome secretion [84].

The regulation of exosome secretion is not only controlled by Rab proteins. Other proteins such as the p53 protein and the transmembrane protein TSAP6 have been shown to be involved in the secretion process [85-86]. In addition, factors such as stress and pH are also involved in the secretion of exosomes [87-88]. The release and the uptake of exosomes by recipient cells have been shown to increase at low pH [88]. Furthermore, stress such as hypoxia has also been shown to increase exosome secretion [87].

The secretion of exosomes has also been suggested to be regulated in a ceramide-dependent pathway, as the inhibition of neutral sphingomyelinase (nSMase), which is an enzyme involved in the breakdown of sphingomyelin to ceramide [68], has been shown to affect the secretion of miRNA containing-exosomes [89-90].

1.3.2 Exosomal Characteristics and Composition

Exosomes have a spherical morphology, with sizes ranging from 30-100 nm in diameter and densities ranging between 1.10-1.21 g/ml [30, 58, 91]. A rigid lipid bilayer, composed of cholesterol-, sphingomyelin-, and ceramide-rich lipid rafts, encases the contents of the exosome, including proteins and RNA [30-31, 69, 92-95]. A large diversity of proteins can be found in exosomes, of which many are common for all exosomes, regardless of their cellular origin [30] (Figure 2).

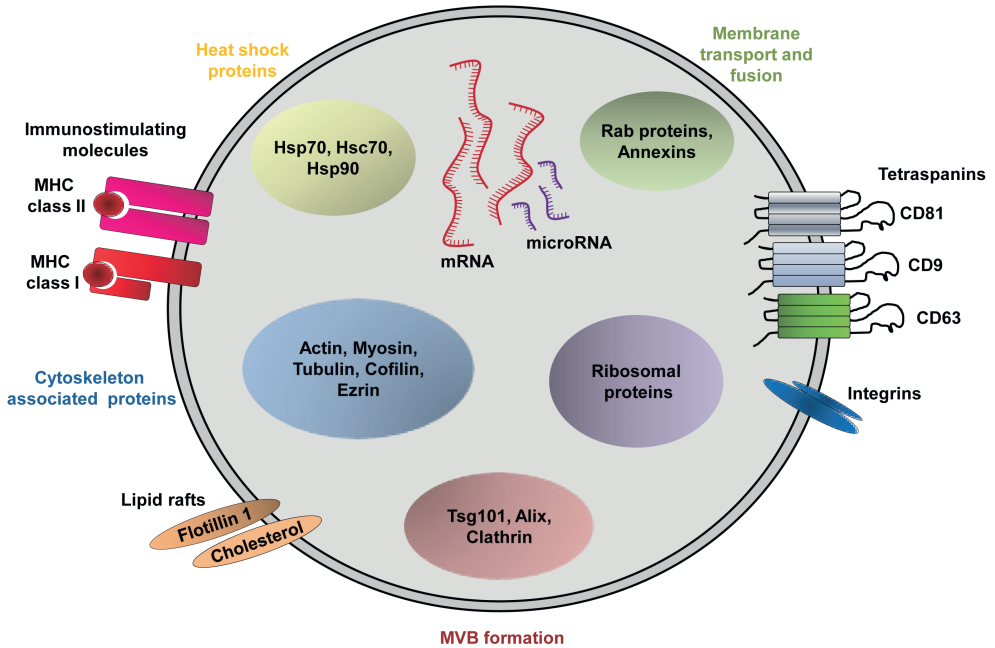


Figure 2. Schematic representation of the molecular composition of exosomes. Image adapted from [16, 30, 57, 96-97].

These conserved proteins, enriched in exosomes, include proteins involved in MVB biogenesis (e.g. Alix, clathrin and Tsg101), membrane transport and fusion proteins (e.g. annexins and Rabs), tetraspanins (e.g. CD9, CD63, CD81), cytoskeleton proteins (e.g. ezrin and actins) and heat shock proteins (e.g. Hsp60, Hsp70 and Hsp90) [30, 91, 98-99]. As well as common proteins, exosomes also contain cell-specific proteins. Exosomes derived from colon epithelial cells contain A33 [43, 100], urinary exosomes harbor aquaporin-2 [47, 101] and exosomes released by antigen presenting cells have been shown to have CD86 and major histocompatibility complex (MHC) class I and II on their surface [35, 102].

Exosomes contain not only proteins, but also a large diversity of RNA, both miRNA and mRNA, with the exosomal mRNA content differing substantially from the mRNA profile of the donor cells [71]. The total RNA profile of the donor cells and exosomes differ, as exosomes show enrichment in lower molecular weight RNA species, such as mRNA and miRNA [71]. Following this discovery, many other studies have shown the presence of RNA in exosomes from many different cellular sources and human body fluids [50, 71, 73, 89-90, 103-109]. Although the mRNA content of exosomes appears to differ from that of its originating cell [71, 73-74, 110], the miRNA content of exosomes has been shown to be similar to the originating cell [104, 110-111]. However, there is data suggesting that the miRNA content of exosomes and their donor cells differ more than the miRNA content between exosomes from different cellular origins [72]. The extensive protein and RNA characterisation of exosomes has generated a lot of data, which has been compiled into online repositories like ExoCarta (<http://www.exocarta.org>) [112-113] and EVPedia (<http://evpedia.info/>).

1.3.3 Isolation and Visualisation of Exosomes

Cell culture supernatants and biological fluids contain a mixture of extracellular vesicles. Experimental procedures to isolate these vesicles cannot distinguish their origin, but can separate the vesicle populations based on properties such as size, density, morphology and composition. It is therefore important to use high quality isolation-, identification- and characterisation methods to be able to distinguish exosomes from other extracellular vesicles. The most commonly used procedure for exosome purification is based on differential centrifugations, with or without a filtration step [30, 57, 114-115]. It consists of a first centrifugation step at a lower speed (around $300 \times g$) to pellet and eliminate cells that might be present in the cell supernatant or biological fluid, followed by a second centrifugation at higher speed (around $10\,000 \times g$) to eliminate larger debris,

apoptotic bodies and other organelles that might be present. The supernatant or biological fluid is then filtered through a 0.2 μm filter for removal of vesicles and particles larger than 200 nm. Finally, the exosomes are pelleted by an ultracentrifugation step (around $100\,000 \times g$). To further eliminate contaminating proteins, e.g. for proteomic analysis of the exosomes, the exosomes can be washed in phosphate buffered saline (PBS) before being ultracentrifuged again to re-pellet the exosomes. For further purification, exosomes can also be isolated based on their density by being placed on a sucrose gradient or cushions, or by immunoaffinity capture where they are bound to antibody-coated beads [30, 114-118]. However, when isolating exosomes based on their density or by immunoaffinity capture, only a subpopulation may be purified. Additional isolation methods may include using a nanomembrane ultrafiltration concentrator [119] or a microfiltration approach [120].

Several companies have developed products aimed to isolate exosomes, such as ExoQuick™ (System Biosciences), which isolates exosomes by precipitating them. Invitrogen™ has developed another exosome purification product called the “Total Exosome Isolation (from Serum)”. Other companies, such as Bioo Scientific, have developed kits that purify exosomes and exosomal RNA or proteins in one protocol, such as the ExoMir™ Kit.

For identification and characterisation of the purified exosomes, a combination of different methods is recommended, including electron microscopy, flow cytometry, and Western blot. Due to the small size of exosomes, they can only be directly visualised by electron microscopy, making this the most reliable method to examine morphological characteristics of exosomes. Furthermore, exosomes cannot directly be analysed by standard flow cytometry, as their small size is unable to be separated from noise and background. This challenge is addressed by attaching exosomes to antibody-coated beads, which can be detected by the laser, thereby allowing analysis of exosomes. Western blot is another method, used to identify and characterise the proteins of exosomes. To date, no exosome-specific protein marker exists, although their specific intracellular origin results in the enrichment of specific proteins, including those associated with the endocytic pathway such as CD63, Tsg101 and Alix, which are often used as “exosomal markers”. Furthermore, the absence of proteins such as the endoplasmic reticulum protein calnexin, indicates little contamination of vesicles from the endoplasmic reticulum, and is often used as a negative control [115, 117].

The exosomal RNA profile fundamentally differs from cells and also from other vesicles, allowing the detection of co-isolation of other vesicles or cell fragments. Cellular RNA largely contains rRNA, seen as two prominent peaks for the 18S and 28S rRNA subunits when analysed by capillary electrophoresis, whereas exosomal RNA lack these subunits and are enriched in lower molecular weight RNA [71].

1.3.4 Exosomes and Their Many Functions

Cell communication can occur in several ways, with or without cell-to-cell contact, including surface-to-surface communication via membrane bound proteins and lipids, hormones, cytokines, chemokines and growth factors. The discovery of exosomes contributed to an increased complexity of cell-to-cell communication. After exosomes have been released into the extracellular environment, they can either act locally or distally by travelling via the circulatory system. However, the mechanism behind the interaction between exosomes and recipient cells is not fully understood, although several hypotheses have been suggested, such as receptor-ligand interactions [35, 121-124], fusion with the plasma membrane [125] or internalisation of the exosomes by endocytosis [90, 126-128]. Exosomes have a diverse range of functions, which depends on both the cell origin and the physiological state of the originating cell, and include eradicating unwanted proteins [32-34], transfer of genetic material between cells [71], immunostimulatory [35] and immunosuppressive functions [129], as well as the promotion of metastasis [36] and spread of infectious agents [130].

Immunostimulatory Functions

Exosomes were first reported to have an immunostimulatory function in 1996, when Raposo *et al* showed that B cells releases MHC class II positive exosomes that can function as antigen presenting vesicles and induce an antigen-specific CD4⁺ T cell response *in vitro* [35]. Two years later, this immunostimulatory function of exosomes was also shown *in vivo* [41]. DC-derived exosomes harboring MHC class I, were shown to eradicate established tumours by the induction of a CD8⁺ T cell response in mice [41]. Following this study, others have shown that exosomes harboring MHC molecules can induce T cell responses, either indirectly or directly. Several studies have showed that DC-derived exosomes can stimulate both CD4⁺ and CD8⁺ T cells, but only in the presence of DCs, thus indirectly activating T cells [99, 121, 124, 128, 131-132]. They *et al* showed that naïve CD4⁺ T cells can be stimulated by DC-derived exosomes *in vivo* [99]. This T cell activation could not be seen *in vitro* unless mature DCs were added, thus

showing that the T cell stimulation was indirectly induced by the exosomes, where they are used by the DCs as a source of antigens [99]. In contrast, it has been shown that this antigen-specific T cell response can be induced directly by DC-derived exosomes [133-134]. Admyre *et al* showed that exosomes derived from human monocyte-derived DCs induced an MHC class I antigen-specific interferon-gamma (IFN- γ) production *in vitro* when added to autologous peripheral CD8⁺ T cells, without the addition of DCs [133]. This direct T cell stimulation was further shown in a cognate T cell-DC interaction study *in vitro* [123]. The investigators showed that the interaction of DC-derived exosomes (MHC class II and intercellular adhesion molecule 1 (ICAM-1) positive) with CD4⁺ T cells is not T cell receptor dependent but rather dependent on the leukocyte function-associated antigen-1 (LFA-1). Activated T cells, efficiently recruit DC-derived exosomes, however T cells in a resting state or T cells with blocked LFA-1 were shown to not recruit exosomes [123].

