Grasmann Eva Christine, BSc

ISOLATION AND CHARACTERIZATION OF PLACENTAL EXOSOMES

MASTERARBEIT

zur Erlangung des akademischen Grades

Master of Science

Masterstudium Biochemie und Molekulare Biomedizin

eingereicht an der

Technischen Universität Graz

Betreuer

Christian Wadsack, Assoc. Prof., PhD

Medizinische Universität Graz
Abteilung für Frauenheilkunde und Geburtshilfe

Graz, Juni 2016

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt, und die den benutzten Quellen wörtlich und inhaltlich entnommenen Stellen als solche kenntlich gemacht habe. Das in TUGRAZonline hochgeladene Textdokument ist mit der vorliegenden Masterarbeit identisch.

30.06.2016
Datum

Grosman Ga Christine, BSc
Unterschrift

Abstract

Isolation and Characterization of Placental Exosomes

Objectives: Extracellular vesicles (EV) are released by many different cell types and play an important role in intercellular communication of cells. Various populations are described and many protocols have been published with varying degrees of vesicle characterization. Microvesicles are deriving from membranous extrusions. The specific exosomal characteristics are the intracellular release by the endosomal pathway via multivesicular bodies, a size from 30 to 100 nm and a density from 1.13 to 1.19 g/ml. Exosomes contain specific cargos and they transport proteins, miRNAs and lipids to impact the signalling in the recipient cell. The human placenta releases syncytiotrophoblast EVs into the maternal circulation and these EVs exhibit many biological activities. *Ex-vivo* dual human placenta perfusion enables to investigate the release of trophoblast derived EVs *ex vivo*. The aim of my thesis was to establish an isolation method for placental exosomes, according to the protocol provided by Yoel Sadovsky, for maternal and fetal placental perfusates. It was hypothesised that maternal-fetal crosstalk during gestation may additionally occur via placental exosomes.

Methods: Human *ex-vivo* dually placental perfusion perfusate was used to isolate exosomes released to the maternal and fetal circuit. Isolation was done by differential centrifugation followed by separation and purification on a density gradient. Isolated exosome fractions were analysed by nanotracking analysis (NTA) to determine size and number of particles of the distinct fractions. The collected fractions were also investigated by Western blotting and probed against specific exosomal and placental marker proteins.

Results: The provided general protocol for exosome isolation enables analysis of placenta derived exosomes from maternal perfusates. The mean size of isolated exosomes after differential centrifugation was 168 ± 69 nm. After three-stepwise centrifugation (1K, 10K and 70K x g) of maternal perfusates all fractions were enriched in CD63, a member of the tetraspanin family and marker for extracellular vesicles. The placenta specificity of the isolated vesicles was shown with PLAP (placental alkaline phosphatase). The placental exosome enriched final pellet contained both intracellular Syntenin-1 (multifunctional adapter protein) and TSG101 (Tumor susceptility gene 101) proteins suggesting that these particles have an intracellular origin but circulating in the mother as a specific subtype of extracelluar vesicles. An additional isopycnic centrifugation step of maternal perfusates decreased the mean size of the exosomal fraction to 130 ± 60 nm and revealed a strong Syntenin-1 band in the immunoblot, which indicates a distinct sorting and excretion of these extracellular vesicles from the human placenta. In contrast, from fetal perfusates neither

microvesicles nor placental derived exosomes could be isolated however, NTA measurements detected distinct particles with a mean size of 125 nm after several centrifugation steps, but with 100-fold less signal intensity compared to maternal perfusates.

Conclusions: In conclusion maternal-placental cross-talk is very likely since trophoblast derived specific exosomes can be isolated and identified from maternal perfusates after perfusion of the placenta. The existence of a maternal-fetal communication via exosomes has to be further investigated, as the existence of placenta derived exosomes in the fetal circulation remains still unclear. The provided protocol is applicable for preparation of placental exosomes since it involves both differential and isopycnic centrifugation steps, characterization of the particle size and verification by endosomal markers.

Zusammenfassung

Isolierung und Charakterisierung von Exosomen der humanen Plazenta

Zielsetzung: Extrazellulären Vesikel (EV), die in der Zell-Zell-Kommunikation eine Rolle spielen, werden von verschiedensten Zelltypen sezerniert. Verschiedene Vesikelpopulationen und Protokolle zur Isolierung und Charakterisierung wurden beschrieben. Mikrovesikel werden direkt von der Zellmembran abgeschnürt. Werden die EVs über das endosomale System abgegeben und weisen eine Größe zwischen 30 und 100 nm auf, werden diese als Exosomen definiert. Eine charakteristische Eigenschaft von Exosomen, die auch als Unterscheidungsmerkmal zu anderen EVs dient, ist die charakteristische Dichtefraktion von 1.13 bis 1.19 g/ml in Körperflüssigkeiten. Exosomen transportieren spezifisch Proteine, Lipide und miRNAs und können an den Empfängerzellen eine Veränderung im Zellmetabolismus oder in Signalkaskaden auszulösen. Die humane Plazenta sezerniert EVs vom Synzytiotrophoblasten in die maternale Zirkulation. Ziel der Masterarbeit war es eine Methode zur Exosomenisolierung aus maternalem und fetalem Plazentaperfusat, mittels eines von Yoel Sadovsky zur Verfügung gestellten Protokolls, zu etablieren. Um einen potentiellen maternal-fetalen „Crosstalk“ während der Schwangerschaft zu untersuchen, wurde die Isolierung von fetalen Exosomen angestrebt.

Methoden: Die duale ex-vivo Perfusion der humanen Plazentawurde zur Gewinnung von Plazentaperfusaten aus maternalem und fetalem Kreislauf verwendet. Aus den Perfusaten wurde mittels differentieller Zentrifugation und einer Auftrennung auf einem Iodixanol-Dichtegradient die Exosomenisolierung durchgeführt. Die Größe und Anzahl der isolierten Exosomen der unterschiedlichen Fraktionen wurde mittels Nanotracking Analyse (NTA) durchgeführt. Zusätzlich wurden die isolierten Exosomen mittels Western Blotting unter der Verwendung exosomenspezifischer Marker charakterisiert.

Resultate: Das Protokoll erlaubte eine spezifische Isolierung von Exosomen aus maternalem Perfusat. Die mittlere Größe der isolierten Exosomen durch differentielle Zentrifugation betrug 168 ± 69 nm. Nach differentieller Zentrifugation (1K, 10K und 70K x g) von maternalem Perfusat konnte eine Anreicherung von CD63, ein Markerprotein für extrazelluläre Vesikel, sowie PLAP (Placental Alkaline Phosphatase), ein Markerprotein für eine plazentale Abstammung der Vesikel, nachgewiesen werden. Das finale Pellet zeigte eine Anreicherung von interzellulärem Syntenin-1 (multifunktionales Adaptorprotein) und TSG101 (tumor susceptility gene 101). Diese Tatsache deutet darauf hin das die isolierten Partikel einen intrazellulären Ursprung haben, aber als spezifische Vesikelpopulation extrazellulär im maternalen Kreislauf zirkulieren. Durch die Kombination von differentieller

Zentrifugation und Auftrennung auf einem Dichtegradienten, kann eine mittlere Größe von isolierten Exosomen bei 130 ± 60 nm. Bei Immunoblot Analysen wiesen die isolierten Partikel eine eindeutige und starke Syntenin-1 Bande auf, was ein Hinweis auf eine spezfische Freisetzung von Exosomen von der Planzenta ist. Im Gegensatz zu den maternalen Ergebnissen konnten aus fetalem Perfusat weder Exosomen noch Mikrovesikel isoliert werden. Jedoch konnten mittels NTA Partikel mit einer Größe von 125 nm in den fetalen Fraktionen detektiert werden. Das Signal war jedoch um einen Faktor 100 geringer als in den vergleichbaren maternalen Fraktionen.

Conclusio: Die Ergebnisse meiner Masterarbeit legen eine Kommunikation zwischen maternalem Kreislauf und Plazenta nahe, da Exosomen mit plazentalem Ursprung aus maternalem Perfusat isoliert werden konnten. Ein maternal-fetaler „Crosstalk“ via Exosomen muss weiter untersucht werden, da die Existenz von plazentalen Exosomen im fetalen Kreislauf nicht nachgewiesen werden konnte. Das Protokoll ermöglicht durch differentielle Zentrifugation und Auftrennung auf einem Iodixanol-Dichtegradient die spezifische Isolierung von plazentalen Exosmen. Eine Charakterisierung mittels NTA und endosomalen Markerproteinen ist möglich.

Danksagung

An dieser Stelle möchte ich mich bei allen bedanken, die mich bei der Anfertigung meiner Masterarbeit unterstützt haben.

Zu allererst möchte ich mich bei meinem Betreuer Assoz. Prof. Dr. Christian Wadsack bedanken, für die Leitung und Unterstützung während der ganzen Dauer meiner Masterarbeit. Zusätzlich möchte ich mich bei Michi und Birgit bedanken, für die Unterstützung und die angeregten Diskussion, mit der Lösungen zu allen meinen Problemen gefunden werden konnten.

Ich freue mich sehr, dass ich meine Masterarbeit hier im Forschungslabor der Frauenklinik absolvieren durfte. Lieben Dank für die herzliche Aufnahme im Labor und für die Hilfsbereitschaft von allen. Besonders bedanken möchte ich mich bei Susi für die Hilfestellungen bei praktischen Problemen.

Ein ganz großer Dank geht an meine Familie, besonders an meine Eltern, die mich nicht nur bei meiner Masterarbeit, sondern bei meiner gesamten Ausbildung hinter mir gestanden sind und immer ein offenes Ohr und eine Schulter zum Anlehnen für mich hatten und haben. Ich bin stolz eure Tochter zu sein! Erwähnen möchte ich hier auch meine Schwester Gabi. Danke für die fachlichen Diskussionen .

Ich möchte mich aber auch bei meinen Freunden, Andi, Ellie, Mathias und Sarah, sowie meinen Mitbewohnern Andi und Denise bedanken, dass ihr mich immer wieder mit einer Tasse Kaffee und netten Gesprächen immer wieder zum Lachen gebracht habt. Ihr habt meine Studentenzeit zu etwas ganze Besonderem gemacht. Ganz besonders erwähnen möchte ich meine beste Freundin Hoa, für die Freundschaft seit der 1. Klasse Gymnasium und die Unterstützung egal wohin der Weg geht.

DANKE!

Abbreviations

BSA	bovine serum albumine
C19MC	chromosome 19 miRNA cluster
DMEM	Dulbecco's Modified Eagle Medium
DMEM+	1x DMEM with 1 % Streptomycin/Penicillin
DMEM++	1x DMEM with 1 % Streptomycin/Penicillin and 10 % FCS
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
ESCRT	endosomal sorting complex required for transport
EV	extracellular vesicle
FasL	Fas ligand
FCS	fetal calf serum
GDM	gestational diabetes mellitus
HBSS	Hank's buffered salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
HSP70	heat shock protein 70
ILV	intraluminal vesicle
MHC	major histocompatibility complex
MIC	MHC class I chain-related protein
MV	microvesicle
MVB	multivesicular body
NFDM	non-fat dry milk
NTA	nanotracking analysis
PBS	phosphate-buffered saline
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PLAP	placental alkaline phospatase
TBE-T	1x tris borate EDTA buffer with 1 % Tween
TNFα	tumor necrosis factor α
tris	tris(hydroxymethyl)aminomethane
TSG101	tumor suceptibility gene 101
ULBP	UL16-binding protein

Content

1. Introduction

1.1. Intercellular communication

The evolution of multicellular organisms from unicellular ones required communication between the cells. In a multicellular organism communication has to take place between neighbouring cells as well as between far distant cells within the same organism. Intercellular communication is also a big player in the development of tissue and groups of specialized cell within organs (1). Several different communication mechanisms are described within the literature (2).

First of all intercellular communication can be effectuated by soluble mediators such as hormones, cytokines, chemokines, bioactive ions, such as calcium, and lipids. These factors are released from cells and can act in a paracrine, which means that they affect a neighbouring cell, or autocrine, which means that the cell which is secreting the information is affected itself, manner (2).

Secondly, intercellular communication can occur via direct adhesion contacts between the signalling and the targeting cell (2). For this purpose gap junctions between cells are formed to mediate the passage of chemical or electrical signals from one cell to another (3). In nerve cells the cell-cell communication is provided via chemical or electric synapses (4).

An additional possibility of communication between cells is the formation of membranous nanotubes between each other. The nanotubes facilitate the intercellular transfer of organelles, cytoplasmic molecules and other membrane components. The formation of nanotubes can bridge cells even over long distances and provide a selective communication between cells (4).

In this thesis the focus will be on the fourth possibility of cell to cell communication, the communication via small extracellular vesicles. Extracellular vesicles are able to act not only in the neighbourhood of the secreting cells but also over longer distances within the multicellular organisms, like the human placenta (2).

1.2. Intercellular communication of the human placenta

In general the placenta is a maternal-fetal organ, which has a short and defined life span. Fetal growth and development extremely depend on the functionality of the placenta (5).

During pregnancy the placenta plays an important role in the intercellular communication. Maternal and fetal cells have to communicate and interact via the placenta with each other to coordinate the exchange of nutrients and oxygen (6). Nutrients have to cross several cell barriers in order to cross the placenta from the maternal side to the fetal circulation. The fetal blood vessels in the placenta

are organised in villous structure, built by endothelial cells (7). The villi are completely covered by the syncytiotrophoblast and are bathed in maternal blood during gestation (8). The transport mechanism of nutrients across these barriers is depending on molecular size, polarity, lipid solubility of the substances, which cross the placenta. Active and facilitated transport mechanisms are described beside diffusion of nutrients and gases (5).

Moreover, the placenta is not only a participant in the intercellular communication between fetus and mother, but it is also communicating directly with the mother. The human syncytiotrophoblast is secreting hormones, cytokines and growth factors into the maternal blood (9). These released substances can then act again as important participants in the cross-talk between mother and fetus (10). Hormones secreted by the human placenta can act either paracrine or endocrine. Examples for hormones released by the placenta are renin, which is involved in the regulation of the blood pressure, or the growth-releasing growth hormone, which regulates fetal and placental growth. There can also be found examples for growth factors released by the placenta, like angiogenic growth factors (11).

Additionally, the placenta secretes immunregulatory factors via the syncytiotrophoblast, which may alter the maternal immune system during pregnancy (2). The alteration is necessary, as the maternal immune system has to accept the fetus for the period of pregnancy (2; 6). Also the growth and development of the fetus is supported by regulatory mechanisms in the maternal circulation (12).

Trophoblasts interact with surface ligands to control the immune reactivity of maternal T cells via cell to cell communication. Another immune control mechanism is active during pregnancy where fetal cells are transported to the maternal circulation in order to promote immune tolerance during major histocompatibility complex (MHC) restriction and maturation of maternal T cells (6).

The intercellular communication mediated by the placenta is also occurring due to the secretion of extracellular vesicles by the syncytiotrophoblast (2). The focus of this thesis will be on the isolation of these extracellular vesicles released from the human placenta.

1.3. Extracellular vesicles

Extracellular vesicles (EV), released by cells, represent an important cell-cell communication mechanism. For a long period of time EVs were considered to be cellular debris. Most of the human cell types such as epithelial cells, fibroblasts, immune cells, stem cells and also tumor cells , release EVs as they play an important role in both physiological and pathophysiological processes in the multicellular organism. EVs are not only involved in the intercellular communication but for example also in inflammation or tumorgenesis (2; 13). Functionality of the EVs depend on type of the

secreting cell, moreover the composition of the EVs strongly depends also on the donor cell. EVs influence the recipient cell in several ways. Membrane receptors and proteins as well as bioactive lipids and miRNAs are transported via EVs, thereby changing the genetic information of the recipient cell (13). Isolation of EVs from most human body fluids such as serum, plasma, urine, amniotic fluid or breast milk has been described (2; 13).

EVs are a very heterogeneous vesicle population as they differ in size, morphology and in their composition. The biogenesis of these EVs is another important differentiation characteristics as they can either be shed from the plasma membrane or be secreted through the endosomal membrane compartment. Basically, EVs can be separated in larger (0.1-2 µm) and smaller (30-100 nm) population. The large EVs are further classified in apoptotic bodies and microvesicles. The small sized EVs are named exosomes (2). An overview about the different populations of EVs and their main characteristics are described in Table 1.

Table 1: Overview of the main characteristics of EVs, modified from Mincheva-Nilsson L. et al. **(14).**

	Exosomes	**Microvesicles**	**Apoptotic Bodies**
Size	30 - 100 nm	0.1 - 2 µm	0.05 – 4 µm
Density in sucrose	1.13 - 1.19 g/ml	not defined	1.16-1.28 g/ml
Sedimentation	100 000 - 110 000 x g	10 000 - 100 000 x g	1 500 – 100 000 x g
Specific markers for identification	Tetraspanins (CD63, CD9, CD81), ESCRT complex members (TSG101)	Integrins, Selectins, CD40	Histones, DNA
Cellular Origin	Endosomal compartment-multivesicular sorting complex	Plasma membrane	Fragments of apoptotic cells
Mechanism of release	Exocytosis when the multivesicular sorting complex fuses with the cell membrane	Plasma membrane blebbing	Plasma membrane blebbing and cellular fragmentation

The terminology of the different subtypes of vesicles is not standardized yet. In the literature EVs are also termed membranous vesicles or membrane particles (15). If the vesicles are isolated from biological fluids, the name of the vesicle is often related to the origin (16). To collect all the molecular data about the different vesicle subpopulations, such as the different names or identified molecules, an own database, Vesiclepedia (http://www.microvesicles.org) was published (17).

1.4. Apoptotic bodies

Normal and cancerous cells can undergo apoptosis, the programmed cell death. Apoptosis is a highly organised process through several stages. In addition different energy-dependent biochemical mechanisms are necessary for cells to undergo apoptosis (18). The initial step is the condensation of chromatin, followed by membrane blebbing in order to disintegrate the cellular content into membrane enclosed vesicles, which are called apoptotic bodies (16; 19).

Those apoptotic bodies are only formed during the cell death, whereas the other described extracellular vesicles are also formed during normal cellular processes. The size of apoptotic bodies are generally between 500 nm and 4 µm. Apoptotic bodies are characterized by the presence of cell organelles such as intact mitochondria or ribosomes within the vesicles (16). Additionally, DNA fragments and histones were determined in apoptotic bodies, which enable apoptotic body identification as well (19). In addition to large vesicles, there is also a release of smaller vesicles, which are between 50 and 500 nm. It is not clear yet if the smaller vesicles derive from membrane blebbing during apoptosis (16).

The blebbing of apoptotic bodies is caused due to changes in the cell membrane composition of apoptotic cells. Phosphatidyl serine determines an asymmetric distribution between the outer and inner membrane surface, compared to normal constituted cells (16; 19).

Apoptotic bodies are detected in the extracellular space, but normally phagocytosed by macrophages of the systemic circulation. The clearance process of apoptotic bodies occurs very locally. The phagocytosis is mediated by the interaction of recognition receptors on the phagocytes altered composition of the membrane of apoptotic bodies (16).

Due to the content of DNA fragments, the uptake of the apoptotic bodies also causes genetic information transfer (16).

1.5. Microvesicles

1.5.1. Morphology

All cells shed vesicles from their plasma membrane which is a general normal cell function. These vesicles are then called microvesicles. Synonyms for vesicles in the literature are ectosomes, shedding vesicles, or microparticles (20).

Microvesicles are a very heterogeneous vesicle population. Their size is between 100 nm and 2 µm (21). Markers, used for a specific characterization of microvesicles are also highly dependent on the donor cell. Common markers for characterization are plasma membrane proteins, such as intergrins,

selectins and CD40 (14; 21). Microvesicles, in comparison to apoptotic bodies, do not contain cytosolic organelles or nuclear fragments (22).

1.5.2. Biogenesis

The biogenesis of the microvesicles is the common characteristic of the vesicle population. The origin of the vesicles is the plasma membrane. The vesicles are formed by outward budding and fission of membrane vesicles from the plasma membrane of the secreting cell, as depicted in Figure 1.

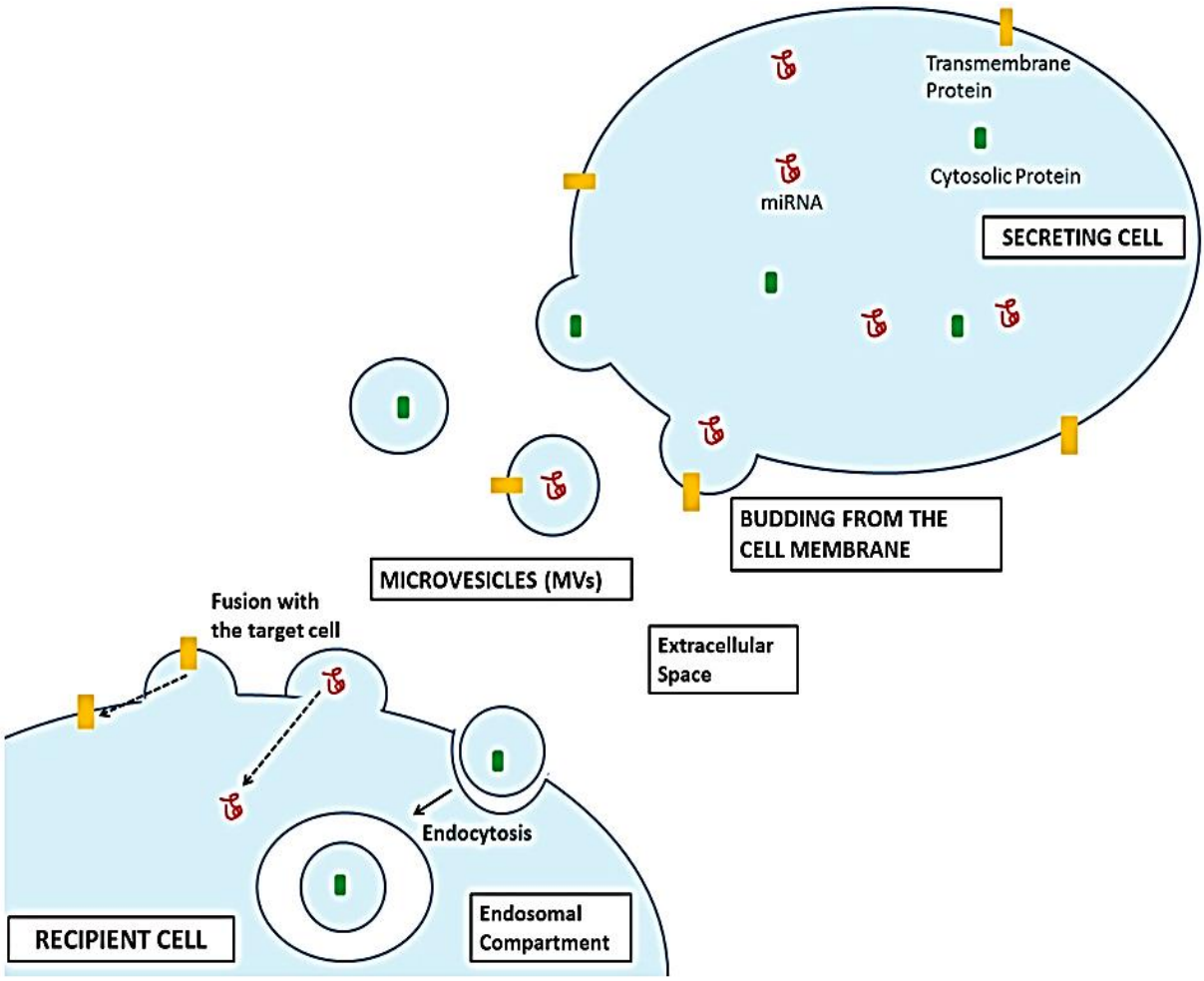

Figure 1: Schematic representation of microvesicle biogenesis. Microvesicles are formed by outward budding and fission of membrane vesicles from the plasma membrane of the secreting cell into the extracellular space. At the recipient cell the vesicles can fuse with the plasma membrane or they are taken up by endocytosis.

