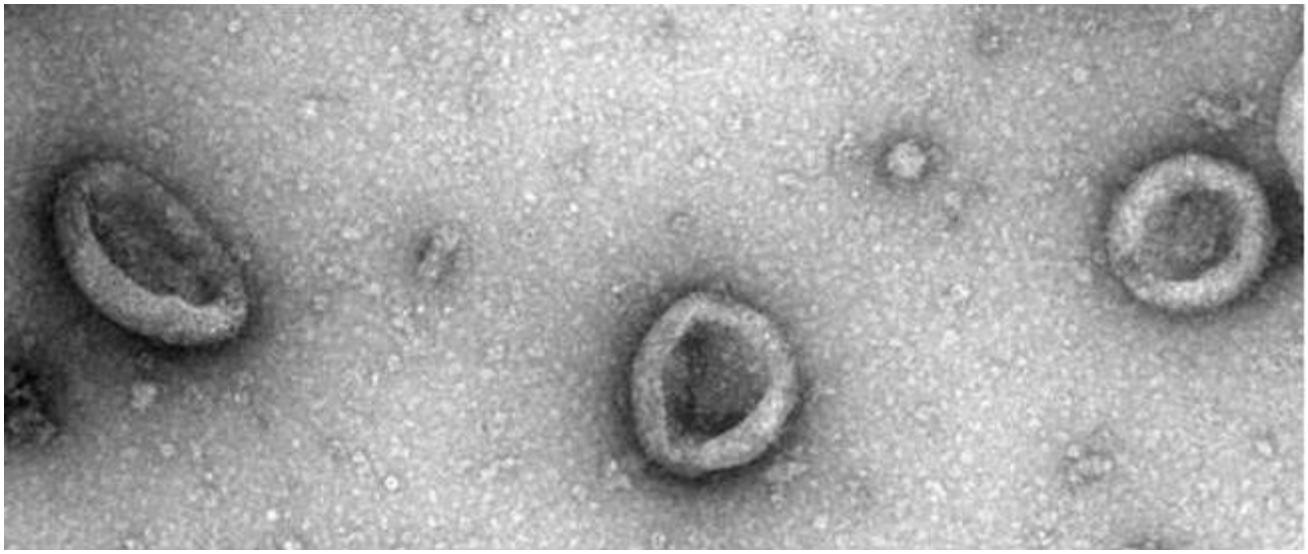
Use of extracellular vesicles as biomarker for disease or as therapeutic agent



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Content

Abbreviations

AD	Alzheimer's disease
AKI	Acute kidney injury
APC	Antigen presenting cell
APP	Amyloid β precursor protein
AQP1	Aquaporin 1
ATF3	Activating transcription factor 3
Aβ peptides	Amyloid β peptide
CSF	Cerebrospinal fluid
CTL	Cytotoxic T-lymphocyte
DC	Dendritic cell
DII4	Delta-like 4
EGFR	Endothelial growth factor
ESCRT	Endosomal complexes required for transport
Exotest	In-house sandwich ELISA
FSGS	Focal segmental glomerulosclerosis
Hsp70	Heat-shock protein 70
I/R	Ischemia/reperfusion
ICAM1	Intercellular adhesion molecule 1
ILV	Intraluminal vesicle
imDex	Exosome-derived from immature dendritic cell
LB	Lewy bodies
LFA1	Lymphocyte function-associated antigen 1
LRG1	Leucine-rich-α-2-glycoprotein
mDex	Exosome-derived from mature dendritic cell
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MiRNA	MicroRNA
MMP	Matrix metalloproteinases
MS	Mass spectrometry
MVB	Multivesicular body
NK	Natural killer
NS	Nephrotic syndrome
NSCLC	Non-small lung cancer
nSMase2	Neutral sphingomyelinase 2
PCa	Prostate cancer
PD	Parkinson's disease
PrP	Prion protein
PS	Presenilin
PSA	Prostate-specific antigen
PSMA	Prostate-specific membrane antigen

Shh	Sonic hedgehog
TCR	T-cell receptor
TEM	Transmission electron microscopy
TIM	T-cell immunoglobulin and mucin domain protein
Treg	Regulatory T-cell
uPA	Urokinase-type plasminogen activator
VEGF	Vascular growth factor
WT-1	Wilms tumor 1
γ-Dex	Exosome-derived from monocyte derived dendritic cell treated with IFN-γ

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Abstract

In recent years, novel ways of intercellular communication have been described involving extracellular vesicles. Cells from various origins actively release small vesicles into the extracellular environment either by pinching off directly from the plasma membrane, which are often referred to as microvesicles, or a consequence of the fusion of the multivesicular body (MVB) with the plasma membrane. The latter process results in the extracellular release of the intraluminal vesicles of MVB, which are now termed exosomes. Cytosolic proteins, mRNAs, miRNAs and membrane bound proteins that are contained by extracellular vesicles can be transferred to target cells, thereby altering the physiology of the target cell. Extracellular vesicles display unique biophysical and biochemical features that are determined by the originating cell, making them of interest for diagnostic and therapeutic purposes. This thesis will focus on the potential of extracellular vesicles as biomarker and therapeutic agent in various diseases.

Introduction

Until recently it was thought that communication between neighboring cells occurred exclusively through direct intercellular contact or through transfer of signaling molecules over larger distances. Recent year revealed new mechanisms by which cells can communicate via extracellular transfer of membrane vesicles (in this thesis referred to as extracellular vesicles)[1]. Extracellular vesicles carry both membrane and cytosolic components. Several extracellular vesicle types have been named after their cell of origin. In general three types of extracellular vesicles can be distinguished; extracellular vesicles that bud directly from the plasma membrane, extracellular vesicles that are secreted upon fusion of intracellular organelles with the plasma membrane and extracellular vesicles that derive from apoptotic cells, named apoptotic bodies. Apoptotic bodies are actively secreted in response to programmed cell death[2] and will not be discussed in this thesis. Large extracellular vesicles (>100 nm) that derive from the plasma membrane are referred in the literature as microvesicles, ectosomes, microparticles or exovesicles. These different names are often used by different research groups and often refer to vesicles with distinct originating cells. Various cell types such, platelets[3], neutrophils[4] and dendritic cells (DCs)[5] , but also tumor cells[6] are able to release extracellular vesicles from their plasma membrane. Extracellular vesicles that are secreted as a consequence of fusion of intracellular organelles with the plasma membrane are referred to as exosomes (50-100 nm in size). Exosomes can often be distinguishes from other small extracellular vesicles through their unique biophysical and biochemical features that resemble intraluminal vesicles of the multivesicular body (MVB). Other, smaller, extracellular vesicles with different biophysical and biochemical features than exosomes are often referred to as nano-particles[7] and exosome-like vesicles[8]. The biophysical and

Table 1: Characteristics of different types of secreted vesicles

Feature	Exosomes	Microvesicles	Ectosomes	Membrane particles	Exosome-like vesicles	Apoptotic vesicles
Size	50-100 nm	100-1,000 nm	50-200 nm	50-80 nm	20-50 nm	50-500 nm
Density in sucrose	1,13-1,19 g/ml	ND	ND	1,04-1,07 g/ml	1,1 g/ml	1,16-1,28 g/ml
Appearance by electron microscopy	cup shape	Irregular shape and electron-dense	Bilamellar round structures	Round	Irregular shape	Heterogeneous
Sedimentation	100,000 g	10,000 g	160,000-200,000 g	100,000-200,000 g	175,000 g	1,200 g, 10,000 g or 100,000 g
Lipid composition	Enriched in cholesterol, sphingomyelin and ceramide; contain lipid rafts; expose phosphatidyl-serine	Expose phosphatidyl-serine	Enriched in cholesterol and diacylglycerol; expose phosphatidyl-serine	ND	Do not contain lipid rafts	ND
Main protein markers	Tetraspanins (CD63, CD9), Alix and TSG101	Integrins, selectins and CD40 ligand	CR1 and proteolytic enzymes; no CD63	CD133; no CD63	TNFRI	Histones
Intracellular origin	Internal compartments	Plasma membrane	Plasma membrane	Plasma membrane	Internal compartments	ND
Main reference	Thery et al, 2006	Heijnen et al, 1999	Gasser et al, 2003	Marzesco et al, 2005	Hawari et al, 2004	Thery et al, 2001

Table 1 is adapted from Thery et al, 2009.

biochemical features of different types of extracellular vesicles are listed in Table 1. All extracellular vesicles contain cytosolic components of their originating cell, which makes them representative for the type and status of the cell of origin. Therefore extracellular vesicles have great potential as future biomarkers of disease, which is further increased by the easily accessibility in body fluids such as blood and urine. Research on extracellular vesicles is focused both on exosomes and microvesicles. Therefore this thesis will be on the function of exosomes and microvesicles and their potential use as biomarker and treatment in various diseases.