DCs have been shown to release exosomes with different properties depending on their physiological state. Mature DCs release exosomes that are enriched in MHC class II, ICAM-1 and CD86, compared to immature DC-derived exosomes, thus making them more immunostimulatory both *in vitro* and *in vivo* [102]. Furthermore, DC-derived exosomes have also been shown to promote natural killer (NK) cell activation and proliferation in an interleukin (IL)-15 α - and natural killer group 2 member D (NKG2D)-dependent way, in both mice and humans [135].

Exosomes with immunostimulatory effects are also released from other antigen presenting cells. Macrophages infected with different bacteria, including *Mycobacterium tuberculosis*, have been shown to release exosomes with proinflammatory functions both *in vitro* and *in vivo* [136]. These exosomes, released from bacteria-infected cells, were shown to contain pathogen-associated molecular patterns (PAMPs), and were able to activate uninfected macrophages, thus stimulating tumor necrosis factor-alpha (TNF- α) production through Toll-like receptors (TLRs) *in vitro*. They were also shown to stimulate TNF- α and IL-2 production *in vivo*, consequently inducing neutrophil and macrophage infiltration in the mice lung [136].

In addition, B cell-derived exosomes contain molecules such as MHC class II, and these have been shown to stimulate T cells [35, 137-138]. Muntasell *et al* further demonstrated that B cell-derived exosomes can stimulate primed CD4⁺ T cells, but not naïve T cells, suggesting a role for these exosomes in an ongoing immune response [137].

Exosomes can be released not only by antigen presenting cells, but are also released by other immune cells, as well as non-immune cells. Mast cell-derived exosomes were first characterised by Raposo *et al* in 1997, who showed that MHC class II-associated exosomes were released upon IgE-antigen trigger [39]. Since then, others have demonstrated that mast cell-derived exosomes have immunostimulatory functions [139-140]. Bone marrow-derived mast cells pretreated with IL-4 have been shown to release exosomes, while mast cell lines can spontaneously release exosomes, which can both activate B cells and T cells *in vitro* and *in vivo* [139]. Mast cell-derived exosomes have also been shown to stimulate DC maturation *in vivo* and to activate endothelial cells to secrete the plasminogen activator inhibitor-1 (PAI-1), leading to pro-coagulant activity *in vitro* [140].

Van Neil *et al* showed that intestinal epithelial cells release exosomes carrying MHC class II/antigen peptide complexes and were able to induce an antigen-specific immune response *in vivo* [100]. It has also been shown that intestinal epithelial cell-derived exosomes, when interacting with DCs, enhance antigen presentation to T cells, therefore suggesting they aid the immune surveillance at mucosal surfaces [141].

Immunosuppressive Functions

Immunostimulation is just one of the functions demonstrated by exosomes. Exosomes have also been shown to be capable of immunosuppression as they have been shown to induce antigen-specific tolerance [129]. Contrary from Van Neil *et al*, Telemo and colleagues showed that serum isolated from a rat feed with ovalbumin, could induce antigen-specific tolerance in naïve recipient animals. They also showed that this tolerogenic effect could be restricted to a ultracentrifugation pellet and were identified as exosomes. These exosomes were hypothesised to be derived from the intestinal epithelium, as exosomes isolated from *in vitro* pulsed intestinal epithelium cells, showed the same characteristics as the tolerogenic serum exosomes. Consequently, these tolerogenic exosomes were named “tolerosomes” [129]. Later, the same group used a mouse model to show that this antigen-specific tolerance, induced by the tolerosomes, was conferred via a MHC class II-dependent pathway [142]. Additionally, they showed that these tolerosomes could inhibit an allergic sensitisation in the recipient mice [143].

Intestinal epithelium-derived exosomes are not the only exosomes to induce tolerance, with exosomes from many cellular origins shown to have this ability. Exosomes isolated from BAL fluid of antigen-specific tolerised mice have been shown to inhibit allergen-induced airway inflammation [144].

Prado *et al* showed that mice pre-treated with BAL-derived exosomes from tolerised animals had reduced levels of IgE antibodies compared to those treated with control exosomes [144]. They also showed that mice pre-treated with tolerogenic exosomes have a significantly reduced production of the Th2 cytokine IL-5 and reduced levels of the regulatory cytokine IL-10. Furthermore, by examining the presence of specific proteins such as MHC class II and surfactant protein B (SP-B) (a protein from alveolar epithelium type II cells), lung epithelial cells were identified as the main source of the tolerising exosomes, as they could identify SP-B but not MHC class II [144].

Exosomes have been shown to have protective effects, with exosomes released by human embryonic stem cell-derived mesenchymal stem cells reducing the infarct size in mice during myocardial ischemia/reperfusion injury by mediating cardioprotection [94]. Another effect of exosomes have been shown during intestinal and cardiac transplantations, in both mouse and rat allograft models, where DC-derived exosomes can induce tolerance [94, 145-148].

Exosomes have been linked with suppression of the host immune response. Similar to tumours evading the immune system by manipulating its host's immune response, fetuses use a similar pathway to evade the maternal immune defense, called placenta-associated immunosuppression [149-154]. Placenta-derived exosomes, which have been shown to suppress the maternal immune system by carrying NKG2D ligands (NKG2DLs) such as MHC class I chain-related proteins (MIC) and the UL-16 binding proteins (ULBP), which can bind and downregulate the NKG2D receptor on NK cells, CD8⁺ and $\gamma\delta$ T cells, consequently reducing the cytotoxicity of these cells *in vitro* [149-150]. It has also been demonstrated, both *in vitro* and *in vivo*, that placenta derived-exosomes carry Fas-ligands (FasL) and induce Fas-mediated apoptosis *in vitro* [151-152]. In accordance with this, Taylor *et al* showed that placenta-derived exosomes from maternal serum carrying FasL can induce T cell suppression by the suppression of CD3- ζ and JAK3 *in vitro* [155]. Moreover, they showed that pregnant women delivering at term have significantly higher levels of placental exosomes in their serum, carrying higher levels of FasL, compared to women delivering preterm. Thus, these placental exosomes from term-delivering pregnancies induce a greater suppression of CD3- ζ and JAK3 [155].

Genetically modified DCs, transduced with adenoviral vectors expressing FasL, IL-10 or IL-4, have been revealed to produce exosomes capable of suppressing inflammation in a mouse model of both collagen-induced arthritis and delayed-type hypersensitivity, in Fas/FasL- and MHC class II-

dependent mechanisms. [156-158]. Endogenous plasma-derived exosomes isolated from immunised mice have also been shown to have anti-inflammatory and immunosuppressive functions that act in an antigen-specific manner, partly through the Fas/FasL pathway [159]. These exosomes were MHC class II, FasL and CD11b positive, but negative for CD11c, indicating that these exosomes might be originating from monocytes/macrophages and not from DCs.

Role of Exosomes in Tumourigenesis

Tumours have many ways of manipulating their environment and escaping the immune system. Tumour-derived exosomes have been shown to be able to assist tumours in two ways; by impairing the immune response, thereby allowing them to escape detection by the immune system, and by creating a tumour friendly environment. Exosomes have been shown to be released by tumour cells both *in vitro* and *in vivo*, as they have been found in peripheral blood [36, 73, 104], malignant effusions [45] and urine [160] from cancer patients. The role of exosomes in tumourigenesis has been a source of debate as some studies show that tumour-derived exosomes do not exert pro-tumourigenic effects, but rather anti-tumourigenic effects, thus, making them good candidates for cancer therapy.

Pro-Tumourigenic Effects of Tumour-Derived Exosomes

Clayton *et al* showed that tumour exosomes, expressing NKG2DLs, can downregulate the NKG2D-surface expression and as a result impair the cytotoxic function of CD8⁺ T cells [161-162]. Other studies have confirmed the role of NKG2DL-carrying tumour exosomes as tumour escape mediators [163-164]. Hedlund *et al* showed that exosomes derived from tumour cells, specifically leukemia and lymphoma cells, carries NKG2DLs which suppress the NK cell cytotoxicity, consequently promoting immune escape for these cells. They also demonstrated that the exosome secretion from these cells was significantly increased upon stress, such as thermal or oxidative stress, and cancer treatments generally expose the body to immense cellular stress [163]. In addition, tumour-derived exosomes have been shown to inhibit the cytotoxic activity of NK cells by blocking IL-2-mediated activation, thus promoting tumour growth [165].

Tumour exosomes can also contribute to tumour immune escape by skewing IL-2 responsiveness, thus driving the immune responses away from cytotoxic T cells and instead favoring regulatory T cells [166]. In addition to impairing

the CD8⁺ T cell function, tumour exosomes can also induce apoptosis of CD8⁺ T cells, mediated via Fas/FasL-interaction [167].

Anti-Tumourigenic Effects of Tumour-Derived Exosomes

In contrast to the pro-tumourigenic effects of tumour exosomes, other studies have established that tumour-derived exosomes do not suppress the immune system, but rather activate it [44-45]. Studies have shown that tumour exosomes from both cell lines and malignant effusions from cancer patients have high levels of MHC class I, heat-shock proteins and carry tumour-specific antigens such as Mart-1/Melan-A and gp100 [44-45]. Tumour-derived exosomes have also been shown to transfer tumour-specific antigen to DCs, subsequently activating tumour-specific cytotoxic T cell reactivity *in vitro*. These exosomes were also demonstrated to exhibit anti-tumour effects against established tumours by inducing T cell mediated anti-tumour immunity *in vivo* [44].

Role of Exosomes in Premetastatic Niche Formation

Tumour-derived exosomes have been shown to both activate and evade the immune system. They have also been shown assist tumour progression by being involved in premetastatic niche formation. The microenvironment surrounding tumours plays an important role in the tumour progression and soluble factors released by cells, such as cytokines and growth factors, have been established to induce cell proliferation, angiogenesis, invasiveness and metastasis of tumours. Many studies have shown results supporting the role of exosomes as mediators of metastasis as they promote tumour vasculogenesis, angiogenesis, invasion and metastasis [36, 168-169].

The “seed and soil” hypothesis [170] proposes that metastatic tumour cells, the “seed”, prepare their specific organ microenvironment, the “soil”, for metastasis. In concordance with this hypothesis, Hood *et al* showed that exosomes released by melanoma cells can act as the “seed” and prepare sentinel lymph nodes, the “soil”, for tumour metastasis [168]. They demonstrated *in vivo* that melanoma-derived exosomes home to the sentinel lymph nodes where they can recruit melanoma cells. Furthermore, they showed that they also increased the expression of extracellular matrix factors and led to the induction of angiogenic growth factors, both trapping and promoting the growth of metastatic melanoma cells [168].