The vesicle cargo possesses transmembrane proteins such as receptors or cytosolic proteins as well as cytosolic miRNAs. The microvesicles are shed directly into the extracellular space (21). The shedding process shows many similarities to the abscission step observed in cytokinesis (13; 23). In order to separate the microvesicles from the plasma membrane the contractile machinery is located in the cleavage furrow and draws the opposing membranes towards each other. In the following step the membrane connection is pinched off (23). The release of the microvesicles takes place at specific

locations at the plasma membrane depending on specific lipids and proteins located in the membrane. For example the membrane has to contain lipids, which stabilize membrane bending, as membrane curvature is the driving force behind the microvesicle formation. The structural asymmetry, necessary for membrane bending and membrane curvature, is caused by translocases, flippases and floppases (16; 23). For finishing the budding process the cytoskeletal structures are necessary, especially the actin-myosin interaction (16). Also the action of small GTPases, such as ARF6 is required for microvesicle formation (20).

After the release of the microvesicles, they circulate in the extracellular space in order to catch a recipient cell adjacent to the releasing cell. Microvesicles may also enter biological fluids, like plasma or urine to reach distant sites. Another possibility of vesicle turnover has been described where vesicles break down immediately once they are released to the extracellular space, thereby its content is diffusing freely in the extracellular space (13).

At the recipient cell the microvesicles interact in different ways as shown in Figure 1. One possibility is that the microvesicles interact with the recipient cell directly by fusion with the plasma membrane. The cargo is released to the recipient cell. As another option the vesicle may be taken up from the target cell by endocytosis also followed by a cargo release to the cell. The third mechanism is an interaction between a receptor on the surface of the recipient cell and a molecule located in the vesicle membrane. The biological consequence here is a signalling process (5).

1.6. Exosomes

Exosomes are the recently most studied extracellular vesicles. The term `exosome' has been introduced 1987 by Dr. Rose Johnstone to underline the endocytic origin of these extracellular vesicles (24). Exosomes are differentiated by the other extracellular vesicles due to their biogenesis. Differences in size, morphology, buoyant density and protein composition are not specific enough criterias for a clear distinction between exosomes and microvesicles or apoptotic bodies (20). Intercellular communication via exosomes provides many advantages in comparison to free diffusion of proteins or miRNAs in the extracellular matrix. This process for example allows to maintain the 3D structure of shuttled proteins and with that they keep their biological activity intact. In addition exosomes provide the possibility to transport molecules with lower mobility and higher concentration (23).

1.6.1. Morphology

Exosomes represent the smallest known family of EVs with a size between 30 and 100 nm (16). The buoyant density of exosomes is 1.13-1.19 g/mL on the sucrose gradient. Isolated exosomes show a cup-shaped form, while exosomes in the lumen of multivesicular bodies in the cell show a uniform

spherical shape. The reason for the change in shape is unclear, probably the isolation procedure causes this change (14).

1.6.2. Content of exosomes

Exosomes are membranous nanovesicles. The architecture of the exosome membrane is a bilayer (25). A schematic representation of exosomes and their content is displayed in Figure 2.

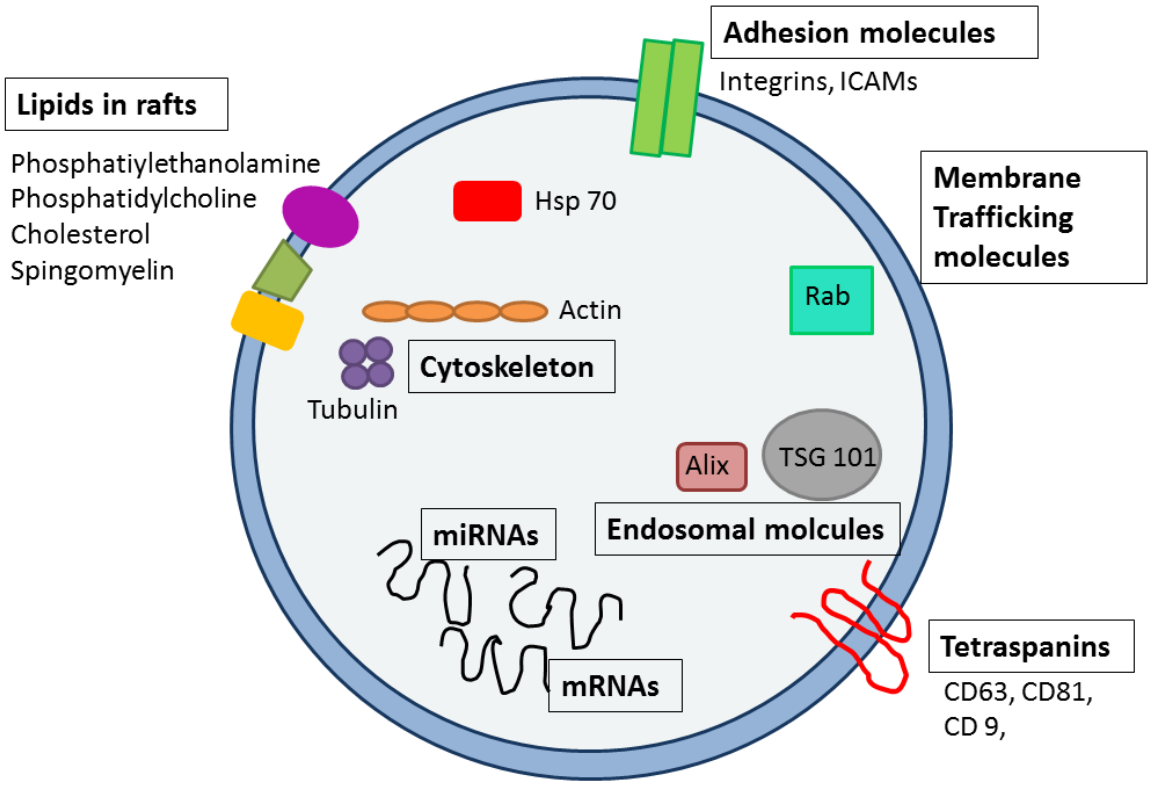

Figure 2: Schematic representation of the common content of exosomes. The representation shows the surface markers, such as adhesion molecules and tetraspanins, as well as the common internal exosomal proteins. Exosomal membranes rich in lipid rafts have a special lipid composition. Also miRNAs and mRNAs belong to the exosomal content.

Exosomal membranes are enriched in sphingomyelin, cholesterol and desaturated species of phosphatidylcholine and phosphatidylethanolamine. Also ceramids can likely be found in exosomal membranes, which causes high rigidity of exosomal membranes (21; 26). In addition exosomal membranes are detergent and low temperature sensitive (14).

The protein/lipid ratio of exosomal membranes is high in comparison with other types or membranes (26). This protein/lipid ratio may be regulated during the exosome biogenesis by the Golgi membranes (27).

Interestingly, higher protein content was found in the exosomal membranes, in particular within lipid rafts (28). The exosomal membrane proteins have either a transmembrane domain or they are

anchored via a glycosylphospinositol linkage (14). Adhesion proteins, such as β-integrins are anchored in the exosomal membranes. A class of proteins, attached to the membrane of exosomes via transmembrane domains, are tetraspanins (2). These proteins are characterized by four transmembrane domains, which separate two extracellular loops form one intercellular loop (29). CD63, CD81, CD9 and CD82 belong all to tetraspanins mainly found in exosomes (2). Tetraspanins show a 7- to 24-fold enrichment in the recipient cells in comparison to the donor cells. That is the main reason why tetraspanins are commonly used as exosomal biomarkers. Areas within the membrane, so called tetraspanin-enriched microdomains, can interact with a large variety of transmembrane and cytosolic signalling proteins. Tetraspanins associate with cholesterol and gangliosides and form special membrane platforms for exosome biogenesis (30).

The content of exosomes consists of proteins, miRNAs and mRNAs (14). After the uptake of exosomes into the target cell the proteins may affect cellular signalling while miRNA and mRNA can modulate gene transcription and translation (31). The exact content of miRNAs and proteins is depending on the cell type from which they originate. The content can even vary within the same cells, depending on the current differentiation and activation state (14). But a common set of conserved exosomal proteins was observed (2).

Intraexosomal proteins are for example parts of the cytoskeleton such as tubulin, actin, and membrane trafficking molecules, such as Rab proteins. Heat shock proteins, G-proteins, and SNAREs are also proteins occurring in all exosomes. Additionally, proteins necessary in the endosomal sorting complex required for transport (ESCRT), which are important for the biogenesis of the exosomes, such as TSG101 and Alix can be found in the exosomes (1; 14; 20).

Also miRNA is detectable on exosomes. The proportion of miRNAs on recipient cells is higher on exosomes than on the parent cells. Since the amount and composition of miRNAs in exosomes differs between patient with disease and healthy individuals, miRNAs serve as biomarkers for diseases. Independently, a specific set of miRNAs which are preferentially sorted to exosomes has been described, e.g. miR-150, miR-142-3p, miR-151 and miR-320 (28). The loading of miRNAs to exosomes is strongly dependent on specific motifs within the miRNAs. The sumoylated ribonucleoprotein A2B1 which is also present in exosomes after the biogenesis, binds directly a set of miRNAs due to their EXO motifs, which are short sequence motifs over-represented in exosomal miRNAs. Due to this binding the loading of miRNAs to the exosomes is regulated (32).

The composition of exosomes plays a very important role for the recipient cell. miRNAs enclosed by exosomes can affect the recipient cell acute or long-term. The transferred miRNAs can change the expression of the transcriptome and therefore alter the whole cellular phenotype. In addition the

exosomal content may lead to a lack in cellular energy production or a loss in the normal membrane phospholipid asymmetry, which leads to the generation of a fusogenic and pro-coaglucant surface (27).

The database ExoCarta (http://www.exocarta.org) catalogues proteins, lipids and miRNAs that have been identified in exosomes (20).

1.6.3. Biogenesis of exosomes

The biogenesis of exosomes is the characteristic feature of this class of EVs. Exosomes originate by the cellular endocytic pathway, as depicted in Figure 3, in particular its development starts in the early endosomes (33). Early endosomes undergo several transformations within the cell to become late endosomal compartments. During this transformation processes, contents, such as proteins or miRNAs, which have to be removed or which should be transported are sorted into small vesicles that bud into the lumen of the late endosomes. The vesicles budded into the late endosomes are called intraluminal vesicles (ILVs) and late endosomes are then known as multivesicular bodies (MVB) (16).

The content of the ILVS can origin from different cell compartments. One possibility is that the content is transported from the plasma membrane to the early and further to the late endosomal compartment. Also proteins which are uptaken to the cell via endocytosis can be transported to the ILVs (2). Molecular constituents of ILVs originate from various cellular compartments such as Golgi apparatus, endoplasmatic reticulum, nucleus or the cytosol. Mitochondrial proteins are not involved in the exosome biogenesis (26).

The late endosomes are targeted to either fuse with lysosomes or with the plasma membrane (16). If the content of the late endosomes is determined to be degraded, the late endosomes fuse with the lysosome, thereby entering the lysosomal pathway. The discriminating factor between MVB sorting, either to lysosomal degradation or to the secretory MVBs, is cholesterol content of the MVB membrane. A cholesterol-poor MVB population is a sign for lysosomal degradation, while a cholesterol-rich MVB population determines exosome secretion (20). If the MVBs are determined to fuse with the plasma membrane they are moved along microtubules toward the cell periphery. There they fuse with the plasma membrane and the ILVs are released to the extracellular space. Due to the release they become exosomes (26; 27). The transport of the MVB along the cytoskeleton and docking of the vesicles to the plasma membrane is controlled by the RAB family of small GTPases (34).

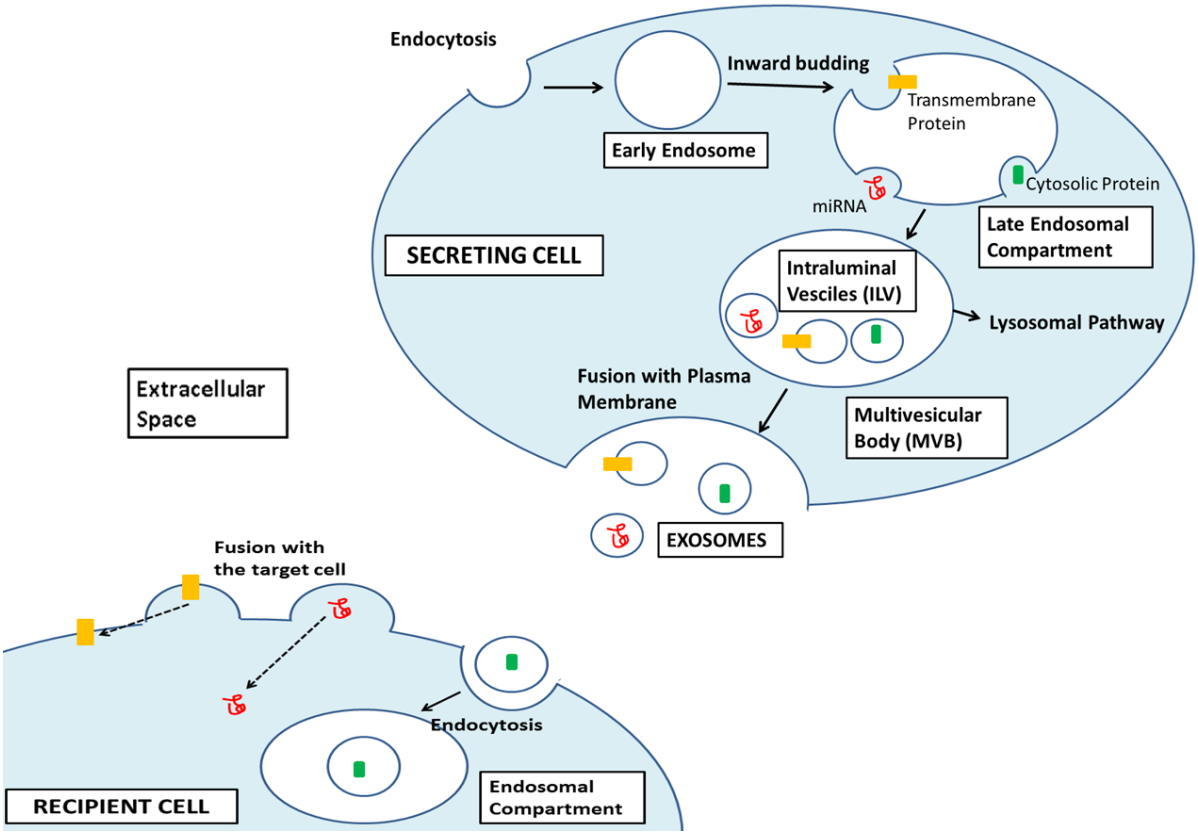

Figure 3: Schematic representation of the biogenesis of exosomes. Early endosomes are transformed to enter the late endosomal compartments. During this process intraluminal vesicles (ILV) in mutivesicular bodies (MVB) are built by inward budding. ILVs are packed with membrane proteins, proteins from various cellular compartments and miRNAs. At the end of this pathway MVBs fuse with the plasma membrane. ILVs are released to the extracellular space and with that renamed as exosomes. At the recipient cell the vesicles may fuse with the plasma membrane or they are taken up by endocytosis.

Exosomes are released to the extracellular matrix and move to the target cell, but they are also capable to enter body fluids such as plasma to bridge bigger distances (13). Like microvesicles, exosomes may also burst after the release to the extracellular space, which causes free diffusion of proteins, miRNAs and lipids.

At the recipient cell exosomes behave similar to microvesicles. The function of the exosomes depends on their ability to interact with the recipient cell via their content of lipids, proteins or miRNAs (20). Exosomes fuse with the plasma membrane of the recipient cell so that the content is released to the cytosol. Another possibility of exchange between exosomes and cells is internalisation of exosomes into the recipient cell by endocytosis (35). Additionally, direct interaction between the cell membrane receptors and target molecules in the exosome membrane results in distinct signalling pathways (13).

1.6.4. Protein sorting for exosome secretion

It is suggested that two main pathways are responsible for the sorting of proteins to the exosomes. One of them is working via a large multiprotein complex, which is called endosomal sorting complex required for transport machinery (ESCRT). The second pathway is an ubiquitin-independent pathway, which is based on sphyingomyelin metabolites, such as ceramides (14).

The ESCRT depicts the predominant process for the ILV formation to the MVB (16). The machinery is conserved from Aarchaea to mammals. It is the same mechanism which is also described in cytokinesis or in budding of enveloped viruses. A schematic representation of the ESCRT pathway for sorting the proteins to the exosomes is depicted in Figure 4.

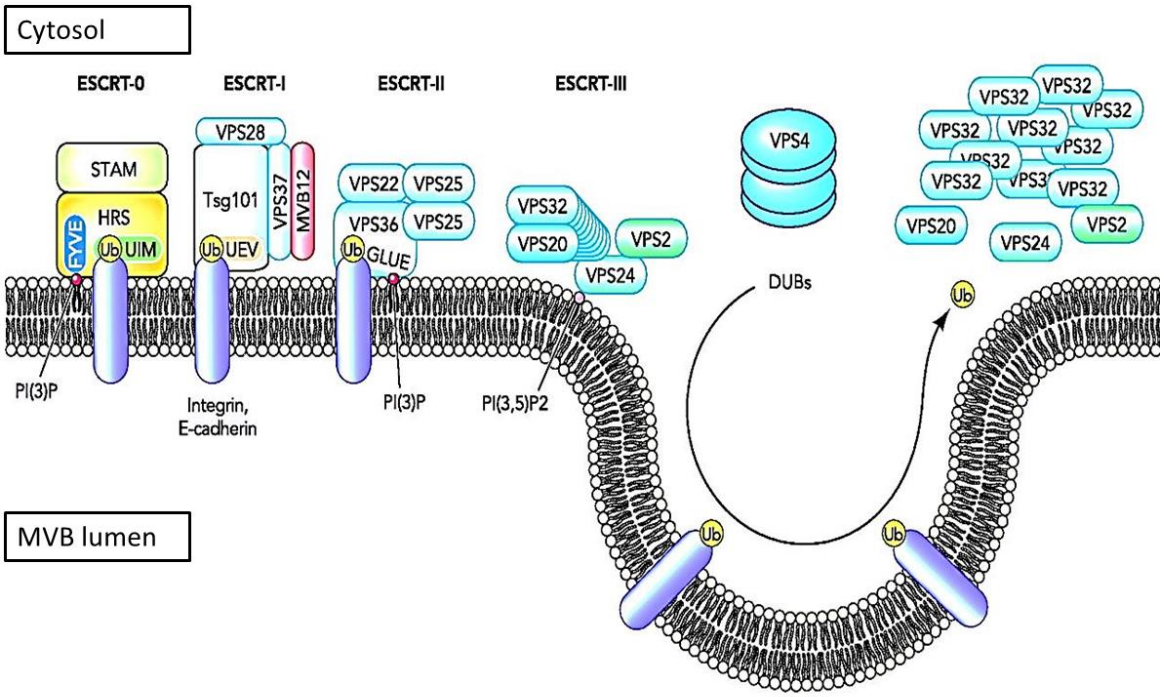

Figure 4: Schematic representation showing protein sorting to exosomes via endosomal sorting complex required for transport machinery (ESCRT), modified from Lobert et.al. (36). Mono-ubiquitinated cargo is captured by the ESCRT-0 complex. ESCRT-II and ESCRT-III are recruited to the membrane of early endosomes. ESCRT-0 and ESCRT-I interact via TSG101. A sequential transfer of the ubiquitinated cargo is provided. ESCRT-III removes the ubiquitin from the cargo before incorporation to the ILVs. ESCRT-III is also responsible for membrane cleavage. At the end Vps4 hydrolyses ATP for recycling the ESCRT-III back to the soluble state.

The ESCRT machinery consists of five protein complexes, ESCRT-0, ESCRT-I, ESCRT- II, ESCRT-III, Vps4-Vta1, and several ESCRT-associated proteins. All the factors are soluble in the cytosol and are recruited to the membrane if necessary. It has to be mentioned that the number of ESCRTs is in the same dimension as the number of ILVs produced. This leads to predictions that a copy of each ESCRT unit is necessary for the shedding of on ILV (37).

Cargo, which should be sent into the ESCRT pathway, is ubiquitylated. Mono-ubiquitination of proteins is a signal for endocytosis and MVB sorting. The ubiquitination plays a role in the interaction of the ESCRT machinery with the proteins determined to be sorted to exosomes (38). If the membrane of the early endosomes is marked with phosphatidylinositol-3-phosphate (PIP3) and the membrane is curved, the ubiquinylated cargo is captured by the ESCRT-0 protein complex which serves also as a recruitment signal for ESCRT-I and ESCRT-II (16). ESCRT-I interacts with ESCRT-0 via the TSG101 protein, which is used for the identification of exosomes since ESCRT-I and ESCRT-II interact directly with each other. This provides a sequential transfer of the ubiquitinated cargo. ESCRT-0, ESCRT-I and ESCRT-II interact with the exosome cargo specifically via the ubiquitylation (38).

The ESCRT-III is anchored in the endosmal membrane and posseses the ability to remove the ubiquitin from the cargo before it is incorporated into the ILV. ESCRT-III is together with the Vps4-Vta1 directly responsible for the membrane cleavage. The soluble ESCRT-III monomers assemble in the membrane and form tightly bound filaments. The formation leads to a conformational change and a recruitment of several other proteins. Additionally ESCRT-III catalyses membrane scission. At the end Vps4 hydrolyses ATP for recycling the ESCRT-III back to the soluble state (37).

As mentioned before the ESCRT process is not required for exosome formation, exosome biogenesis and secretion, but for ILV- formation (14). The ESCRT-mechanism involves lipids, tetraspanins and heat shock proteins. Ceramides are proposed to influence the curvature of the membrane of MVB to form ILVs. The phospholipase D2 is necessary in the ESCRT- independent exosome biogenesis. Phospholipase D2 hydrolyses phospatidylcholine to phosphatic acid, which is an inducing factor for membrane curvature. Tetraspanin CD63 participates in the sorting of proteins into the ILVs in an ESCRT-independent manner. Additionally, the heat shock protein 70 (HSP70) binds to cytosolic proteins containing a KFERQ motif, thereby proteins were sorted to the ILVs (34). The ESCRT-independent sorting mechanism causes dramatic alterations in the morphology of the components of the endocytic pathway. One consequence is the formation of more heterogeneous ILVs (39).

1.7. Exosomes of the human placenta

The placenta plays a big role in mediating the maternal adaption to pregnancy as well as regulating fetal growth and development. During pregnancy exosomes are important players as they provide an important and common way of cell-to-cell communication under normal and pathological conditions, such as gestational diabetes or pre-eclampsia (40). They induce changes in the mother during pregnancy (25).

Exosomes play a also relevant role in in the development of autoimmunity of the mother during pregnancy (27). This is important as the placenta is a transient organ and plays a major role in the

successful development of the fetus (2). The immune system of the mother has to tolerate the semiallogeneic fetus and has to support the development and growth of the fetus (12). The release of exosomes during pregnancy provides different mechanisms to inhibit the maternal immune system so that the fetus survives (33).

The release of placental exosomes is also an important factor in the invasion of placental cells to the maternal tissue. Therefore, beside the maternal immune system also the metabolic and cardiovascular activities of the maternal body have to be adapted, which may also take place via exosomal communication. Exosomes are released from the placenta in response to changes in the milieu, such as oxygen tension, so that cell invasion and migration can take place (25). In the literature it is reported, that the placental exosome concentration in the maternal blood stream is increasing during a normal pregnancy. The placenta releases exosomes into the maternal circulation beginning at 6 weeks of pregnancy. The number of exosomes in the maternal plasma correlates with the weight of the placenta and the blood supply within the placenta. Whilegestation progresses the number of placental exosomes increases. In particular the level of exosomes was about 4.8 and about 13.5 fold higher during the second trimester and the third trimester respectively compared to the exosome concentration in the first trimester. Interestingly, the exosome number is 50 fold higher in pregnant women than in non-pregnant women. Although the number of exosomes is rising during pregnancy the bioactivity of exosomes is highest in the first trimester of pregnancy (40).