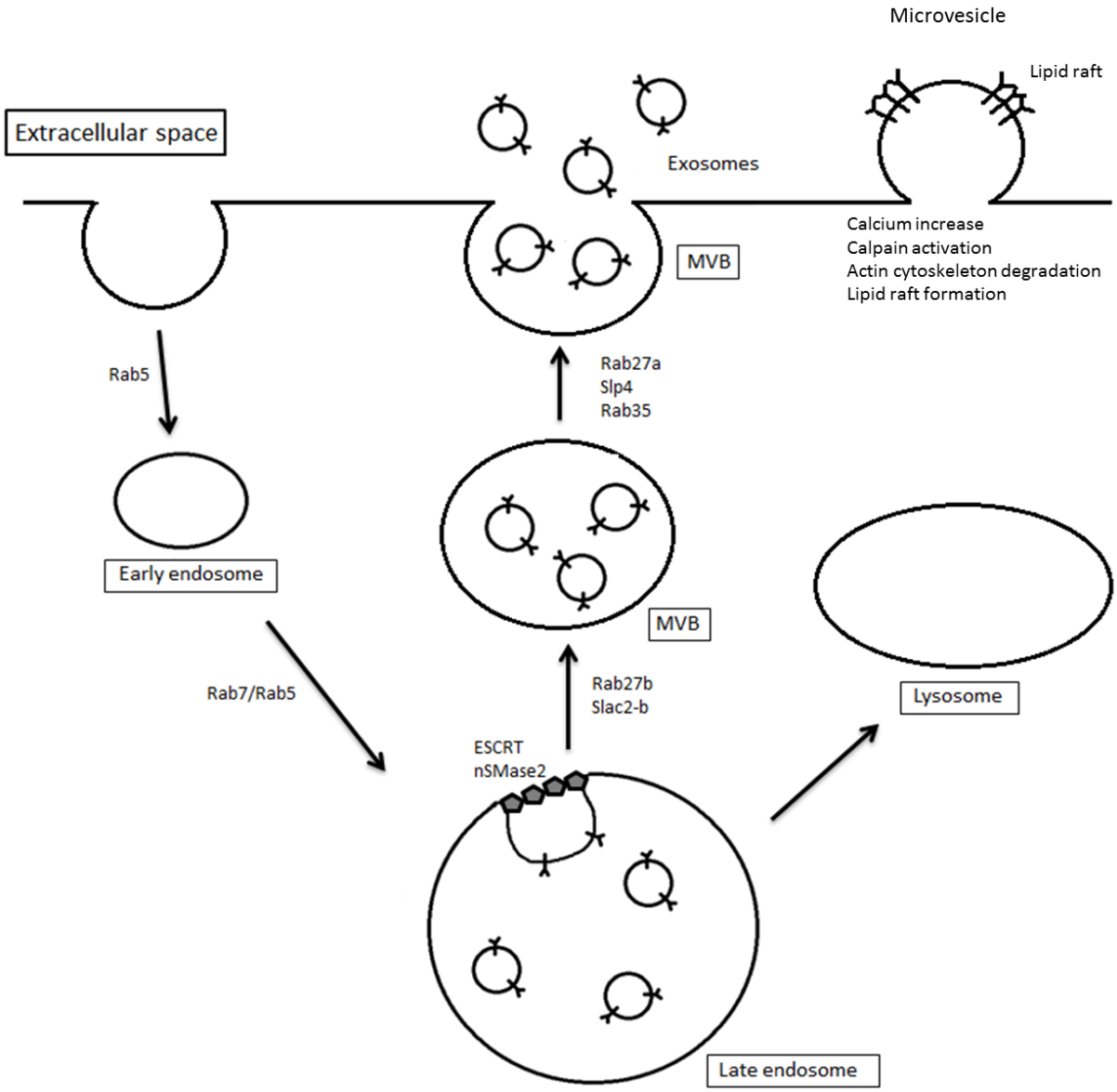
Biology of Exosomes

Exosomes are secreted by many cell-types, including epithelial cells[9], DC[10], T-lymphocytes[11], B-lymphocytes[12,13,14], mast cells [15,16], various stem cells[17] and platelets[18]. Exosomes are present in physiological body fluids like blood[19], urine[20], semen[21,22], breast milk[22], saliva[23, 24], pleural fluid [25], cerebrospinal fluid (CSF)[26] and amniotic fluid[24]. Exosomes contain cytosolic and membrane associated proteins, as well as mRNA and microRNA (miRNA)[27,28] Highly enriched ubiquitous proteins of exosomes include the chaperones Hsc73 and Hsc90, tetraspanins (CD9, CD63, CD81 and CD82), and Tsg101[29]. Besides such ubiquitous proteins, exosomes also contain membrane associated proteins, cytosolic

proteins, mRNA and miRNA that are specific to the cell of origin. Not much is known about physiological functions of exosomes, due to the limited amount of studies performed *in vivo*. Also the mechanism of exosome formation is not fully elucidated, complicating interference studies on the function of *in vivo* generated exosomes, for example by the use of knock-out mice. However, studies that can be performed *in vivo* have shown for example that isolated exosomes from *in vitro* cultured DCs can modulate the immune system when injected into mice[30]. Exosomes derived from antigen presenting cells (APCs), such as DCs display major histocompatibility complex II (MHC II) and MHC class I (MHC I) on their membrane, allowing them to function as an immune response modulator. Immature DCs display low levels of ubiquitinated MHC II on their plasma membrane. In mature DCs ubiquitination of the MHC II is abrogated, leading to increased levels of MHC II on the plasma membrane. Ubiquitination of the MHC II complexes drives the sorting of MHC II on ILVs inside the MVB towards lysosomal degradation. DCs loaded with antigen display a distinct MHC II sorting, which is independent of ubiquitination. In antigen loaded DCs, ILVs are generated in the MVB that contain MHC II and CD9, independent of ubiquitination, that are secreted as exosomes and can activate antigen-specific CD4+ T-lymphocytes. This reveals two separate MVB pathways, the ubiquitination pathway that induces lysosomal degradation and the ubiquitination independent pathway that induces exosomal secretion[31,32]. Depending on the presence of other membrane proteins, exosomes may either activate or inhibit immune responses. For example, the number of extracellular vesicles (both exosomes as microvesicles) isolated from the plasma of pregnant women was increased in the women that delivered at full term. These extracellular vesicles induced a decrease in the number of T-lymphocytes and in the T-lymphocyte responsiveness, leading to a suppression of the immunity against the unborn baby[33,34]. These results show that exosomes can exert both inhibitory as activating effects on the immune system depending on their content and origin.

Exosomes are generated at and stored inside MVBs. The intraluminal vesicles (ILVs) of MVB are formed by invagination of the late endosomal membrane[29]. During invagination of the membrane, the lumina of these future exosomes is filled with selected soluble proteins, mRNAs, miRNAs and other cytosolic molecules. RNA and miRNA species present in exosomes are also named exosomal shuttle RNA (esRNA) due to their potential function in altering gene expression in the target cell[28].

Currently it is not fully known which proteins are involved in the formation of exosomes. Some reports showed that endosomal complexes required for transport (ESCRT complexes) regulate the inward budding of the late endosome, resulting in the formation of ILVs. Two members of the ESCRT complexes were identified more than ten years ago by proteomic analysis, Tsg101/Vps23 and AIP1/Alix/Vps31, indicating that exosome biogenesis could be dependent of ESCRT complexes[35]. However, the role of ESCRT complexes in exosome formation and secretion is not fully confirmed by other studies, therefore it remains unclear if ESCRT complexes are indeed involved in the



Figuur 1: Extracellular vesicle formation. Plasma membrane components are endocytosed and fuse together to form the early endosome, mediated by Rab5. The conformation of the early endosome to the late endosome is regulated by a convertion of Rab5 expression to Rab7 expression. It is suggested that inward budding of the late endosome and pinching off the ILVs is mediated by ESCRT complexes, however this is not confirmed in the literature. Incorporation of miRNAs in ILVs is mediated by ceramide, which is regulated by SMase2. The late endosome containing ILVs is now named the MVB. Rab27b and its effector Slac2-b mediate the transport of the MVB via microtubules to the actin-dependent docking at the plasmamembrane. Rab27a and its effector Slp3 regulate the docking of the MVB to the plasmamembrane and the fusion of the MVB with the plasma membrane, where the exosomes are released in the extracellular space. The secretion of exosomes is regulated by Rab35. The formation of microvesicles occurs by the increase of cytosolic calcium concentration and calpain activation, which leads to a degradation of the actin cytoskeleton. Shedding of microvesicles from the plasma membrane occurs upon lipid raft formation in the plasma membrane, resulting in plasma membrane blebbing and finally microvesicle secretion.

exosomal biogenesis[36]. The incorporation of miRNAs in the lumen of ILVs is dependent of ceramide which is regulated by neutral sphingomyelinase 2 (nSMase2) and independent of ESCRT complexes[37]. Formation of the late endosome in which ILVs are formed is regulated by members of the Rab family GTPase proteins. The Rab family GTPase proteins also regulate the docking and fusion of the MVB at the plasma membrane. Early endosomes are formed by Rab5, which regulate the fusion of endocytic vesicles with the early endosome. Rab5 also regulates the fusion of multiple smaller early endosomes to form one larger early endosome. The shift from Rab5 binding to Rab7 recruitment, together with the recycling of endocytosed material to the plasma membrane initiates the maturation of the early endosome into the late endosome[38]. Rab11 regulates the transfer of the MVB towards the plasma membrane instead of the lysosome[39], and promotes a calcium dependent docking and fusion of the MVB at the plasma membrane[39]. Rab27b participates in the transfer of membranes from the trans-golgi network towards the MVB and regulates the transfer of the MVB by microtubules to the actin-dependent docking at the plasma membrane. Rab27a exerts important functions in the docking of the MVB at the plasma membrane and the fusion of vesicles[40]. TBC1D10A-C regulates the secretion of exosomes in a catalytic activity-dependent way. Inhibition of Rab35, which is the target of TBC1D10A-C leads to the intracellular accumulation of endosomal vesicles[41]. Knockdown of Rab2b, Rab9a, Rab5a, Rab27a or Rab27b inhibited exosome secretion only partially, indicating that their activities are not crucial for exosome secretion and that more essential regulatory mechanisms still remain to be discovered[40]. Another complication is that besides being secreted by MVB, exosomes may also bud directly from the plasma membrane at endosome-like domains, as was demonstrated for T-lymphocytes (Figure 1)[42].

The secretory pathways of both exosomes as microvesicles can be hijacked by infectious agents such as retroviruses and prions, that use this pathway for their release from the producing cell and enable their transfer to other unaffected cells[43].

Biology of microvesicles

Microvesicles shed directly from the plasma membrane of several cell types have an origin-specific content such as membrane receptors, lipids, proteins, mRNA[44,45]. Microvesicle secretion is dependent on an increase in the calcium concentration in the cytosol, activation of calpain and degradation of the actin cytoskeleton[3]. Microvesicle that shed from the plasma membrane contain lipid rafts that are formed at the plasma membrane and that in addition to certain lipids also contain specific membrane proteins, such as membrane-associated receptors. The formation of such lipid rafts may contribute to microvesicle shedding[46]. The amount of secreted microvesicles depends on several factors such as: activation status of the cell, hypoxia/irradiation, oxidative injury, shear stress exposure and exposure to activated components of the complement system[47, 48].

Microvesicles have different alternative names. For example, microparticles are microvesicles secreted by activated blood platelets[49]. Microparticles exert differential functions in blood, such as, assembling of

clotting factors due to the high concentration of phosphatidylserine on the membrane[50]. Defects in the secretion of microparticles are related to clotting or coagulation disorders such as Scott syndrome, Glanzmann's thromboastenia and Castaman's defect[50,51]. Microparticles in high concentration can also function as a chemoattractant for monocytes, natural killer (NK) cells, T-lymphocytes and B-lymphocytes to damaged tissue sites[52]. Argosomes are microvesicles derived from the basolateral membrane of epithelial cells, and contain morphogens that are essential for the creation of morphogen gradient needed for proper tissue development[53]. Ectosomes are microvesicles originated from human polymorphonuclear leukocytes[4], of which the function is still unknown. Besides the above described effect of microvesicles on the immune system, coagulation and tissue development, microparticles and microvesicles derived from tumor cells are also important for tumor growth and metastasis, which will be described later in the thesis.