Tumour growth has also been shown to be promoted by exosomes inducing angiogenesis *in vitro* and *in vivo* [74, 171-172]. Gesierich *et al* reported that tumour exosomes bearing the tetraspanin D6.1A/CO-029 (Tspan8), which

has previously been shown to be a strong angiogenesis inducer, induced endothelial cell branching *in vitro* and angiogenesis *in vivo* [171]. In addition, Peinado *et al* showed *in vivo* that exosomes derived from a highly metastatic cancer cell line could induce a larger metastatic tumour burden and tissue distribution compared to exosomes derived from a poorly metastatic cell line. They also showed that these tumour exosomes can promote a metastatic niche by educating bone marrow-derived cells towards a pro-vasculogenic and pro-metastatic phenotype through the upregulation of the MET oncoprotein [36]. Tumour-derived exosomes are not the only exosomes shown to be involved in the tumour progression, exosomes from IL-4 activated macrophages have been revealed to increase the invasiveness of breast cancer cells by the transfer of oncogenic miRNAs, like miR-223 [173]. Angiogenic activity, both *in vitro* and *in vivo*, has been shown to be induced by exosomes derived from CD34⁺ stem cells [174].

The Role of Exosomal RNA in Cell-to-Cell Communication

Exosomes have many different functions, which depend on both their cellular origin as well as the current state of that cell, as it influences the cargo of the released exosomes. However, these functions have primarily been associated with the exosomal proteins. The novel finding that exosomes contain RNA [71] has prompted many researchers to address the biological role of exosomal RNA, which further increases the complexity of the understanding of cell-to-cell communication. Valadi *et al* did not only demonstrate the presence of RNA in exosomes, but also demonstrated that the exosomal RNA could be transferred to recipient cells. This was demonstrated by culturing mouse mast cells with radioactive uridine prior to exosome isolation, thus incorporating radioactive RNA into the exosomes released by these cells. Radioactive RNA could be measured in recipient cells following co-culture with the exosomes, demonstrating RNA transfer from exosome to cell. Since exosomes themselves do not contain the complete machinery to produce proteins, the functionality of the RNA content was shown by using an *in vitro* translation assay, where mouse mast cell-derived exosomes were used as templates. Subsequently, after adding mouse mast cell-derived exosomes to human mast cells, newly produced mouse proteins could be identified in the human cells. These proteins were shown to be present only as mRNA, not as proteins, in the mouse mast cell-derived exosomes. Thus, this translation demonstrated the functionality of the exosomal mRNA [71].

The fact that exosomal mRNA can affect the protein production of recipient cells, and that it is predicted that one miRNA can interfere with 100-200 mRNA [175-176], suggests a potential biological role for exosomal RNA.

Subsequently, numerous studies have been published describing RNA in exosomes from numerous cellular sources [50, 71-73, 75, 89-90, 103-105, 107-109, 111, 160, 177-179] and functional transfer of exosomal RNA [50, 71, 73, 89, 107, 180-182] in various research fields including cell biology, reproduction [178], immunology [108, 183] and cancer.

In order for exosomal RNA to be functional, exosomes must not only be internalised by the recipient cell, but the exosomal RNA must also be transported to the cytosol where the mRNA can be translated and miRNA function as an inhibitor of translation. Montecalvo *et al* demonstrated that exosomes can interact with recipient cells by fusion with the cells plasma membrane, thus releasing the exosomal cargo into the cytosol of the recipient cell [107]. This was demonstrated by capturing luciferin inside the exosomes and co-culturing these with transgenic luciferase recipient cells. As luciferin is not able to cross lipid membranes, the measurement of luciferase activity in recipient cells, only 8 minutes after the cells received exosomes, indicates the delivery of the exosomal luminal cargo, including miRNA and mRNA [107].

The functionality of mRNA has been further shown in other studies by the use of luciferase reporter genes. Skog *et al* showed that the exosomal mRNA derived from glioblastoma cells could be both transferred and expressed in recipient cells [73]. They demonstrated this by transducing glioblastoma cells with a lentivirus vector encoding a luciferase reporter gene, thus producing exosomes containing luciferase activity. These exosomes were then added to endothelial recipient cells, where luciferase activity could be measured. Furthermore, they also showed that this activity increased over time, indicating an ongoing translation [73]. A similar study confirmed that exosomes could transfer mRNA coding for a luciferase reporter gene, leading to a luciferase activity in recipient cells. Furthermore, they showed that this activity was dose dependent [90].

Messenger RNA is not the only RNA species to be transferred by exosomes. MicroRNA has been shown to not only be transferred to recipient cells, but also to be functional [107, 180, 182]. Pegtel *et al* showed that Epstein-Barr virus (EBV)-transformed B cells release exosomes that contain mature EBV-miRNAs, which can be transferred to recipient cells [180]. They also showed that when incubated with EBV-miRNA containing B cell-derived exosomes, there was an 80% reduction in luciferase activity in recipient cells transfected with a luciferase vector carrying an EBV-miRNA-regulated sequence. However, cells expressing disrupted EBV-miRNA binding sites were shown to have significantly less reduction in luciferase activity. This study suggested that exosomes can regulate functional gene repression of specific

mRNAs targets in recipient cells by the transfer of functional miRNAs [180]. Montecalvo *et al* also demonstrated the delivery of functional miRNA via exosomes, by transfecting recipient cells with a luciferase reporter gene containing copies of the complementary target sequence for a specific miRNA. When exosomes containing the specific miRNA were incubated with these cells, the result was inhibited luciferase activity in these cells [107]. The functionality of exosomal miRNA has also been demonstrated with the transfection of cells with lentiviral vector short hairpin RNA (LV-sh) [182]. Exosomes released from cells transfected with LV-shCD81 resulted in the downregulation of CD81 surface expression in the recipient cells [182].

The complexity of cell-to-cell communication involving exosomal RNA was demonstrated by Zhang *et al* in 2011, with the discovery of exogenous plant miRNA in human serum, which could regulate mammalian gene expression [184]. MicroRNA-156a (miR-156a) and miR-168a, which have been shown to be enriched in rice, were found in high concentrations in serum of healthy Chinese subjects. Furthermore, they demonstrated an upregulation of these miRNA in both serum and liver of mice fed with a rice diet. They also showed that most of this miRNA found in mouse serum was contained within microvesicles. The authors also demonstrated that microvesicles released by transfected colon cells, thus containing miR-168a, could be taken up by liver cells and consequently upregulate their miR-168a concentration *in vitro*. In addition, the binding site for miR-168a was demonstrated, by bioinformatics analysis, to be the low-density lipoprotein (LDL) reporter adaptor protein 1 (LDLRAP1). The regulation of mammalian gene expression by exogenous plant miRNAs was demonstrated in mice fed with rice, as these mice showed an enrichment of miR-168a in both serum and liver and an inhibition of the LDLRAP1 in the liver, resulting in a decreased removal of LDL from the plasma in these mice. These results suggest that regulatory endogenous miRNA taken up by intestinal epithelial cells could be packed into vesicles, travel to different organs and cells and exert an effect, such as the liver [184].

The Role of Exosomes in the Transfer of Infectious Agents

Exosomes have not only been shown to be involved in the metastasis of cancers but have also been implicated in the spread of infectious agents. Prior to the knowledge about exosomes, HIV particles were demonstrated to accumulate in late endosomes of macrophages [185-186] and the fact that retroviruses and exosomes had biochemical similarities [187] led to the “The Trojan exosome hypothesis” [188]. “The Trojan exosome hypothesis”

proposes that retroviruses can hi-jack the exosomal biogenesis pathway for their replication and release, and consequently escape the host's immune system [188]. Contentiously, despite the similarities between retroviruses and exosomes, differences between the two have been demonstrated that argue against the "The Trojan exosome hypothesis" [189]. Furthermore, exosomes have been suggested to transfer the infectious prion proteins and β -amyloid peptides between cells [37, 130, 190].

The Potential of Exosomes in Diagnostics and Therapeutics

The Use of Exosomes in Diagnosis and Prognosis

Patients with different malignant diseases have been shown to have circulating tumour-derived exosomes containing tumour-associated antigens, mRNA and miRNA previously related to tumours, thus reflecting the tumour profile in the tumour tissue [36, 73, 104, 110-111, 191-193]. Circulating tumour exosomes can be easily sampled from patients (urine, plasma) using relatively non-invasive means. This quality and the fact that their content can reflect their origin, makes these circulating tumour exosomes excellent candidates as both diagnostic and prognostic markers for many different malignant diseases [36, 111, 160, 191-193].

The first study to show the potential of exosomes as biomarkers was conducted by Skog *et al*, who showed that 25% of plasma exosomes from patients with glioblastoma contain the mRNA for the mutated protein EGFRvIII, which is seen in about 50% of the tumours and is absent in healthy controls. They also showed that the mRNA could not be detected in the plasma exosomes two weeks after removal of the tumour, indicating that the tumour was the source of the exosomes [73]. Furthermore, the mRNA content of tumour-derived exosomes found in urine has been shown to be a potential source of biomarkers for prostate cancer [160].

It has been suggested that the miRNA content of exosomes could be used as diagnostic markers for lung cancer [111]. Rabinowits *et al* reported that serum exosomes from patients with lung cancer contain miRNA previously associated with lung tumours, suggesting that the miRNA isolated from the serum exosomes reflected the miRNA profile of the tumour tissue. The concentration of exosomes and the exosomal miRNA were both upregulated in the serum of patients with lung cancer, compared to control group, making them good diagnostic candidates [111]. In addition, the miRNA content of extracellular vesicles has also been shown to be useful as prognostic markers

to predict survival. Silvia *et al* demonstrated that the presence of let-7 miRNA in plasma-derived vesicles is related to non-small cell lung cancer. Furthermore, they showed that these cancer patients could be divided into let-7f low and let-7f high groups, with the low let-7f group having an overall survival rate of approximately 20% higher compared to the let-7f high level group at 46 months [194].

The RNA content of exosomes is not the only potential diagnostic and/or prognostic marker, the exosome concentration and the exosomal proteins may also serve as markers. Logozzi *et al* demonstrated that melanoma patients had a significantly higher concentration of plasma exosomes compared to healthy controls. They were also shown to have a significantly higher amount of exosomes expressing the tumour specific markers caveolin-1, compared to healthy controls. Circulating tumour-specific exosomes were shown to be decreased in patients undergoing chemotherapy [191]. Peinado *et al* also showed a melanoma-specific signature in the circulating exosomes of subjects with advanced melanoma [36]. They demonstrated that the VLA-4, Hsp70 and the melanoma-specific protein TYRP2, were increased in exosomes from subjects with advanced melanoma. Furthermore, they presented that the exosomal protein concentrations of circulating exosomes in subjects with advanced melanoma was higher than in normal controls, and that the exosomal protein concentration was related with survival prognosis, where protein-poor exosomes were associated with survival advantages compared to protein-rich exosomes [36].

Exosome-Based Immunotherapy

The immunostimulatory and immunosuppressive functions of exosomes, specifically DC-derived exosomes, have made them very attractive as therapeutic tools in clinical research, particularly for vaccines, allograft survival and for the treatment of inflammatory and autoimmune diseases such as arthritis [41, 147, 156-157, 195-197].