Exosomes released by the syncytiotrophoblast are important in the control of the immune mechanisms during pregnancy. These immune mechanisms necessary for the acceptance and support of the fetal allograft, include cytotoxicity, T-cell response not only in the local vicinity of the placenta but also at a distance from the maternal-fetal interface (2; 3).

Placental exosomes are released from the apical side of the syncytiotrophoblast directly into the maternal bloodstream (14; 33). This specific high protein turnover rate argues for a high number of MVBs released by the placenta (14). *In vitro* it has been shown that the release of exosomes strongly depends on different oxygen levels in primary trophoblast cells (41).

1.7.1. Composition of placenta derived exosomes

Placenta derived exosomes show the same characteristics and content as normal exosomes, which is depicted in Figure 2. Exosomes carry beside common proteins, lipids and miRNAs, also molecules strongly depending on their cell of origin (14).

Proteins:

Placental exosomes contain different classes of proteins e.g. immunactive proteins, vasoactive proteins and proteins involved in thrombosis (8). Immunomodulatory proteins of exosomes, are B7 family immunmodulators, with the specific members CD274 and CD276, and HLA-G5, all of them expressed by syncytiotrophoblasts. Moreover, B7 family members and HLA-G are carried by placental-derived exosomes they can function systemically (42).

Placental exosomes also carry Syncytin-1, which is able to reduce the secretion of proinflammatory cytokines such as TNFα (Tumor necrosis factor) from leukocytes, which leads to a repression of the proinflammatory cytokines in healthy pregnancies (8).

In the syncytiotrophoblast of the human placenta the proteins FasL (Fas Ligand) and TRAIL are expressed. These two molecules are apoptosis inducing proteins which can be also found bound on exosome membranes . FasL is inducing cell death by binding to it receptor Fas. Beside apoptosis FasL is also a player in organ development. Also TRAIL is inducing apoptotic cell death. TRAIL induced cell death is important in other physiological process such as suppression of autoimmunity and immune surveillance. FasL and TRAIL build an oligomerized complex in form of death inducing signalling complexes on the exosomal membrane and trigger therefor exosome-induced apoptosis of T-cells (12).

Placental exosomes also show the expression of MHC class I chain-related proteins (MICs) and UL-16 binding protein (ULBPs). ULBPs and MICs are constitutively expressed by the syncytiotrophoblast and can be found on the membrane of exosomes. The transport of those proteins in exosomes leads to a down-regulation of NKG2D receptor located on lymphocytes such as T-cells, B-cells and natural killer cells, and monocytes. This down-regulation process leads to an additional immune system inhibition (43). The expression of NKG2D correlatesalso with the level of TNFα (44). As already mentioned before the level of secreted TNFα can also be reduced by other placental exosome components.

Placental exosomes also show syncytiotrophoblast-specific proteins such as PLAP (placental alkaline phosphatase) as their cargo. PLAP can be found bound to the membrane of placental exosomes (40).

miRNAs:

miRNAs have an important function in the placenta, as a maternal-fetal interface. Therefore it is important to know the pattern of miRNAs transported in placental exosomes (45).

30-40 % of all miRNAs found in the placenta, are located on chromosome 19 (46). This cluster of miRNA genes is termed chromosome 19 miRNA cluster (C19MC). These miRNAs are detectable in the maternal circulation during pregnancy. The selection of certain C19MC to exosomes is done via a specific mechanism (45).

Two placenta-specific miRNAs, miRNA517A and miRNA21, were detected in exosome-enriched fractions. miRNA517A is included in the C19MC. It is also proposed, that the miRNA517A is also involved in TNF signalling (46), which is also a part of the maternal immune system.

About the role of miRNAs during pregnancies under healthy and pathological conditions not many things are known right now (46).

1.7.2. Placental exosomes in pregnancy diseases

The release of exosomes during pregnancy correlates with pregnancy outcome. Specific exosome expression profiles were observed for pathologies of pregnancies such as pre-eclampsia, intrauterine growth restriction and gestational diabetes mellitus (GDM) (27).

Pre-eclampsia

Pre-eclampsia is one of the most common reasons for maternal mortality in the developed world (47). It can also have effects on the long-term health of mother and fetus (48). The first stage of pre-eclampsia is poor spiral artery remodelling, which leads to a dysfunctional placenta perfusion and oxidative stress in the placenta. The second stage is caused by a release of placental factors into the blood. The maternal syndrome is characterized by a systemic vascular inflammation. The inflammation is accompaniedby the symptoms of hypertension, proteinuria and oedema (49). Increased levels of exosomes can be found in the plasma from women suffering from pre-eclampsia (50). The exosome levels correlate with the severity of the disease severity (47). Also the molecular cargo carried by exosomes is different and this can cause an alteration in the biological function of the exosomes (49). For example the syncytiotrophoblast specific protein, Syncytin 2 is down regulated in exosomes of pregnant women suffering from pre-eclampsia. Syncytin 2 takes part in the cell fusion of the trophoblast (51).

Exosomes isolated from supernatant of placental explant culture supernatant of women suffering from pre-eclampsia, showed a shift in the lipid composition in comparison to exosomes isolated from healthy ones. Phosphatidylserine is significantly increased, while sphingomyelin and ceramides showed a similar but non-significant trend to be increased. It is also described in the literature, that proinflammatory cytokines such as TNFα promote the lipid dysregulation by insulin resistance (52).

Gestational Diabetes Mellitus (GDM)

Insulin resistance during normal pregnancy is induced near mid-pregnancy, and progresses through the third trimester. This resistance declined rapidly after delivery. If the maternal insulin release to the circulation is not high enough for the compensation of the insulin resistance, Gestational Diabetes Mellitus (GDM) is developed.(41). Women with GDM show higher levels of inflammatory

markers, such as TNFα (53). In addition GDM can cause oxidative stress, which has bad consequences for mother and baby (54). During pregnancy at 24 to 28 weeks of gestation a screening for GDM is done by an oral glucose tolerance test (41).

The exosome concentration is 1.5-fold higher in a GDM pregnancy than in a normal one. Nevertheless the number of exosomes in pregnant women is already increased in comparison to non-pregnant women. GDM is occurring also in combination with a proinflammatory state. Exosomes isolated from the plasma of women with GDM are bioactive. They modulate the proinflammatory cytokines, as for example TNFα, released from the endothelial cells. The inflammatory environment can alter the maternal glucose metabolism (41).

Preterm labour

Exosomes may also play an importantrole in preterm labour. The concentration of exosomes in the plasma of women who deliver preterm is reduced at 28 to 30 weeks of gestation in comparison to mothers who deliver at term. These exosomes differ from normal exosomes since they are associated with less FasL, which results in reduced T-cell activity suppression (49).

1.7.3. Characterization of placental exosomes

In order to characterize the isolated exosomes, specific proteins have to be used. Due to the fact that not all proteins are exosome specific, different markers have to be chosen to characterize exosomes specifically and to show their placental origin.

Placental alkaline phosphatase (PLAP):

Exosomes derived from the placenta are characterized by PLAP (placental alkaline phosphatase) since PLAP is expressed by syncytiotrophoblasts. The 55.51 kDa surface glycoprotein belongs to the human alkaline phosphatases protein family. The protein possesses two glycosylation sites, which explains why the protein is detected at 70 kDa in the Western blot (55). Human PLAP is polymorphic and three main isoforms and more than 15 rare forms have been published. These isoforms do not correlate either with fetal sex or with placenta weight (56).

PLAP protein was detected on the membrane of exosomes (40). It has been shown that PLAP is associated with fetal development in the third trimester of pregnancy (57) as it acts as an F_c antibody fragment. The fragment can capture IgG antibodies, supporting the fetal uptake of maternal IgG during the late phase of gestation (56).

CD63:

The lysosomal membrane glycoprotein CD63 belongs to the family of tetraspanins and consists of four transmembrane domains (58). The molecular weight of CD63 is 25 kDa (59). It has three N-linked

glycosylation sites, so that the molecular weight depends on its glycosylation pattern. CD63 is encoded by a single copy gene (58).

CD63 serves as one exosome unspecific marker located in the membrane of exosomes generated via the lysosomal pathway (15). However, as CD63 is located in the cell membrane, it can also be found in microvesicles and thus described as a general marker for extracellular vesicles (60).

Tumor susceptibility gene 101 (TSG101):

Tumor susceptibility gene 101 (TSG101) is described as a specific marker protein for exosomes. TSG101 is located in different cell compartments depending on the cell cycle phase. The protein is either located in cell nucleus or in the cytoplasm but was also detected in the periplasmic membrane. TSG101 participates on cell growth, proliferation, gene expression and endosomal trafficking (61). The protein is part of the ESCRT-I complex (38). TSG101 shows a domain which is characteristic for ubiquitin conjugases, but TSG101 lacks a site which is necessary as an agent for ubiquitination (61). Additionally, the protein shows a proline-rich domain and a proline-serine-alanine-proline motif. These motifs assure a sequential transfer of the ubiquitinated cargo selected for the sorting to the exosomes along the ESCRT (38). TSG101 has been also detected in released exosomes (21), and therefore TSG101 serves as an exosomal marker protein.

Beside full-length protein of 43 kDa a second isoform of 31 kDa has been dscribed (62).

Syntenin:

The protein encoded by SDCBP gene was initially identified as molecule linking syndecan –mediated signalling to the cytoskeleton and now used as an exosome specific marker protein as it is enriched in exosomes. The protein consists of 298 amino acids (32 kDa) (63) and contains two PDZ modules (63) which are abundant interaction modules recognizing short amino acid motifs at the C-termini of target proteins (64). Syntenin forces the budding of exosomes into the MVB. The budding process depends on the interaction of Syntenin with intercellular ALIX (65). Furthermore, syntenin interacts with the tetraspanin CD63 and is also located in the tetraspanin-enriched microdomains in the membrane of exosomes (66).

2. Hypothesis

The human placenta releases multiple extracellular vesicles, mainly derived from the syncytiotrophoblast into the maternal circulation. The role of nanovesicles also named exosomes has been attracted increasing interest due to their role in intercellular communication. More recently exosomes have been shown to carry molecules involved in placental physiology. If placental exosomes have an impact on maternal physiology or even if placental exosomes are released to the fetal circulation is still unclear.

Aim of this thesis is to establish a protocol for exosome isolation of maternal and fetal perfusates which were collected during an *ex-vivo* dual human placenta perfusion experiment. The objectives of this work are (i) to validate the method by characterisation of the exosomes with comprehensive methods and (ii) to compare the isolated maternal and fetal exosomes from different perfusates.

3. Materials and Methods

3.1. *Ex-vivo* dual human placenta perfusion

The *ex-vivo* dual perfusion of the human term placenta presents a technique to study transplacental transfer, metabolism and storage of substances, acute toxicity and the potential role of transporters in the placenta (5). With this technique also exosomes on the maternal and fetal side of the placenta and a transport of exosomes across the placenta can be studied.

For the *ex-vivo* perfusion of the tissue of the human term placenta was collected directly after a vaginal birth or a cesarean section. Placentae from uncomplicated term pregnancies were used. A first visual control ensured that the cotyledon, which should be perfused, was macroscopically not damaged on the maternal side. Small ruptures in the tissue may lead to leakage either on the maternal side of the tissue or in the corresponding fetal blood vessels of the placenta. Preferentially, a cotyledon in the marginal zone of the placenta was chosen. A fetal vein and a corresponding fetal artery were cannulated with polyethene cannulas (artery: fine bore polyethene tubing, Portex, 800/100/300, 1.02 mm ID; vein: fine bore polyethylene tubing, Portex, 800/100/500, 2 mm ID, Thermo Scientific Inc., Waltham, MA, USA). The cannulas were fixed with surgical suture material (PremiCron, Braun, Melsungen, Germany). Vessel branches had to be closed by knots with surgical suture material to exclude perfusion of a wrong cotyledon. The cannulated cotyledon was rinsed by using a syringe (Injekt, Braun, Melsungen, Germany) with 20 ml 37°C prewarmed perfusion media. The composition of perfusion media and Earl's buffer is shown in Table 2 and Table 3.

Table 2: Composition of perfusion media DMEM: Dulbecco's Modified Eagle Medium; BSA: bovine serum albumin. The used Earl's buffer was prepared in the laboratory. The BSA was added to the perfusion media short time before usage.

Amount per liter	Compound	Purtity	Company
666 mL	DMEM		Gibco, Life Technologies
333 mL	Earl's Buffer	composition displayed in Table 3	
1,33 g	D-Glucose	For microbiology	Merck KGaA
250 mg	Amoxicilin	≥900 µg/mg	Sigma Aldrich
10 g	Dextran FP 40	research grade	Serva Electrophoresis GmbH
5 g	BSA	≥ 96 %,	Sigma Aldrich

Table 3: Composition of Earl's Buffer used for perfusion media

Concentration (g/L)	Compound	Purtity	Company
6.8	NaCl	p.a.	Merck KGaA
0.4	KCl	p.a.	Merck KGaA
0.14	NaH_2PO_4	p.a.	Merck KGaA
0.2	$MgSO_4.7\ H_2O$	p.a.	Merck KGaA
0.2	$CaCl_2$	p.a.	Merck KGaA
2.2	$NaHCO_3$	p.a.	Merck KGaA
Fill up with 1 liter MilliQ water			

The placenta was fixed in a so called tissue holder in a way that the perfused cotyledon was not squeezed or damaged. The remaining placenta tissue was removed. Thereafter, the tissue holder was embedded into the perfusion chamber, with the maternal side up. The perfusion chamber was warmed at 37 °C, so that the placenta was able to be kept at physiological conditions.

The tubes of the fetal circuit was connected to an infusion pump (Argus 707v®, Codan, Salzburg, Austria) with an adjusted flow rate of 4 ml/min which was checked regularly during perfusion. The maximum tolerated loss of fetal perfusate volume was 0.2 ml/min. During perfusion experiment the back pressure of the arterial fetal vessels was measured in time by a catheter (Micro-Cath™ diagnostic pressure catheter, Millar Inc., Houston, Texas). In order to avoid air bubbles in both circulations bubble traps were included. The fetal perfusion media was treated with 95 % N_2, 5 % CO_2 using an membrane oxygenator (LSI-OX®, Living Systems, St. Albans, VT, USA) to provide a physiological pH-value and approximate physiological O_2 saturation in the perfusate.

After the establishment of the fetal perfusion circuit also the maternal side was perfused. Therefore, three blunt stainless steel needles were positioned into the intervillous space by puncturing the decidual plate, serving as the maternal arteries. As the maternal side has no veins, a suction tube was used to collect the perfusate after leaking from the intervillous space. The maternal arterial perfusion rate was 8 ml/min which was provided by an infusion pump (Argus 707v®, Codan, Salzburg, Austria). The maternal perfusion media was preconditioned with 75 % N_2, 20 % O_2, 5 % CO_2 by using an oxygenator (LSI-OX®, Living Systems, St. Albans, VT, USA).

After 10 minutes wash out phase in open circuits, maternal and fetal circuits were closed, as depicted in Figure 5. 150 ml perfusion media for both the maternal and fetal circuit was applied for the start of the experiment.

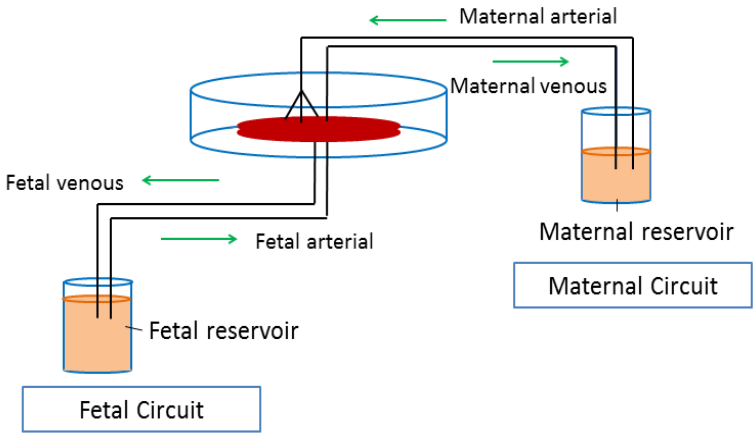

Figure 5: Schematic representation of the *ex-vivo* human placental perfusion model. Maternal and fetal circuit are closed. During the perfusion tissue viability control (level of pH, pO_2, pCO_2, lactate and glucose) was done maternal arterial and venous and fetal arterial and venous.

Perfusion with the closed circuit was done for 120 minutes. Throughout the time of the experiments the whole perfusion the tissue viability was controlled by sampling perfusates from both circulations. Levels of pH, pO_2, pCO_2, lactate and glucose was analysed by a blood gas analyser (ABL 800 Basic®, Drott Medizintechnik GmbH, Wiener Neudorf, Austria).

Data obtained from the blood gas analyser and the pressure catheter was monitored and documented by the Placental Perfusion Laboratory Data Logger Software Version 2.

After 120 minutes the perfusion media was collected and stored at 4 °C, isolation of the exosomes from the perfusates should be started within two days after collection.

3.2. Cell Culture

3.2.1. Isolation of primary human trophoblasts from term placenta

After delivery of the placenta, tissue was placed on a plate with the maternal side up. With sterile scissors and forceps membranes, cord and basal plate were removed. The placenta was cut into pieces, which were put into cold 0.9 % sodium chloride (NaCl) solution (Fresenius Kabi, Bad Homburg, Germany). The pieces were washed with NaCl for several times in order to remove all the blood, and finally dried on cheesecloth (Setpack® Toptex® lite RK, Lohmann & Rauscher GmbH & Co.KG, Rengsdorf, Germany). Thereafter, the villi were gently removed from the vessels with a scalpel (surgical blade 22, Braun, Melsungen, Germany). The removed tissue was placed in cold NaCl solution. On the top of a sterile beaker a new sterile cheesecloth was fixed with a rubber band. The scrapped tissue was moved from the sterile saline onto the cheesecloth, where it was washed with sterile NaCl solution again. After the washing step the tissue was dried for some minutes and the weight of the tissue was determined. The tissue was stored in 200 ml 1x DMEM (Dulbecco's Modified Eagle Medium, Gibco, Life Technologies, Carlsbad, CA, USA) with 1 % Streptomycin/Penicillin (Gibco, Life Technologies, Carlsbad, CA, USA) and 2.5 % 1 M HEPES (≥ 99.5 %, Carl Roth GmbH+Co.KG, Karlsruhe, Germany) over night at 4 °C.

150 mL of the digest buffer (compounds shown in Table 4) were used for the enzymatic digestion of the tissue, which was carried out for 15 minutes at 37 °C under constant stirring. After the incubation the homogenate was filtered across sterile cheesecloth into a sterile beaker, containing 50 ml fetal calf serum (FCS, Hyclone™, GE Healthcare Life Sciences, Chalfont St. Giles, UK). The tissue, which was not digested was washed with with NaCl solution and was then again incubated in 150 ml digest buffer for 30 minutes at 37 °C. After filtering the homogenate, the rest of the tissue was incubated with 150 ml digest buffer a third time for 30 minutes at 37°C. The homogenate was filtered again. In the end the cheesecloth used for filtration was pressed to collect all isolated cells.

Table 4: Compounds of the Trypsin/DNase digest buffer used for the isolation of human trophoblasts from the term placenta.

Volume	Solution	Purity	Company
50 ml	10x HBSS		Gibco, Life Technologies
12.5 ml	1 M HEPES	≥ 99.5 %	Carl Roth GmbH+Co.KG
1 ml	1 M $CaCl_2$	≥ 98 %	Merck KGaA
1 ml	0.4 M Mg_2SO_4	≥ 98 %	Merck KGaA
2.5 ml	2 M NaOH	p.a.	Merck KGaA
450 ml	Distilled water	sterile	Fresenius Kabi
2 ml	DNaseI (2000 U/mg)		Roche, Sigma Aldrich
50 ml	2.5 % (10x) Trypsin		Gibco, Life Technologies

The solution was centrifuged at 300 x g for 20 minutes at 4 °C (centrifuge 5810R and rotor A-4-81, Eppendorf AG, Hamburg, Germany), pellet was resuspended in 5 mL 1x DMEM with 1 % Streptomycin/Penicillin and 10 % FCS (DMEM++) and pooled in four 50 mL tubes which were filled up with DMEM++. This suspension was centrifuged again at 300 x g for 20 minutes at 4 °C. The pellet was again resuspended in 3 ml 1x DMEM with 1 % Streptomycin/Penicillin (DMEM+). These samples were placed on a Percoll gradient.

The Percoll gradient was prepared by mixing 20 mL 10x HBSS (Gibco, Life Technologies, Carlsbad, CA, USA), 5 ml 1M HEPES, before 80 ml Percoll (pH 8.5-9.5, Sigma Aldrich, St. Louis, MO, USA) and 95 ml sterile distilled water were added. The solution was mixed thoroughly.

Each of the four cell pellets gained by the centrifugation step before and dissolved in 3 ml DMEM+, 25 ml Percoll gradient solution were placed in a sterile ultra-centrifuge tube (Polycarbonate, 38.5 mL, Herolab GmbH Labware, Wiesloch, Germany). Samples were centrifuged at 23000 x g for 50 minutes at 4 °C. (Optima XE-90 Ultracentrifuge and Type 70 Ti rotor, Beckman Coulter Inc., Brea, CA, USA).

The trophoblast layer was transferred by a sterile syringe into a 50 ml tube filled with DMEM+. The samples were centrifuged for 15 minutes at 300 x g at 4 °C, and the pellet was resuspended in 20 mL DMEM++. The number of cells was counted with a cell counter (CASY® Cell Counter + Analyser System, Roche Innovatis AG, Bielefeld, Germany).

2 x 10^7 cells in 1.5 mL DMEM ++ with 10 % DMSO (Dimethyl sulfoxide, Serva Electrophoresis GmbH, Heidelberg, Germany) were frozen and stored in liquid nitrogen.

3.2.2. Purification of primary human trophoblasts

In order to remove all non-trophoblastic cells, which remained still after the isolation procedure, cells were further purified.

Per vial trophoblast cells which was thawed, two 15 ml round bottom tubes were filled with 500 µl Dynabeads ® Goat anti-Mouse IgG (Invitrogen, Life Technologies, Carlsbad, CA, USA). 5 ml washing

buffer containing 1x HBSS (Gibco, Life Technologies, Carlsbad, CA, USA) with 0.1 % BSA (≥ 96 %, Sigma Aldrich, St.Louis, MO, USA) and 200 mM EDTA (EDTA Ultra-Pure, 0.5 M, pH 8.0,Invitrogen, Life Technologies, Carlsbad, CA, USA) were added for washing the particles. After shaking the tubes thoroughly, the supernatant is removed by separation in the magnetic separator (Dynal®, Invitrogen, Life Technologies, Carlsbad, CA, USA). The beads are resuspended in 500 µL washing buffer. 25 µl antibody (mouse anti-human HLA ABC MCA81, BioRad laboratories Inc., CA, USA) were added. The suspension is incubated at room temperature for 60 minutes. Afterwards the beads were washed with the wash buffer three times with 2 ml washing buffer. For each frozen vial of trophoblasts 5 mL DMEM++ were warmed at 37 °C. The cells were thawed quickly at 37 °C in the water bath. Cells are put into the pre-warmed media. The suspension was centrifuged at 350 x g for 7 minutes at 4°C. The supernatant was removed, while the cells were found in the pellet, which was dissolved in 24 mL media. The cell number was counted by CASY® Cell Counter.

6 mL of cell suspension were mixed with 500 µL of the Dynabeads. The tubes were incubated at 4 °C at the shaker (Unimax 1010, Heidolph Instruments GmbH, Schwabach, Germany) for 30 minutes. The non-trophoblastic cells bound to the beads via the antibody. By magnetic separation, the supernatant, which was free from non-trophoblastic beads, can be transferred to a new 50 mL tube. The suspension was filled up to 50 mL with media. Afterwards the cells were centrifuged at 350g for 7 minutes at 4 °C. The pellet was dissolved in 10 mL media and the cell number was determined again by CASY® Cell Counter.

