**Diploma Thesis**

# The role of Siglecs (2,3 and 6) in the cellular internalization of placental exosomes

submitted by

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*Graz, 17.05.2025* *Jasmin Karner m.p.*

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# Zusammenfassung in Deutsch

## Hintergrund:

Plazentare Exosomen, die auf der mütterlichen Seite der Plazenta freigesetzt werden (pEXsM) sind in die materno-fetale Kommunikation während der Schwangerschaft involviert. Sie können in Abhängigkeit von ihrer Zusammensetzung zu unterschiedlichen funktionellen Veränderungen in ihren Zielzellen führen und somit einen Einfluss auf physiologische und pathophysiologische (z.B. Präeklampsie, Gestationsdiabetes) Prozesse während der Schwangerschaft haben. Die Plazenta produziert jedoch auch Exosomen (EXs) die auf der fetalen Seite der Plazenta (pEXsF) freigesetzt werden und möglicherweise ähnliche Funktionen wie pEXsM haben könnten. Bisher sind pEXsF jedoch weitestgehend unerforscht, weshalb es das Ziel dieser Arbeit ist neue Erkenntnisse über die Eigenschaften von pEXsF zu gewinnen.

## Hypothese und Zielsetzung:

Durch die Interaktion mit fetalen Zielzellen könnten pEXsF eine wichtige Rolle als Stoffwechselsensoren spielen. Da die fetale Leber neben der Plazenta das zentrale stoffwechselaktive Organ des Fetus ist und als erstes mit dem von der Plazenta kommenden Blut in Kontakt kommt, könnte es sein, dass Leberzellen mit pEXsF interagieren. Frühere Experimente haben bereits gezeigt, dass HepG2-Zellen, eine immortalisierte Leberkrebszelllinie, mit pEXsF interagieren. Darüber hinaus wurden in einigen Studien bereits Interaktionen zwischen EXs und Sialinsäure-bindenden Immunoglobulin-ähnlichen Lektinen (Siglecs) nachgewiesen und pEXsF exprimieren α2-6-verknüpfte Sialinsäure, einen Liganden für verschiedene Siglec-Rezeptoren. Ziel dieser Arbeit ist es daher, folgenden Fragen zu beantworten:

- Werden Siglec-2, -3 oder -6-Rezeptoren von HepG2-Zellen exprimiert?
- Gibt es einen temperaturabhängigen Unterschied in der Aufnahme von pEXsF in HepG2-Zellen?

## Materialien und Methoden:

Western-Blot-Analysen mit Antikörpern gegen Siglec-2, -3 und -6 wurden durchgeführt, um die Siglec-Expression auf HepG2-Zellen, Plazentagewebe und pEXsF zu untersuchen. Zusätzlich wurden Zellkulturstudien und

Immunfluoreszenzfärbungen bei 4°C und 37°C durchgeführt, da ausschließlich bei 37°C eine rezeptorvermittelte Internalisierung von pEXsF zu erwarten ist.

### Ergebnisse:

Mittels Western-Blot konnte gezeigt werden, dass Siglec-3 im Plazentagewebe und auf HepG2-Zellen exprimiert wird. Zur Bestimmung der Expression von Siglec-6 wurden zwei verschiedene ABs ausgetestet. Beide zeigten eine starke und spezifische Expression von Siglec-6 im Plazentagewebe. Zusätzlich zeigte der Siglec-6 AB von R&D Systems eine Siglec-6 Bande bei ~55kDa auf den pEXsF und bei ~55 sowie ~70kDa auf den HepG2-Zellen. Während mittels Immunfluoreszenzaufnahmen ebenfalls eine Expression von Siglec-3 und Siglec-6 gezeigt werden konnte, blieb eine eindeutige Ko-Lokalisierung dieser Proteine mit der GFP-transfizierten HepG2-Zellmembran aus. Außerdem waren in den Immunfluoreszenzaufnahmen nur sehr wenige pEXsF sichtbar, und eine direkte Assoziation zwischen einem Siglec-Rezeptor und einem pEXF konnte nicht beobachtet werden.