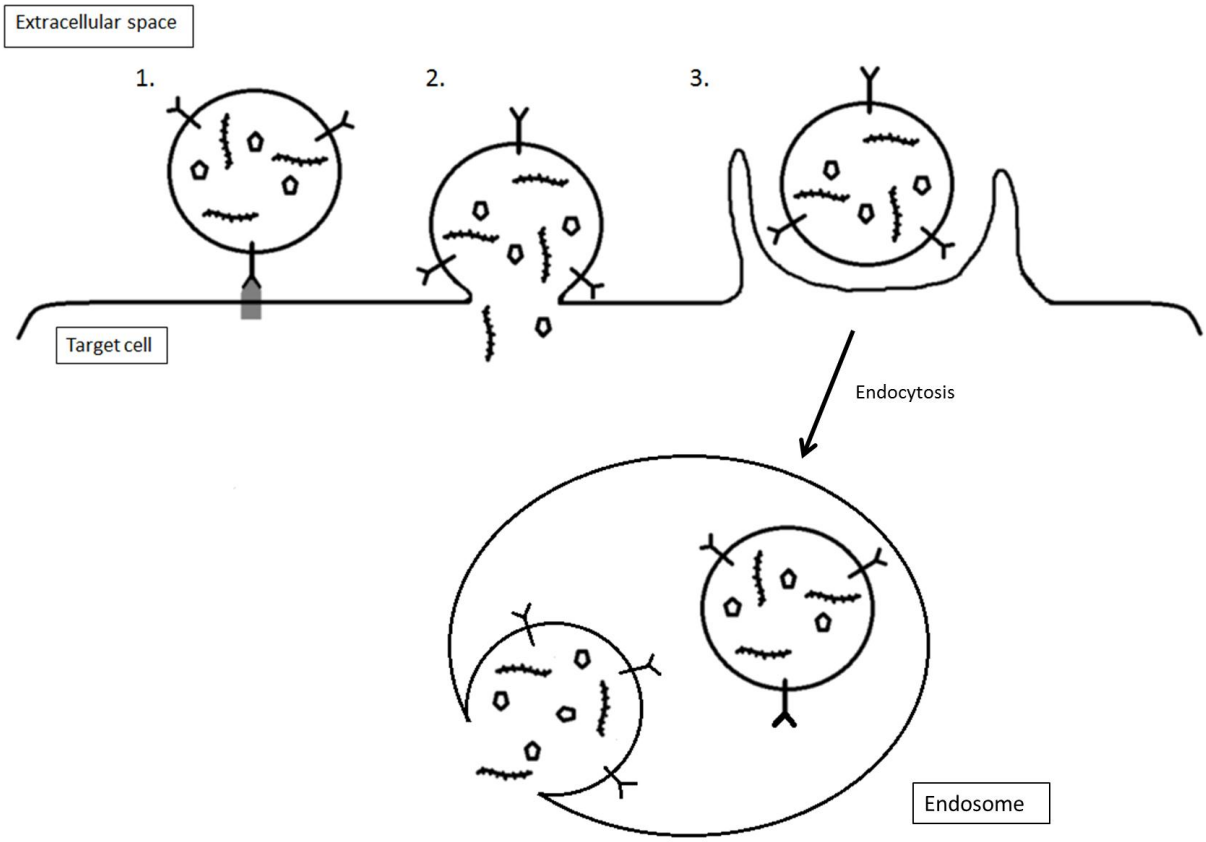
Inhibition of microvesicle secretion can lead to better understanding of the role of microvesicles *in vivo*. Currently, formation of microvesicles can be inhibited in four different manners. At first administration of calcium blockers can result in inhibition of microvesicle secretion, since microvesicle shedding is dependent on an increased calcium concentration[54]. Secondly lipid raft formation inhibitors like statins and polyene antibiotics can inhibit or reduce the formation of lipid rafts and thereby the secretion of microvesicles[55]. Thirdly, microvesicles display phosphatidylserine on their membrane, which binds to annexin-V. Adsorption columns that contain annexin-V matrix could therefore mediate depletion of microvesicles from blood[56]. Finally administration of a CD41 antagonist (abciximab) can inhibit or reduce microparticle secretion[57], since research showed that the adhesion molecule CD41 is involved in microparticle secretion from activated platelets[52, 58].

Interaction of extracellular vesicles with the target cell

Extracellular vesicles can deliver their content and membrane components to their target cells in at least three different manners (Figure 2):

1. Extracellular vesicles attach to the target cell by adhesion molecules and receptors present on their membrane. This may induce signaling cascades within the target cell[59, 60].
2. Extracellular vesicles can fuse directly with the plasma membrane of the target cell, and release their content[61]. DC-derived exosomes have shown to be able to release their content directly upon fusion with the plasma membrane of the recipient cell[62].
3. Extracellular vesicles can be incorporated by the target cell via endocytosis. The content of the extracellular vesicle is then released into the cytoplasm upon fusion of the exosome with the endosomal delimiting membrane[28, 63].

Extracellular vesicles express several adhesion molecules on their membrane, by which they can bind to the receptors and ligands on the plasma membrane of the target cell. DC-derived exosomes express intercellular adhesion molecule 1 (ICAM1) on their



Figuur 2: Mechanisms of cell-cell communication mediated by extracellular vesicles. Extracellular vesicles adhere to the recipient cell via adhesion molecules and receptors present on the membrane, such as intercellular adhesion molecule 1 (ICAM1) on the exosomal membrane that can adhere to lymphocyte function-associated antigen 1 (LFA1) on the plasma membrane of the recipient cell. Besides the adhesion molecule ICAM1 on the exosomal membrane, extracellular vesicles can also bind to the recipient cell via phosphatidylserine, which can bind to T cell immunoglobulin domain and mucin domain protein 1 (TIM1 or TIM4) present on recipient T lymphocytes (1). Extracellular vesicles can fuse directly with the plasma membrane of the recipient cell. Fusion of the extracellular vesicle membrane with the plasma membrane of the recipient cell results in the release of their content into the cytoplasma of the recipient cell and incorporation of membrane bound receptors in the plasma membrane (2). Extracellular vesicles can also be incorporated by the recipient cell via endocytosis. After the endocytosis, the extracellular vesicles can fuse with the membrane of the endosome, leading to release of the content of the extracellular vesicles in the cytoplasma of the recipient cell (3).

membrane, by which they bind to lymphocyte function-associated antigen 1 (LFA1) on the plasma membrane of activated T-lymphocytes[64]. Attachment of exosomes to the target cell can also be mediated by β1 and β2 integrins that have been found on the membrane of exosomes. β1 and β2 integrins bind to ICAM1[60] and fibronectin (extracellular matrix protein)[65], which are present on the recipient cell. Activated lymphocytes and phagocytes express T cell immunoglobulin domain and mucin domain protein 1 (TIM1 and TIM4) on their membrane which are described as phosphatidylserine binding molecules[66]. Indicating that target cells such as lymphocytes and phagocytes efficiently capture extracellular vesicles by the receptors and ligand expressed on their plasma membrane, that bind to the extracellular vesicles via the receptors and ligands present on the extracellular vesicle membrane[67]. After binding of the extracellular vesicle to the

recipient cell, via receptor mediated binding or incorporation of the extracellular vesicle by endocytosis, the content of the extracellular vesicle is released inside the cytoplasma of the target cell and exert their functions. Intracellular signaling cascades can be activated by proteins that originated from the extracellular vesicles leading to activation of transcription factors and signaling molecules. Extracellular vesicular miRNAs can down-regulate already activated signaling pathways and thereby influence the intrinsic signaling cascades. For cancer it is known that downregulation of miRNAs can result in tumorigenesis, tumor progression and metastasis[68], and some research groups hypothesized that surrounding cells compensate the downregulated miRNAs during the tumorigenesis, by secreting extracellular vesicles containing these downregulated miRNAs[37].

Isolation and purification of extracellular vesicles

Different protocols are currently used for the isolation of exosomes and microvesicles. Many of these isolation protocols lead to contaminated exosomal and microvesicle fractions that are used for protein and RNA determinations, finally leading to confusing and contradicting results[69]. At present four different principles are used to isolate exosomes from body fluids and cell culture medium. Of these four, differential ultracentrifugation is the most often used method. At first whole cells and cell debris are removed using a low force centrifugation at 500-2000 x *g*. A high force centrifugation step at 100.000 x *g* is used to sediment the exosomes. The centrifugation step at a low force can also be replaced by filtration (0.1 µm or 0.22 µm pore sizes) or by including a centrifugation step at an intermediate force (60.000 x *g*) to remove microvesicles that are larger in size as exosomes[70]. Isolation of exosomes from body fluids requires an extra dilution step which reduces the viscosity of the sample, and an increase in centrifugation time and force[71]. One other way to isolate exosomes is by buoyant density-based separation, for example by centrifugation into linear sucrose gradients. Exosomes have an equilibrium density of 1.13-1.19 g/mL[72]. Both these methods have drawbacks. Differential ultracentrifugation results in contamination of the exosomal sample with protein aggregates that co-sediment with the exosomes, leading to conflicting results in the exosomal protein identification using mass spectrometry (MS)[73]. The contamination of protein aggregates can be resolved by complementing the procedure with floatation of vesicles from pelleted material into a linear sucrose gradient. A thirdly described method is using an iodixanol 6-18% gradient that efficiently removes contaminating retroviruses from the exosomal fraction[74]. Finally, exosomes can be isolated by immunoadsorbtion. For example, tumor-derived exosomes that display EpCAM on the membrane can be isolated using immunoisolation with anti-EpCAM antibodies attached to magnetic beads. The tumor-derived exosomes in the sample bind to the anti-EpCAM antibodies in the column, resulting in a more pure exosomal fraction compared to exosomal fractions isolated following ultracentrifugation and density-based separation[69]. This method can therefore be used to specifically isolate tumor-derived exosomes, without co-isolation of nontumor-derived exosomes. After these isolation protocol the isolated fraction is

checked for exosomal size (50-100nm), which can be measured using transmission electron microscopy (TEM)[75]. Western blot and MS are often used to determine the presence of exosome proteins, e.g. CD9, CD63, CD81, LAMP1 and TSG101[76].

Microvesicles from body fluids such as blood are often larger than exosomes, and require different isolation procedures. For example, a centrifugation step at 160 x *g*, may be followed by two centrifugation steps at 4000 x *g*, to remove irrelevant material. The collected plasma is then filtered through a filter with a pore size of 0.1µm. Exosomes and proteins freely pass the filter while the microvesicles that are larger than 0.1µm in size are retained. Microvesicles can then be collected with a final centrifugation step at 25.000 x *g*, by which the exosomes are sedimented and the microvesicles remain in the supernatant[77].

Several reports have addressed the concern for pre-analytical variables especially for the use of microvesicles isolated from blood. It has already been shown that microvesicles may be released from platelets and erythrocytes during storage, in a time and temperature dependent manner[78]. This indicates that platelets and erythrocytes are very sensitive to environmental factors leading to variable results[79]. Exosomes on the other hand seem less sensitive to pre-analytical variables. Except for isolation of exosomes from urine fractions, where the exosomes are entrapped by the polymeric Tamm-Horsfall protein, leading to a loss of exosomes[80]. Mitchell *et al*[81], also reported a high variation in both quantity and quality of exosomes isolated from urine, even though every sample was immediately handled after collection. Taken together a generalized protocols to isolate exosomes or microvesicles would reduce conflicting and confusing results between studies. This is of great importance for the implication of extracellular vesicles as diagnostic parameter in the clinic[79].

Function of extracellular vesicles in cancer

Several roles of tumor-derived extracellular vesicles have been described in the literature. Both anti-tumor effects as pro-tumor effects are described, which are most likely dependent on the content of the extracellular vesicles.