According to the number of cells measured by CASY® Cell Counter, cells were seeded. They were kept at 21 % O_2 at 37 °C for 67 hours with the same media, before the supernatant was collected. To study a difference in the exosome amount trophoblast cells were additionally kept in culture for only 22 hours before collection of the supernatant. From the collected supernatant placental exosomes were isolated.

3.2.3. Characterization of primary human trophoblasts

All cell preparations were subjected an immunocytochemical characterization. The antibodies Vimetin and Cytokeratin 7 were used for identification of the trophoblasts.

For this purpose isolated and purified trophoblasts were seeded to chamber slides and cultured either for 22 hours or 67 hours. After removing the supernatant, the cells were washed with 1x HBSS three times. The chamber slides were dried and afterwards fixed with 70 % acetone (p.a., Merck KGaA, Darmstadt, Germany). After drying and marking them with a hydrophobic pen (Dako Pen, Dako, Agilent Technologies, Inc., Santa Clara, CA, USA).

For the staining of the cells the Lab Vision™ UltraVision™ LP Detection System: HRP Polymer (Thermo Scientific Inc., Waltham, MA, USA) is used.

The chamber slides were washed four times with tris-borate-EDTA (TBE) buffer pH 7.3 (Apotheke LKH Univ. Klinikum Graz) with 0.1% (v/v) Tween 20 (Sigma Aldrich, St. Louis, MO, USA), short TBE-T, for 3 minutes, before being incubated with the primary antibodies for 60 minutes.

The primary antibodies were diluted with antibody diluent (Dako, Agilent Technologies Inc., Santa Clara, CA, USA). All the incubation steps were performed in a wet chamber.

Table 5: Host, clone, company and dilution of antibodies used for the characterization of human trophoblasts isolated from term placenta.

Antibody specificity	Host/clone	Company	Dilution
Negative Control Mouse IgG 1	Mouse/ DAK-GO1	Dako/ X0931	1:250
Vimetin	Mouse/V9	Dako/GA630	1:250
Cytokeratin 7	Mouse/OV-TL12/30	Dako/M7018	1:750

Afterwards the slides were washed again with 1x TBE-T four times for 3 minutes. Primary antibody enhancer is placed on the slides for 10 minutes before the slides were washed four times with 1x TBE-T again. Then the slides were incubated with the HRP polymer followed by an additional washing process, four times with 1x TBE-T for 3 minutes. Afterwards the large volume AEC chromogen single solution (Thermo Scientific Inc., Waltham, MA, USA) is applied to the chamber slides. After a short washing step with distilled water, the chamber slides are incubated for 5 minutes with hemalaun (Gatt-Koller GmbH, Absam, Austria) in order to stain the nuclei of the cells. The slides are blued with tap water for 5 minutes. They are mounted with mounting media (Aquatex®, Merck KGaA, Darmstadt, Germany).

Isolation and purification of trophoblasts was accepted when the isotype control and Vimentin showed no staining. Cells stained with Cytokeratin 7 showed strong staining.

3.3. Exosome Isolation

3.3.1. Isolation of exosomes by a three step differential centrifugation procedure

Isolation of the placental exosomes from maternal and fetal perfusates collected after ex-vivo placenta perfusion experiments by differential centrifugation was followed by the protocol of Gupta et. Al. (67).

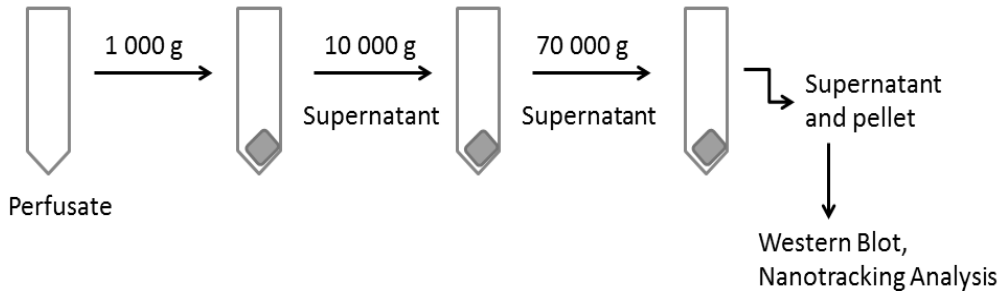

Figure 6: Isolation of exosomes following the protocol of Gupta et. Al. (67). The protocol is based on a three-step centrifugation technique. First, the perfusate was centrifuged for 10 minutes at 1000xg. The supernatant was then centrifuged at 10000xg for 10 minutes in the ultracentrifuge. Finally the so received supernatant was centrifuged again at 70000 x g for 90 minutes. Supernatant and pellet were kept for further characterization by Western Blot and Nanotracking Analysis.

The protocol is based on three-step centrifugation procedure (Figure 6). All centrifugation and pipetting steps were done at 4 °C to make sure that the desired vesicles are not damaged. 50ml of maternal or fetal perfusates were centrifuged using an Allegra® X-12R benchtop centrifuge (Beckman Coulter Inc., Brea, CA, USA) at 1000 x g for 10 minutes. The pellet containing all the cells and cellular debris was discarded. Ultracentrifuge tubes (Quick-Seal™ Centrifuge Tubes, 16x76 mm, Beckman Coulter Inc., Brea, CA, USA) were filled with the supernatant. The samples were again centrifuged in an Optima XE-90 ultracentrifuge (Beckman Coulter Inc., Brea, CA, USA) at 10 000xg for 10 minutes with a Type 70 Ti rotor (Beckman Coulter Inc., Brea, CA, USA). The supernatant was transferred into a new ultracentrifuge tube and filled up with 1 x PBS (phosphate-buffered saline, Medicago AB, Sweden) before being centrifuged in the ultracentrifuge at 70 000 x g for 90 minutes.

An aliquot of the supernatant was kept for further analysis. The pellet was dissolved in 1 ml 1x PBS. Samples were stored at 4 °C until Nanotracking analysis and immunoblotting analysis.

3.3.2. Isolating exosomes by differential centrifugation and density gradient:

The golden standard for isolation of the exosomal populations can be achieved by differential centrifugation combined with isopyknic centrifugation.

For this reason an unpublished protocol of Yoel Sadovsky, which he kindly provided to us, was used. A graphical scheme is depicted in Figure 7.

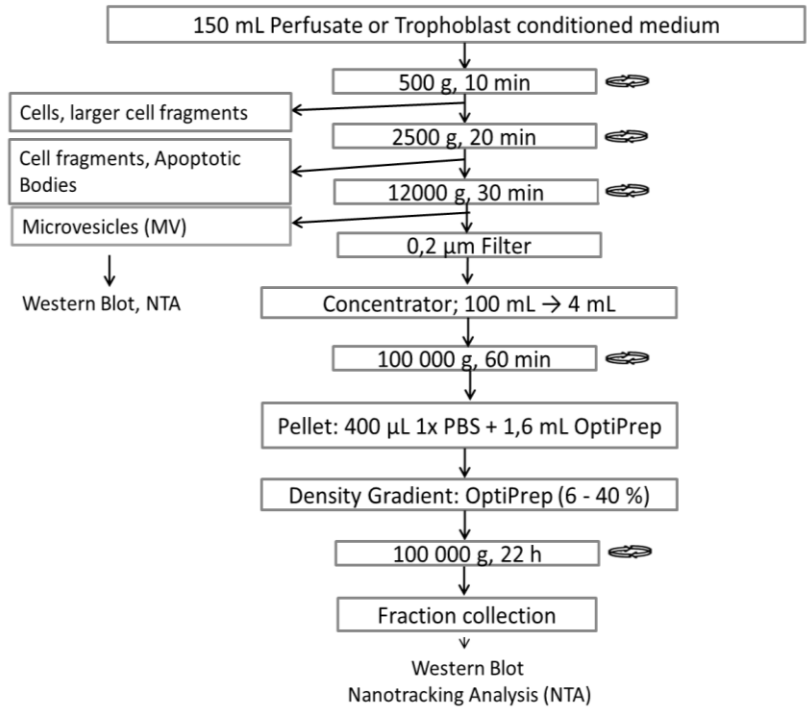

Figure 7: Schematic representation of the exosome isolation method combined of differential and isopyknic centrifugation. During the differential centrifugation cells, cell fragments and apoptotic bodies were separated. Also the microvesicle fraction can be isolated by 12 000xg centrifugation. The isopkynic isolation step was done with a 6-40 % Optiprep density gradient with 22 hours centrifugation at 100 000xg. Fractions were analysed by Western Blot and Nanotracking Analysis.

All the different centrifugation and pipetting steps were done at 4 °C. In order to isolate exosomes either maternal or fetal perfusates which were collected after ex-vivo placenta perfusion of a cotyledon or term trophoblast conditioned media was used.

Differential centrifugation:

For the isolation of exosomes an initial volume of 150 ml perfusate/media was required. The samples were centrifuged in the Allegra® X-12R benchtop centrifuge (Beckman Coulter Inc., Brea, CA, USA) at 500 x g for 10 minutes to pellet the cells and the cell debris. The supernatant was transferred while the pellet was discarded. The transfer of the whole supernatant has to be done carefully so that a carry-over of the cell pellet is avoided. Another centrifugation step was carried out at 2 500 x g for 20 minutes in the benchtop centrifuge. The pellet contains the smaller cell fragments and the apoptotic bodies which were discarded. 132 ml supernatant were transferred into ultracentrifuge tubes (Ultra-ClearTM Centrifuge Tubes, 38.5 mL, Beckman Coulter Inc., Brea, CA, USA) and centrifuged in the Optima XE-90 ultracentrifuge at 12 000xg for 30 minutes with a Type 70 Ti rotor. The pellet after this centrifugation step contained the microvesicle population. It was resuspended in 1.5 ml PBS and was stored at 4 °C until further analysis. From this step on an aliquot was taken after each step for further analysis and characterization of the intermediate fractions.

The supernatant was filtered through a 0.2 µm filter (Nalgene® Syringe Filter, 0.2 µm, Thermo Scientific Inc., Waltham, MA, USA) in order to remove any particles in the sample larger than 200 nm. To concentrate the exosomes containing fraction by reducing the volume a concentrator with a cut-off of 100 kDa (Vivacell® 100 Centrifugal Concentrator, PES membrane, 100 000 MWCO; Sartorius Stedim Biotech, Goettingen, Germany) was used. The samples were centrifuged in the benchtop centrifuge at 1 800 x g until the samples were concentrated to 4 ml. As the exosomes are larger than 100 kDa they are not passing the membrane. The supernatant was pipetted to an ultracentrifuge tube (Quick-Seal™ Centrifuge Tubes, 16x76 mm, Beckman Coulter Inc., Brea, CA, USA) and it was diluted with 9 ml 1x PBS. Centrifugation was done for 60 minutes at 100 000 x g in the ultracentrifuge with the Type 70Ti rotor to pellet the exosomal population. The supernatant was removed and the pellet containing the exosomes was dissolved in 400 µl 1x PBS.

Isopyknic centrifugation

The further exosome isolation was done by density gradient centrifugation on a 6-40 % Optiprep (60 % Iodixanol (w/v) in water, Sigma Aldrich, St. Louis, MO, USA) step gradient. The gradient fractions where prepared with 4.4% (w/v) mannitol (≥ 98 %, Sigma Aldrich, St. Louis, MO, USA), 1 mM EDTA (Ethylenediaminetetraacetic acid, 99.0-101.0 %, Sigma Aldrich, St. Louis, MO, USA), 10 mM Tris (Tris(hydroxymethyl)nitromethane, p.a., Merck, Darmstadt, Germany), pH 7.4

The sample was filled up with 60 % Optiprep™ solution to a volume of 2 ml and pipetted into the bottom of an ultracentrifuge tube (Ultra-Clear™ Centrifuge Tubes, 38.5 ml). The sample was overlayed by a solution of 6-40 % Optiprep™ gradient starting with the highest density coming to the lower ones at the top. 1.5 ml of each gradient step (one step per 2 % change) was pipetted to the tube with a syringe. The gradient was centrifuged in the ultracentrifuge for 22 hours at 100 000 x g. During the centrifugation a continuous density gradient is formed, so that the exosomes can float upward to the position corresponding to their theoretical density (1.13 – 1.19 g/ml) (13).

After centrifugation in 1.5 ml steps fractions were removed from the top to the bottom of the gradient. As the protein concentration in the fractions collected from the gradient was too low for further analysis another concentration step had to be done. Therefor a concentrator with a cut-off of 100 kDa (VivaSpin6, PES membrane, 100 000 MWCO, Sartorius Stedim Biotech, Goettingen, Germany) was used. The 1.5 mL sample were diluted with the 3 fold 1x PBS, and centrifuged at the benchtop centrifuge at 3 000xg for 8 minutes. The sample was filled up with 1 x PBS to 6 mL and was again centrifuged at 3 000 x g for approximately 5 minutes until 100 µL of each fraction sample were left.

The samples were stored at 4 °C until Western Blot and Nanotracking analysis.

3.4. Western Blot

The intermediate fractions and the gradient fractions collected during the isolation of exosomes were directly analysed by Western blot. In order to quantify total protein content of each sample without any further treatment Pierce® BCA Protein assay (Thermo Scientific Inc., Waltham, MA, USA) was conducted. The samples were mixed with the same amount of Sample Buffer (Laemmli 2x concentrate, Sigma Aldrich, St. Louis, MO, USA). Afterwards the proteins were denaturated by incubating them at 95 °C for 5 minutes.

10 µg/well of total protein per sample were separated on precast 10% SDS gels (Mini-Protean® TGX™ gels, BioRad laboratories Inc., CA, USA) at 120 V for 60 minutes. Proteins were electrotransferred with the Trans-Blot® Turbo™ Transfer System (BioRad laboratories Inc., CA, USA) at 25 V for 7 minutes onto nitrocellulose membranes (Trans-Blot® Turbo Transfer Pack, BioRad laboratories Inc., CA, USA). Membranes where stained with Ponceau S (Sigma Aldrich, St. Louis, MO,USA) to ensure proper protein separation and correct blotting. The membranes were blocked with 5% (w/v) non-fat dry milk powder (BioRad laboratories Inc., CA, USA) in 1x TBE-T, for 1 hour at room temperature. The blocking solution was also used as an antibody diluent. The used antibodies and there origin as well as the used dilutions are shown in Table 6. Membranes were incubated with the corresponding primary antibody on a shaker overnight at 4 °C. Afterwards they were washed for 1 hour at room temperature with TBE-T changing the buffer every 10 minutes. Membranes were then incubated with host-specific horseradish peroxidase (HRP) conjugated secondary antibody (goat anti-rabbit IgG and goat anti-mouse IgG, BioRad laboratories Inc., CA, USA) for 1 hour at room temperature, followed by 1 hour of washing with TBE-T changing the buffer every 10 minutes. Protein bands were visualized using an chemiluminescent substrate for detection of HRP (Super Signal® West Pico Chemiluminescent Substrate or Super Signal® West Femto Maximum Sensitivity Substrate, ThermoScientific, Waltham, MA, USA) and imaged on the ChemiDoc XRS system (BioRad laboratories Inc., CA, USA; Software: Quantity One – 4.6.9)

Table 6: Host, clone, company, dilution and dilution media of antibodies used for Western blotting. NFDM: Non-fat dry milk

Antibody specificity	Host/clone	Company	Dilution	Dilution medium
Placental alkaline phosphatase (PLAP)	Rabbit/EPR6141	Abcam/ab133602	1:500	5 % NFDM
CD63	Mouse/MEM-259	Abcam/ab8219	1:500	5 % NFDM
Syntenin	Rabbit polyclonal	Abcam/ ab19903	1:750	5 % NFDM
TSG101	Mouse/4A10	Abcam/ ab83	1:1000	5 % NFDM

3.5. Nanotracking Analysis (NTA)

General method description

Nanoparticle tracking analysis (NTA) is a method for visualizing and analysing nanoparticles such as exosomes and microvesicles in liquids. NTA uses Brownian motions of particles, which is directly associated with the size of these distinct particles. The particles are visualised in liquids by light scattering after the laser beam has illuminated them (25; 68).

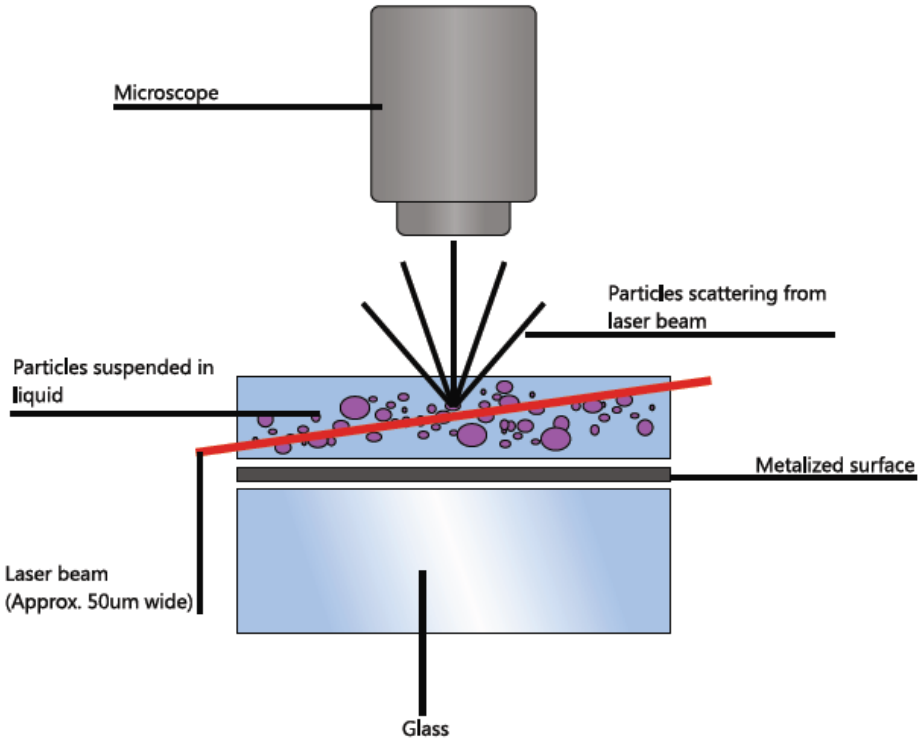

Figure 8: NanoSight Setup. The laser beam is refracted at a low angle before entering the sample. The collimated light beam is scattered by hitting particles which are suspended in the liquid. For the detection a conventional microscope and a camera fixed normally to the laser beam are used. (Taken from Malvern UK **(68)**).

The system uses basically a laser beam which is focused with a glass prism. Once the laser beam enters the sample, a low angle refraction of the focused beam takes place as it can be seen in Figure 8.

A thin beam of laser light is generated which is illuminating the particles in the sample. For the visualization and analysation of the particles a conventional microscope is used. The microscope is coupled to a video camera and is set in a right angle to the laser beam. The particles are illuminated for 60 to 90 seconds with 25 frames per second. To analyse the video Nanoparticle Tracking Analysis (NTA) software was used (25).

The software is able to identify and analyse the movement of the particle. For the calculation of the size of the particles via the velocity of particle movement the two-dimensional Stokes-Einstein equation is applied:

$$< x, y >^2 = \frac{K_B T}{3 \pi \eta d_h}$$

$< x, y >^2$ mean square displacement, K_B Boltzmann's constant, T temperature of the solvent in Kelvin, η viscosity and d_h hydraulic diameter of the particle (69; 70).

Nanoparticle tracking analysis of exosomes

For size distribution measurements of the different maternal and fetal fractions and gradient steps gained during the isolation procedure size distribution profiles were obtained using the NanoSight LM10 instrument with a 488 nm laser (Malvern UK), camera C11440 (Hamatsu Photonics K.K., Hamatsu, Japan) and Nanoparticle Tracking Analysis (NTA) software Version 2.3, Build 0025 (Malvern UK).

The samples were diluted with 1x PBS to be able to detect between 10 and 100 particles per image. The PBS was filtered across a sterile 0.02 µm filter (Whatman, GE Healthcare Life Sciences,). Before applying the samples to the measuring chamber the samples were strongly mixed with a syringe.

The settings for measurements are displayed in Table 7. The settings were kept constant between all measurements in order to compare the experiments.

Table 7: NanoSight set up: The settings for size distribution measurements of the different samples are shown in this Table.

Viscosity	0.92 cP (Centipoise; 1 cP = 10^{-3} Ns/m^2)
Temperature	23.5 °C
Camera Level	11
Polydispersity	Medium
Capture duration	90 seconds
Frames per second	25
Frames processed	2500

Per sample three videos were captured and then analysed. Each video was analysed to get information about the size distribution, the concentration of particles per frame and a calculation of the number of particles in the sample.

4. Results

4.1. Isolation of exosomes by a three step differential centrifugation protocol

Placental exosomes isolated from maternal and fetal perfusate, obtained during an *ex-vivo* human placental perfusion experiment and isolated by stepwise differential centrifugation (1 000 x g, 10 000 x g and 70 000 x g), were analysed by Western blot with specific marker proteins, CD63, PLAP, Syntenin or TSG101 (Figure 9).

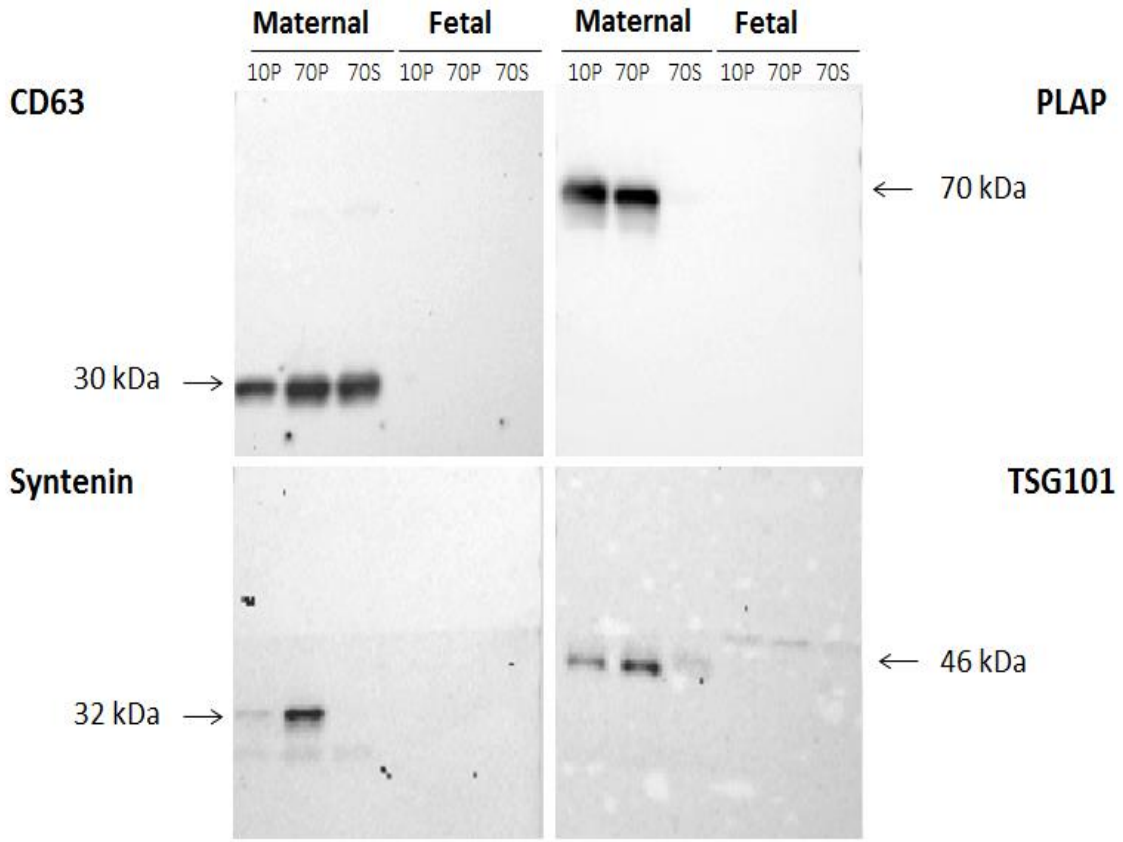

Figure 9: Exosomes characterized by specific marker protein expression. Placental exosomes were sequentially isolated by 1 000 x g, 10 000 x g and 70 000 x g centrifugation steps, respectively. The pellets after 10 000 x g (10P), 70 000 x g centrifugation (70P), and corresponding supernatants (70S) were analysed from the maternal and fetal perfusates. Protein load was [10 µg/well].