A successful cancer vaccine requires the induction of an antigen-specific T cell response and a long-term memory in the patients, which is both costly and time consuming to produce. The difficulty is due to the fact that to avoid MHC mismatch sequelae, autologous tumour samples from individual cancer patients are needed [198]. Several different approaches to deliver tumour antigens to patients have been developed and various antigen sources have been evaluated, such as antigenic peptides, recombinant proteins, recombinant microbial vectors, DNA or RNA constructs, irradiated whole

tumour cells and tumour cell lysates [199]. There is clearly a need for a safer, easier and less costly vaccine, such as a cell-free vaccine.

In 1998, exosomes were shown to have an anti-tumour effect, and thus of potential use in the immunotherapy of cancer. Zitvogel *et al*, showed that exosomes produced by mouse DCs, pulsed with tumour-peptides, suppress the growth of established tumours in mice [41]. After this discovery, others have explored the potential use of exosomes in cancer therapy. Several phase I studies using exosomes, have been undertaken. The first took place in 2005 by Escudier *et al* where autologous DCs-derived exosomes directly loaded with antigens, were injected into patients with malignant melanoma [197]. The results were promising and demonstrated that exosome administration was safe. In addition, another phase I study and a phase II study have been performed using DCs-derived exosomes immunotherapy in patients with advanced non-small cell lung cancer with promising results [196, 200].

Tumour-derived exosomes are natural carriers of tumour peptides and have been shown to stimulate T cells, both *in vitro* and *in vivo*, when loaded onto DCs [44-45]. As a result, tumour-derived exosomes have been studied for their potential as cell-free cancer vaccines. However, tumour-derived exosomes have not been shown to induce satisfactory anti-tumour effects *in vivo*, so numerous strategies have been attempted to improve their immunogenicity. Yang *et al* demonstrated that IL-2 gene modified tumour cells release IL-2 containing exosomes which are more efficient at inducing an immune response and consequently inhibiting tumour growth [201]. In another study, it was shown that heat-shocked lymphoma cells release exosomes that contain more Hsps and immunogenic molecules like RANTES, IL-1 β , MHC class I, MHC class II, CD40 and CD86. These exosomes were shown to be better at eliciting anti-tumour immune responses [202]. Furthermore, autologous ascites-derived exosomes combined with granulocyte-macrophage colony-stimulating factor (GM-CSF) as an adjuvant, have been used in the immunotherapy of colon cancer with a beneficial reaction in a cytotoxic T cell mediated response [203]. DC-derived exosomes have been shown to induce a more efficient anti-tumour response, compared to tumour-derived exosomes [204], and they have also been shown to induce a more potent immunogenicity when combined with adjuvants such as CpG [195].

Exosomes have also been shown to be useful for vaccination against both parasitic and viral infections. DCs pulsed with parasitic *Toxoplasma gondii*-derived antigens can induce an immune response specific to the *Toxoplasma gondii* infection, while exosomes spiked with the S proteins of the severe

acute respiratory disease coronavirus consequently lead to high levels of neutralising antibodies against the virus [205-207].

In addition, exosomes have been shown to be potentially useful in other types of immunotherapy. Exosomes released by chronic myelogenous leukemia cells have been shown to stimulate angiogenesis, both *in vitro* and *in vivo* in a nude mouse assay [172]. The potential therapeutic use of these exosomes in leukaemia patients has been demonstrated in a mice model, with carboxyamidotriazole-ototate, an orally bioavailable signal transduction inhibitor, shown to reduce exosome-stimulated angiogenesis which might increase survival in patients [208]. An alternative approach to inhibiting the natural angiogenesis ability of exosomes is to exploit this function as a potential therapy. This angiogenic function of exosomes could be used to improve the outcome and recovery of ischemic injuries, as CD34⁺ cell-derived exosomes have been shown to independently induce angiogenic activity, both *in vitro* and *in vivo* [174] Furthermore, exosomes released by FasL genetically modified or IL-10 treated DCs have been demonstrated to have immunosuppressive and anti-inflammatory functions. As a consequence, they may potentially be used for the treatment of inflammatory and autoimmune diseases such as rheumatoid arthritis [156-157, 159].

The immunosuppressive functions of tumour-exosomes have prompted a different approach to treating cancer. A phase I clinical trial is planned by Aethon Medical, who developed a medical device, The Aethlon Hemopurifier[®], to selectively remove immunosuppressive exosomes from the circulatory system, thus restoring the cancer patients immune system [114, 209].

Exosomes as Gene Delivery Vehicles

The delivery of genetic material, such as RNA, has been revealed to have potential therapeutic use for numerous diseases. However, using naked small interfering RNA (siRNA) is limited by its low efficiency rate as a result of a short half-life, negative charge and hydrophilicity, which impair their cellular uptake. The delivery of naked siRNA poses another difficulty as it normally accumulates unspecifically in the spleen, liver and kidney, rather than at a specific site for the best effect. To overcome these problems, siRNA can be encapsulated in a vector such as viruses, nanoparticles or liposomes. However, vectors such as viruses will eventually be detected by the immune system, triggering an immune response [210-212]. Exosomes are naturally occurring vectors capable of transferring both antigens and genetic material between cells. Compared to other vectors, exosomes have the advantage of

being endogenous and therefore are not detected by the immune system as harmful. This feature has made them attractive candidates as vectors for gene therapy.

The first proof of concept study for the use of exosomes as vectors for siRNA was demonstrated by a group in Oxford in a mouse model [213]. They reported that exosomes can not only be loaded with a specific siRNA, but that they can also be modified to be delivered to a specific cell/organ. This was demonstrated by constructing a plasmid with a neuron cell targeting peptide, RVG, fused to a membrane protein, Lamp2b, which is known to be enriched in exosomal membranes. The plasmid was then transfected into mouse bone marrow-derived immature DCs and resulted in the release of exosomes with the RVG peptide on their surface. The modified exosomes were then loaded using electroporation, with therapeutic siRNA designed to knock down the BACE1 gene, which is a gene implicated in Alzheimer's disease. Mice receiving these exosomes displayed a reduced level of BACE1 in the brain, demonstrating the specificity of the RNA-loaded exosomes and the therapeutic potential [213].

1.4 Uveal Melanoma

Uveal melanoma is a tumour that arises in the melanocytes of the uveal tract and is the most common primary cancer of the eye [214] and is the most common melanoma after cutaneous melanoma [215]. Despite successful control of the primary tumour, about 30% of patients with primary uveal melanoma develop distant metastases with the most common site of metastasis being the liver in approximately 90% of cases [216]. In two-thirds of the uveal melanoma cases, the retinoblastoma is inactivated by hyperphosphorylation as a consequence of cyclin D over-expression. The mechanism behind the over-expression of cyclin D is yet not fully understood, but one route could be through the constitutive activation of the MAPK pathway as a result of an oncogenic mutation [214]. These mutations can be on different genes in different cancers, as for cutaneous melanoma where mutations in the BRAF, RAS and KIT genes result in a constitutive activation of the MAPK pathway. Interestingly, these mutations are not found in uveal melanoma [214, 217-220], although the activation of the MAPK pathway is common [218], however it rarely occurs through mutation of BRAF or RAS. In uveal melanoma, the most common mutation occurs in the GNAQ gene [221-223]. In addition, GNA11, a paralogue gene to GNAQ, has also been found to be frequently mutated in uveal melanoma [221]. Mutations in GNAQ or GNA11 occur in about 80% of the uveal melanoma cases [221]. Aberrant regulation of miRNAs has also been associated with the regulation of uveal melanoma, such as the cyclin D2, MITF and BCL2 suppressor miR-182, which have been shown to be downregulated in uveal melanoma [224]. Furthermore, miR-34a which is also a tumour suppressor in uveal melanoma, by inhibition of c-Met, has been presented to be downregulated in uveal melanoma [225]. Additionally, the PI3K–AKT pathway, which is a major mediator of cell survival, has been shown to be activated in many uveal melanomas. PTEN, which is a negative regulator of the PI3K–AKT pathway, is also downregulated or completely inactivated in many uveal melanomas [226].

2 AIMS

The overall aim of this thesis is to evaluate the biological role of exosomes and exosomal RNA.

The specific aims of this thesis are:

- To determine the exosomal RNA profile variation using different isolation methods.
- To determine whether exosomes can influence the response of other cells during oxidative stress.
- To determine whether oxidative stress changes the RNA content of exosomes.
- To determine whether the exosomal RNA content is involved in cell-to-cell signalling.
- To determine whether liver metastases from uveal melanoma releases exosomes.

3 METHODOLOGY

3.1 Cell Culture

The mouse mast cell line MC/9 (ATCC, Manassas, VA) was used in paper I and II, and they were cultured in DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol (all from Sigma-Aldrich, St Louis, MO, USA) and 10% Rat T-Stim (BD Biosciences, Erembodegem, Belgium).

In paper II, the MC/9 cells were subjected to oxidative stress, which was induced by exposing cells to 125 µM hydrogen peroxide (H₂O₂) (Sigma-Aldrich) for 24 hours under cell culture conditions.

In paper III, the human mast cell line HMC-1.2 (Dr. Joseph Butterfield, Mayo Clinic, Rochester, MN, USA), the human malignant melanoma cell line A375 (ATCC), the human malignant melanoma cell line MML-1 (CLS, Eppelheim, Germany), the human breast cancer cell line HTB-133 (ATCC) and the human lung carcinoma cell line HTB-177 (ATCC) were used.

The A375 cells were cultured in DMEM medium, 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (all from Sigma-Aldrich) and 1 % Non-Essential Amino Acids (PAA laboratories, Pasching, Austria).

The MML-1 cells, HTB-133 cells and the HTB-177 cells were all cultured in RPMI-1640 containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (all from Sigma-Aldrich). In addition, the HTB-133 complete growth medium also contained 0.2 units/ ml insulin (Sigma-Aldrich)

The HMC-1.2 cells were cultured in IMDM, 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 1.2 mM alpha-thioglycerol (all from Sigma-Aldrich).

All complete growth media used for cell cultures in this thesis (paper I, II and III) include serum, which contain exosomes. To eliminate these serum exosomes, present in both Rat T-Stim (paper I and II) and FBS (paper I, II and III) the serum was ultracentrifuged at 120 000 x g, 4°C (Ti45 rotor, Beckman Coulter, Brea, CA, USA). In paper I and II this was done for 90

minutes and for paper III the time was increased to over night. Furthermore, all cells were cultured at 37°C and 5% CO₂.

3.2 Patients

In paper III, twelve patients with isolated liver metastases from uveal malignant melanoma, during the period of November 2010 to October 2012, underwent treatment with isolated hepatic perfusion (IHP). Inclusion criteria included less than 50% of the liver replaced by tumour and no extra hepatic tumour manifestations. Patient characteristics are summarised in Table 1 in paper III. The study was approved by the Regional Ethical Review Board at the University of Gothenburg.