Currently it has been described that extracellular vesicles can exert anti-tumor effects by induction of the immune system and induction of tumor cell apoptosis[82]. Tumor-derived exosomes display an increased expression of Bax and a decreased expression of Bcl-2, which leads to the activation of the mitochondrial apoptosis pathway in tumor cells. Induction of PTEN and GSK-3β expression and decreased expression of pyruvate dehydrogenase is also initiated by tumor-derived exosomes, which will drive the tumor cells to apoptosis[7]. Tumor-derived exosomes decrease Notch-1 expression, which will inhibit the Notch survival pathway and will therefore activate apoptosis pathways[83]. On the other hand, induction of the immune system by tumor-derived exosomes is exerted at different manners. At first, exosomes derived from DCs display tumor antigens that induce tumor specific $CD8^+$ T-lymphocytes[84]. Secondly heat-shocked tumors release exosomes with increased

levels of MHC I and co-stimulatory molecules. that may induce anti-tumor T-lymphocyte immunity[85]. Other reports have shown that exosomes derived from heat-shocked tumors contain high levels of heat-shock protein 70 (Hsp70). Hsp70 enhances the Th1 polarized immune response independent of MHC and co-stimulatory molecules[86]. Tumor-derived exosomes that are engineered with a high concentrations of Hsp70 have been shown to stimulate the Th1 and the cytotoxic T-lymphocyte (CTL) anti-tumor immunity even more potently than exosomes derived from heat-shocked tumors[87]. DC and T-lymphocyte attraction and activation is also driven by chemokines released from exosomes originating from heat-shock tumors[88].

Besides these anti-tumor effects of tumor-derived extracellular vesicles, tumor-derived vesicles may also exert immunosuppressive properties as the induction of tumor invasiveness and metastasis via angiogenesis and the modulation of stromal cells. Tumor-derived microvesicles exert their immunosuppressive properties by the expression of FasL and TRAIL molecules on the membrane of the microvesicles, which induces the apoptosis of T-lymphocytes[89,90]. Tumor-derived extracellular vesicles down-modulate the expression of the CD3-ζ-chain, which impairs T-cell receptor (TCR) signaling and therefore downregulates the activity of effector T-cells[91]. Besides the effects of tumor-derived extracellular vesicles on T-lymphoctyes, tumor-derived exosomes can also suppress the function of NK cells[92], and modulate the differentiation and function of myeloid cells. Tumor-derived microvesicles induce monocyte differentiation from DCs to myeloid-derived suppressor cells (MDSCs). Thereby the tumor-derived exosomes induce TGF-β1 expression that results in suppression of T cell activity[93]. MDSCs express prostaglandin E2 in combination with TGF-β1, which promotes tumor growth[94]. On the other hand, tumor-derived exosomes also stimulate the function of regulatory T cells (Tregs) in multiple ways. Tregs are T-lymphocytes that suppress pro-inflammatory responses e.g. in cancer rejection. Tumor-derived microvesicles support the function of Tregs and enhance resistance of Tregs to apoptosis via TGF-β and IL-10[95]. It is also observed that tumor-derived exosomes increase the numbers of tumor specific Tregs[96]. Besides the effects of tumor-derived exosomes on the immune system, tumor-derived exosomes also enhance tumor invasiveness and metastasis, by modulation of the function of stromal cells, induction of angiogenesis, remodeling of extracellular matrix and the creation of a premetastatic niche[82]. The function of stromal cells is modulated by the expression of TGF-β on the exosomal membrane. TGF-β induces the differentiation of fibroblasts into myofibroblasts, which results in altered stroma, tumor growth, vascularization and metastasis of the tumor[97]. Tumor-derived exosomes contain matrix metalloproteinases (MMPs) that degrade the extracellular matrix leading to invasion of the tumor into the stroma[98]. The expression of CD44 by tumor-derived exosomes leads to the formation of a more soluble matrix[99]. This soluble matrix can serve as a reservoir that contains growth factors, chemokines and proteases needed for tumor embedding and tumor growth[100]. Tumor-derived exosomes contain proteins that induce angiogenesis. Tspan8, an exosomal protein was found to be selectively incorporate CD106 and CD49d into the tumor-

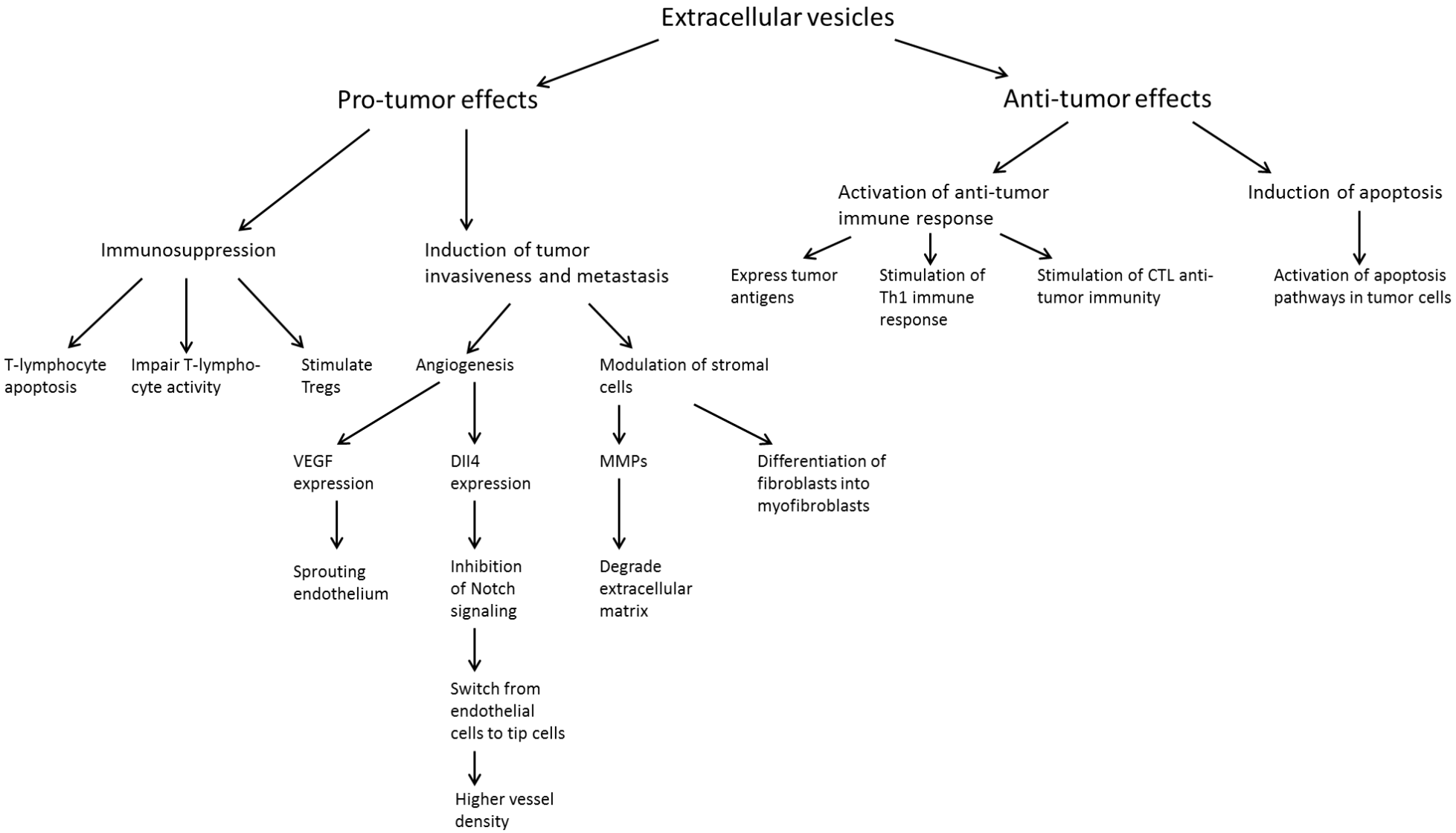


Figure 3: Functions of extracellular vesicles in cancer. Extracellular vesicles exert both pro-tumor effects as anti-tumor effects. Extracellular vesicles induce pro-tumor effects by immunosuppression and the induction of tumor invasiveness and metastasis in several ways. Anti-tumor effects are induced by extracellular vesicles by activation of the anti-tumor immune response and the induction of apoptosis in tumor cells.

derived exosomes. CD106 and CD49d are associated with the binding and internalization of exosomes by endothelial cells, increasing the metastatic properties of the tumor. Tspan8 also increases the secretion of MMPs and urokinase-type plasminogen activator (uPA). Increased levels of MMPs and uPA lead to an increased expression of vascular endothelial growth factor (VEGF) in fibroblasts, the endothelial growth factor receptor (EGFR) and Tspan1 in the sprouting endothelium, thereby inducing vascular growth[101,102]. Tumor-derived exosomes contain the Notch ligand Delta-like 4 (DII4). DII4 inhibits Notch signaling which induces in a switch from endothelial cells to tip cells, resulting in a higher vessel density and more vessel branches[102]. Tumor-derived microvesicles are able to induce fibroblast activation via the upregulation of extracellular signal-regulated kinase phosphorylation and MMP-9. Activation of fibroblasts leads to an increased release of fibroblast-derived microvesicles, which in turn leads to an increased migration of metastatic tumor cells through the interaction of CX3CL and fractalkine ligands that bind to CX3CR1[103].

MRNA and miRNA that originate from tumor-derived exosomes have effects on the microenvironment of the tumor[104]. The transport of these mRNAs and miRNAs from metastatic tumor cells to benign tumor cells can induce growth and proliferation of the benign tumor cells, as seen in exosomes containing miRNA derived from glioblastoma cells[105]. The let-7 miRNA family was found in metastatic gastric tumor cells that target oncogenes such as RAS and HMGA2, genes that normally exert a tumorsuppressive role. The take-up of let-7 by recipient cells of the metastatic gastric tumor cell derived exosomes leads to the suppression of RAS and HMGA2, therefore inducing metastasis in the recipient cells[106]. Tumor-derived exosomes also contain apoptosis inhibiting factors such as surviving. Transfer of surviving from one tumor cell to the neighboring tumor cells will lead to inhibition of apoptosis in these cells[107, 108]. The reports that showed that tumor-derived extracellular vesicles display several pro- and anti-tumor effects, have led to increased interest in the clinical relevance of tumor-derived extracellular vesicles in cancer as both therapeutic agent and as diagnostic parameter.

Extracellular vesicles as biomarker in several diseases

As the content and amount of extracellular vesicles secreted is closely related to the cell of origin and displays disease-specific information, extracellular vesicles could become potent diagnostic biomarkers, both for early determination of disease as well as for monitoring drug responsiveness. The easy accessibility of extracellular vesicles in body fluids can only increase their potential as biomarker compared to biopsies, that are currently used for diagnosis of disease. This thesis will summarize the increasing number of papers published that discuss the extracellular vesicle amount and/or content are disease specific and can therefore be used for diagnostics.