The extracellular cellular vesicle specific marker protein, CD 63, was expressed in all maternal fractions. It is described that the pellet obtained after the 10 000 x g centrifugation (10P) represents the microvesicle fraction, while the pellet after the 70 000 x g centrifugation should have included exosomes (71). Both, maternally derived pellet fractions (10P, 70P) and the corresponding supernatant (70S) were CD63 positive.

In order to check the placental origin of the isolated exosomes and microvesicles the syncytiotrophoblast specific protein PLAP was used for immunoblots. PLAP positive were the maternal microvesicle and exosome fraction (10P and 70P). The 70S fraction lacks of PLAP indicating these supernatants were free from placental proteins. The Exosome specific markers, Syntenin and TSG101, which both are proteins biogenesis of exoxomes, were detectable in pellet fraction of maternal perfusates (70P). As the supernatant showed no signal, it is assumed that the exosomes are really pelleted during the 70 000 x g centrifugation step, which is crucial for getting a maximum yield.

In contrast, fetal fractions, collected in parallel with the maternal perfusates, showed no specific signals suggesting that these samples did not contain any placental or vesicle specific proteins. Fetal samples measured by NTA showed no signal and in line with the immunoblots; these results together reassured the lack of any placental vesicles in fetal perfusates. The fractions obtained from isolation out of maternal perfusates were measured with NTA (Figure 10).

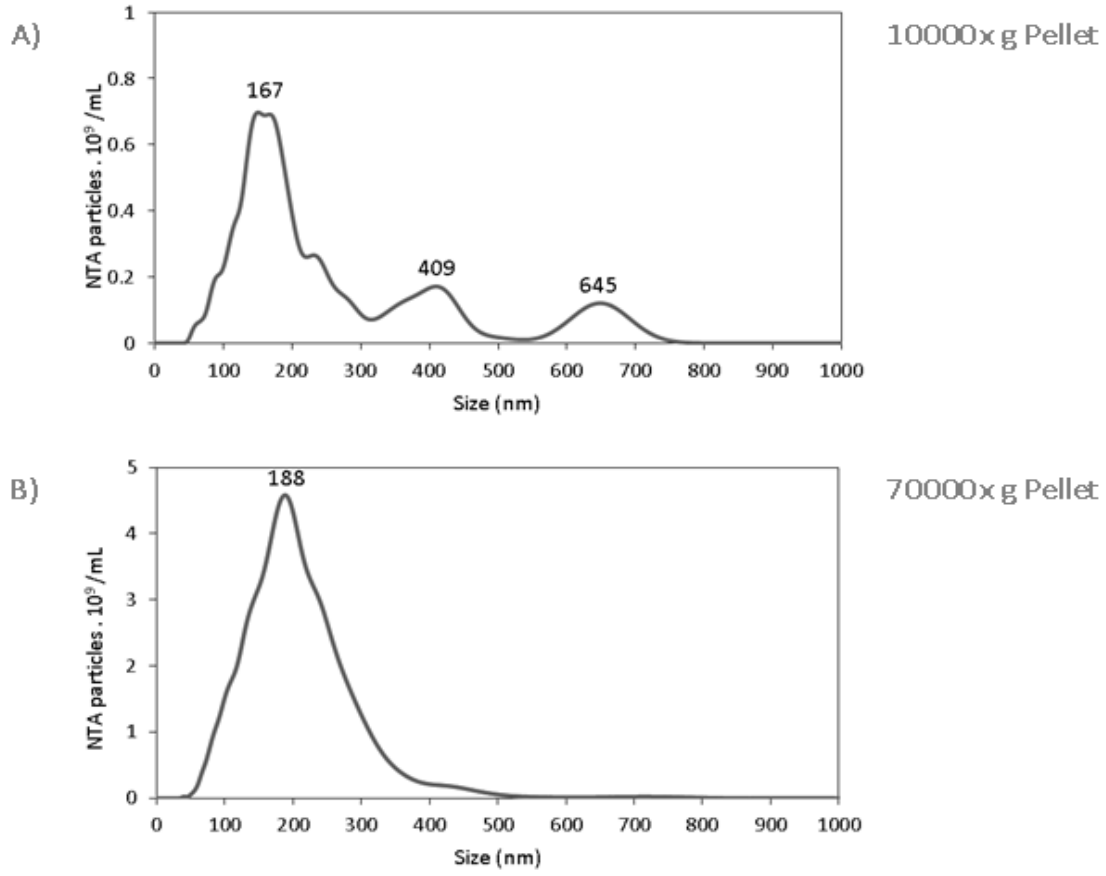

Figure 10: Size distribution of isolated microvesicle and exosome fractions measured by Nanoparticle tracking analysis (NTA). A three step differential centrifugation protocol with 1 000 x g, 10 000 x g and 70 000 x g centrifugation was done, respectively from maternal perfusates. A) Microvesicles (10P) after centrifugation showed a maximum size of 167 nm. B) The NTA analyses showed a single band with a maximum size of 188 nm for maternally derived exosomes.

The pellet after the 10 000 x g centrifugation, which represents the microvesicles showed a disperse size contribution profile. The main peak was detected at 167 nm, followed by two additional peaks at 409 nm and 645 nm. The total mean value out of three measurements was 264 ± 150 nm. The placental microvesicles derived from the plasma membrane of the syncytiotrophoblats are heterogenous in size and shape (21), which was confirmed by NTA measurements.

Analysis of the 70P pellet, which represents the exosomal fraction, showed a single peak with the maximum at 188 nm. The mean size out of three measurements per sample was 208 ± 86 nm. The mean size of the exosomes was smaller than the mean size of the microvesicle fraction (264 ± 150 nm), but still above the normal size of exosomes (<100nm), according to the literature (26).

In summary, the results showed, that the three step differential centrifugation protocol to isolate exosomes is not specific enough in order to get accurate exosomes for further experiments. The results are displayed in Table 8.

Table 8: Yield of microvesicles and exosomes received after isolation out of maternal and fetal perfusate. Percentage of yield for microvesicles was calculated as the relation between the protein concentration of the pellet after the 10 000 x g centrifugation step and total perfusate protein content , or of the 70 000 x g centrifugation fraction for the yield of exosomes.

Isolation	Microvesicle fraction (%)	Exosome fraction (%)
Maternal Perfusate, closed circuit, t = 90 min	0.35	0.18

Out of 1,994 µg/µl total maternal perfusate protein only 0.35 % microvesicles and 0.18 % exosomes could be isolated. In general the microvesicle yield was about 2-fold higher than the exosome fraction

4.2. Storage and its impact on the isolated microvesicel fractions

The impact of freezing, storage and subsequent processing of the 10P, 70P, as well as 70S fractions was studied. The directly after the centrifugation obtained fractions were compared to the same fractions but stored for seven days under -20°C. Results of the Western Blot are shown in Figure 11 and the NTA results in Figure 12.

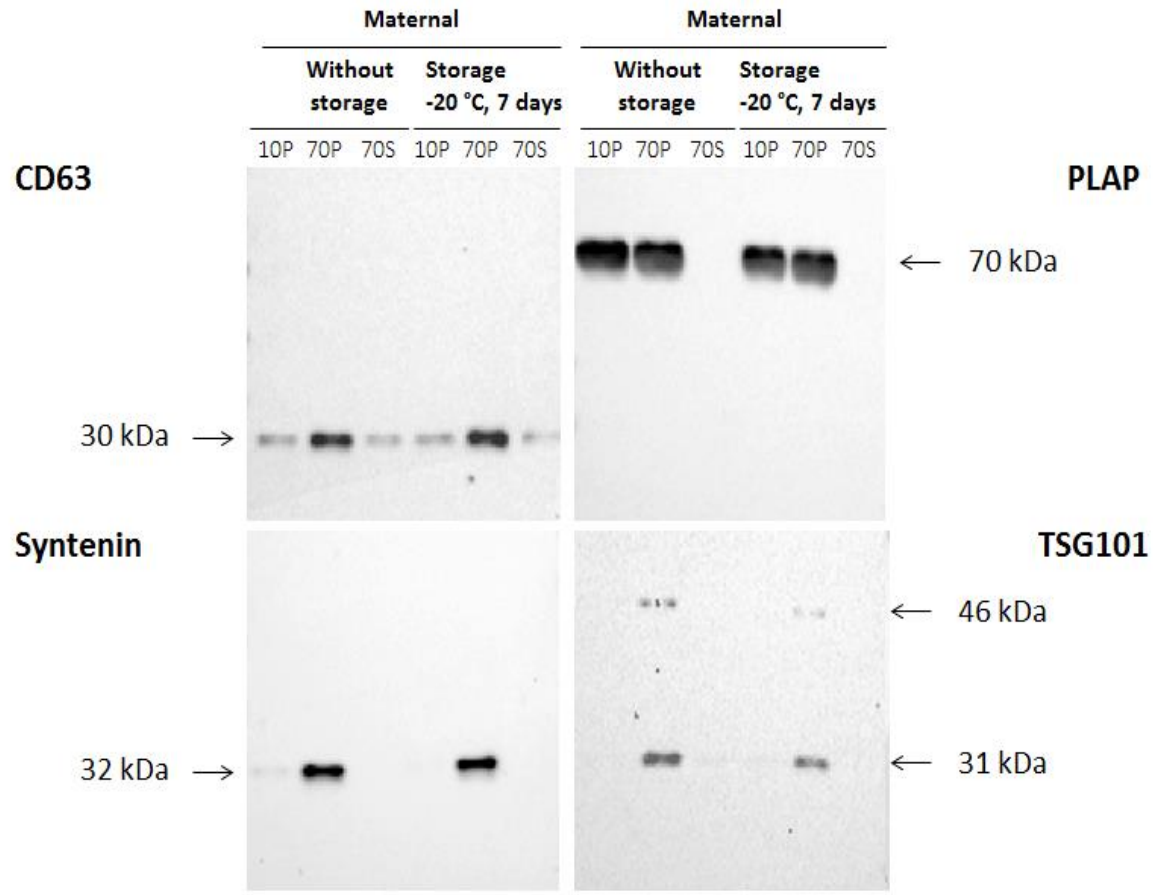

Figure 11: The influence of particle storage after isolation was studied by comparing marker protein expressions (of placental microvesicles and exosomes. Integrity of the obtained pellet (10P, 70P) and supernatant fraction (70S) were analysed right after the centrifugation steps of the perfusates or after 7 days freezing and thawing procedure of the same fractions. Protein load = 10 µg/well.

Independently, whether the samples were frozen at -20 °C for 7 days or directly used after centrifugation, the protein pattern of all fractions were very similar. There was no difference in the density of the specific proteins PLAP, TSG101 or Syntenin of all Westerns detectable. Additionally, TSG101 showed both isoforms at 31 kDa and 46 kDa, which has already been described (62). Interestingly, Syntenin could also be detected in the microvesicle fraction (10P), but only in the

protein fraction directly used for immunoblot. While no freezing or storage differences of the specific placental and exosome proteins could be observed, the NTA measurement showed significant differences (Figure 12).

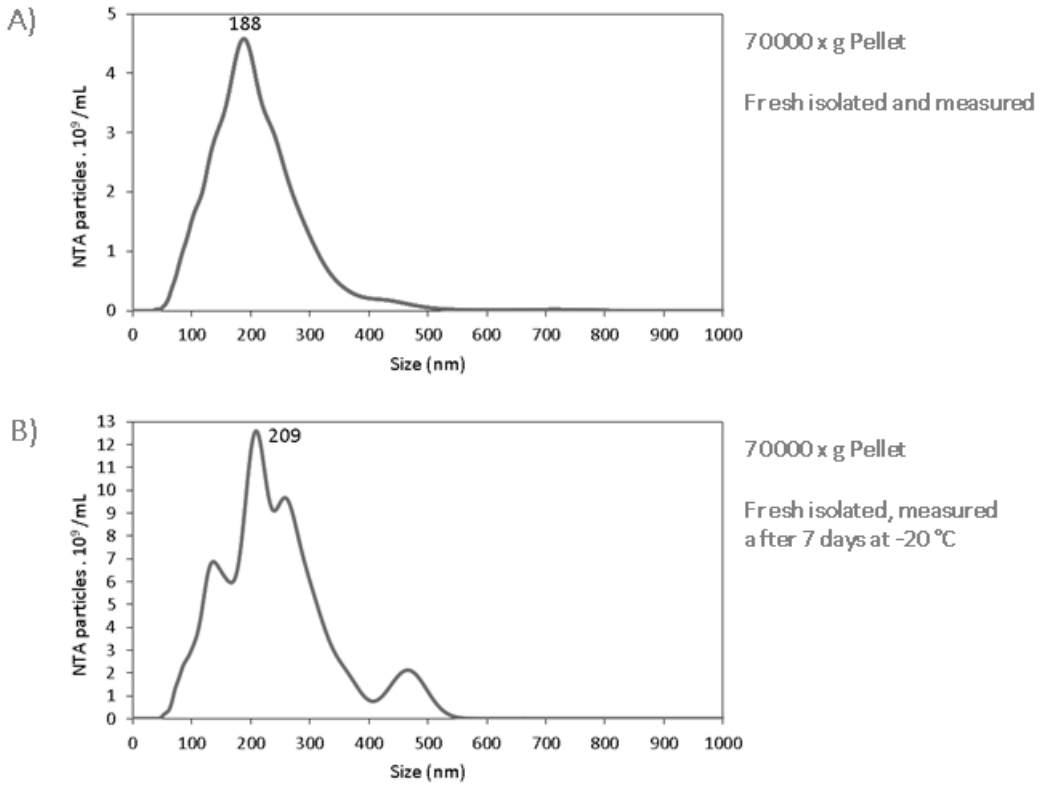

Figure 12: Size distribution plots of isolated placental derived exosomes isolated from maternal perfusates measured by Nanoparticle tracking analysis (NTA). A) Exosomes were isolated directly after placenta perfusion. Samples were measured by NTA without being frozen. The maximum size peak was detected at 188 nm. B) Exosomes were isolated directly after placenta perfusion. Samples were stored at -20 °C for 7 days before analysation by NTA. The maximum size peak was detected at 209 nm.

The fresh isolated and directly afterwards measured pellet collected after the 70 000 x g centrifugation (70P) showed a single peak at a maximum size of 188 nm, which is only roughly in accordance with the literature (40-120µm). The same pellet (70P) representing exosomes, which was stored at -20 °C for 7 days in the freezer showed a more disperse size distribution. The main peak shifted from 188 nm to 209 nm. In addition, peaks at 138 nm, 258 nm and a dispersal peak between 400 and 500 nm were detected. The higher size peaks can be explained by aggregation of exosomes, when the isolated fractions were stored over a longer period of time at -20 °C. It has to be mentioned, that the number of particles per millilitre, recognized by NTA after the storage, was in general bigger than the number of particles per millilitre observed in the fresh analysed sample. It is possible that this was also influenced by the storage process. Storage at -20 °C for 7 days should be avoided for NTA analysis as it is influencing the size distribution. In conclusion, in order to use the exosomal fractions for further investigations storage at -20 °C should be avoided.

4.3. Exosome isolation by differential centrifugation and density gradient

One major objective of my master thesis was to compare different isolation methods for placental exosomes since for further experiments only well characterized and pure particles are valuable to use. An isopyknic centrifugation process with a density gradient after the differential centrifugation isolation was established to purify the exosomal fraction.

4.3.1. Investigation of the differential centrifugation process

Primarily the obtained fractions after differential centrifugation steps according to the protocol were investigated by Western blotting and NTA (Figure 13).

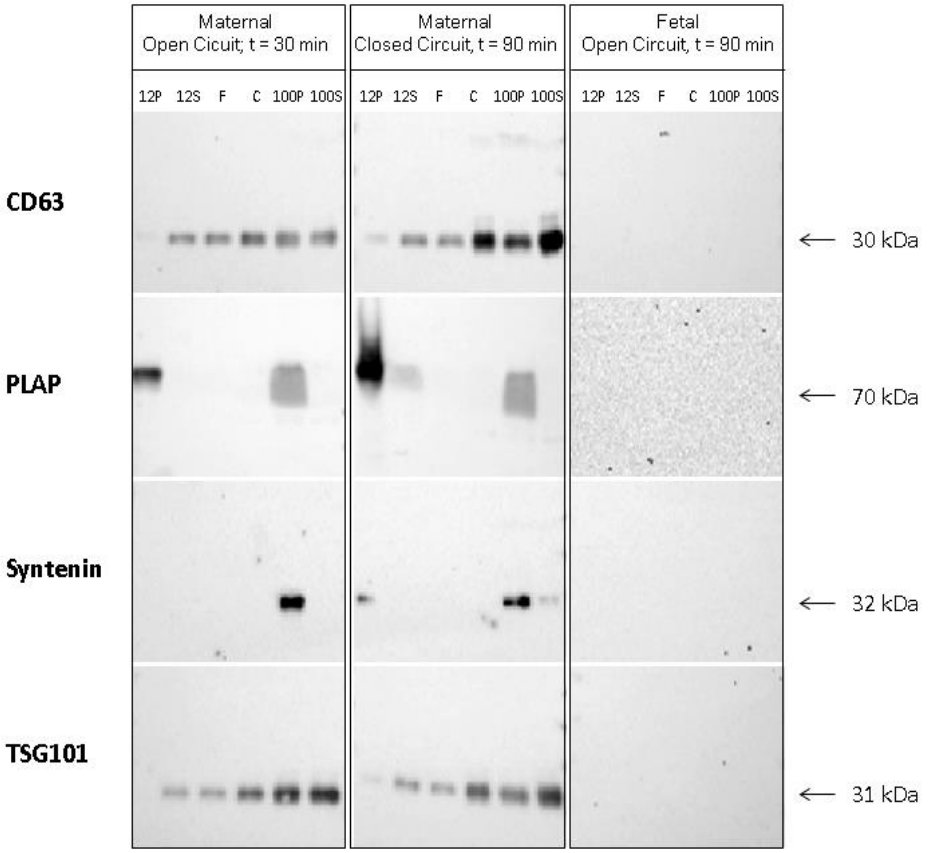

Figure 13: Exosomal marker protein expression (CD63, PLAP, Syntenin and TSG101) of the different fractions received during the differential centrifugation part from maternal and fetal perfusate. The perfusate is gained by ex-vivo human placenta perfusion. The exosome isolation is done by 500 x g centrifugation, followed by 2500 x g centrifugation and 12 000 x g centrifugation. The supernatant is filtered through a 0.2 µm filter and concentrated. A 100000 x g centrifugation step was done to pellet the exosomes. The pellet fraction after the 12 000 x g centrifugation (12P) containing the microvesicles, as well as the corresponding supernatant (12S) was analysed. Also the sample after filtration (F) and concentration (C) was investigated as well as the 10 0000 x g pellet, containing the exosomal fraction, and the corresponding supernatant (100S). The isolation was done with maternal perfusate with open circuit for 30 minutes, maternal perfusate with closed circuit for 90 minutes and fetal perfusate with open circuit for 90 minutes. Protein load = 10 µg/well.

Fetal and maternal perfusate were used for the isolation. Additionally, we also examined if there is a difference in exosome enrichment when using perfusates for isolation from open maternal circuit compared to closed circuit. All three isolations (maternal open, maternal closed and fetal) were done in parallel and perfusates derived from the same placenta perfusion.

PLAP, Syntenin1 and TSG101 showed the same results, independent the opened or closed circuit or the perfusion time. CD63 showed a clear enrichment in the closed circuit. CD63, a marker specific for extracellular vesicles showed bands for all fractions. The microvesicle fraction (12P) showed slight bands. The band after 90 minutes perfusion in a closed circuit was stronger. Additionally there were still extracellular vesicles contained in the supernatant, and they were maintained during filtration and concentration. After the concentration the bands were stronger even so the same amount of protein was loaded onto the Western blot. During the concentration with a cut off of 100 kDa the BSA contained in the perfusion media with a molecular weight of 66,43 kDa (72) was shed off, which had an impact onto the protein concentration. CD63 also showed bands for the pellet and the supernatant after the 10 000 x g centrifugation step.

The syncytiotrophoblast specific marker PLAP, showing the placental origin of the extracellular vesicles, was positive for the microvesicle fraction (12P) and the exosome fraction (100P). This suggests that the vesicles were concentrated in the pellet fractions. Perfusing the maternal side in a closed circuit for 90 minutes showed a slight band in the supernatant obtained after the 12 000 x g centrifugation. After this step the exosomes are still included in the supernatant. Filtrate and concentrate showed no bands as an effect of too high dilution.

Also the exosome specific marker proteins Syntenin and TSG101 revealed the same bands, suggesting that yield of microvesicles and exosomes are independent of the perfusion setup on the maternal side. As shown in Figure 13 fetal perfusates contained no detectable microvesicles or exosomes. In contrast, to the maternal fractions neither placenta specific proteins nor any exosomal proteins were detectable in factions of the fetal side. Samples were also analysed additionally by NTA (Figure 14) in order to characterize the isolated vesicles from perfusates by their size.

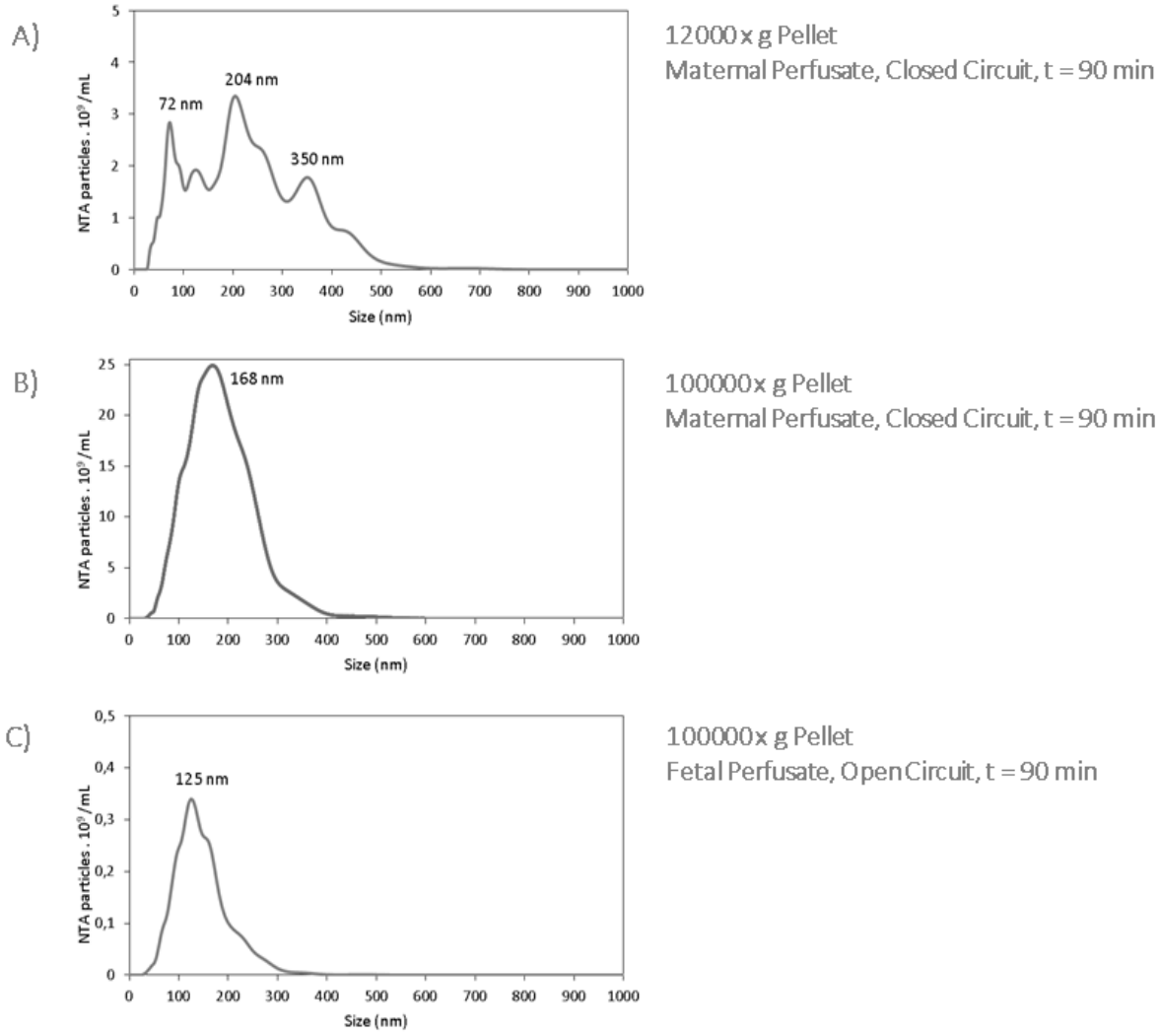

Figure 14: Size distribution plots of isolated microvesicles and exosomes from maternal perfusate and exosomes of fetal perfusate measured by Nanoparticle Tracking Analysis (NTA). Maternal and fetal perfusate were collected after ex-vivo human placenta perfusion. . The exosome isolation is done by 500 x g centrifugation, followed by 2500 x g centrifugation and 12 000 x g centrifugation. The supernatant is filtered through a 0.2 µm filter and concentrated. A 10 0000 x g centrifugation step was done to pellet the exosomes. A) Microvesicle fraction, pellet of the 12 000 x g centrifugation step of the maternal perfusate. The blot was dispersed with maxima at 72, 204 and 350 nm. B) Pellet of the 10 000 x g centrifugation step of the maternal perfusate, representing the exosome fraction with a maximum at 168 nm. C) Pellet of the 10 0000 x g centrifugation step, representing the exosome fraction with a maximum at 125 nm.