3.3 Isolated Liver Perfusion and Sample Collection

In paper III, the IHP technique, which has been described previously [227], was used. In brief, the liver was isolated by clamping and cannulation of the hepatic artery and the inferior caval vein, and the portal vein was clamped. The hepatic artery and inferior caval vein cannulas were then connected to an oxygenated extracorporeal circuit and the liver was perfused with a priming solution. Thereafter, melphalan (1 mg/kg body weight) was administered into the perfusion system. The temperature was held at 40°C with a total perfusion time of 60 minutes. After perfusion, the liver was washed with 1 L of Ringer's solution (Baxter, Deerfield, IL, USA) and one unit of erythrocytes was added. Liver perfusate (approximately 200 ml) was collected after 10 minutes of perfusion with priming solution, before melphalan was added. In addition to the liver perfusate, peripheral blood samples were taken before surgery, 4 weeks and 3 months after surgery. Plasma was obtained from both liver perfusate and peripheral blood by centrifuging at $400 \times g$ for 10 minutes followed by a second centrifugation at $1\,880 \times g$ for 10 minutes.

3.4 Exosome Isolation

In all papers (I, II and III), exosomes were isolated using the purification procedure based on differential centrifugations together with membrane filtration, using a Beckman Coulter Ultracentrifuge (rotor Ti45 or Ti70). For isolation of exosomes from cell culture (paper I, II and III), the cell suspensions were centrifuged for 10 minutes at $300 \times g$, to pellet the cells, and the exosomes were prepared from the supernatant. The cell supernatant was first centrifuged at $16\,500 \times g$ for 20 minutes, to remove remaining cells

and cell debris. It was then filtered through 0.2 μm filters, to remove any molecules larger than 200 nm. The exosomes were then pelleted by an ultracentrifugation at $120\,000 \times g$ for 70 minutes, all steps at 4°C .

In paper III, exosomes were also isolated from human blood plasma. The first high-speed centrifugation was then raised to $29\,500 \times g$ for 20 minutes, and the final ultracentrifugation, to pellet the exosomes, was extended to 90 minutes.

3.5 RNA Extraction

In paper I, seven different RNA isolation methods were used to extract RNA from both cells and exosomes; Trizol[®] (Invitrogen, Paisley, UK), Trizol[®] followed by cleanup using the modified RNeasy[®] Mini Kit (Qiagen, Hilden, Germany), RNeasy[®] Mini Kit, modified RNeasy[®] Mini Kit, miRNeasy Mini Kit (all three from Qiagen), miRCURY[™] RNA Isolation Kit (Exiqon, Vedbaek, Denmark) and mirVana[™] miRNA Isolation Kit (Ambion, Austin, TX, USA). Each exosomal sample was isolated from a large volume of cells, and the exosome pellet was dissolved in nuclease free water and subsequently split and transferred to seven different RNase free tubes for RNA isolation. Both cells and exosomes were immediately lysed in respective lysing solution and continued for RNA purification. Trizol[®], RNeasy[®] Mini Kit, miRNeasy Mini Kit, miRCURY[™] RNA Isolation Kit and mirVana[™] miRNA Isolation Kit were all used according to the manufacturer's protocol but with the double volume lysing buffer for the RNeasy[®] Mini Kit. A modified version of the RNeasy[®] Mini Kit was used (<http://www1.qiagen.com/>), to extract small RNA under 200 nucleotides. In brief, cells and exosomes were disrupted and homogenised in 700 μl RLT buffer containing 1 % β -mercaptoethanol and 3.5 volumes of 100% ethanol were added to the samples prior to the use of the RNeasy[®] mini spin column. The samples were washed twice in 500 μl RPE buffer and eluted in RNase free water. For Trizol[®] followed by modified RNeasy[®] Mini Kit, the Trizol[®] protocol was followed until the phase separation and it was then continued according to the modified RNeasy[®] Mini Kit. The elution volume for mirVana[™] miRNA Isolation Kit was 100 μl , for the other methods the RNA was eluted in a volume of 50 μl .

In paper II Trizol[®] (Invitrogen) was used to extract RNA from both cells and exosomes, and in paper III the miRCURY[™] RNA Isolation Kit (Exiqon) was used to extract exosomal RNA. Both were used according to manufacturer's protocol.

3.6 RNA Analyses

3.6.1 Bioanalyzer

In paper I and III, the total RNA quality, yield and size distribution pattern of exosomal and cellular total RNA was analysed using chip-based capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, Foster City, CA, USA) with the total RNA 6000 Nano and the 6000 Pico Kit, according to manufacturer's protocol. Additionally, in paper I small RNA was analysed using the small RNA kit in the Agilent 2100 Bioanalyzer. For the small RNA analysis, both RNA diluted to contain 10 ng total RNA or diluted 10X for all except mirVana™, which was eluted in the double volume and therefore diluted 5X, was analysed according to manufacturer's protocol. Bioanalyzer electropherograms were analysed using the Agilent 2100 Expert B.02.07 software that includes data collection, presentation, and interpretation functions

Also, in paper I total RNA was in separate experiments, treated with or without RNase A (final concentration of 5 µg/µL, Fermentas, St. Leon-Rot, Germany) for 90 minutes at 37°C and analysed using the total RNA 6000 Nano Kit.

3.6.2 Spectrophotometric Analysis

In paper I, the cellular and exosomal RNA purity was evaluated using ultraviolet (UV) absorption using a SPECTRAMax PLUS 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at the absorbance 230, 260 and 280 nm. The average A260/230 and A260/280 ratios were used to assess the presence of peptides, phenols, aromatic compounds or carbohydrate and proteins.

3.6.3 cDNA Synthesis

In paper I and III, total RNA was DNase treated using Turbo DNase Free kit (Ambion) prior to cDNA synthesis. In paper I, exosomal RNA isolated by two different RNA extraction methods was analysed, and in paper III exosomal RNA from both patient liver perfusate and from five different cell lines were analysed.

In paper I, miRCURY™ LNA™ micro PCR System (Exiqon) was used for first strand cDNA synthesis and real-time PCR according to manufacturer's protocol. In brief, each cDNA synthesis was performed in duplicates using a

fixed volume of total RNA, miR-451 specific reverse primer and first strand cDNA synthesis kit reagents, and incubated for 30 minutes at 50°C followed by 10 minutes at 85°C. Each cDNA sample was then diluted 1:10.

In paper III, the RT² miRNA First Strand Kit (Qiagen) was used for cDNA synthesis according to manufacturer's protocol. In brief, total RNA was mixed with the first strand cDNA synthesis kit reagents and incubated for 120 minutes at 37°C followed by 5 minutes at 95°C. Each cDNA sample was then diluted 1:10.

3.6.4 Real-Time PCR

In paper I, each cDNA sample was used in duplicates together with miRCURY[™] LNA[™] SYBR[®] Green master mix, Universal primer and LNA[™] PCR miR-451 specific primer (all from Exiqon).

In paper III, a panel of 88 cancer-related miRNA using RT² miRNA PCR arrays (MAH-108A) and RT² SYBR[®] Green qPCR Mastermix (both from Qiagen, Germantown, MD, USA) were used according to manufacturer's instructions.

In both paper I and III, the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) was used for real-time PCR reactions and the results were analysed using the CFX Manager[™] software (Bio-Rad).

For further information regarding the real-time PCR analyses, please refer to paper I and III respectively.

3.6.5 Bioinformatic Analyses

Affymetrix Microarray

In paper II, RNA was analysed by Affymetrix microarray. The Mouse Genome 430A 2.0 microarray (Affymetrix, Santa Clara, CA, USA) was performed by SweGene (www.swegene.org/) according to Affymetrix microarray DNA chip analysis (Affymetrix). Gene expression profiles were analysed using the MAS5.0 software (Affymetrix). The microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO). Details can be found at <http://www.ncbi.nlm.nih.gov/geo> (the GEO accession number is: GSE24886).

Clustering and Pathway Analyses

In paper III, miRNAs were clustered using Cluster 3.0, and the analysis was performed with a hierarchical method using an average linkage and Euclidean distance metric to illustrate relationships between the different patients and cell lines. Also, a heat map of the clustering data was visualised using Microsoft Excel. Furthermore, enriched KEGG pathway analyses for miRNAs was conducted via DIANA-miRPath v.2.0 software based on predicted targets by DIANA-microT-CDS [228]. Targets of miRNAs with a score of more than 0.8 were selected. Only KEGG pathways including at least 5 genes and displaying an enrichment p-value of less than 0.05 were considered significant.

3.7 Protein Isolation and Measurement

In paper II and III, total protein was extracted from cells and exosomes using modified RIPA buffer or 20 mM TrisHCL1%SDS lysis buffer, followed by sonication and vortex. In paper II, total protein concentration was measured from the supernatant using Dc protein assay reagents A and B (BioRad), the proteins were precipitated by TCA prior to the measurement. In paper III, exosomes were measured for their total protein content using the BCA[™] protein assay kit (Thermo Scientific Pierce, Rockford, IL, USA) according to manufacturer's recommendations.

Furthermore, the total exosomal protein measurement was also used for exosome quantification in paper III, as this is often used for this purpose as currently there is no well-established and reliable method for this.

3.8 Immunostaining

3.8.1 Western Blot

Two different systems were used for Western blot in paper II and III. In both papers, exosomal proteins were separated on 1D gels, polyacrylamide gels in paper II and on Mini-Protean TGX precast gels (Any kD, Bio-Rad Laboratories, Hercules, CA, USA) in paper III. The polyacrylamide gels were then blotted onto nitrocellulose membranes (Bio-Rad) and when the TGX precast gels were used they were transferred to Trans-Blot Mini PVDF membranes using the Trans-Blot[®] Turbo[™] Transfer system (Bio-Rad). Caspase-3 (paper II) and Melan-A (paper III) was detected by using anti-

Caspase-3 (1:150; clone 269518, R&D Systems, Minneapolis, MN, USA) and anti-Melan-A (1:250; clone FL-118, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and as secondary antibody a peroxidase-conjugated donkey-anti-rabbit IgG F(ab')₂ Fragment was used (1:10 000; Jackson Immuno Research, Baltimore, PA, USA or GE Healthcare, Little Chalfont, UK). The proteins were then visualised and analysed with the Amersham™ ECL Plus™ Western Blotting Detection System (GE Healthcare), VersaDoc™ 4000 MP Imaging System (Bio-Rad) and the Quantity One® software (Bio-Rad).

3.8.2 Detection of Oxidized Proteins

In paper II, OxyBlot™ oxidized protein detection kit (Millipore, Billerica, MA, USA) was used according to the manufacturer's recommendations for the detection and quantification of oxidized proteins. In brief, the protein carbonyl groups, which are a consequence of the oxidative stress modifications, were derivatised. Equal amounts of protein were separated on polyacrylamide gels and blotted onto nitrocellulose membranes (Bio-Rad). The oxidized proteins were then detected using primary antibody specific to the derivatised carbonyl group on the proteins, followed by horseradish peroxidase-coupled secondary antibody (both specific to the OxyBlot™ kit; Millipore). The proteins were then visualised using Amersham™ ECL Plus™ Western Blotting Detection System (GE Healthcare) and VersaDoc™ 4000 MP Imaging System (Bio-Rad). The Quantity One® software (Bio-Rad) was used for relative quantification.