Extracellular vesicles as biomarker in cancer

Biomarker potential of extracellular vesicles has gained increased interest by many researchers. The high mortality rates in cancer increased the interest in biomarkers that can achieve early diagnosis and a reliable

discrimination between benign and metastatic cancers. In most cancer types currently used biomarkers lack specificity, which leads to unnecessary biopsies and treatment. Prostate cancer (PCa) is one of the few cancers with a clinically used biomarker. Prostate-specific antigen (PSA) is used for both diagnosis of PCa and the follow-up after treatment. At this moment PSA is the golden standard for the detection of PCa[109]. Unfortunately there are some downsides to PSA as biomarker. The sensitivity of PSA detection is acceptable, but the specificity is not sufficient to discriminate between a benign prostate disease and an aggressive PCa or indolent PCa, which can result in unnecessary biopsies and an insufficient treatment. Therefore the need increases for new biomarker detection of PCa[110]. Current biomarker research focussed mainly on urinary exosomes or prostasomes in seminal/prostatic fluid or cultured cells[111]. Prostasomes are extracellular vesicles secreted by the prostate gland in semen. Exosomes and prostasomes share many features. The mean diameter of prostasomes is 150 nm and the mean diameter of exosomes is 100 nm[112]. The protein content of prostasomes is comparable to the content of exosomes derived from PCa cell lines[21]. The best known function of prostasomes is their positive effect on the motility of spermatozoa[113]. It is also suggested that prostasomes protect the spermatozoa from the phagocytes of the immune system of the female[21]. It is most likely that prostasomes are exosomes that originate from prostate tissue[111]. Two studies have recently shown that the prostasome level in blood plasma was increases in patients with prostate cancer compared to control. Prostasome levels of prostate cancer patients correlated with a Gleason score of 7 or higher, while the prostasome levels of healthy controls was correlated with a Gleason score of 6 or lower, suggesting that prostasome level in blood plasma can be used as a biomarker in early diagnosis of prostate cancer[114,115]. Proteomic studies on the prostasome content, revealed the presence of proteins involved in cancer progression, such as: Akt, caveolin-1, pyruvate kinase M2, poly(A)-binding protein 1 and programmed cell death 6 interacting protein. These proteins act on angiogenesis and increase the vasculature nearby the tumor[116]. The content of exosomes isolated from patient samples were studied for their biomarker potential. Mitchell *et al*[81]. studied exosome quantity, presence of prostate tumor markers, PSA and prostate-specific membrane antigen (PSMA) and tumor-associated marker 5T4 in urine samples of 10 PCa patients and 10 healthy controls that underwent hormonal therapy preceding radical radiotherapy. The quantity and the quality of the exosomes present in urine was highly variable, and showed no differences between PCa patients and healthy controls. PSA and PSMA were detected in the majority of the urinary exosomes of PCa patients and not in the urinary exosomes of healthy controls. Only one patient showed a treatment related decrease in the prostate markers. Nilson *et al*[20], showed that previously discovered PCa biomarkers, PCA-3 and TMPRSS2:ERG were detectable in tumor-derived exosomes isolated from urine. These potential biomarkers could give an indication of the androgen deprivation therapy response, where low biomarker expression indicates tumor reduction.

Several studies have addressed the diagnostic potential of exosomes found in serum and urine of non-small lung cancer (NSCLC) patients[117]. One study revealed that the miRNA content in circulating exosomes is the similar with the miRNA pattern of the lung tumor, indicating that the circulating exosomes isolated were indeed lung tumor-derived. Also the amount of circulating exosomes was higher in lung cancer patients compared to healthy controls, however there was no correlation between the amount of circulating exosomes and cancer stage[118]. Li *et al*[119]. performed proteomics on urinary exosomes from NSCLC patients and compared this to urinary exosomes of healthy controls. A total of 18 proteins were identified in the urinary exosomal fraction. Leucine-rich α-2-glycoprotein (LRG1) was expressed at a higher level in urinary exosomes of NSCLC patients compared to healthy controls. LRG1 was also highly expressed in the tumor tissue by 65% of the NSCLC patients, indicating that high levels of LRG1 found in urinary exosomes may be derived from the lung tumor. Welton *et al*[120], studied the exosomal content of a cell line that represents a transitional bladder cell carcinoma. They identified 353 proteins in the exosomal content, of which 72 that were not described in previous exosomal proteomics studies. Some of these proteins were positively identified in exosomes isolated from a bladder cancer patient[120], indicating that these proteins are indeed bladder cancer specific. Rupp *et al*[121]. found that CD24 and EpCAM were present on exosomes derived from ascites of ovarian cancer patients. Serum derived exosomes of breast cancer patients only displayed CD24 expression, while EpCAM is cleaved from the exosomes by MMPs in the serum. This indicates that exosomal CD24 could be a potential serum biomarker indicating both breast cancer as ovarian cancer. Taylor *et al*[122]. studied the expression of 8 miRNAs (miRNA-21, miRNA-141, miRNA-200a, miRNA-200c, miRNA-203, miRNA-205 and miRNA-214) in serum- and cellular-derived exosomes of patient with benign ovarian cancer and several other stages of ovarian cancer. The expression of these eight miRNAs in exosomes isolated from serum and ovarian cancer cells were similar, indicating that the circulating exosomes originated from the tumor tissue. The expression of the miRNAs between the patients with benign ovarian cancer was significantly different from the patients with various other stages of ovarian cancer. The studied miRNAs were undetectable in healthy controls, indicating that the expression profile of these miRNAs combined can discriminate between benign ovarian cancer and other more severe stages of ovarian cancers. Logozzi *et al*[123]. designed an in-house sandwich ELISA (Exotest) which captures tumor derived exosomes in plasma based on the expression of tumor-associated marker, caveolin-1 and the housekeeping proteins CD63 and Rab5b, with a sensitivity of 68%. The Exotest was tested on exosomes in plasma of melanoma patients and controls. Expression of caveolin-1 was significantly increased in melanoma patients compared with the controls. Also the caveolin-1 expression was significantly lower in melanoma patients receiving chemotherapy compared to melanoma patients without chemotherapy. This indicates that Exotest is capable of both diagnosis of melanoma as the effectiveness of chemotherapy. In contrast to all these promising results, Skog *et al*[105] revealed that only 7 of the 25 patients studied

expressed tumor-specific EGFRvIII in serum exosomes of patients with glioblastoma.

Extracellular vesicles as biomarker for renal disease

Besides the potential of extracellular vesicles as biomarker in cancer, other researchers have investigated the potential of extracellular vesicles as diagnostic parameter in renal disease. Extracellular vesicles that are secreted in urine are mainly derived from the kidneys. Therefore many research is performed at extracellular vesicles of patients suffering from various renal diseases.

Only 3% of the total amount of protein in urine is derived from exosomes[124]. Therefore isolating exosomes from urine fractions results in increased sensitivity and more precise protein determination. Zhou *et al*[125]. identified fetuin-A as exosomal protein that was increased in patients suffering from acute kidney injury (AKI) compared to healthy controls, suggesting fetuin-A as a potential biomarker for AKI. They also discovered the presence of two transcription factors, activating transcription factor 3 (ATF3) and Wilms Tumor 1 (WT-1) in urine from patients with AKI and focal segmental glomerulosclerosis (FSGS). ATF3 was detected 0-24 hours after ischemia/reperfusion (I/R) injury, and prior to the elevation of serum creatinine level, indicating that ATF3 could be used for early detection of AKI. WT-1 is a marker for podocyte injury indicating the onset of FSGS. Urinary exosomal WT-1 was detected prior to proteinuria and histological glomerular damage. WT-1 was tested as biomarker in patients with childhood idiopathic nephrotic syndrome (NS) predicting steroid responsiveness or renal pathological conditions. Unfortunately WT-1 was only detected in some patients suffering of NS, and therefore WT-1 seems unsuitable as diagnostic parameter detecting renal pathological conditions or steroid responsiveness in patients with childhood NS[126]. These results suggest that WT-1 could only be used for podocyte injury detection in patients with FSGS but not for childhood NS. Sonoda *et al*[127]. studied changed exosomal expression of aquaporin-1 (AQP1) after renal allograft transplantation in one recipient, and the expression of AQP1 in rats after I/R injury. AQP1 levels were decreased as early as 48 hours after allograft transplantation. In rats the AQP1 expression was already decreased 6 hours after I/R injury, and remained low until 96 hours after I/R injury, suggesting potential of AQP1 as diagnostic parameter for I/R injury. Additional studies with larger patient numbers will be needed to evaluate AQP1 as a potential biomarker predicting AKI development after I/R injury.

Extracellular vesicles as biomarker for neurodegenerative disease

Early detection of neurodegenerative diseases is difficult. Visualization techniques are currently the most often used methods for diagnostics purposes in neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD)[128]. Unfortunately PET-scans and other visualization techniques are expensive. Therefore low-cost and better applicable diagnostic parameters are well studied. Currently the importance of exosomes in the development of several neurodegenerative diseases is studied, and some reports showed promising results for exosomes as diagnostic parameter for neurodegenerative disease.

AD is the most common form of dementia in human[129]. AD is caused by extracellular deposition of the amyloid β-peptide (Aβ) and aggregation of hyperphosphorylated tau protein inside the cytoplasma of neurons. The majority of familial AD is caused by mutations in the presenilin (PS) genes. Mutations in the PS genes cause an increased production of Aβ peptides, that are derived from the amyloid-β precursor protein (APP)[130]. Multiple studies investigated the role of exosomes in the pathology of AD. One study showed that over-expression of mutated PS genes results in reduced levels of cystatin C (which has a neuroprotective role in AD) and a modulation of APP metabolites in exosomes[131]. A previous study already revealed that low serum cystatin C levels can predict development of AD in humans without dementia[132]. Both studies indicate that cystatin C level measured in circulating exosomes could become a potential biomarker in early discovery of AD. Saman *et al*[26], showed that tau secretion can occur in an exosome dependent manner. The major component of tau measured in CSF, is exosomal secreted tau. This indicates that tau secretion via exosomes plays an important role in the abnormal tau processing in neurons and is therefore important in the development of AD. Increased exosomal tau expression in CSF of early AD patients can become a biomarker indicating AD development in elderly people.

PD is a neurodegenerative disease characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta and the presence of Lewy bodies (LB). LB are inclusions that contain high levels of alpha-synuclein, present inside the cytoplasma of neurons due to dysfunction of the lysosome[133]. Aggregation of alpha-synuclein in neurons plays an important role in the pathology of PD. It was recently reported that alpha-synuclein secretion was associated with exosomes, implicating a role in the spread of alpha-synuclein by exosomes[134]. Alvarez-Erviti *et al*[135]. showed that cells that overexpressed alpha-synuclein secrete alpha-synuclein via exosomes. When lysosomal inhibition was induced in alpha-synuclein producing cells, both the amount of alpha-synuclein in the cytosol as in the exosome was increased, indicating that alpha-synuclein detection in exosomes isolated from CSF could predict PD. Unfortunately no definite evidence of alpha-synuclein aggregates in the cytosol was observed, indicating that other processes besides exosomal deposition of alpha-synuclein contribute to PD development.

Recent research revealed the importance of exosomes in the transmission of infectious agents such as prions. Prions induce the neurodegenerative disease Creutzfeldt-Jakob disease in human or bovine spongiform encephalopathy in cattle[136]. Prion diseases are induced by conversion of the host-encoded prion protein (PrP^{C}) into an abnormal isoform of the of this prion protein (PrP^{Se}). Vella *et al*[137], revealed that both PrP^{C} as PrP^{Se} are spread by exosomes derived from both peripheral and neuronal cells. Exosomes containing PrP^{C} and PrP^{Se} are capable of inducing prion disease when injected into healthy mice. Therefore it can be concluded that exosomes play a key role in the prion spread.