The microvesicle fraction, gained by the 12 000 x g centrifugation step, of the maternal perfusate with the closed circuit for 90 minutes, showed a very heterogeneous population. Maxima were detected at 72, 204 and 350 nm. The mean value, determined by three measurements was 223 ± 109 nm. The concentration of all detected particles in this fraction was 7 x 10^{11} particles/ml.

In addition the isolated exosomes from the same maternal perfusate showed a single peak at 168 nm when measured with NTA. According to the literature the size of exosomes is located between 30 and 100 nm (26). This would mean that the mean peak was not included in this area. After a filtration

with a 0.2 µm filter there should also be no particles bigger than 0.2 µm in the solution. An aggregation of exosomes can be an explanation. With a concentration of 3 x 10^{12} particles/mL, the particle concentration in the exosome fraction was higher than the concentration of the microvesicle fraction.

Also in the fetal perfusate in the pellet after the 10 0000 x g centrifugation a peak was detectable. The overall concentration of particles found in the pellet was 4 x 10^{10} particles/mL, which is 100-fold less than in the maternal pellet after the 10 0000 x g centrifugation. There were also perfusions where no particles were detectable in the pellet after the 10 0000 x g centrifugation of the fetal perfusate.

To estimate the yield of microvesicles and exosomes isolated from maternal and fetal perfusate the relation between the protein contents of the pellets after the 12 000 x g centrifugation for microvesicles or after the 10 0000 x g centrifugation for exosomes and the protein content of the corresponding perfusate. An overwiew over the reached yields can be seen in Table 9.

Table 9: Yield of microvesicles and exosomes received by isolation out of maternal and fetal perfusate. The yield was calculated as the relation between the protein content of the pellets after the 10 000 x g centrifugation for the microvesicles or of the 70 000 x g centrifugation for the exosome and the protein content of the perfusates. Yield was estimated for maternal perfusate with an open circuit for 30 minutes, for maternal perfusate with a closed circuit for 90 minutes and for fetal perfusate in an open circuit for 90 minutes.

Isolation	Microvesicle fraction (%)	Exosome fraction (%)
Maternal Perfusate, open circuit t = 30 min	0.36	0.19
Maternal Perfusate, closed circuit t = 90 min	0.29	0.30

The isolation of the extracellular vesicles from the maternal perfusate with the open circuit for 30 minutes shows a relation of microvesicles to exosomes of 2:1, while for the perfusate with a closed circuit for 90 minutes this relation was 1:1. An enrichment of exosomes due to the closed cicuit was observable. In general the yield of the distinct vesicle fractions varied between the different perfusions. Therefor it was not possible to set fix numbers.

4.3.2. Optimization of the centrifugation step for exosomes

As seen in Figure 13, the Western Blot showed bands in the supernatant after the 100000 x centrifugation step. One explanation could be that the pelletizing of the exosomal fraction was not complete due to too short centrifugation of the fraction. It was investigated if 5 hours compared to 1 hour of 100000 x g centrifugation leads to higher yield of exosomes. All isolation steps before were the same as outlined in the protocol. The results are depicted in Figure 15.

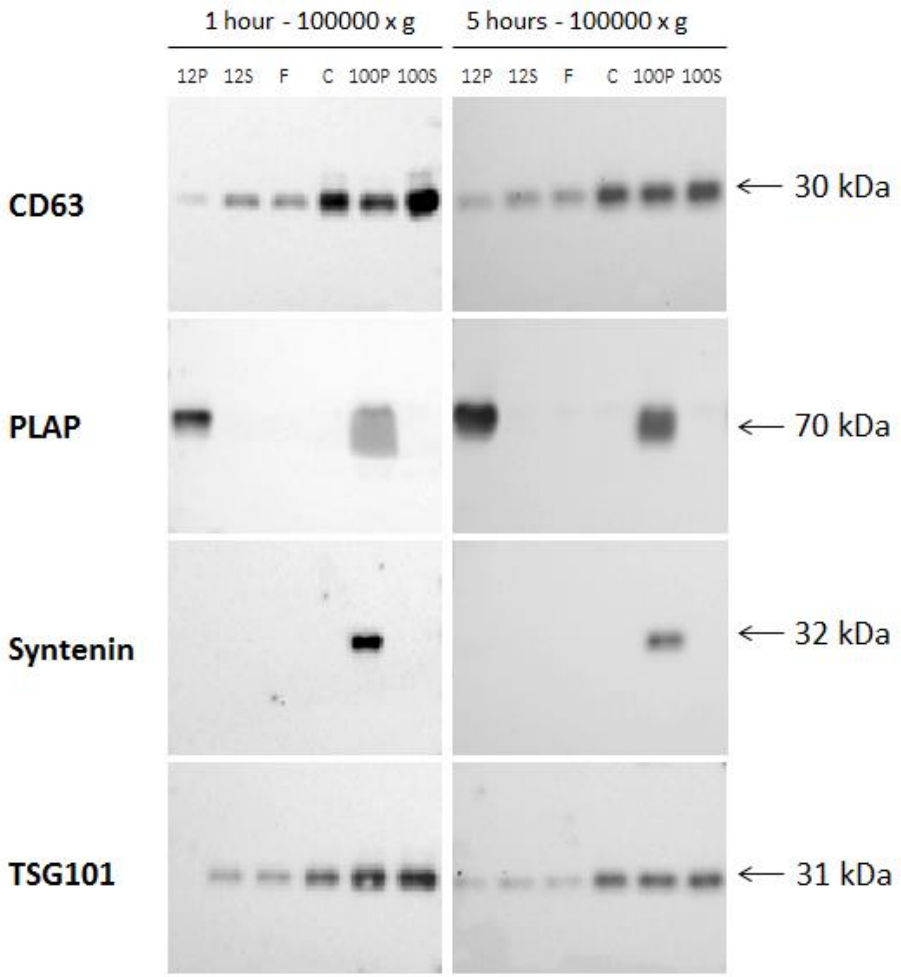

Figure 15: Analysis of exosome marker protein expression from maternal perfusates after different centrifugation times. The exosome isolation was done by 500 x g centrifugation, followed by 2500 x g and 12 000 x g centrifugation. The supernatant of the 12 000 x g centrifugation step was filtered through a 0.2 µm filter and concentrated. A 10 0000 x g centrifugation step was done to pellet the exosomes. The effect of the 10 0000 x g centrifugation for 1 hour or 5 hours was compared. Protein load = 10 µg/well.

The marker proteins, used for the identification of EVs and exosomes, as well as their placental origin, showed the same protein pattern in all fractions. Out from these results it seems to be not more efficient to extend the 100 000 x g centrifugation step for more than one hour.

4.3.3. Isopyknic centrifugation on a density gradient for exosome isolation, bottom-up approach

As the pellet after the 100000 x g centrifugation was not pure enough for further studies or investigations, an additional purification step via isopyknic centrifugation was added to the protocol. It has been described in the literature that beside the characteristic of the size of exosomes also the defined buoyant density of exosomes (1.12 – 1.19 g/mL) can be used as an additional criteria for isolation of pure exosomes (51).

The fractions obtained after the isopyknic centrifugation were investigated by Western blot and NTA as it can be seen in Figure 16 and Figure 17.

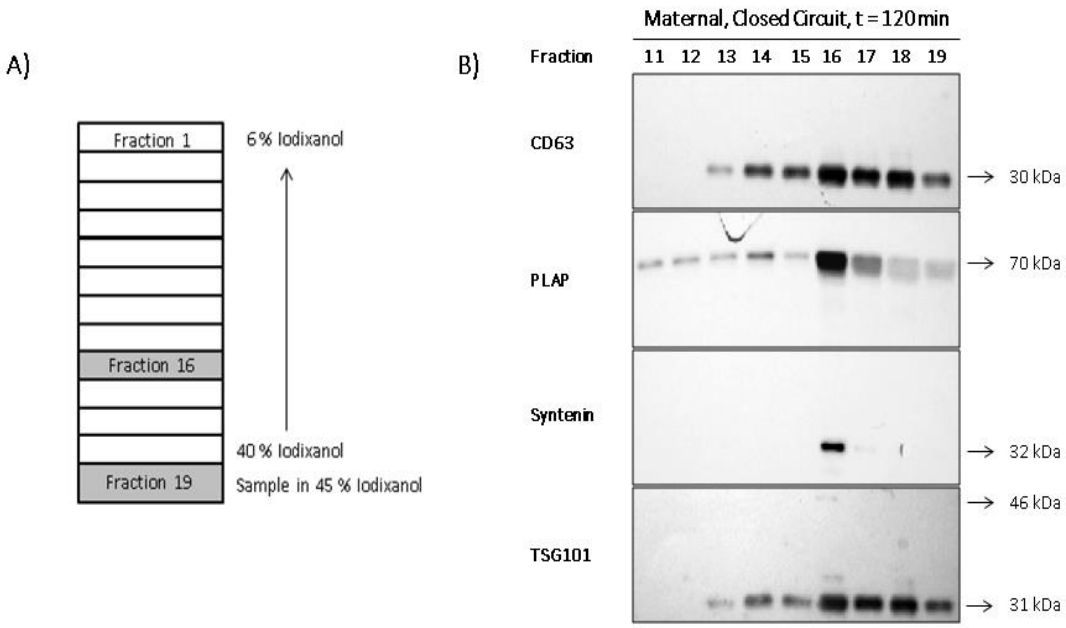

Figure 16: Western blot analysis of exosome marker protein expression (CD63, PLAP, Syntenin and TSG101) of the different fractions gained during the isopyknic centrifugation part from maternal perfusate. The 10 0000 x g pellet was dissolved in 400 µL PBS and mixed with 60% Iodixanol. The gradient was centrifuged at 10 0000 x g for 22 hours. After a concentration step, the fractions of the gradient were analysed by Western blot. A) The gradient for isopyknic centrifugation. Fraction 16 showed the strongest bands in the Western Blot analysis. B) Western blot results for the fractions with the highest protein concentrations. Protein load: 10 µg/well.

The gradient fractions with the highest protein concentrations were analysed by Western blot. Different marker proteins were used for the identification of placental exosomes. The extracellular vesicle marker CD63 showed bands for fractions 13 – 19. The strongest band is detected for fraction 16. Also the syncytiotrophoblast marker PLAP showed the strongest band for fraction 16. The other samples had slighter bands. The anti-Syntenin antibody, which is an exosome specific marker revealed a single band for fraction 16. Therefore this antibody was very specific in the identification

of the fraction with the highest exosome concentration. With the second exosome specific antibody, anti-TSG101 the result was able to be confirmed. The 31 kDa band was detectable in gradient fractions 13 to 19 with the most intense band in fraction 16. In contrast the 46 kDa band was only observed in fraction 16. The Western blot showed a clear result, that fraction 16 was the fraction containing the most exosomes.

The fetal gradient fractions, which were processed parallel to the maternal samples, contained small amounts of protein and the analyzation by Western blot was not possible because no bands were detected.

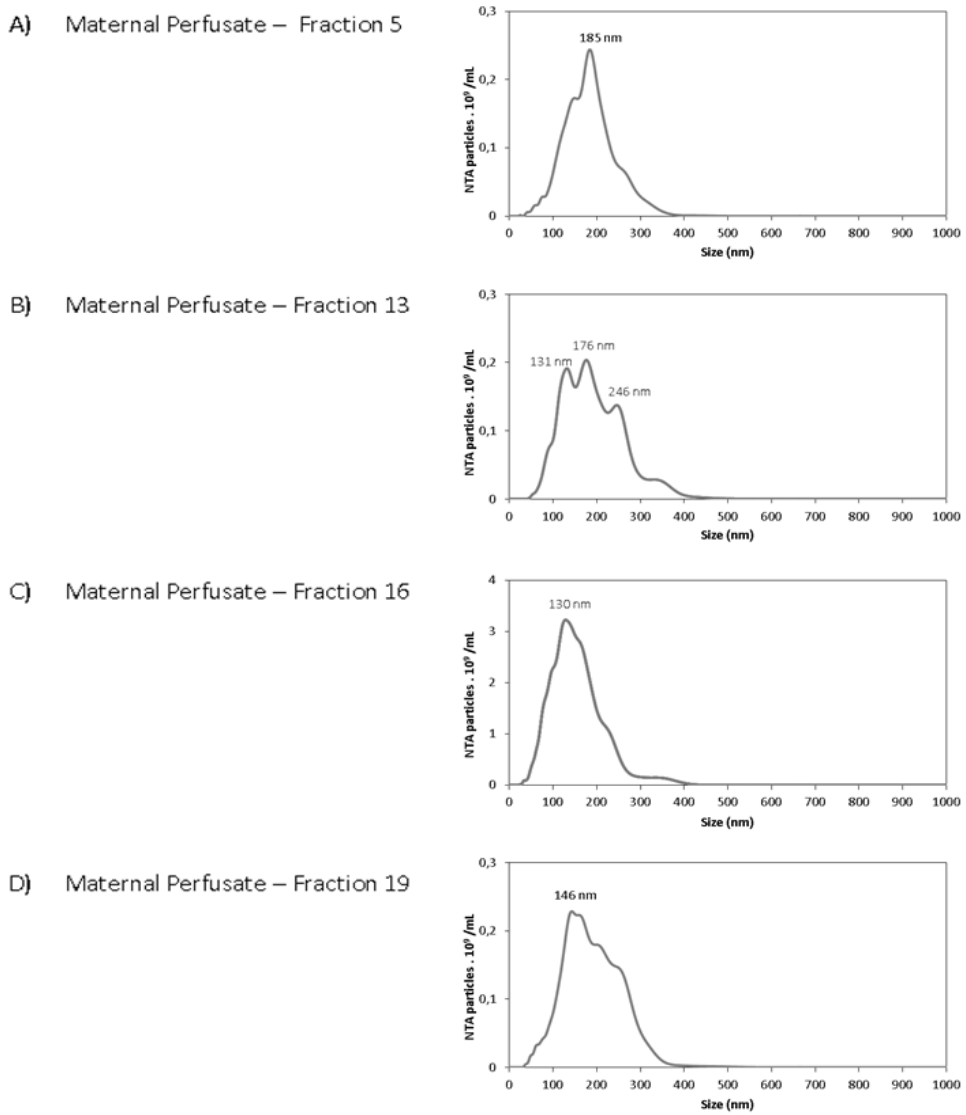

Figure 17: Size distribution plots of isolated exosomes from maternal perfusate measured by Nanoparticle Tracking Analysis (NTA). The pellet after the 10 0000 x g centrifugation was separated on a gradient. The fractions were collected after centrifugation of the gradient for 22 hours by 10 0000 x g. After concentration of the samples they were analysed. A) Maternal perfusate gradient fraction 13; B) Maternal perfusate gradient fraction 5; C) Maternal perfusate gradient fraction 16; D) Maternal perfusate gradient fraction 19.

To confirm and visualize the results of the Western blot maternal and fetal gradient steps were additionally analysed by NTA. (Figure 17)

Maternal gradient fraction 16, which showed the strongest bands on the Western Blot also showed with 3.1 x 10^{11} particles/ mL the highest particle concentration. The particle concentration was 10-fold increased compared to the other maternal gradient fractions.

The maximum of gradient fraction 16 was found at 130 nm, while the mean size is 161 ± 60 nm. The other gradient fractions, represented by fraction 5, 12 and 19 showed increased mean values of the particles. Gradient fraction 13 shows three maxima at 131, 176 and 246 nm.

Also the fetal gradient fractions were measured. None of the fractions showed particles even when measuring the sample undiluted.

To estimate the maternal and fetal yield of exosomes in pellet gained after the 10 0000 x g centrifugation step and the subsequent gained gradient fractions the protein content in relation to the whole protein content of the perfusate was calculated. The maternal results of the maternal gradient fractions also used for the Western blot can be seen in Table 10.

Table 10: Yield of placental derived exosomes received after isolation out of maternal perfusate. The yield was calculated as the relation between the protein content for the respective gradient fractions or the pellet received after the 10 0000 x g centrifugation for 1 hour and taking into account the protein content of the perfusates.

Maternal perfusate sample; closed circuit, t=120 min perfusion time	Yield for exosomes (%)
10 0000 x g Pellet	0.13
Fraction 11	0.00
Fraction 12	0.00
Fraction 13	0.00
Fraction 14	0.01
Fraction 15	0.01
Fraction 16	0.02
Fraction 17	0.03
Fraction 18	0.04
Fraction 19	0.02

The calculation showed that the 0.13 % yield of exosomes in the pellet after the 10 0000 x g was separated on the gradient between the fractions 14 and 19. The yield found in the gradient fractions also summed up to 0.13 %. The highest yield can be found in fraction 18. It was a different result than Western Blot and NTA indicated where gradient fraction 16 was the most intense one.

4.3.4. Isopyknic centrifugation on a density gradient for exosome isolation, top-down approach

It was also investigated if there is a change of separation of the exosome fraction on the Iodixanol gradient, when the sample is placed on the top of the gradient. The pellet was dissolved in 6 % Iodixanol. The centrifugation with 10 0000 x g for 22 hours was ident to the centrifugation of the gradient, where the pellet was dissolved in 45 % Iodixanol and placed on the bottom of the centrifugation tube.

As it can be seen in Figure 18, the Western Blot showed a similar result to the one were the sample was placed in the bottom of the centrifugation tube. The difference is the gradient fraction in which the highest protein concentration and the strongest band in the Western blot was detectable.

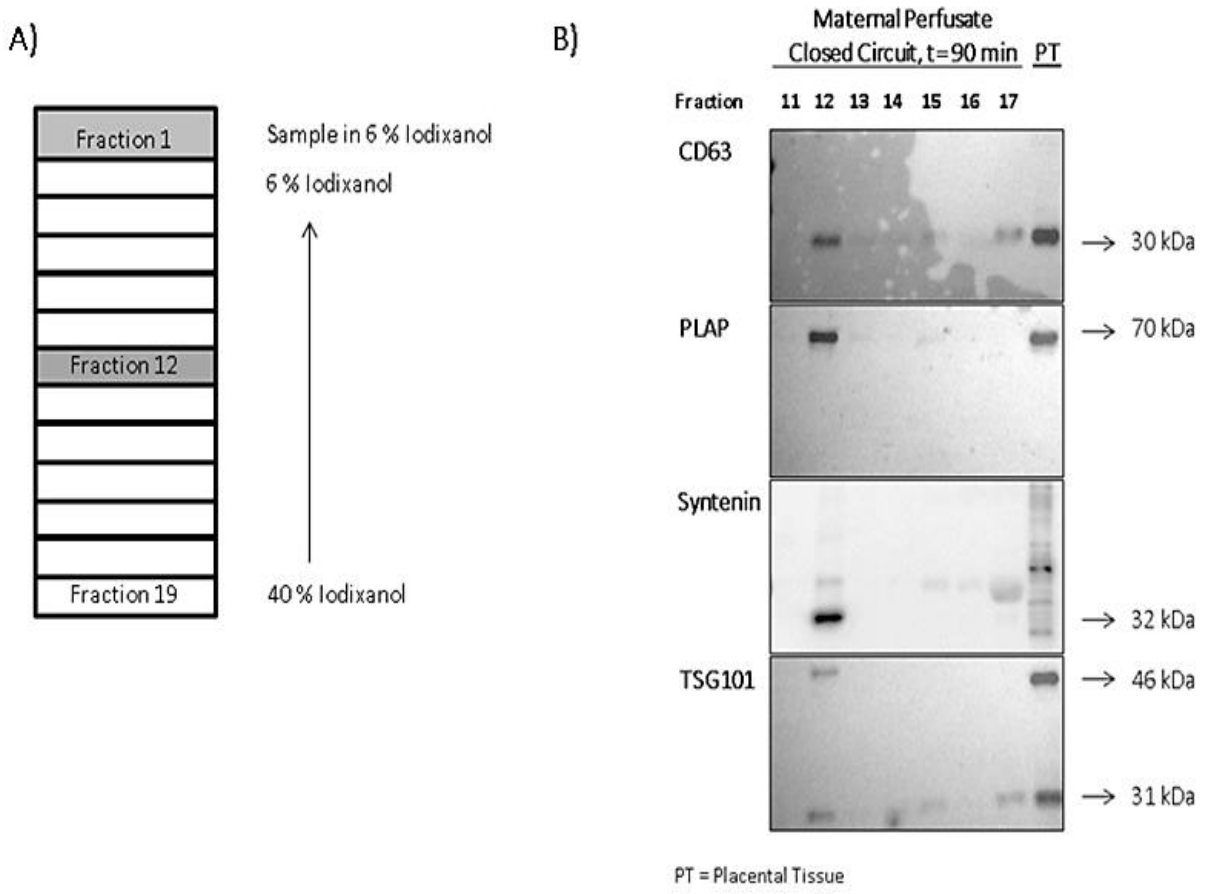

Figure 18: Western blot analysis of exosome marker protein expression (CD63, PLAP, Syntenin and TSG101) of the different fractions gained during the isopyknic centrifugation part from maternal perfusate where the sample was placed on top of the gradient. The 10 0000 x g pellet was dissolved in 400 µL PBS and mixed with 6% Iodixanol. The gradient was centrifuged at 10 0000 x g for 22 hours. After a concentration step, the fractions of the gradient were analysed by Western blot. A) The gradient for isopyknic centrifugation. Fraction 12 showed the highest protein concentration. B) Western blot results for the fractions with the highest protein concentrations. Protein load: 10 µg/well. PT = placental tissue as positive control

The extracellular vesicle marker CD63 protein showed bands in fraction 12-17. The clearest band was observed in fraction 12. This was confirmed by the syncytiotrophoblast marker PLAP. There was a single band visible for fraction 12. Syntenin and TSG101, the exosome markers revealed the same results. For Syntenin a specific band was detectable within fraction 12. The 31 kDa band of TSG101 can be detected within the fractions 12 and 17, while the 46 kDa band was specifically observed in fraction 12. The different antibodies proved fraction 12 to contain the most exosome specific proteins beside the highest protein content in the fraction. To assure that the Western blot showed no wrong positives, placenta tissue was used as a positive control.