3.8.3 Flow Cytometry Analysis

Exosomes are too small to be detected by available flow cytometry methods, and are therefore attached to antibody-coated beads before being analysed (Figure 3).

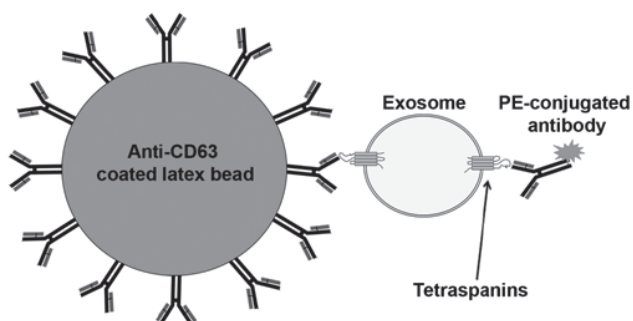


Figure 3. Schematic representation showing the coupling of exosomes to antibody-coated beads for flow cytometry analysis.

In paper III, latex beads (Interfacial Dynamics, Portland, OR, USA) coupled with anti-CD63 antibody (BD Biosciences, Erembodegem, Belgium) were used for flow cytometry analysis of patient liver perfusate-derived exosomes. In brief, 25 μl 4- μm -diameter aldehyde/sulphate latex beads (30×10^6 beads; Interfacial Dynamics) were washed twice in 100 μl MES buffer, $3\,000 \times g$ for 15 minutes and the pellet was redissolved in 100 μl MES buffer. The antibody mixture was then prepared by mixing 12.5 μg of anti-CD63 antibody (BD Biosciences) and equal volume of MES buffer. The beads were then incubated with the antibody mixture under gentle agitation at room temperature (RT) overnight, washed three times with PBS, $3\,000 \times g$ for 20 minutes. The pellet was then dissolved in 100 μl storage buffer (0.1% glycine and 0.1% sodium azide in PBS with a pH of 7.2) with a final concentration of 300 000 beads/ μl . Exosomes were resuspended in PBS, and 30 μg of exosomes were incubated with 1.5×10^5 anti-CD63-coated beads for 15 minutes at RT. PBS was then added up to 300 μl and the beads were incubated at 4°C overnight under gentle agitation after which the reaction was stopped by incubating with 100 mM glycine for 30 minutes at RT. The exosome-bead complexes were then washed twice in FACS buffer (3% exosome depleted FBS in PBS) and incubated in human IgG (Sigma-Aldrich) at 4°C for 15 minutes, followed by incubation with PE-conjugated anti-CD9 (clone M-L13), anti-CD63 (clone H5C6), anti-CD81 (clone JS-81) antibody or isotype control (all antibodies were from BD Biosciences) for 40 minutes at RT under gentle agitation. The exosome-bead complexes were then washed twice, resuspended in 300 μl FACS buffer and analysed by flow cytometry using a FACSAria (BD Biosciences, San Jose, CA, USA) and analysed using FlowJo Software (Tri Star Inc, Ashland, OR, USA).

3.9 Electron Microscopy

In paper III, electron microscopy was performed on liver perfusate derived-exosomes from one patient. Exosomes, re-suspended in PBS, were loaded and incubated for 10 minutes on formvar/carbon-coated nickel grids (Ted Pella Inc, Redding, CA, USA) that been UV-treated for 5 minutes prior. The exosomes were then fixed in 2% paraformaldehyde and washed with PBS. The exosomes were immunostained with anti-CD63 antibody (BD Bioscience) or isotype control (Sigma-Aldrich), followed by staining with a 10 nm gold-labeled secondary antibody (Sigma-Aldrich). The exosomes were then fixed in 2.5% glutaraldehyde, washed in ddH₂O and contrasted in 2% uranyl acetate. The exosomes were examined using a LEO 912AB Omega electron microscope (Carl Zeiss NTS, Jena, Germany).

3.10 Transfer Experiment

In paper II, exosomes isolated from MC/9 donor cells, exposed to H₂O₂ (125 μM) or vehicle (complete medium) for 24 hours, were redissolved in complete medium and added to MC/9 recipient cells in the ratio of 1.7:1. The recipient cells were subsequently challenged with H₂O₂ (125 μM) and harvested after 0, 2, 12 and 24 hours. The cell viability was assessed by using the trypan blue dye exclusion method.

In separate experiments, exosomes were redissolved in PBS, and exosomes isolated from cells exposed to H₂O₂ were then subjected to ultraviolet (UV)-light (254 nm) for 1 hour at 0-4°C prior to transfer. As controls, exosomes released by cells exposed to H₂O₂ or vehicle, not subjected to UV-light, were kept at 4°C for 1 hour prior to transfer. The recipient cells were harvested after 0, 2 and 12 hours.

3.11 Statistical Analyses

Data are expressed as mean ±SEM where appropriate. In paper II, when comparing more than two groups, statistical analysis was performed using one-way ANOVA. When comparing two conditions, and also the differences in gene expression between normal conditions and oxidative stress, paired t-test analyses were used. In paper III, statistical analysis was performed using Student t-test. A p-value less than 0.05 was regarded as statistically significant.

4 RESULTS AND DISCUSSION

In this section, I will present and discuss the main findings obtained in papers I, II and III. For more detailed information please see the respective papers.

4.1 Extraction of Exosomal RNA (paper I)

The finding that exosomes can carry and transfer RNA [71] resulted in a great deal of interest in this field, which has rapidly expanded as a consequence. In the initial years after this novel finding, several papers were published describing RNA in exosomes from different cellular origins [50, 71, 73, 89-90, 104-105, 108-109, 180]. The exosomal RNA was described to be enriched in small RNA in some reports and in others, it was shown to have a much broader RNA size distribution pattern. The variety of reports about exosomal RNA revealed a need to determine whether this difference observed in the RNA size distribution was a true variation or a consequence of the different RNA extraction methods used.

Unlike other cell lines, some of which require stimulation to release exosomes, the mouse mast cell line MC/9 spontaneously release exosomes, removing any artificial effects. The MC/9 cell-derived exosomes were used to evaluate seven different RNA isolation methods (Table 1, paper I) with different extraction techniques from four different companies, to determine their efficiency in extracting exosomal RNA. To accurately compare the seven methods, each exosomal sample was isolated from a large volume of cells, and the exosome pellet subsequently split into seven fractions for the seven different RNA isolation methods (Figure 1, paper I).

All extraction methods were able to extract RNA with high quality and purity (Table 2 and Figure 5A-G, paper I), but showed a vast variation in both yield and size distribution of exosomal RNA, even though the exosomes were harvested from the same cell line. The quality of exosomal RNA could not be analysed directly as exosomal RNA lacks the two prominent 18S and 28S rRNA subunits, which are needed for the algorithm for calculating RNA quality. Therefore, cells and exosomes were run in parallel and the cellular RNA quality was used as a reference for the exosomal RNA quality.

The RNA yield, of both exosomes and their donor cells, was determined using chip-based capillary electrophoresis (Agilent 2100 Bioanalyzer). The results showed that the RNA yield differed markedly between the different

RNA isolation methods for both cells and exosomes (Figure 3A-B and Figure 4A-B, paper I) with the cellular RNA yield ranging from 1.0 ± 0.7 to 6.3 ± 1.1 $\mu\text{g}/\text{million}$ cells (Table 2, paper I) and the exosomal RNA yield ranging from 13.0 ± 7.7 to 107.7 ± 25.7 $\text{ng}/\text{million}$ donor cells (Table 3, paper I). The pure column based method, miRCURY™, resulted in the highest total RNA yield for both cells and exosomes, with a cellular RNA yield of 6.3 ± 1.1 $\mu\text{g}/\text{million}$ cells and 107.7 ± 25.7 $\text{ng}/\text{million}$ donor cells for exosomes (Table 2 and 3, paper I).

Interestingly, this variation in total exosomal RNA yield was shown to correlate with the different extraction techniques used by the different RNA isolation methods. The methods that showed the lowest yield were all obtained using phenol-based methods (including the combined phenol and column based techniques), while the methods that showed the highest yield were all column based approaches. However, this correlation was not found for the cells. This, together with the fact that all methods, except one (miRCURY™), showed a difference when comparing the cells and exosomes, might be explained by the difference in their membranes. Exosomes have been shown to have very rigid membranes [93], enriched in tetraspanins [98], sphingomyelin [229] and cholesterol [69], compared to the less rigid donor cells plasma membrane.

We could also see that the exosomal RNA size distribution pattern, also assessed using the Bioanalyzer, differed substantially between the different RNA isolation methods evaluated (Figure 4B). In cells however, this difference is difficult, or impossible, to detect as the mRNA and small RNA expression is masked by the high rRNA content (Figure 4A). In the Bioanalyzer electropherograms of the exosomal RNA we could see an extensive variation in the relative presence of smaller and larger RNA molecules, thus some methods would give the impression that exosomes are enriched in lower molecular weight RNA. The column based RNA extraction techniques resulted in a broader size distribution pattern compared to the phenol based methods, which were shown to be relatively more efficient at extracting small RNA rather than total RNA.

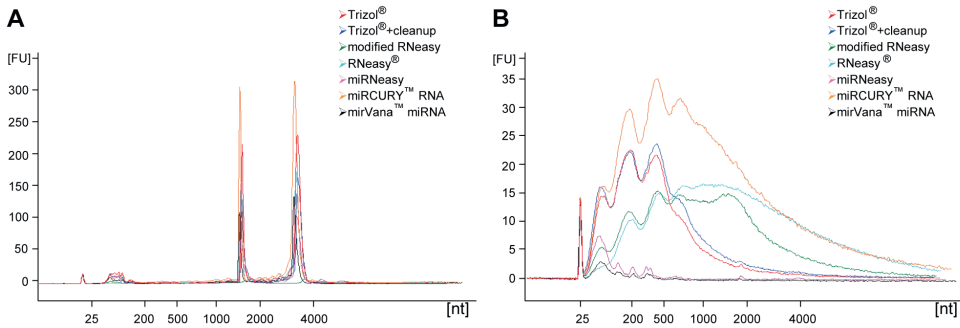


Figure 4. Bioanalyzer electropherogram showing the total RNA size distribution of cellular (A) and exosomal (B) RNA. Reprinted from Eldh et al, *Molecular Immunology*, 2012;(50):278–286 with permission from Elsevier.

Given the variation in the exosomal total RNA size distribution, where some methods seemed more efficient than others at extracting small RNA, we further investigated their ability to extract small RNA, again by using the Bioanalyzer. We could see that all methods extracted small RNA (Figure 6A, paper I), but again, with different efficiencies. The extraction of small RNA was most efficiently extracted by the Trizol[®] and miRCURY[™] methods, which had the highest yield. To confirm the variation in exosomal small RNA size distribution, we determined the relative amount of small RNA extracted by the different methods. This was achieved by loading the same amount of total RNA onto the Bioanalyzer chip, rather than the same volume (Figure 6B, paper I). This showed that the miRNeasy and mirVana[™] extracted a higher relative amount of small RNA compared to the other methods.