Extracellular vesicles as potential therapeutic agent

Extracellular vesicles display both immunosuppressive and immunogenic properties in cancer. Both experimental therapies focusing on the immune activating properties of extracellular vesicles as studies that target extracellular vesicles due to their immunosuppressive properties have been performed.

Of DC-derived exosomes is described that they activate tumor-specific $CD8^+$ T-lymphoctyes *in vitro*. Therefore many researchers exploited the possibility of exosomes derived from DCs (Dex) as vaccine for immunotherapy in the last decade[138]. DCs have a high immunogenic capacity as APC by priming naïve T-lymphocytes. Tumor antigens become immunogenic upon presentation to T-lymphocytes by DCs or Dex[139]. Dex remain stable for a long period and can be stored for 12 months at -80°C providing multiple vaccines from one leukapheresis, which is an advantage compared to vaccination with DCs[140].

The first reports described the use of exosomes derived from immature DCs (imDex) as a cell-free vaccine that inhibited tumor growth in mice[141]. Unfortunately imDex cannot activate $CD4^+$ and $CD8^+$ T-lymphocytes, without the help of mature DCs. MHCs II present on imDex must be transferred to mature DCs, enabling the mature DCs to activate the naive $CD4^+$ lymphocytes, resulting in differentiated effector T-lymphocytes[142-144]. Biochemical composition of imDex showed that MHC molecules and tetraspanin family members (CD9, CD63, CD81 and CD82) were present in high quantitites, but costimulatory molecules like B7.2/CD86 and adhesion molecules like CD11b, CD11c, CD58 and ICAM-1/CD54 were expressed in low quantities on the surface of imDex[145]. CD83, B7.1/CD80 and CD40, proteins that are normally expressed by DCs were undetectable in imDex[145]. Two clinical trials in end-stage metastatic melanoma patients, and nonoperable NSCLC patients were performed using imDex. These clinical trials showed increased amounts and function of NK cells, indicating that imDex had a stimulating effect on the innate immune system. Unfortunately no response against tumor epitope spread was detected using MAGE3.A1 and MAGE3.DP04 peptide-specific T-lymphocytes[146,147]. Segura *et al*[148,149], showed that exosomes derived from mature DCs (mDex) are capable of direct activation of T-lymphocytes in contrast to imDex, caused by the enrichment of costimulatory molecules and adhesion molecules on the surface of mDex compared to imDex. Viaud *et al*[140], showed that exosomes secreted from monocyte-derived DCs treated IFN-γ, called γ-Dex expressed higher levels of costimulatory molecules and adhesion molecules like CD40, CD80, CD86 and ICAM-1 compared to imDex. Therefore γ-Dex, just as mDex can induce direct activation of T-lymphocytes. Currently γ-Dex are tested in phase II trials on NSCLC patients, by Viaud *et al*[140].

Besides the immune activating properties of exosomes derived from DCs, it has also been described that removal of tumor-derived exosomes from blood of cancer patients could improve the function of the anti-tumor immune response. Removal of tumor-derived exosomes could even inhibit metastasis, due to the immunosuppressive effects of tumor-

derived exosomes[82]. A hollow-fiber cartridge (Hemopurifier) system is developed that selectively depletes circulating viruses from the blood circulation by lectin-based resin, which binds to the glycosylated viral particles on the surface of the virus[150]. Exosomes also display these glycosylated particles on their membrane, and are similar in size as viruses, suggesting that this system could also be used to deplete tumor-derived exosomes from the blood circulation of cancer patients. A reasonable concern is if the system is capable of distinguishing tumor-derived exosomes from nontumor-derived exosomes. Depletion of both tumor-derived exosomes and nontumor-derived exosomes could cause serious defects in normal cell-cell communication[82]. However this problem could be overcome by incorporation of anti-EpCAM antibodies, as EpCAM is selectively expressed on tumor-derived exosomes.

Angiogenisis can be initiated or inhibited using microvesicles derived from T-lympocytes. Microvesicles derived from activated T-lymphocytes cell lines express the morphogen Sonic hedgehog (Shh) which promotes angiogenesis[151,152], while microvesicles shed from apoptotic T-lymphocytes cell lines lack expression of Shh and inhibit angiogenesis[152]. Therefore microvesicles containing Shh can be used in diseases such as atherosclerosis or after myocardial infarction to induce angiogenesis, while microvesicles lacking Shh can be used in patients with cancer, inhibiting angiogenesis and therefore tumor spread[153].

Conclusion

Extracellular vesicles have great potential both as diagnostic biomarker and as therapeutic agent in several diseases. Extracellular vesicles have the advantage of easy accessibility over currently used diagnostic parameters such as biopsies. Extracellular vesicles used as therapeutic agent can be produced in cell cultures, and the content can be adjusted. Drawbacks in implementation of extracellular vesicles in the clinic as biomarker or therapeutic agent are a result of impure fraction used for biomarker studies. These impure extracellular fractions lead to conflicting and confusing results. Therefore a generalized extracellular vesicle isolation protocol should replace the various protocols that are currently used, leading to reliable comparisons between studies. When these technical problems are overcome the full potential of extracellular vesicles as biomarker and therapeutic agent can be exploited, and this will hopefully lead to implementation of extracellular vesicles in the clinic.

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References

1. Davis, D. M. Intercellular transfer of cell-surface proteins is common and can affect many stages of an immune response. *Nat. Rev. Immunol.* **7**, 238-243 (2007).

2. Rak, J. Microparticles in cancer. *Semin. Thromb. Hemost.* **36**, 888-906 (2010).

3. Heijnen, H. F., Schiel, A. E., Fijnheer, R., Geuze, H. J. & Sixma, J. J. Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood* **94**, 3791-3799 (1999).

4. Hess, C., Sadallah, S., Hefti, A., Landmann, R. & Schifferli, J. A. Ectosomes released by human neutrophils are specialized functional units. *J. Immunol.* **163**, 4564-4573 (1999).

5. Obregon, C., Rothen-Rutishauser, B., Gitahi, S. K., Gehr, P. & Nicod, L. P. Exovesicles from human activated dendritic cells fuse with resting dendritic cells, allowing them to present alloantigens. *Am. J. Pathol.* **169**, 2127-2136 (2006).

6. Al-Nedawi, K. *et al*. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat. Cell Biol.* **10**, 619-624 (2008).

7. Ristorcelli, E. *et al*. Human tumor nanoparticles induce apoptosis of pancreatic cancer cells. *FASEB J.* **22**, 3358-3369 (2008).

8. Hawari, F. I. *et al*. Release of full-length 55-kDa TNF receptor 1 in exosome-like vesicles: a mechanism for generation of soluble cytokine receptors. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 1297-1302 (2004).

9. Arteaga, R. B. *et al*. Endothelial microparticles and platelet and leukocyte activation in patients with the metabolic syndrome. *Am. J. Cardiol.* **98**, 70-74 (2006).

10. Miksa, M. *et al*. Immature dendritic cell-derived exosomes rescue septic animals via milk fat globule epidermal growth factor-factor VIII [corrected. *J. Immunol.* **183**, 5983-5990 (2009).

11. Wang, G. J. *et al*. Thymus exosomes-like particles induce regulatory T cells. *J. Immunol.* **181**, 5242-5248 (2008).

12. Escola, J. M. *et al*. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J. Biol. Chem.* **273**, 20121-20127 (1998).

13. Wubbolts, R. *et al*. Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. *J. Biol. Chem.* **278**, 10963-10972 (2003).

14. Saunderson, S. C. *et al*. Induction of exosome release in primary B cells stimulated via CD40 and the IL-4 receptor. *J. Immunol.* **180**, 8146-8152 (2008).

15. Skokos, D. *et al*. Mast cell-dependent B and T lymphocyte activation is mediated by the secretion of immunologically active exosomes. *J. Immunol.* **166**, 868-876 (2001).

16. Skokos, D. *et al*. Nonspecific B and T cell-stimulatory activity mediated by mast cells is associated with exosomes. *Int. Arch. Allergy Immunol.* **124**, 133-136 (2001).

17. Lai, R. C. *et al*. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell. Res.* **4**, 214-222 (2010).

18. Sellam, J. *et al*. Increased levels of circulating microparticles in primary Sjogren's syndrome, systemic lupus erythematosus and rheumatoid arthritis and relation with disease activity. *Arthritis Res. Ther.* **11**, R156 (2009).

19. Orozco, A. F. *et al*. Placental release of distinct DNA-associated micro-particles into maternal circulation: reflective of gestation time and preeclampsia. *Placenta* **30**, 891-897 (2009).

20. Nilsson, J. *et al*. Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer. *Br. J. Cancer* **100**, 1603-1607 (2009).

21. Poliakov, A., Spilman, M., Dokland, T., Amling, C. L. & Mobley, J. A. Structural heterogeneity and protein composition of exosome-like vesicles (prostasomes) in human semen. *Prostate* **69**, 159-167 (2009).

22. Admyre, C. *et al*. Exosomes with immune modulatory features are present in human breast milk. *J. Immunol.* **179**, 1969-1978 (2007).

23. Houali, K. *et al*. A new diagnostic marker for secreted Epstein-Barr virus encoded LMP1 and BARF1

oncoproteins in the serum and saliva of patients with nasopharyngeal carcinoma. *Clin. Cancer Res.* **13**, 4993-5000 (2007).

24. Keller, S. *et al*. CD24 is a marker of exosomes secreted into urine and amniotic fluid. *Kidney Int.* **72**, 1095-1102 (2007).

25. Bard, M. P. *et al*. Proteomic analysis of exosomes isolated from human malignant pleural effusions. *Am. J. Respir. Cell Mol. Biol.* **31**, 114-121 (2004).

26. Saman, S. *et al*. Exosome-associated tau is secreted in tauopathy models and is selectively phosphorylated in cerebrospinal fluid (CSF) in early Alzheimer's Disease. *J. Biol. Chem.* (2011).

27. Luo, S. S. *et al*. Human villous trophoblasts express and secrete placenta-specific microRNAs into maternal circulation via exosomes. *Biol. Reprod.* **81**, 717-729 (2009).

28. Valadi, H. *et al*. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* **9**, 654-659 (2007).

29. Stoorvogel, W., Kleijmeer, M. J., Geuze, H. J. & Raposo, G. The biogenesis and functions of exosomes. *Traffic* **3**, 321-330 (2002).

30. Camussi, G., Deregibus, M. C., Bruno, S., Cantaluppi, V. & Biancone, L. Exosomes/microvesicles as a mechanism of cell-to-cell communication. *Kidney Int.* **78**, 838-848 (2010).

31. Buschow, S. I. *et al*. MHC II in dendritic cells is targeted to lysosomes or T cell-induced exosomes via distinct multivesicular body pathways. *Traffic* **10**, 1528-1542 (2009).

32. Gauvreau, M. E. *et al*. Sorting of MHC class II molecules into exosomes through a ubiquitin-independent pathway. *Traffic* **10**, 1518-1527 (2009).