Also the fetal gradient samples were blotted and analysed. Except of the bands of the positive control no bands were visible on these blots. The fetal gradient was pipetted and centrifuged parallel to the maternal one. The result observed already before was confirmed.

Also for this investigation the samples were analysed with NTA. The results for the maternal gradient fractions are displayed in Figure 19, while the results for the fetal gradient fractions are shown in Figure 20.

It has to be mentioned that the particle concentration in the maternal gradient fractions was generally 10 fold lower compared to the one where the pellet after the 10 0000 x g centrifugation was located in the bottom of the gradient. Fraction 12, which showed the most specific and strongest bands in the Western blot, showed a 10 fold increase compared to the other fractions tested by NTA. The particle concentration in the maternal gradient fraction 12 was 1.39×10^{10} particles/ml.

The size distribution blot of gradient fraction 12 showed three different maxima at 122, 168 and 219 nm. The mean value was 197 ± 85 nm. The value was higher compared to the one of fraction 16. The mean value of fraction 16 was 152 ± 93 nm. In fraction 16 a maximum at 91 nm was detected, which is very close to the size of exosomes (30-100 nm), described in the literature (26). The peak between 450 and 650 nm can occur because of the aggregation of exosomes. Fraction 5 was dispersed and the mean value was 178 ± 102 nm. The standard deviation was very high. Several peaks were detected. Maxima were found at 108, 203 and 390 nm. The total particle concentration with 2.37×10^{9} particles/mL is 2 fold less than in fraction 16.

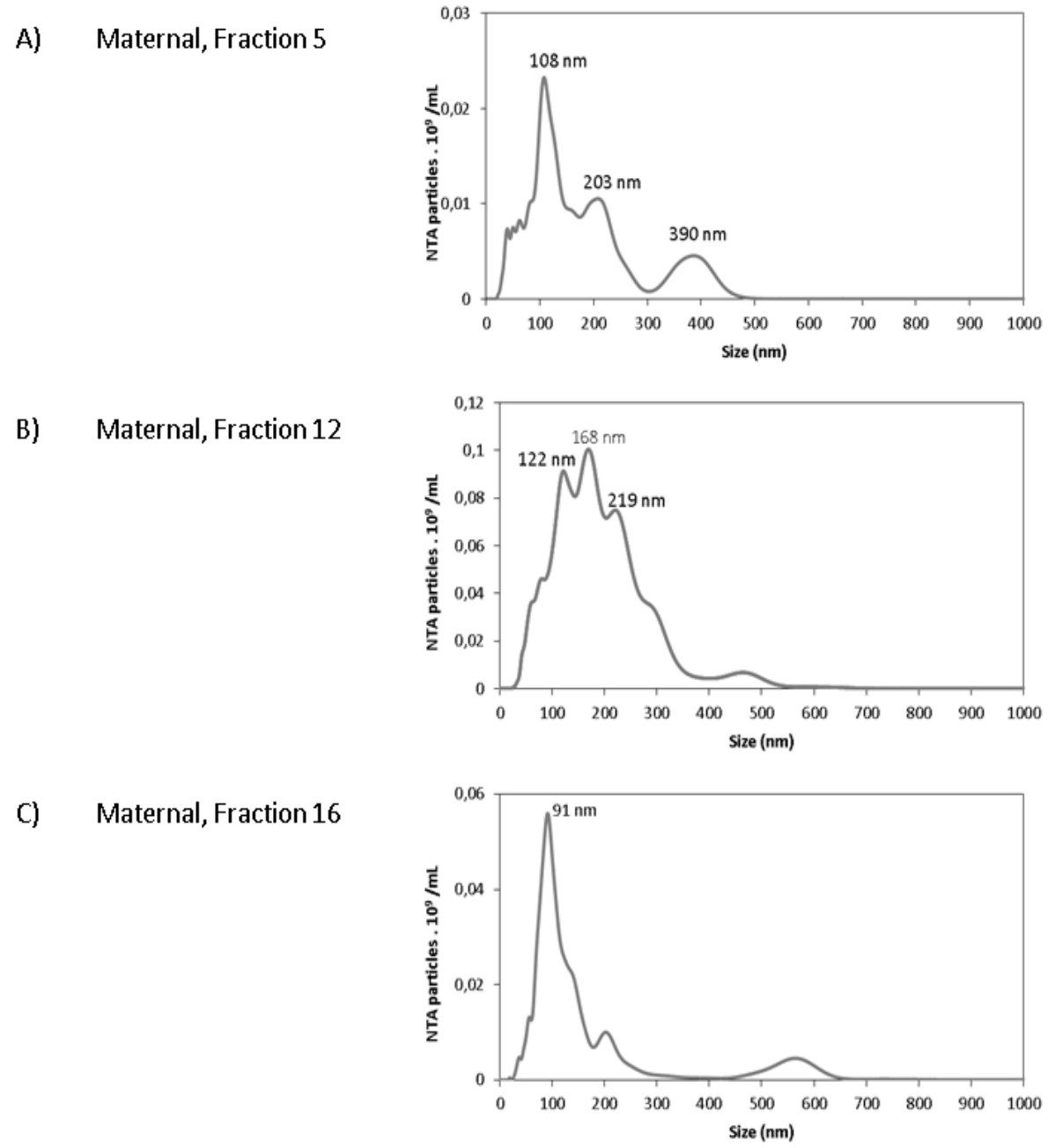

Figure 19: Size distribution of exosomes from maternal perfusate measured by NTA. The pellet after the 10 0000 x g centrifugation was separated on a gradient. The pellet was dissolved in 6 % Iodixanol. The fractions were collected after centrifugation of the gradient for 22 hours by 10 0000 x g. After concentration of the samples they were analysed. A) Maternal perfusate gradient fraction 5; B) Maternal perfusate gradient fraction 12; C) Maternal perfusate gradient fraction 16.

The fetal gradient fractions were analysed by NTA (Figure 12). The samples showed a dispersed size distribution, but there were particles detectable. The detection of particles in the fetal pellets and gradient fractions cannot be predicted. In comparison to the maternal gradient fraction 12, which showed the highest particle concentration in the parallel isolation experiment, the particle of the fetal gradient fraction 12 is 10 fold decreased. The particle concentration of the fetal gradient fraction 12 is 3.9 x 10^9 particles/ml. The plot of the fraction had two peaks at 90 nm and 185 nm. The

distribution was scattered. The same observation was done in fraction 16 of the fetal gradient. Maxima at 92 nm and 137 nm were detected. The largest particles found in fraction 16 was about 320 n, while in fraction 12 there were also larger particles. The mean size of fraction 12 was 184 ± 72 nm and the mean size of fraction 16 was 132 ± 52 nm. These values are in the size range also seen in the maternal fractions. The particle finding was a contrast to the Western blot results shown previously (Figure 13).

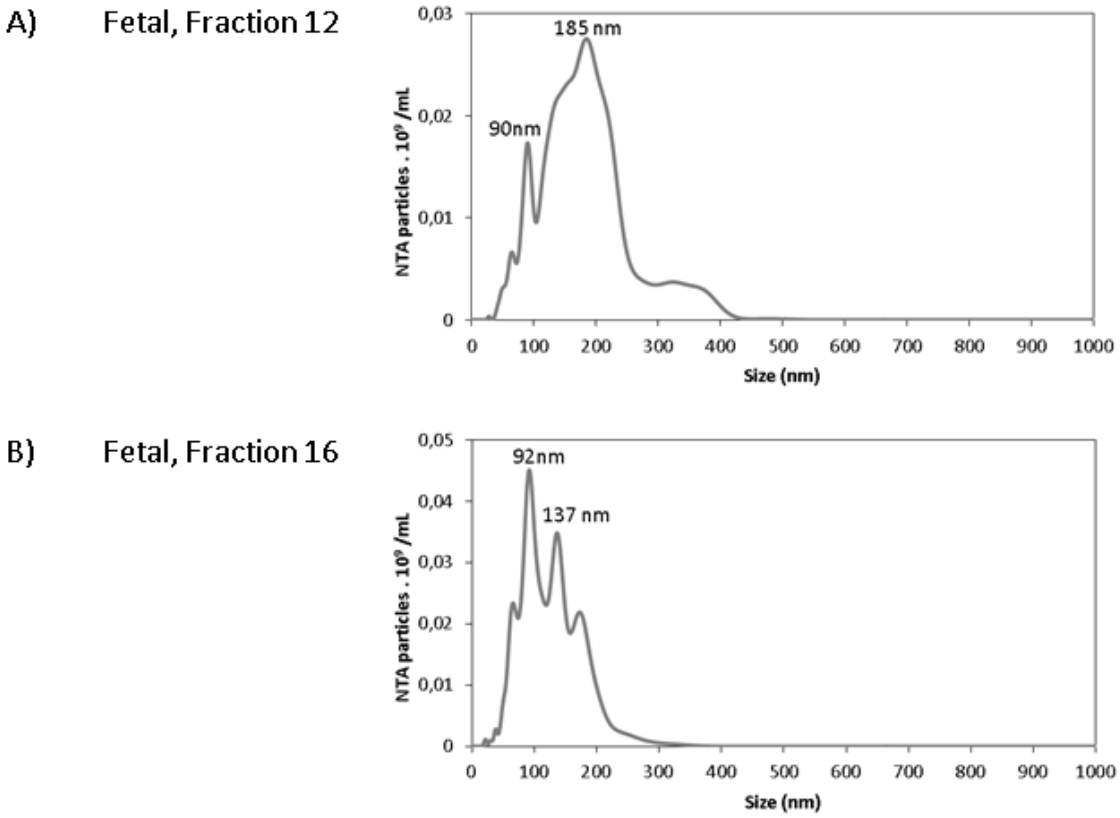

Figure 20: Size distribution of isolated vesicles isolated from fetal perfusates. The pellet after the 10 0000 x g centrifugation was separated on a gradient. The pellet was dissolved in 6 % Iodixanol. The fractions were collected after centrifugation of the gradient for 22 hours by 100000 x g. After concentration of the samples they were analysed. **A)** Fetal perfusate gradient fraction 12; **B)** Fetal perfusate gradient fraction 16.

4.4. Isolation of exosomes released from primary trophoblasts

Exosomes have also been isolated by differential centrifugation followed by isopyknic centrifugation from cell culture supernatants of *in vitro* cultivated human term trophoblasts. The supernatants were collected after 22 and 67 hours of trophoblast cultivation. The protein pellets after the 100 000 x g centrifugation step were analysed by Western blotting. Results are depicted in Figure 21 and Table 11.

As identification and characterization of the isolated fractions the specific marker proteins PLAP, CD63, TSG101 and Syntenin were investigated on the Western blot.

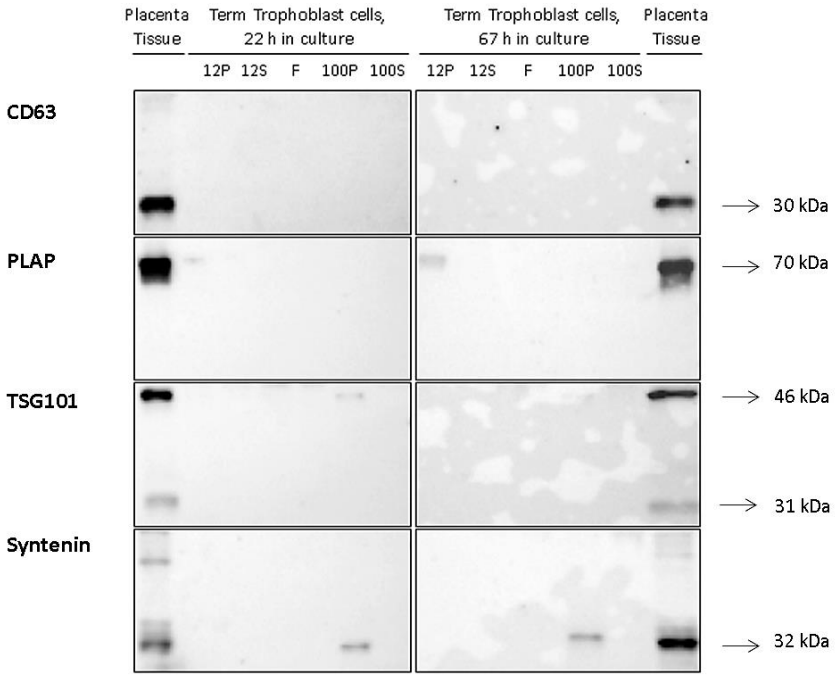

Figure 21: Western blot analysis of exosome marker protein expression (CD63, PLAP, Syntenin and TSG101) of samples isolated from the supernatant of human placental term trophoblast cells. The cell culture supernatant was centrifuged at 500 x g, followed by 2500 x g centrifugation and 12 000 x g centrifugation. The supernatant is filtered through a 0.2 µm filter and concentrated. A 10 0000 x g centrifugation step was done to pellet the exosomes. The pellet fraction after the 12 000 x g centrifugation (12P) containing the microvesicles, as well as the corresponding supernatant (12S) was analysed. Also the sample after filtration (F) was investigated as well as the 10 0000 x g pellet, containing the exosomal fraction, and the corresponding supernatant (100S).

The extracellular vesicle specific marker protein CD63 did not show any band in any fraction. There is also no difference between the different cultivation times.

The anti- PLAP antibody, directed against a syncytiotrophoblast specific protein, detected bands only in the pellet after the 12 000 x g centrifugation. PLAP is located in the plasma membrane of the trophoblast cells (59). The protein is not reaching the endosomal compartment to be sorted to the

exosomes in a high enough amount for Western blot detection. In contrast the microvesicles are shed from the plasma membrane and therefor include PLAP in the plasma membrane.

The exosome specific marker protein TSG101 revealed a slight signal for the pellet gained after the 100 000 x g centrifugation step from the supernatant of term trophoblasts kept in culture for 22 hours. The band was detected at 46 kDa. No band was detected at 31 kDa in the same fraction. It has to be mentioned that term trophoblasts in culture for 22 and 67 hours were not used from the same isolation and not from the same placenta.

Beside TSG101 also Syntenin was very specific for the exosome fraction. Specific bands were detectable in the pellets after the 100 000 x g centrifugation step from the supernatant of term trophoblasts kept in culture for 22 hours and 67 hours. The result is confirming the observations done with the exosome isolation from maternal perfusate, that TSG101 and Syntenin are very specific for exosomes.

To prove that the Western blot results can be used a placenta tissue was applied to each Western blot as a positive control. All antibodies showed positive results for the positive controls.

In order to estimate the yield of isolated microvesicles and exosomes obtained by isolation of the cell culture supernatants of trophoblasts the protein ratio was calculated. In aprticular, the microvesicle yield the ration of the protein concentration of the pellet after the 12 000 x g centrifugation and the protein concentration of the cell culture supernatant before centrifugation was calculated. The exosome yield was calculated the same way, replacing the 12 000 x g pellet concentration with the 100 000 x g pellet concentration. The numbers can be seen in Table 11.

Table 11: Exosomes and microvesicles received by isolation of the supernatant of placental human term trophoblasts in culture. The yield was calculated as the relation between the protein content for the pellet received after the 12 000 x g centrifugation for the microvesicles or the pellet received after the 10 0000 x g centrifugation for the exosomes in relation to the total protein content of supernatant after 22 or 67 hours.

Isolation	Microvesicle fraction (% of total protein)	Exosome fraction (% of total protein)
Supernatant of trophoblasts after 22 hours cultivation	0.36	0.13
Supernatant of trophoblasts after 67 hours cultivation	0.51	0.11

After cultivating the cells for 22 hours the collected supernatants yielded in 0.36% of microvesicles of the total protein concentration of the supernatant whereas only 0.13% of the total protein concentration of the supernatant exosomes could be detected after centrifugation. The extension of

cell cultivation time- up to 67 hours did not increase the number of released exosomes however, the amount of isolated microvesicles was increased (Table 11). It has to be mentioned that the term trophoblasts kept in culture for different time course experiments were not isolated from the same placenta, which may cause the biological dependent differences.

In comparison to the yield achieved by isolating exosomes and microvesicles from the maternal perfusate, the ratio of total protein and protein concentration in the exosome and microvesicle fraction, respectively were comparable.

4.5. TSG101-Isoforms

Two isoforms of TSG101 has been described in the literature (62). The the full-length protein exhibits a size of 46 kDa. In the isoform amino acids 15 to 119 are deleted thereby encoded by a molecular weight of 31 kDa. Both isoforms are expressed in human placental tissue, but the full length of the protein is preferentially expressed (62), as we also could demonstrate (Figure 22).

In order to get new insights about the specificity of the exosomal marker protein TSG101 and if its expression is dependent on the isolation procedure, we compared the protein distribution within obtained fractions of the two protocols.

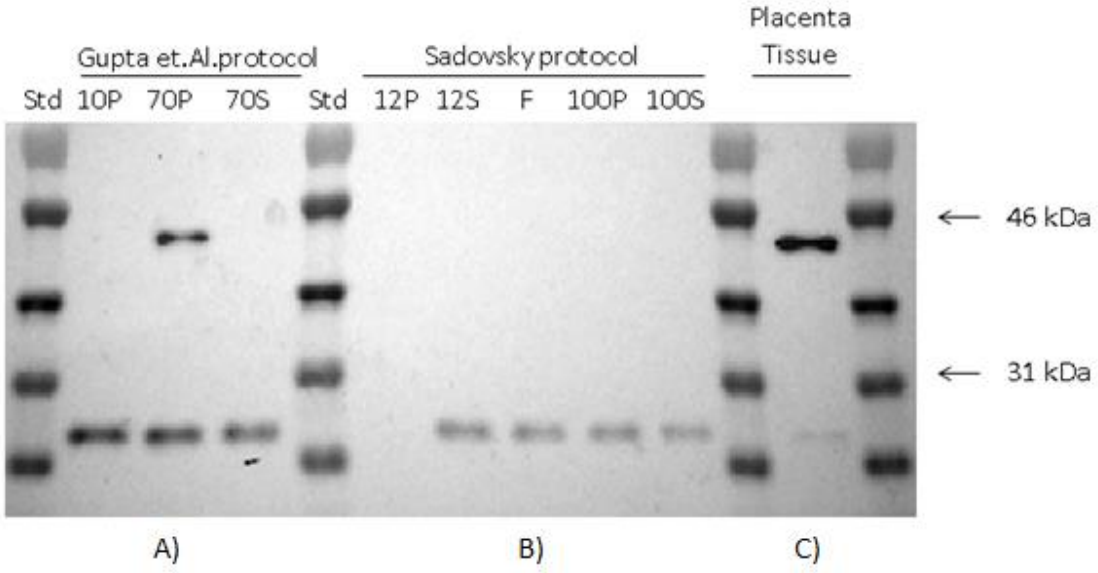

Figure 22: Endosomal marker protein TSG101 isoform distribution in different fractionated maternal perfusates. **A)** All three fractions of Gupta´s isolation method (10P, 70P and 70S) showed the 31 kDa protein isoform of TSG101, whereas exclusively in the 70P microvesicle/exosome pellet fraction both the 31 and 46 kDa isoform was detectable. **B)** Based on Sadowsky´s protocol the pellet 100P and supernatant fractions (12S, 100S, F) of maternal perfusates showed exclusively the 31 kDa TSG101 isoform. The 12P pellet lacks of TSG101 protein. **C)** Term placental tissue served as a positive control and both isoforms were detectable, total protein load was 10 µg/well in each of the lanes.

The TSG101 isoform analyses by Western blotting experiments based on the Gupta et. al., protocol for microvesicle/exosome isolation showed specific the 31 kDa protein form in each fraction. In addition, the pellet fraction (70P) revealed also the 46 kDa isoform of TSG101. Interestingly, the 31 kDa isoform is much more prominent in the 70P fraction as observed in placental tissue, where the full protein expression of TSG101 prevailed.In all generated fractions according to the extended Sadovsky protocol we detected only the 31 kDa isoform. TSG101 was not detectable in the pellet obtained after the 12 000 x g centrifugation step. This fraction (12P) should contain only the microvesicles, which is confirmed by lack of TSG101 protein as shown in Figure 14. In contrast, the supernatant after this 12 000 x g centrifugation step (12S) shows one single band, which means that the exosomes are still in the supernatant. Also during the filtration step no significant amount of protein is lost since similar signals for TSG101 protein (12S vs F). After the final 100 000 x g centrifugation step in which the pellet should be enriched with placental derived exosomes the 31 kDa TSG101 isoform was still detectable in the pellet and the supernatant. This finding indicates either an insufficient centrifugation procedure or due to cracked exosomes obtained during procedure TSG101 is distributed in pellet and supernatant.

In summary, the 46 kDa full protein can only be detected, if the number of exosomes is high enough within the fraction.

5. Discussion

The secretion of extracellular vesicles (EVs) is an important biological process for cell to cell communication and this process plays a critical role in normal and complicated pregnancies as well (26).

The main objective of my thesis was to establish and to verify a protocol for exosome isolation deriving from the human placenta. This protocol was already established for the isolation of exosomes from cell cultivation media of term primary human trophoblasts. The aim was to translate and modify this protocol for exosome isolation (kindly provided by Prof. Yoel Sadovsky), for maternal and fetal perfusate - collected after two hours of *ex-vivo* dually human placental perfusion. For further investigations of this isolated placental exosomes a validation of the method and characterization of the obtained particles are mandatory. Characterization of isolated exosomes includes more than one parameter e.g. size and size-distribution, a number of marker proteins should be used for the definition of isolated vesicles.

With the combination of differential centrifugation and isopyknic centrifugation, the exosomal fraction of maternal perfusates revealed a mean size of 130 ± 60 nm. Additionally CD63, marker for extracellular vesicles and PLAP (placental alkaline phosphatase), showing the placenta specificity, were enriched in the exosomal fractions. Enrichment of Syntenin-1, an intercellular adaptor protein, and TSG101 (Tumor sus susceptility gene 101) proteins suggested an intercellular origin of the exosomes circulating in the mother. In contrast, from fetal perfusates neither microvesicles nor placental derived exosomes could be isolated however, NTA measurements detected distinct particles with a mean size of 125 nm after several centrifugation steps, but with a 100-fold less signal intensity compared to maternal perfusates.

Isolation of any subtypes of EVs or exosomes via ultracentrifugation only allows enrichment of isolated subtypes, because vesicles of similar size and protein aggregates may co-sediment at 100 000 x g. Therefore, we tested whether an additional sucrose gradient centrifugation of the perfusion samples may lead to a higher purity of the placental enriched exosomal fraction. Secondly, regardless of the protocol used, each technique must be validated for any given cell type – or biological fluid. Thus, the second objective was to confirm the identity of purified vesicles from maternal and fetal media samples collected during *ex vivo* perfusion of the human placenta. This requires the use of a combination of several methods to determine the characteristics of the isolated particles.

Exosomes isolated from maternal perfusate

In order to verify the Sadovsky protocol characterization of the isolated exosomes generated from maternal perfusate were compared with exosomal features released by isolated primary human term trophoblasts. For cultivation media of trophoblasts isolation protocol for nanoparticles has already been verified. The characterization results for exosomal particles from maternal perfusate were in agreement with particles obtained from cell media supernatant of trophoblast cultures, which indicates that the protocol is also valid for placenta perfusion media. Moreover, the comparison of the results of the protocol combined of differential centrifugation and separation on a density gradient to differential centrifugation alone showed that the separation on the density gradient is very sufficient. Gradient fractions collected after the isopyknic centrifugation process showed a decreased mean size of the exosomal fraction analysed by NTA. This additionally centrifugation step is necessary to yield a higher enriched fraction for exosomes. Otherwise non-exosomal particle and protein aggregations may float in the same size range and are then used for continuous characterizations and experiments.

On the other hand it has to be mentioned that with the increasing number of centrifugation, filtration and concentration steps a loss of yield in exosomes has to be accepted. The yield was shrinking with every step and especially the 100 000 x g centrifugation step were rough for the placental exosomes. Therefore it was important to show that an extension of the 100 000 x g centrifugation step in order to isolate exosomes was not increasing the pelletizing amount of exosomes. Western blotting revealed that there were still remaining vesicles in the supernatant. This suggests that a compromise between purity of exosomes and yield has to be done.