All methods evaluated were shown to extract small RNA, including RNA with the size of miRNA. These results were confirmed by performing real-time PCR on two distinct different methods, the pure column based method miRCURY[™] and the combined phenol and column based method mirVana[™]. In a previous study [71], miR-451 was shown to be one of the most abundant miRNA in MC/9 cell-derived exosomes, therefore this miRNA was selected for the miRNA validation. We chose to use a fixed volume of RNA rather than a fixed concentration of RNA, which is usually used for cDNA synthesis. A fixed concentration of RNA is usually used when comparing two different samples, making the fixed volume of RNA a better method for comparing the same sample using two different methods. The results confirmed that both extraction methods do indeed isolate miRNA, with the pure column based method resulting in a higher yield.

In conclusion, the results in paper I show unequivocally that the exosomal RNA pattern largely depends on the extraction method used, and shows that exosomal RNA results cannot be directly compared when different methods have been used. As demonstrated by these results, there is a need to standardise the methodology for isolating RNA from exosomes and a need for careful consideration of the method choice, which would depend on the research question at hand. It should be acknowledged that exosomes from different sources, in addition to the method chosen, may result in different RNA patterns. So even if a consensus is reached regarding the methodology, exosomes derived from different cellular origins and other extracellular vesicles may still not be directly comparable. All methods evaluated are good methods for extracting RNA from exosomes as the current methods for analysing RNA are all very sensitive. As working with exosomes often means working with small quantities, it is advisable to choose a method that results in a high yield of RNA, in order to retain sufficient material for answering research questions that arise. Additionally, it is advisable to choose a method that allows the analysis of both larger and smaller molecular weight RNA such as the pure column based method miRCURY™.

4.2 Role of Exosomes and Exosomal RNA in Oxidative Stress (paper II)

Many different biological functions had been described for exosomes at the start of this study. These functions were shown to depend on both their cellular origin and the cellular conditions under which they are released. These functions included antigen presentation [35, 133], induction of tolerance [129] and promotion of cancer cell growth [161], suggesting infinite effects *in vivo*. In addition, the exosomal RNA content from different cellular origins, and the ability of exosomes to transfer functional RNA to recipient cells had been described [71, 73]. It had also been shown that the exosomal RNA content differed to that of the originating cell, suggesting a selective packaging of the RNA into the exosomes [71]. At this time however, there were no published papers describing the exosomal RNA derived from either different physiological states of the originating cells or the biological role of exosomal RNA. We hypothesised that the exosomal RNA content would differ, not only depending on the cellular origin, but also on the physiological states of the originating cells and furthermore, these exosomes, derived from different conditions, would elicit different effects on the recipient cells.

As a model to study this hypothesis, we chose oxidative stress, which can be induced by H₂O₂ [230-231]. This model is a well established stress model and oxidative stress has been implicated in many diseases including asthma [232-233] and cardiovascular disease [234]. Furthermore, it has been documented that cells pre-treated with a low H₂O₂ dose develop a resistance to higher doses of H₂O₂ [235-237]. Therefore, for this study we chose normal conditions and oxidative stress as a model of different physiological states. In addition, we decided to work with the mouse mast cell line MC/9 which had previously been shown to spontaneously release exosomes [71], unlike many cell lines that require stimulation to release exosomes, which might add extra stress.

To determine if the exosomal RNA content of exosomes differed depending on which cellular conditions they were derived from, the RNA content of the exosomes and their donor cells, from both normal conditions and oxidative stress, were evaluated using Affymetrix microarray analysis. This analysis confirmed the findings of previous studies, with a poor correlation observed between cellular mRNA and exosomal mRNA [71], under either condition, indicating a difference in mRNA content of exosomes and their donor cells (Figure 4D and 4E, paper II).

We also showed for the first time that the mRNA content of exosomes, derived from cells grown under different conditions, is substantially different (Figure 4F, paper II). One speculation for the different mRNA content of exosomes derived from different conditions could be that the RNA-binding proteins may differ in cells exposed to stress, compared to cells grown under normal conditions, which might result in different mRNA being packed into the exosomes. Further investigation is required to confirm this hypothesis. The mRNA that do correlate between the exosomes derived from different conditions might be explained by the fact that the exosome population derived from oxidative stress also contains exosomes produced before the cells were exposed to stress.

The results also revealed that the relationship between significantly changed mRNA transcripts in exosomes changed vastly when released under different conditions, which was not observed for the cells (Figure 5, paper II). The specific mRNA transcripts that were induced in exosomes released under oxidative stress, compared to those in exosomes released under normal conditions, were unexpected, as we anticipated that some of the most induced mRNA transcripts would be stress-related, which was not the case (Table 1, paper II).

The RNA content of exosomes is not a random sample of the cellular mRNA but is specifically packed into the exosomes. This concept is supported by the evidence that the exosomal mRNA content differs from that of the originating cell [71, 73, 110] and also between the different conditions, as we have shown in this study. In addition, the oxidized protein content of these exosomes derived under different conditions was not a random sample, further supporting the specific packaging concept. This was demonstrated by comparing the degree of oxidization of cellular and exosomal proteins by studying the carbonyl groups introduced by the H₂O₂ exposure. As expected, in the cells grown under different conditions, we could see an increase in oxidized proteins in cells exposed to H₂O₂ (Figure 3A, paper II). However, this difference was not seen in the exosomes derived from the different conditions (Figure 3B, paper II). Together, these results indicate that the molecular content of exosomes is closely regulated depending on the physiological state and/or function of cells.

To determine if the conditions under which exosomes are produced and released affect their biological function, exosomes harvested from cells cultured under normal conditions and under oxidative stress for 24 hours, were incubated with untreated recipient cells, which were then exposed to oxidative stress at the same concentration as the donor cells. The viability of

the recipient cells was then examined by trypan blue dye exclusion at 0, 2, 12 and 24 hours after H_2O_2 exposure. The results showed that exosomes released under oxidative stress reduce cell death in recipient cells exposed to oxidative stress, as cells pre-treated with exosomes released under oxidative stress (oxi exo) were shown to have a higher viability at all time points compared to cells pre-treated with exosomes released by cells grown under normal conditions (norm exo) (Figure 5).

In addition, Western blot analysis of endogenous levels of cleaved caspase-3, an apoptosis marker, revealed that recipient cells pre-treated with oxi exo, had a reduced level of apoptosis upon oxidative stress treatment compared to those receiving norm exo (Figure 6). Thus, exosomes released by cells grown under oxidative stress can mediate a tolerising effect against stress to the recipient cells. Therefore, the physiological condition of the originating cell alters the biological function of the exosomes released by the cells.

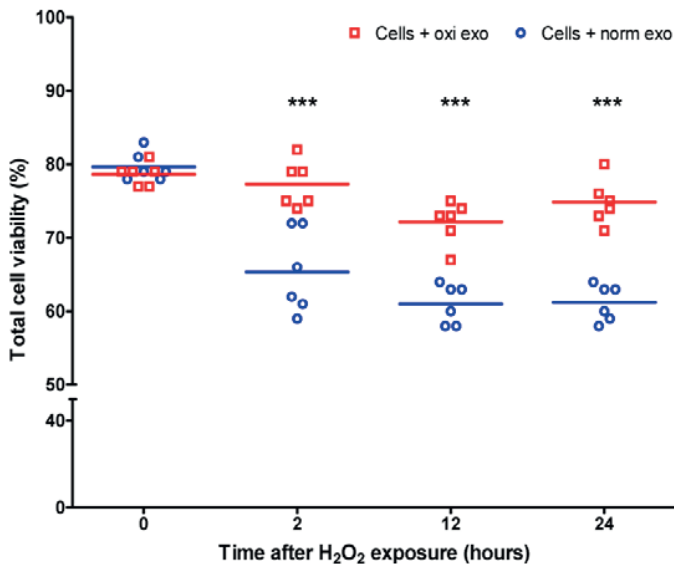


Figure 5. Time course of MC/9 recipient cell viability after exposure to H_2O_2 when pre-treated with exosomes released from normal conditions (norm exo) or under oxidative stress (oxi exo). Reprinted from Eldh et al, *PLoS One*, 2010;5(12):e15353 under the Creative Commons Attribution License.

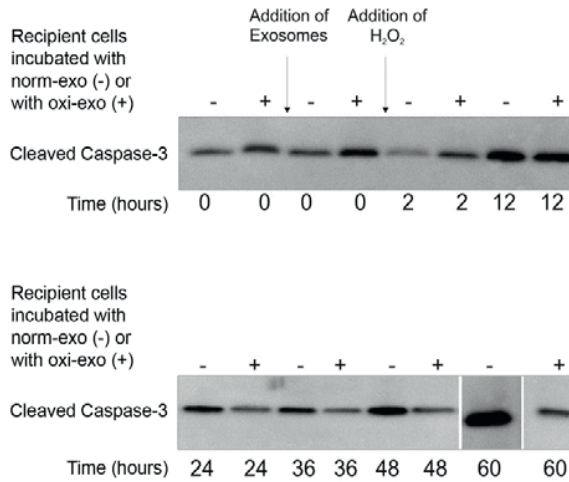


Figure 6. Western blot analysis of cleaved caspase-3 expression in MC/9 recipient cells pre-treated with exosomes released under normal conditions (norm exo) or under oxidative stress (oxi exo) then subsequently exposed to oxidative stress.

Our results showed that the exosomal mRNA content of exosomes derived from cells grown under oxidative stress differs substantially to that of exosomes derived from normal conditions, and that these stress-derived exosomes can alter the recipient cells ability to handle stress. Previously published data demonstrated that exosomal RNA can be transferred to recipient cells and be functional [71], prompting us to hypothesise that the stress-tolerising effect we observed might be mediated by the exosomal RNA. To study this, we exposed oxi exo to UV-C radiation (254 nm) for one hour to inactivate the exosomal RNA, as this treatment is known to have an inactivating and damaging effect on nucleic acids [238-240]. Control exosomes from both normal and stressed conditions were treated identically, however they were not exposed to UV-light.

The UV-C exposed exosomes and the control exosomes were added to untreated recipient cells, which were then exposed to oxidative stress at the same concentration as the donor cells, and their cell viability was determined at 0, 2 and 12 hours. The results revealed that UV-C eliminated the exosomal protective signal, as oxi exo exposed to UV-light were shown to lose their protective effect at the 12 hour time point (Figure 7). These results suggest a possible role for the exosomal RNA in the communication of protective messages. However, as proteins can also be damaged by UV-C exposure [241] the possibility that exosomal proteins play a role in the protection cannot be excluded.