33. Pap, E. *et al*. T lymphocytes are targets for platelet- and trophoblast-derived microvesicles during pregnancy. *Placenta* **29**, 826-832 (2008).

34. Taylor, D. D., Akyol, S. & Gercel-Taylor, C. Pregnancy-associated exosomes and their modulation of T cell signaling. *J. Immunol.* **176**, 1534-1542 (2006).

35. Thery, C. *et al*. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J. Immunol.* **166**, 7309-7318 (2001).

36. Bobrie, A., Colombo, M., Raposo, G. & Thery, C. Exosome secretion: molecular mechanisms and roles in immune responses. *Traffic* **12**, 1659-1668 (2011).

37. Kosaka, N. *et al*. Secretory mechanisms and intercellular transfer of microRNAs in living cells. *J. Biol. Chem.* **285**, 17442-17452 (2010).

38. Spang, A. On the fate of early endosomes. *Biol. Chem.* **390**, 753-759 (2009).

39. Savina, A., Fader, C. M., Damiani, M. T. & Colombo, M. I. Rab11 promotes docking and fusion of multivesicular bodies in a calcium-dependent manner. *Traffic* **6**, 131-143 (2005).

40. Ostrowski, M. *et al*. Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nat. Cell Biol.* **12**, 19-30; sup pp 1-13 (2010).

41. Hsu, C. *et al*. Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A-C. *J. Cell Biol.* **189**, 223-232 (2010).

42. Booth, A. M. *et al*. Exosomes and HIV Gag bud from endosome-like domains of the T cell plasma membrane. *J. Cell Biol.* **172**, 923-935 (2006).

43. Pelchen-Matthews, A., Raposo, G. & Marsh, M. Endosomes, exosomes and Trojan viruses. *Trends Microbiol.* **12**, 310-316 (2004).

44. Ratajczak, J. *et al*. Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia* **20**, 847-856 (2006).

45. Spees, J. L., Olson, S. D., Whitney, M. J. & Prockop, D. J. Mitochondrial transfer between cells can rescue aerobic respiration. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 1283-1288 (2006).

46. Del Conde, I. *et al*. Microvesicle-associated tissue factor and Trousseau's syndrome. *J. Thromb. Haemost.* **5**, 70-74 (2007).

47. Beaudoin, A. R. & Grondin, G. Shedding of vesicular material from the cell surface of eukaryotic cells: different cellular phenomena. *Biochim. Biophys. Acta* **1071**, 203-219 (1991).

48. Greenwalt, T. J. The how and why of exocytic vesicles. *Transfusion* **46**, 143-152 (2006).

49. George, J. N., Thoi, L. L., McManus, L. M. & Reimann, T. A. Isolation of human platelet membrane microparticles from plasma and serum. *Blood* **60**, 834-840 (1982).

50. Zwaal, R. F., Comfurius, P. & Bevers, E. M. Scott syndrome, a bleeding disorder caused by defective scrambling of membrane phospholipids. *Biochim. Biophys. Acta* **1636**, 119-128 (2004).

51. Hugel, B., Martinez, M. C., Kunzelmann, C. & Freyssinet, J. M. Membrane microparticles: two sides of the coin. *Physiology (Bethesda)* **20**, 22-27 (2005).

52. Distler, J. H. *et al*. Microparticles as regulators of inflammation: novel players of cellular crosstalk in the rheumatic diseases. *Arthritis Rheum.* **52**, 3337-3348 (2005).

53. Greco, V., Hannus, M. & Eaton, S. Argosomes: a potential vehicle for the spread of morphogens through epithelia. *Cell* **106**, 633-645 (2001).

54. Lee, Y. J. *et al*. Elevated platelet microparticles in transient ischemic attacks, lacunar infarcts, and multiinfarct dementias. *Thromb. Res.* **72**, 295-304 (1993).

55. Zhuang, L., Kim, J., Adam, R. M., Solomon, K. R. & Freeman, M. R. Cholesterol targeting alters lipid raft composition and cell survival in prostate cancer cells and xenografts. *J. Clin. Invest.* **115**, 959-968 (2005).

56. Krailadsiri, P. *et al*. The effects of leukodepletion on the generation and removal of microvesicles and prion protein in blood components. *Transfusion* **46**, 407-417 (2006).

57. Reverter, J. C. *et al*. Inhibition of platelet-mediated, tissue factor-induced thrombin generation by the mouse/human chimeric 7E3 antibody. Potential implications for the effect of c7E3 Fab treatment on acute thrombosis and "clinical restenosis". *J. Clin. Invest.* **98**, 863-874 (1996).

58. Barry, O. P., Pratico, D., Lawson, J. A. & FitzGerald, G. A. Transcellular activation of platelets and endothelial cells by bioactive lipids in platelet microparticles. *J. Clin. Invest.* **99**, 2118-2127 (1997).

59. Denzer, K., Kleijmeer, M. J., Heijnen, H. F., Stoorvogel, W. & Geuze, H. J. Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J. Cell. Sci.* **113 Pt 19**, 3365-3374 (2000).

60. Clayton, A. *et al*. Adhesion and signaling by B cell-derived exosomes: the role of integrins. *FASEB J.* **18**, 977-979 (2004).

61. van Balkom, B. W., Pisitkun, T., Verhaar, M. C. & Knepper, M. A. Exosomes and the kidney: prospects for diagnosis and therapy of renal diseases. *Kidney Int.* **80**, 1138-1145 (2011).

62. Montecalvo, A. *et al*. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *Blood* **119**, 756-766 (2012).

63. Fevrier, B. & Raposo, G. Exosomes: endosomal-derived vesicles shipping extracellular messages. *Curr. Opin. Cell Biol.* **16**, 415-421 (2004).

64. Nolte-'t Hoen, E. N., Buschow, S. I., Anderton, S. M., Stoorvogel, W. & Wauben, M. H. Activated T cells recruit exosomes secreted by dendritic cells via LFA-1. *Blood* **113**, 1977-1981 (2009).

65. Rieu, S., Geminard, C., Rabesandratana, H., Sainte-Marie, J. & Vidal, M. Exosomes released during reticulocyte maturation bind to fibronectin via integrin alpha4beta1. *Eur. J. Biochem.* **267**, 583-590 (2000).

66. Miyanishi, M. *et al*. Identification of Tim4 as a phosphatidylserine receptor. *Nature* **450**, 435-439 (2007).

67. Thery, C., Ostrowski, M. & Segura, E. Membrane vesicles as conveyors of immune responses. *Nat. Rev. Immunol.* **9**, 581-593 (2009).

68. Bonci, D. *et al*. The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. *Nat. Med.* **14**, 1271-1277 (2008).

69. Tauro, B. J. *et al*. Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes. *Methods* (2012).

70. Mathias, R. A., Lim, J. W., Ji, H. & Simpson, R. J. Isolation of extracellular membranous vesicles for proteomic analysis. *Methods Mol. Biol.* **528**, 227-242 (2009).

71. Lenassi, M. *et al*. HIV Nef is secreted in exosomes and triggers apoptosis in bystander CD4+ T cells. *Traffic* **11**, 110-122 (2010).

72. Raposo, G. *et al*. B lymphocytes secrete antigen-presenting vesicles. *J. Exp. Med.* **183**, 1161-1172 (1996).

73. Kovacsovics-Bankowski, M. & Rock, K. L. Presentation of exogenous antigens by macrophages: analysis of major histocompatibility complex class I and II presentation and regulation by cytokines. *Eur. J. Immunol.* **24**, 2421-2428 (1994).

74. Cantin, R., Diou, J., Belanger, D., Tremblay, A. M. & Gilbert, C. Discrimination between exosomes and HIV-1: purification of both vesicles from cell-free supernatants. *J. Immunol. Methods* **338**, 21-30 (2008).

75. Thery, C., Amigorena, S., Raposo, G. & Clayton, A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr. Protoc. Cell. Biol.* **Chapter 3**, Unit 3.22 (2006).

76. Mathivanan, S., Ji, H. & Simpson, R. J. Exosomes: extracellular organelles important in intercellular communication. *J. Proteomics* **73**, 1907-1920 (2010).

77. Grant, R. *et al*. A filtration-based protocol to isolate human plasma membrane-derived vesicles and exosomes from blood plasma. *J. Immunol. Methods* **371**, 143-151 (2011).

78. Rubin, O., Crettaz, D., Tissot, J. D. & Lion, N. Pre-analytical and methodological challenges in red blood cell microparticle proteomics. *Talanta* **82**, 1-8 (2010).

79. Gyorgy, B. *et al*. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell Mol. Life Sci.* **68**, 2667-2688 (2011).

80. Fernandez-Llama, P. *et al*. Tamm-Horsfall protein and urinary exosome isolation. *Kidney Int.* **77**, 736-742 (2010).

81. Mitchell, P. J. *et al*. Can urinary exosomes act as treatment response markers in prostate cancer? *J. Transl. Med.* **7**, 4 (2009).

82. Yang, C. & Robbins, P. D. The roles of tumor-derived exosomes in cancer pathogenesis. *Clin. Dev. Immunol.* **2011**, 842849 (2011).

83. Ristorcelli, E., Beraud, E., Mathieu, S., Lombardo, D. & Verine, A. Essential role of Notch signaling in apoptosis of human pancreatic tumoral cells mediated by exosomal nanoparticles. *Int. J. Cancer* **125**, 1016-1026 (2009).

84. Bu, N. *et al*. Exosome-loaded dendritic cells elicit tumor-specific CD8+ cytotoxic T cells in patients with glioma. *J. Neurooncol.* **104**, 659-667 (2011).

85. Chen, W. *et al*. Efficient induction of antitumor T cell immunity by exosomes derived from heat-shocked lymphoma cells. *Eur. J. Immunol.* **36**, 1598-1607 (2006).

86. Cho, J. A., Lee, Y. S., Kim, S. H., Ko, J. K. & Kim, C. W. MHC independent anti-tumor immune responses induced by Hsp70-enriched exosomes generate tumor regression in murine models. *Cancer Lett.* **275**, 256-265 (2009).

87. Xie, Y. *et al*. Membrane-bound HSP70-engineered myeloma cell-derived exosomes stimulate more efficient CD8(+) CTL- and NK-mediated antitumour immunity than exosomes released from heat-shocked tumour cells expressing cytoplasmic HSP70. *J. Cell. Mol. Med.* **14**, 2655-2666 (2010).

88. Chen, T., Guo, J., Yang, M., Zhu, X. & Cao, X. Chemokine-containing exosomes are released from heat-stressed tumor cells via lipid raft-dependent pathway and act as efficient tumor vaccine. *J. Immunol.* **186**, 2219-2228 (2011).

89. Andreola, G. *et al*. Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. *J. Exp. Med.* **195**, 1303-1316 (2002).

90. Huber, V. *et al*. Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape. *Gastroenterology* **128**, 1796-1804 (2005).

91. Taylor, D. D., Gercel-Taylor, C., Lyons, K. S., Stanson, J. & Whiteside, T. L. T-cell apoptosis and suppression of T-cell receptor/CD3-zeta by Fas ligand-containing membrane vesicles shed from ovarian tumors. *Clin. Cancer Res.* **9**, 5113-5119 (2003).