In terms of the purity and specificity of the isolated exosomes it remains to be confirmed, if the isolated exosomes were located in the proper range of density after the separation on the iodixanol density gradient. Density of fractions can be determined by measuring the refraction index. According to the literature the buoyant density of exosomes is 1.13 to 1.19 g/ml (14). Relying on the data relating to the iodixanol gradient done with mannitol as a dilution media, a gradient from 40 to 10 % iodixanol spans a density range 1.22 to 1.07 g/ml (73), which overlaps the density of single isolated exosomes.

Nanotracking analysis (NTA) for characterization of exosomes:

+Nanotracking analysis (NTA) relates on the rate of Brownian motion of particles which is in direct relation with the particle size. Size profiles done by NTA can also be used analysation of isolated exosomes (69).

We have also shown that the separation of pelletized exosomes from maternal perfusate on an iodixanol density gradient was leading to a decrease in the mean diameter of the particles. Nevertheless the mean values for the maternal exosomes measured by NTA were above the diameter of exosomes published in the literature wit 30 to 100 nm (2), even after the separation on the density gradient.

This increase can have different reasons. Firstly exosomes are lipid vesicles, which tend to aggregate (74). The isolation procedure comprises several steps and is very time consuming. So that exosomes can aggregate over long periods of time. The conventional NTA, used in this thesis for the characterization of placental exosomes, cannot be used for predictions about the vesicle phenotype (69), whereby discrimination between large vesicles and aggregated exosomes is not possible.

Nevertheless one has to keep in mind, that in the case of the long exosome preparation protocol, exosomes may already undergo some loss of function. In the literature it is discussed that due to the long exosome isolation protocol a shift towards larger size can be observed. Exosomes can swallow when stored at 4 °C, the temperature which is used for isolating exosomes. The swallowing can be an initial step for vesicle disruption (75).

Additionally with the conventional NTA vesicle size and particle concentration can be determined but it is not possible to determine the origin of vesicles. (Ref ID 10) For the further validation of the isolation method an investigation of the isolated exosomal fraction with fluorescent NTA should be considered. For the fluorescent NTA exosomes are labelled with fluorophores attached to antibodies. To prove the placental origin the exosomes isolated from perfusates could be labelled with PLAP. Fluorescence NTA can also be used to distinguish between exosomes and particles in the same size range. Therefore the marker proteins already used for Western blot characterization, CD63, TSG101 and Syntenin, can be used.

An explanation for the increased size of the exosomal fraction can also be due to the formation of a biocorona around the exosomes. When incubating defined silica nanoparticles in bovine and human serum the characteristics of the particles are altered. Proteins such as apolipoproteins, thrombin or albumin form a stable biocorona around the silica nanoparticles. Due to the biocorona formation around the silica nanoparticles show increased size when analysed by NTA (76). It is imaginable that also biocorona formation around exosomes is occurring causing a size increase of exosomes. Further investigations have to be done on this topic, because a potential corona formation cannot be prohibited as exosomes are isolated from biological fluids also containing apolipoproteins or albumin. Of course the biocorona formation also has an impact on the interaction of the particles with receptors or ligands (76).

Storage of exosomes

For the further handling and for characterization of isolated exosomes and microvesicles it is also important to have knowledge about the storing conditions of exosomal fractions. We demonstrated by the NTA performance of the particles that isolated exosomes cannot be stored for 7 days at -20 °C. The mean size of exosomes was increased and the size distribution plot showed dispersed peaks. The storage of exosomes at -20 °C for 7 days allows no proper characterization of exosomal size distribution by NTA. In the literature the shift in size distribution is also described (75). Interestingly, the total particle concentration in the exosome fraction was increased about 2-fold after storing the sample at -20 °C. A decrease in particle number was expected, as vesicles can burst as a consequence of freezing and thawing. An aggregation of exosomes leads also to a loss in total vesicle number in the sample. An explanation for the findings can be that larger particles show higher light intensities, which leads to an overestimation of the number of smaller particles (74).

In the literature it is discussed, that if storage is necessary, exosomes should be kept at -80 °C but not longer than 7 days (74). To be sure that the exosomes are not degraded during the storage it is advised to add protease inhibitor to the samples According to the literature multiple freezing and thawing cycles have no impact on the size of exosomes (77).

The storage of the isolated exosomes from maternal perfusate at -20 °C for 7 days had no impact on the results yielded by immunoblotting. The results were ident. In the literature it is described that the total protein concentration of the samples is rising when storing the isolated exosomes at -20 °C (74).

Generally long storage of isolated exosomes should be avoided not only for NTA but also for further characterizations and investigations done with exosomes.

TSG101 isoforms

For the validation of the exosome isolation procedure different exosomal marker proteins were used. One of those proteins was TSG101, a protein participating in the endosomal sorting complex required for transport machinery (ESCRT). Databases are describing two different TSG101 isoforms, one isoform with 46 kDa and the second with 31 kDa (62).

Total placenta tissue revealed strong expression of the 46 kDa isoform and a weak expression of the 31 kDa isoform. In the exosomal fractions it was the other way round. The 31 kDa was clear present, while the 46 kDa isoform was only very weak detectable in fractions with high exosome concentrations. It was also demonstrable that TSG101 is an exosome specific marker protein, because the microvesicle fractions showed lack of TSG101. These facts mean that TSG101 is not only

an exosome specific marker protein, but that the isoform with 31 kDa is higher specific for exosomes. A possible explanation for this is that the 31 kDa isoform is the isoform participating in the ESCRT complex, while the 46 kDa isoform plays in viral budding or regulation of cell growth amore important role. As the TSG101 can be found in different cellular components, such as cytoplasm, peripheral plasma membranes, nucleus and endosomal compartment (62) it is also possible that the isoform type is depending on the cellular localization within the cell. The 31 kDa isoform would then be expressed in the endosomal compartment as a participant in the exosome biogenesis. The presence of the larger isoform in exosomes is due to the sorting of proteins from the cytosol or the nucleus to the exosomes.

The distribution of the different TSG101-isoforms and their roles in the biogenesis of exosomes is not discussed in the literature until now.

Particles isolated from fetal perfusate:

Disparate results were observed when trying to isolate exosomal fractions from fetal perfusate after two hours of ex-vivo dual human placenta perfusion. When characterizing the fractions collected during the isolation and the fractions collected after the separation on the density gradient by NTA divergent results were observable. After some exosomes isolations from fetal perfusate particles were detectable, but there existed also perfusions where no particles were detectable. The particles detected in the fetal fractions showed a distinct size pattern in the same size as exosomes. The number of particles in the fraction was 100-fold lower than in the corresponding maternal fractions isolated from the maternal perfusate from the same placenta perfusion experiment.

By immunoblotting no bands were detectable after any exosome isolation from fetal perfusate. Even the perfusate itself, without centrifugation steps before, showed no results positive for the chosen marker proteins.

It is likely that the particles found in the fetal perfusates are no exosomes, as they show no positive signals in immunoblotting. The origin, characteristics and role of these particles found in the fetal perfusate remains unclear.

Additionally, the reason why sometimes particles in the size of exosomes can be detected by NTA and sometimes not remains to be established. Several factors are possible. Potential explanations can be influences of fetal sex or variations in the vital parameters of the mother, such as the blood pressure or lipid levels.

Further perspectives:

Exosomes are important participants in the cell to cell communication. The placenta releases exosomes from the trophoblast layer of the placenta, which serve as one biological communication tool between the placenta and the mother. Although we demonstrated a successful method for isolation of exosomes from perfusates obtained after *ex-vivo* dually human placental perfusion experiments, and to characterize the isolated exosomal fraction, several questions remain not to be answered.

First, the characterization of the isolated enriched EV´s has to be expanded since NTA is a fast and cheap way to analyse particles simultaneously thereby getting information about size and concentration distribution of the nanoparticles. Second, as exosome levels are altered in the plasma of mothers suffering from pregnancy pathologies, such as pre-eclampsia and gestational diabetes mellitus (GDM) (27) they could have the potential for early diagnosis in the future. Therefore a concrete and confident isolation method and validation is indispensable. A diagnosis in an early stage of gestation may lead to an early medication and lead to a positive pregnancy outcome (25).

Beside the potential of exosomes as marker in pathologies, exosomes may also be used as drug delivery systems in the future (51). For this purpose the mechanism of protein sorting to the exosomes and the protein interactions of exosomes and target cells have to be understood.

In case of the placental exosomes a proteomic and lipidomic analysis would give overall information about the composition and the specific lipid/protein cargo of placental nanoparticles. Furthermore, comparison of the particle protein composition between normal and pathologic pregnancies would help to understand the biological role of them. As placental exosomes carry miRNA as well (27), miRNA profiling would elucidate functional mechanisms of exosomes at the recipient cells.

References

1. **Mittelbrunn, M. und Sanchez-Madrid, F.** Intercellular communication: diverse structures for exchange of genetic information. *Nat.Rev.Mol.Cell Biol.* 2012, Bd. 13, 5, S. 328-335.

2. **Mincheva-Nilsson, L. und Baranov, V.** The role of placental exosomes in reproduction. *Am.J.Reprod.Immunol.* 2010, Bd. 63, 6, S. 520-533.

3. **Alberts, B., Johnson, A., Lewis, J., et al.** Cell junctions. *Molecular Biology of the Cell.* New York : Garland Science, 2002, Vol. 4th Edition.

4. **Gurke, S., Barroso, J.F. und Gerdes, H.H.** The art of cellular communication: tunneling nanotubes bridge the divide. *Histochem.Cell Biol.* Bd. 129, 5, S. 539-550.

5. **Mose, T. und Knudsen, L.E.** Placenta Perfusion - A Human Alternative. 2006, Bd. 23(Suppl), S. 358-363.

6. **Hsiao, E.Y. und Patterson, P.H.** Placental regulation of maternal-fetal interactions and brain development. *Dev.Neurobiol.* 2012, Bd. 72, 10, S. 1317-1326.

7. **Burton, G.J. und Jauniaux, E.** What is the placenta? *Am.J.Obstet.Gynecol.* 2015, Bd. 213, 4 Suppl, S. S6.e1-4.

8. **Tong, M. und Chamley, L.W.** Placental extracellular vesicles and feto-maternal communication. *Cold Spring Harb Perspect.Med.* 2015, Bd. 5, 3.

9. **Knipp, Gregory T., Audus, Kenneth L. und Soares, Michael J.** Nutrient transport across the placenta. *Adv.Drug Deliv.Rev.* 1999, Bd. 38, 1, S. 41-58.

10. **Clifton, V.L., et al., et al.** Review: The feto-placental unit, pregnancy pathology and impact on long term maternal health. *Placenta.* 2012, Bd. 33 Suppl, S. 37-41.

11. **McNamara, J.M. und Kay, H.H.** Placental Hormones: Physiology, Disease, and Prenatal Diagnosis. [Buchverf.] H. Kay, D.M. Nelson und Y. Wang. *The Placenta: From Development to Disease.* s.l. : John Wiley & Sons, 2011, S. 57-65.

12. **Stenqvist, A.C., et al., et al.** Exosomes secreted by human placenta carry functional Fas ligand and TRAIL molecules and convey apoptosis in activated immune cells, suggesting exosome-mediated immune privilege of the fetus. *J.Immunol.* 2013, Bd. 191, 11, S. 5515-5523.

13. **Turturici, G., et al., et al.** Extracellular membrane vesicles as a mechanism of cell-to-cell communication: advantages and disadvantages. *Am.J.Physiol.Cell.Physiol.* 2014, Bd. 306, 7, S. C 621-633.

14. **Mincheva-Nilsson, L. und Baranov, V.** Placenta-Derived Exosomes and Their Role in the Immune Protection of the Fetus. [Buchverf.] Dr. Jing Zheng (Ed.). *Recent Advances in Research on the Human Placenta.* s.l. : InTech, 2012.

15. **Gyorgy, B., et al., et al.** Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell Mol.Life Sci.* 2011, Bd. 68, 16, S. 2667-2688.

16. **Akers, J.C., et al., et al.** Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. *J.Neurooncol.* 2013, Bd. 113, 1, S. 1-11.

17. **Kalra, H., et al., et al.** Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. *PLoS Biol.* 2012, Bd. 10, 12.

18. **Elmore, S.** Apoptosis: A Review of Programmed Cell Death. 2007, Bd. 35, 4, S. 495-516.

19. **Lawson, C., et al., et al.** Microvesicles and exosomes: new players in metabolic and cardiovascular disease. *J.Endocrinol.* 2016, Bd. 228, 2, S. R57-71.

20. **Raposo, G. und Stoorvogel, W.** Extracellular vesicles: exosomes, microvesicles, and friends. *J.Cell Biol.* 2013, Bd. 200, 4, S. 373-383.

21. **Lee, Y., El Andaloussi, S. und Wood, M.J.** Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. *Hum.Mol.Genet.* 2012, Bd. 21, R1, S. R125-134.

22. **McDaniel, K., et al., et al.** Functional role of microvesicles in gastrointestinal malignancies. *Ann.Transl.Med.* 2013, Bd. 1, 1, S. 4.

23. **Muralidharan-Chari, V., et al., et al.** Microvesicles: mediators of extracellular communication during cancer progression. *J.Cell.Sci.* 2010, Bd. 123, Pt 10, S. 1603-1611.

24. **Johnstone, R.M., et al., et al.** Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J.Biol.Chem.* 1987, Bd. 262, 19, S. 9412-9420.

25. **Sarker, S., et al., et al.** Placenta-derived exosomes continuously increase in maternal circulation over the first trimester of pregnancy. *J.Transl.Med.* 2014, Bd. 12.

26. **Record, M.** Intercellular communication by exosomes in placenta: a possible role in cell fusion? *Placenta.* 2014, Bd. 35, 5, S. 297-302.

27. **Salomon, C. et. al.** The Role of Placental Exosomes in Gestational Diabetes Mellitus. [Buchverf.] Dr. Luis Sobrevia (Ed.). *Gestational Diabetes - Causes, Diagnosis and Treatment.* s.l. : InTech, 2013.

28. **Zhang, J. et. al.** Exosome and Exosomal MicroRNA: Trafficking, Sorting, and Function. *Genomics, Proteomics & Bioinformatics.* 2015, Bd. 13, S. 17-24.

29. **Liang, Y., et al., et al.** Complex N-linked glycans serve as a determinant for exosome/microvesicle cargo recruitment. *J.Biol.Chem.* 2014, Bd. 289, 47, S. 32526-32537.

30. **Andreu, Z. und Yanez-Mo, M.** Tetraspanins in extracellular vesicle formation and function. *Front.Immunol.* 2014, Bd. 5.

31. **Henderson, M.C. und Azorsa, D.O.** The genomic and proteomic content of cancer cell-derived exosomes. *Front.Oncol.* 2012, Bd. 2.

32. **Villarroya-Beltri, C., et al., et al.** Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs. *Nat.Commun.* 2013, Bd. 4.

33. **De Toro, J., et al., et al.** Emerging roles of exosomes in normal and pathological conditions: new insights for diagnosis and therapeutic applications. *Front.Immunol.* 2015, Bd. 6.

34. **Kowal, Joanna, Tkach, Mercedes und Théry, Clotilde.** Biogenesis and secretion of exosomes. *Curr.Opin.Cell Biol.* 2014, Bd. 29, S. 116-125.

35. **Mulcahy, L.A., Pink, R.C. und Carter, D.R.** Routes and mechanisms of extracellular vesicle uptake. *J.Extracell Vesicles.* 2014, Bd. 3.

36. **Lobert, V.H. und Stenmark, H.** Cell polarity and migration: emerging role for the endosomal sorting machinery. *Physiology (Bethesda).* 2011, Bd. 26, 3, S. 171-180.

37. **Wollert, T., et al., et al.** The ESCRT machinery at a glance. *J.Cell.Sci.* 2009, Bd. 122, Pt 13, S. 2163-2166.

38. **Nobuyuki, T et. al.** Endosomal sorting complex required for transport proteins in cancer pathogenesis, vesicular transport, and non-endosomal functions. *Cancer Sci.* 2008, Bd. 99, 7, S. 1293-1303.

39. **Stuffers, S., et al., et al.** Multivesicular endosome biogenesis in the absence of ESCRTs. *Traffic.* 2009, Bd. 10, 7, S. 925-937.

40. **Salomon, C., et al., et al.** A gestational profile of placental exosomes in maternal plasma and their effects on endothelial cell migration. *PLoS One.* 2014, Bd. 9, 6.

41. **Salomon, C., et al., et al.** Gestational Diabetes Mellitus Is Associated With Changes in the Concentration and Bioactivity of Placenta-Derived Exosomes in Maternal Circulation Across Gestation. *Diabetes.* 2016, Bd. 65, 3, S. 598-609.

42. **Kshirsagar, S.K., et al., et al.** Immunomodulatory molecules are released from the first trimester and term placenta via exosomes. *Placenta.* 2012, Bd. 33, 12, S. 982-990.

43. **Hedlund, M., et al., et al.** Human placenta expresses and secretes NKG2D ligands via exosomes that down-modulate the cognate receptor expression: evidence for immunosuppressive function. *J.Immunol.* 2009, Bd. 183, 1, S. 340-351.

44. **Mincheva-Nilsson, L., et al., et al.** Placenta-derived soluble MHC class I chain-related molecules down-regulate NKG2D receptor on peripheral blood mononuclear cells during human pregnancy: a possible novel immune escape mechanism for fetal survival. *J.Immunol.* 2006, Bd. 176, 6, S. 3585-3592.

45. **Ouyang, Y., et al., et al.** Review: Placenta-specific microRNAs in exosomes – Good things come in nano-packages. *Placenta.* 2014, Bde. 35, Supplement, S. S 69-73.

46. **Luo, S.S., et al., et al.** Human villous trophoblasts express and secrete placenta-specific microRNAs into maternal circulation via exosomes. *Biol.Reprod.* 2009, Bd. 81, 4, S. 717-729.

47. **Goswami, D., et al., et al.** Excess syncytiotrophoblast microparticle shedding is a feature of early-onset pre-eclampsia, but not normotensive intrauterine growth restriction. *Placenta.* 2006, Bd. 7, 1, S. 56-61.

48. **Tannetta, D. und Sargent, I.** Placental disease and the maternal syndrome of preeclampsia: missing links? *Curr.Hypertens.Rep.* 2013, Bd. 15, 6, S. 590-599.

49. **Tannetta, D., et al., et al.** Extracellular vesicles and reproduction-promotion of successful pregnancy. *Cell.Mol.Immunol.* 2014, Bd. 11, 6.

50. **Salomon, C., et al., et al.** The possible role of extravillous trophoblast-derived exosomes on the uterine spiral arterial remodeling under both normal and pathological conditions. *Biomed.Res.Int.* 2014, Bd. 2014.

51. **Mitchell, M.D., et al., et al.** Placental exosomes in normal and complicated pregnancy. *Am.J.Obstet.Gynecol.* 2015, Bd. 213, 4 Suppl, S. 173-181.

52. **Baig, S., et al., et al.** Lipidomic analysis of human placental syncytiotrophoblast microvesicles in adverse pregnancy outcomes. *Placenta.* 2013, Bd. 34, 5, S. 436-442.

53. **Buchanan, T.A. und Xiang, A.H.** Gestational diabetes mellitus. *Joural Clin Invest.* 2005, Bd. 115, 3 .

54. **Lappas, M., et al., et al.** The role of oxidative stress in the pathophysiology of gestational diabetes mellitus. *Antioxid.Redox Signal.* 2011, Bd. 15, 12, S. 3061-3100.

55. **Millán, J.L.** Molecular Cloning and Sequence Analysis of Human Placental Alkaline Phosphatase. *J Biol Chem.* 1986, Bd. 261, 7, S. 3112-3115.

56. **Sartori, M.J., et al., et al.** Role of placental alkaline phosphatase in the interaction between human placental trophoblast and Trypanosoma cruzi. *Exp.Mol.Pathol.* 2002, Bd. 72, 1, S. 84-90.

57. **Kam, W., et al., et al.** Cloning, sequencing, and chromosomal localization of human term placental alkaline phosphatase cDNA. *Proc.Natl.Acad.Sci.U.S.A.* 1985, Bd. 82, 24, S. 8715-8719.

58. **Metzelaar, M.J. et. al.** CD63 Antigen - a novel lysosomal membrane glycoprotein, cloned by a screening procedure for intracellular antigens in eukaryotic cells. 1991, Bd. 266, 5, S. 3239-3245.

59. http://www.uniprot.org/uniprot/P08962. *UniProt CD63.* [Online] 03 2016.

60. **Pols, Maaike S. und Klumperman, Judith.** Trafficking and function of the tetraspanin CD63. *Exp.Cell Res.* 2009, Bd. 315, 9, S. 1584-1592.

61. **Lin, Y.S., et al., et al.** Identification of TSG101 functional domains and p21 loci required for TSG101-mediated p21 gene regulation. *PLoS One.* 2009, Bd. 8, 11.

62. http://www.uniprot.org/uniprot/Q99816. *UniProt Tumor susceptibility gene 101 protein.* [Online] 03 2016.

63. http://www.uniprot.org/uniprot/O00560. *UniProt Syntenin-1.* [Online] 03 2016.

64. **Lee, H.J. und Zheng, J.J.** PDZ domains and their binding partners: structure, specificity, and modification. *Cell.Commun.Signal.* 2010, Bd. 8.

65. **Ghossoub, R., et al., et al.** Syntenin-ALIX exosome biogenesis and budding into multivesicular bodies are controlled by ARF6 and PLD2. *Nat.Commun.* 2014, Bd. 5.

66. **Latysheva, N., et al., et al.** Syntenin-1 is a new component of tetraspanin-enriched microdomains: mechanisms and consequences of the interaction of syntenin-1 with CD63. *Mol.Cell.Biol.* 2006, Bd. 26, 20, S. 7707-7718.

67. **Gupta, A.K., et al., et al.** A comparative study of the effect of three different syncytiotrophoblast micro-particles preparations on endothelial cells. *Placenta.* 2005, Bd. 26, 1, S. 59-66.

68. NanoSight Range - Visualize and measure nanoparticles size and concentration. [Online] Malvern Instruments Limited, UK;. [Zitat vom: 15. 12 2015.]

69. **Dragovic, R.A., et al., et al.** Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis. *Nanomedicine.* 2011, Bd. 7, 6, S. 780-788.

70. **Filipe, V., Hawe, A. und Jiskoot, W.** Critical evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates. *Pharm.Res.* 2010, Bd. 27, 5, S. 796-810.

71. **Szatanek, R., et al., et al.** Isolation of extracellular vesicles: Determining the correct approach (Review). *Int.J.Mol.Med.* 2015, Bd. 36, 1, S. 11-17.

72. **Hirayama, K., et al., et al.** Rapid confirmation and revision of the primary structure of bovine serum albumin by ESIMS and Frit-FAB LC/MS. *Biochem.Biophys.Res.Commun.* 1990, Bd. 173, 2, S. 639-646.

73. **Graham, J.** OptiPrepTM Density Gradient Solutions for Mammalian Organelles. *The Scientific World Journal.* 2002, Bd. 2, S. 1440-1443.

74. **Muller, L., et al., et al.** Isolation of biologically-active exosomes from human plasma. *J.Immunol.Methods.* 2014, Bd. 411, S. 55-65.

75. **Lorincz, A.M., et al., et al.** Effect of storage on physical and functional properties of extracellular vesicles derived from neutrophilic granulocytes. *J.Extracell Vesicles.* 2014, Bd. 3.

76. **Izak-Nau, E., et al., et al.** Altered characteristics of silica nanoparticles in bovine and human serum: the importance of nanomaterial characterization prior to its toxicological evaluation. *Part Fibre Toxicol.* 2013, Bd. 10, 1.

77. **Oosthuyzen, W., et al., et al.** Quantification of human urinary exosomes by nanoparticle tracking analysis. *J.Physiol.* 2013, Bd. 591, 23, S. 5833-5842.

Figures

Tables