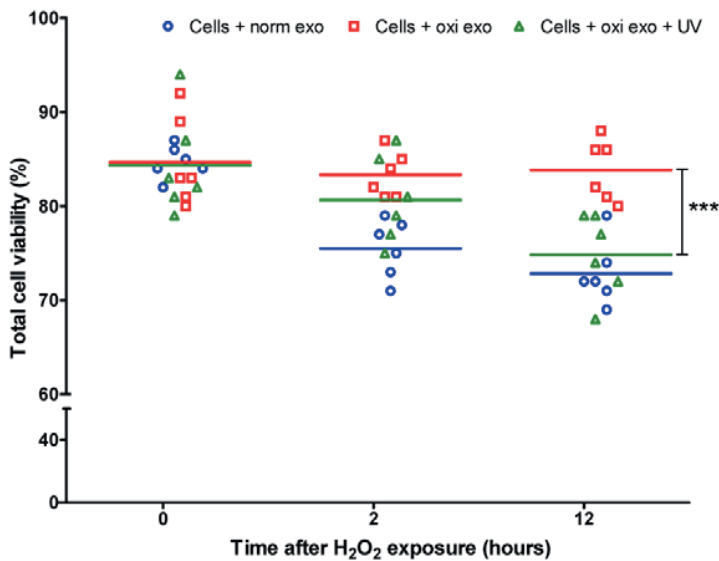


Figure 7. Time course of MC/9 recipient cell viability after exposure to H₂O₂ when pre-treated with exosomes released under normal conditions (norm exo), under oxidative stress (oxi exo) or UV treated oxi exo. Reprinted from Eldh et al, PLoS One, 2010;5(12):e15353 under the Creative Commons Attribution License.

4.3 Exosomes in Uveal Melanoma Liver Metastases (paper III)

The research field of exosomes is a fast growing field with extensive possibilities. Exosomes have been suggested to be involved in tumourigenesis as they have been shown to influence the metastatic capacity and immune evasion of tumours [36, 161-162, 165-169]. Patients with different malignant diseases have been shown to have an increased amount of circulating exosomes in their peripheral blood and furthermore these exosomes have been shown to contain tumour-associated antigens, mRNA and miRNA [36, 73, 104, 110-111, 191-193]. This has sparked an interest in exosomes as diagnostic markers and therapeutic targets. In this study, we wanted to determine if exosomes were present in liver perfusate isolated during isolated hepatic perfusion (IHP) treatment from patients with metastatic uveal melanoma. Furthermore, we aimed to characterise these exosomes to determine if exosomes from these patients could be a potential source of diagnostic markers.

To ascertain if the exosome-pellet isolated from liver perfusate did indeed contain exosomes, we examined their size, morphology, specific protein markers and RNA content. The electron microscope was used to determine both size and morphology, and small spherical membrane vesicles around 50 nm in diameter were observed and were positive for CD63 (Figure 2A, paper III). Furthermore, flow cytometry analysis using anti-CD63-coated latex beads revealed that these vesicles were positive for CD9, CD63 and CD81, which are proteins commonly enriched in exosomes and thus used as exosomal markers (Figure 2B, paper III). We could also see an individual difference in the positivity of these different protein markers. The total RNA profile of the vesicles was analysed using capillary electrophoresis. This analysis showed that the vesicles were enriched in lower molecular weight RNA and lacked the prominent 18S and 28S rRNA subunits, which is a typical exosomal RNA profile (Figure 4A, paper III). These results showed that the exosome-pellet isolated from liver perfusate did contain exosomes.

Next, we wanted to establish if these exosomes from the liver perfusate were of tumour origin. To determine this, we performed a Western blot analysis on the exosomal proteins using a melanoma-specific antibody, Melan-A. The liver perfusate exosomes were positive for Melan-A, but again showed individual differences (Figure 2C, paper III). These results suggested that at least a portion of the liver perfusate exosomes are of melanoma origin.

Other studies have shown that tumour-exosomes have a potential diagnostic value, as they have been shown to exhibit both specific tumour signatures and to exist at higher levels in cancer patients compared to healthy controls [36, 104, 111]. Therefore, we wanted to determine if patients with metastatic uveal melanoma also had higher levels of circulating exosomes in their peripheral blood compared to healthy controls. Currently, there is no well established and reliable method for exosome quantification, therefore the total exosomal protein measurement is often used for this purpose. Using this method, the total exosomal protein concentration per ml of peripheral blood plasma was used to determine the level of circulating exosomes for this study. The results revealed that patients with metastatic uveal melanoma had a significantly higher level of exosomes circulating in their peripheral blood compared to healthy controls (Figure 3, paper III), suggesting that these exosomes may potentially be used as diagnostic markers.

Studies have shown tumour-specific miRNA profiles in cancer patients [104, 111], which may be useful as diagnostic markers in the future. The liver perfusate exosomes from uveal melanoma patients contained RNA, including RNA the size of miRNA. To verify the presence of miRNA and to further investigate if the exosomes have a melanoma associated miRNA profile, we performed real-time PCR on liver perfusate-derived exosomes from five different patients. Uveal melanoma is a relatively rare cancer and to date, there is no uveal melanoma-specific miRNA PCR array, therefore we used a PCR plate of 88 brain cancer miRNA, as this contained several miRNA previously associated with uveal melanoma. Another limitation with performing real-time PCR on these samples is that there are no good controls, as liver perfusions on healthy controls would be unethical, and there is low exosomal RNA concentrations in the peripheral blood of healthy controls. For these reason, we used five different cell lines as controls. We chose two human malignant melanoma cell lines (A375 and MML-1), one human breast cancer cell line (HTB-133), one human lung cancer cell line (HTB-177) and one human mast cell line (HMC-1).

The results of the PCR array revealed that the liver perfusate exosomes do indeed contain miRNA. The lack of reference genes for exosomes and miRNA means that the concentration of each individual miRNA cannot be compared between patients. To address this challenge, we ranked the miRNA in each patient, thereby making it feasible to compare the individual patients miRNA profiles (Supplementary Table 1, paper III). When using the cluster analysis software (Cluster 3.0) to analyse the miRNA profiles, we could see that there was a strong grouping of the five patients, but not of the patients and the melanoma cell lines (Figure 4B, paper III). Of interest, we could

observe six miRNA in one of the miRNA clusters mainly represented in the patients and barely represented in the cell lines (Figure 4C, paper III). These six miRNAs (miR-370, miR-210, miR-320a, miR-124, miR-107, miR-486-5p) were entered into a KEGG pathway analysis with DIANA-miR Path software, which revealed melanoma, together with glioma, hedgehog signaling and prostate cancer, as the most significant pathways associated with these miRNAs (Table 2, paper III).

In the literature, miR-182 and miR-34a have both been demonstrated to function as tumour suppressors in uveal melanoma [224-225] and were of particular interest in this study as they were both shown to be low or absent in patient material. This suggested that the miRNA profiles seen in the patients might be associated with uveal melanoma. Taken together, these findings imply that circulating exosomes from patients with liver metastases from uveal melanoma could be a potential source of diagnostic markers to detect metastasis at an earlier stage. In the clinic today, there is no treatment for these patients and the only thing that might increase a patient's life expectancy is the detection of the tumour at an earlier stage.

5 CONCLUSIONS

Paper I. This study highlights the importance of the choice of RNA isolation method when working with exosomes, as different extraction methods result in variations in both RNA yield and size distribution.

Paper II. Exosomes derived from cells cultured under different conditions contain different mRNA content and have the ability to affect recipient cells in different ways. Exosomes released under oxidative stress have the ability to communicate a stress resistance to the recipient cells, which is eradicated with UV-C exposure, indicating that the exosomal RNA might have a possible role in the altered biological function.

Paper III. In this study, we showed that liver perfusate from patients with metastatic uveal melanoma contain melanoma-specific exosomes. Furthermore, we showed the potential of exosomes from these patients to function as possible diagnostic markers, as patients were shown to have a significantly higher exosome concentration in their peripheral blood compared to healthy controls. In addition, liver perfusate-derived exosomes were shown to contain miRNA profiles with a strong similarity between the patients, but not between patients and cell lines. Several miRNA which were found to be clustered together and mainly expressed in the patients, were shown to be associated with the KEGG pathway of melanoma. Together, these findings indicate that exosomes from patients with metastatic uveal melanoma could be a potential source of diagnostic markers.

In summary, exosomes are intricately involved in cell-to-cell communication and their RNA cargo appears to be of biological importance. Furthermore, exosomes and their RNA may potentially be used for determining diagnosis and prognosis in uveal melanoma.

6 FUTURE PERSPECTIVES

This thesis has examined some of the important methodological questions within the field of exosome research, as well as the biological function of exosomes and their RNA content.

In the first paper of this thesis, we highlight the importance of the RNA extraction method when investigating the field of exosomal RNA. We showed that RNA methodology affects the RNA profile obtained, thus results from different methods cannot be directly compared. As such, we ascertained that it is essential to consider the methodology when studying exosomes, as the observations may not represent the whole truth. Given that the RNA of exosomes from different sources and conditions also differ, it may be difficult to find a method that best answers all questions and allows comparisons between different research groups. Additionally, the same cell line have been shown to release different subpopulations of extracellular vesicles [82] and exosomes released from the same cell line may differ in size, composition and therefore function [82, 242]. Collectively, these results emphasise the complexity of extracellular vesicle research and highlights the significance of ensuring the methodology is correct so that a level of certainty can be achieved. We have only examined the methodology of the extraction of exosomal RNA, however there are still many more methodology questions to be answered.

Different RNA isolation methods and different cellular origins will contribute to varying RNA results. In paper II, we showed that the condition under which the originating cell is grown under also has an impact on exosomes and their RNA content. The RNA content of exosomes from various cellular sources has been widely studied. The exosomal RNA has been shown to be able to be transferred to recipient cells where it can affect the protein synthesis of that cell. However, the biological function of exosomal RNA and how the exosomal RNA and its function differ under different physiological states requires more research. While this study examined the differing mRNA content of exosomes derived from different conditions, it would be of interest to examine the mRNA content of the recipient cells, to determine if this also differs depending on which exosomes they receive. Additionally, the exosomal proteins and enzymes should not be disregarded, as they may also play a part of the exosomes conditioning function. The investigation of the role of exosomal RNA and protein in conditioning may provide some answers on the mechanism behind the tolerising effect of exosomes.

The packaging of the exosomal RNA is another critical area to be explored. The results from paper II, together with findings by other groups, suggests that exosomes are specifically packed. The mechanism behind this is an important area of research as it may help in the future manipulation of the exosomal RNA content and consequently, the function of the exosomes. It may also be beneficial for both the understanding and treatment of different diseases. Today, there is a great deal of research on the use of exosomes in diagnostics and therapy. There is still substantial research needed, although the potential as gene delivery vehicles have been explored, with promising results in mice [213]. Many important questions still remain and extensive research is needed before exosomes may be used as vectors in humans. The research conducted on the use of exosomes as a diagnostic tool is relatively more progressed compared to their use as therapy vehicles. The discovery of new biomarkers can make a discernible difference and have an impact for many people, as there is no good treatments for many cancers and an early detection can drastically change the patients prognosis. Most research performed on exosomes has been conducted *in vitro*, which may have some inherent uncertainty, as they often have mutations and do not reflect the true biological situation. In paper III, we examined exosomes from patients with metastatic uveal melanoma. The results suggest that exosomes may be used as diagnostic markers for uveal melanoma, however the use of exosomes in diagnostics and therapy still requires substantial additional investigation.

The findings in this thesis have answered some of the underlying questions in the field of exosomes, however it has also raised further questions for additional investigation. The quest to find the biological function of exosomal RNA requires the study of exosomes from many different conditions and from many different cell origins, both *in vivo* and *in vitro*.

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