92. Clayton, A. & Tabi, Z. Exosomes and the MICA-NKG2D system in cancer. *Blood Cells Mol. Dis.* **34**, 206-213 (2005).

93. Valenti, R. *et al*. Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes. *Cancer Res.* **66**, 9290-9298 (2006).

94. Xiang, X. *et al*. Induction of myeloid-derived suppressor cells by tumor exosomes. *Int. J. Cancer* **124**, 2621-2633 (2009).

95. Szajnik, M., Czystowska, M., Szczepanski, M. J., Mandapathil, M. & Whiteside, T. L. Tumor-derived microvesicles induce, expand and up-regulate biological activities of human regulatory T cells (Treg). *PLoS One* **5**, e11469 (2010).

96. Wada, J. *et al*. Surface-bound TGF-beta1 on effusion-derived exosomes participates in maintenance of number and suppressive function of regulatory T-cells in malignant effusions. *Anticancer Res.* **30**, 3747-3757 (2010).

97. Webber, J., Steadman, R., Mason, M. D., Tabi, Z. & Clayton, A. Cancer exosomes trigger fibroblast to myofibroblast differentiation. *Cancer Res.* **70**, 9621-9630 (2010).

98. Nieuwland, R., van der Post, J. A., Lok, C. A., Kenter, G. & Sturk, A. Microparticles and exosomes in gynecologic neoplasias. *Semin. Thromb. Hemost.* **36**, 925-929 (2010).

99. Stoeck, A. *et al*. A role for exosomes in the constitutive and stimulus-induced ectodomain cleavage of L1 and CD44. *Biochem. J.* **393**, 609-618 (2006).

100. Jung, T. *et al*. CD44v6 dependence of premetastatic niche preparation by exosomes. *Neoplasia* **11**, 1093-1105 (2009).

101. Nazarenko, I. *et al*. Cell surface tetraspanin Tspan8 contributes to molecular pathways of exosome-induced endothelial cell activation. *Cancer Res.* **70**, 1668-1678 (2010).

102. Sheldon, H. *et al*. New mechanism for Notch signaling to endothelium at a distance by Delta-like 4 incorporation into exosomes. *Blood* **116**, 2385-2394 (2010).

103. Castellana, D. *et al*. Membrane microvesicles as actors in the establishment of a favorable prostatic tumoral niche: a role for activated fibroblasts and CX3CL1-CX3CR1 axis. *Cancer Res.* **69**, 785-793 (2009).

104. Zomer, A. *et al*. Exosomes: Fit to deliver small RNA. *Commun. Integr. Biol.* **3**, 447-450 (2010).

105. Skog, J. *et al*. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat. Cell Biol.* **10**, 1470-1476 (2008).

106. Ohshima, K. *et al*. Let-7 microRNA family is selectively secreted into the extracellular environment via exosomes in a metastatic gastric cancer cell line. *PLoS One* **5**, e13247 (2010).

107. Khan, S. *et al*. Extracellular, cell-permeable survivin inhibits apoptosis while promoting proliferative and metastatic potential. *Br. J. Cancer* **100**, 1073-1086 (2009).

108. Khan, S. *et al*. Survivin is released from cancer cells via exosomes. *Apoptosis* **16**, 1-12 (2011).

109. Stamey, T. A. *et al*. Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. *N. Engl. J. Med.* **317**, 909-916 (1987).

110. Crawford, E. D. & Abrahamsson, P. A. PSA-based screening for prostate cancer: how does it compare with other cancer screening tests? *Eur. Urol.* **54**, 262-273 (2008).

111. Duijvesz, D., Luider, T., Bangma, C. H. & Jenster, G. Exosomes as biomarker treasure chests for prostate cancer. *Eur. Urol.* **59**, 823-831 (2011).

112. Jansen, F. H. *et al*. Exosomal secretion of cytoplasmic prostate cancer xenograft-derived proteins. *Mol. Cell. Proteomics* **8**, 1192-1205 (2009).

113. Stegmayr, B. & Ronquist, G. Promotive effect on human sperm progressive motility by prostasomes. *Urol. Res.* **10**, 253-257 (1982).

114. Lance, R. S., Drake, R. R. & Troyer, D. A. Multiple recognition assay reveals prostasomes as promising plasma biomarkers for prostate cancer. *Expert Rev. Anticancer Ther.* **11**, 1341-1343 (2011).

115. Tavoosidana, G. *et al*. Multiple recognition assay reveals prostasomes as promising plasma biomarkers for prostate cancer. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 8809-8814 (2011).

116. Ronquist, K. G., Ronquist, G., Larsson, A. & Carlsson, L. Proteomic analysis of prostate cancer metastasis-derived prostasomes. *Anticancer Res.* **30**, 285-290 (2010).

117. Jemal, A. *et al*. Cancer statistics, 2003. *CA Cancer. J. Clin.* **53**, 5-26 (2003).

118. Rabinowits, G., Gercel-Taylor, C., Day, J. M., Taylor, D. D. & Kloecker, G. H. Exosomal microRNA: a diagnostic marker for lung cancer. *Clin. Lung Cancer.* **10**, 42-46 (2009).

119. Li, Y., Zhang, Y., Qiu, F. & Qiu, Z. Proteomic identification of exosomal LRG1: a potential urinary biomarker for detecting NSCLC. *Electrophoresis* **32**, 1976-1983 (2011).

120. Welton, J. L. *et al*. Proteomics analysis of bladder cancer exosomes. *Mol. Cell. Proteomics* **9**, 1324-1338 (2010).

121. Rupp, A. K. *et al*. Loss of EpCAM expression in breast cancer derived serum exosomes: role of proteolytic cleavage. *Gynecol. Oncol.* **122**, 437-446 (2011).

122. Taylor, D. D. & Gercel-Taylor, C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecol. Oncol.* **110**, 13-21 (2008).

123. Logozzi, M. *et al*. High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. *PLoS One* **4**, e5219 (2009).

124. Zhou, H. *et al*. Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. *Kidney Int.* **69**, 1471-1476 (2006).

125. Zhou, H. *et al*. Exosomal Fetuin-A identified by proteomics: a novel urinary biomarker for detecting acute kidney injury. *Kidney Int.* **70**, 1847-1857 (2006).

126. Lee, H. *et al*. Urinary exosomal WT1 in childhood nephrotic syndrome. *Pediatr. Nephrol.* (2011).

127. Sonoda, H. *et al*. Decreased abundance of urinary exosomal aquaporin-1 in renal ischemia-reperfusion injury. *Am. J. Physiol. Renal Physiol.* **297**, F1006-16 (2009).

128. Frisoni, G. B. *et al*. Virtual imaging laboratories for marker discovery in neurodegenerative diseases. *Nat. Rev. Neurol.* **7**, 429-438 (2011).

129. Selkoe, D. J. Amyloid beta protein precursor and the pathogenesis of Alzheimer's disease. *Cell* **58**, 611-612 (1989).

130. Masters, C. L. & Beyreuther, K. Alzheimer's centennial legacy: prospects for rational therapeutic intervention targeting the Abeta amyloid pathway. *Brain* **129**, 2823-2839 (2006).

131. Ghidoni, R. *et al*. Cystatin C is released in association with exosomes: a new tool of neuronal communication which is unbalanced in Alzheimer's disease. *Neurobiol. Aging* **32**, 1435-1442 (2011).

132. Sundelof, J. *et al*. Serum cystatin C and the risk of Alzheimer disease in elderly men. *Neurology* **71**, 1072-1079 (2008).

133. Spillantini, M. G. *et al*. Alpha-synuclein in Lewy bodies. *Nature* **388**, 839-840 (1997).

134. Emmanouilidou, E. *et al*. Cell-produced alpha-synuclein is secreted in a calcium-dependent manner by exosomes and impacts neuronal survival. *J. Neurosci.* **30**, 6838-6851 (2010).

135. Alvarez-Erviti, L. *et al*. Lysosomal dysfunction increases exosome-mediated alpha-synuclein release and transmission. *Neurobiol. Dis.* **42**, 360-367 (2011).

136. Vella, L. J., Sharples, R. A., Nisbet, R. M., Cappai, R. & Hill, A. F. The role of exosomes in the processing of proteins associated with neurodegenerative diseases. *Eur. Biophys. J.* **37**, 323-332 (2008).

137. Vella, L. J. *et al*. Packaging of prions into exosomes is associated with a novel pathway of PrP processing. *J. Pathol.* **211**, 582-590 (2007).

138. Chaput, N. *et al*. The potential of exosomes in immunotherapy of cancer. *Blood Cells Mol. Dis.* **35**, 111-115 (2005).

139. Yang, L. & Baltimore, D. Long-term in vivo provision of antigen-specific T cell immunity by programming hematopoietic stem cells. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 4518-4523 (2005).

140. Viaud, S. *et al*. Updated technology to produce highly immunogenic dendritic cell-derived exosomes of clinical grade: a critical role of interferon-gamma. *J. Immunother.* **34**, 65-75 (2011).

141. Zitvogel, L. *et al*. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat. Med.* **4**, 594-600 (1998).

142. Andre, F. *et al*. Exosomes as potent cell-free peptide-based vaccine. I. Dendritic cell-derived exosomes transfer functional MHC class I/peptide

complexes to dendritic cells. *J. Immunol.* **172**, 2126-2136 (2004).

143. Chaput, N. *et al*. Exosomes as potent cell-free peptide-based vaccine. II. Exosomes in CpG adjuvants efficiently prime naive Tc1 lymphocytes leading to tumor rejection. *J. Immunol.* **172**, 2137-2146 (2004).

144. Thery, C. *et al*. Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes. *Nat. Immunol.* **3**, 1156-1162 (2002).

145. Lamparski, H. G. *et al*. Production and characterization of clinical grade exosomes derived from dendritic cells. *J. Immunol. Methods* **270**, 211-226 (2002).

146. Escudier, B. *et al*. Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of thefirst phase I clinical trial. *J. Transl. Med.* **3**, 10 (2005).

147. Morse, M. A. *et al*. A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. *J. Transl. Med.* **3**, 9 (2005).

148. Segura, E., Amigorena, S. & Thery, C. Mature dendritic cells secrete exosomes with strong ability to induce antigen-specific effector immune responses. *Blood Cells Mol. Dis.* **35**, 89-93 (2005).

149. Segura, E. *et al*. ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming. *Blood* **106**, 216-223 (2005).

150. Ichim, T. E. *et al*. Exosomes as a tumor immune escape mechanism: possible therapeutic implications. *J. Transl. Med.* **6**, 37 (2008).

151. Soleti, R. *et al*. Microparticles harboring Sonic Hedgehog promote angiogenesis through the upregulation of adhesion proteins and proangiogenic factors. *Carcinogenesis* **30**, 580-588 (2009).

152. Martinez, M. C. & Andriantsitohaina, R. Microparticles in angiogenesis: therapeutic potential. *Circ. Res.* **109**, 110-119 (2011).

153. Yang, C. *et al*. Lymphocytic microparticles inhibit angiogenesis by stimulating oxidative stress and negatively regulating VEGF-induced pathways. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **294**, R467-76 (2008).