### Schlussfolgerung und Diskussion:

Es konnte erstmals gezeigt werden, dass HepG2-Zellen Siglec-3 und -6 exprimieren. Die Ergebnisse der temperaturabhängigen Zellkulturstudien entsprach jedoch nicht den Erwartungen, und es konnten keine klaren Assoziationen zwischen den Siglec-Rezeptoren, den pEXsF und den HepG2-Zellen gezeigt werden, sodass noch weitere experimentelle Untersuchungen notwendig sind. Zukünftige Experimente sollten die Verwendung verschiedener Zelllinien und spezifischer Inhibitoren für Siglec-Rezeptoren sowie die Optimierung des Experimentprotokolls für die Immunfluoreszenzfärbung hinsichtlich der pEXsF-Menge, der Inkubationszeiten und der Zellmembranfärbung in Betracht ziehen. Darüber hinaus sollten die Herkunft der pEXsF und die Untersuchung von pEXsF aus venösem Nabelschnurblut erwogen werden.

# Abstract in Englisch

**Background:**

Placental exosomes released to the maternal side of the placenta (pEXsM) are involved in the materno-fetal communication during pregnancy. Depending on their composition, they can cause a variety of functional changes in their target cells, thus inducing adaptions in physiological and pathophysiological pregnancies (preeclampsia, gestational diabetes etc.). The placenta, however, also releases pEXs to the fetal side of the placenta (pEXsF), which could potentially have similar functions as pEXsM, but are largely unexplored until now. Hence, the objective of this thesis is to provide new insights into the characteristics of pEXsF.

**Hypothesis and Aims:**

By interacting with target cells in the unborn child, pEXsF could play an important role as metabolic sensors. As the fetal liver is, besides the placenta, the central fetal metabolically active organ, being the first in line to have contact with blood coming from the placenta, it could be possible that liver cells interact with pEXsF. Previous experiments have already shown that HepG2 cells, an immortalized liver cancer cell line, interact with pEXsF. In addition, some studies have demonstrated specific interactions between Sialic acid-binding immunoglobulin-like lectins (Siglecs) and EXs and previous experiments have indicated that pEXsF carry α2-6-linked sialic acid, a ligand for various Siglec receptors. Therefore, the aim of this thesis is to contribute to the exploration of the fate of pEXsF by answering the following questions:

- Are Siglec-2, -3 or -6 receptors expressed on HepG2 cells?
- Is there a temperature-dependent difference in the uptake of pEXsF in HepG2 cells?

**Material and Methods:**

We analysed the Siglec expression on HepG2 cells, placental tissue and pEXsF by employing western blot analyses with antibodies targeting Siglec-2, -3 and -6. To visualise the Siglec receptors' expression on HepG2 cells and to further investigate their potential role in the uptake of pEXsF, cell culture studies and

immunofluorescence staining were carried out at 4°C and 37°C, given that the receptor-mediated uptake should only occur at 37°C.

## Results:

The results of the immunoblotting experiments demonstrated the expression of Siglec-3 only on placental tissue and HepG2 cells. To determine the expression of Siglec-6 we tested two different ABs. Both demonstrated a strong and specific expression of Siglec-6 in placental tissue. Additionally, the Siglec-6 AB from R&D Systems showed an expression of Siglec-6 at ~55kDa in pEXsF and at ~55 as well as ~70kDa in HepG2 cells. While immunofluorescence imaging revealed Siglec-3 and Siglec-6 AB-binding, a clear co-localization of these proteins with the GFP transfected HepG2 cell membrane failed. Furthermore, there were very few pEXsF visible in the immunofluorescence imaging, and a direct association between a Siglec receptor and a pEXF could not be observed.

## Conclusion and Discussion:

We could show that HepG2 cells express Siglec-3 and -6, which had not been previously described. However, the temperature-dependent uptake did not match expectations, and associations between Siglec-3 and -6, pEXs and HepG2 cells were inconclusive and require further investigations. Future experiments should consider using different cell lines and specific inhibitors for Siglec receptors, as well as optimizing the staining protocol for immunofluorescence staining regarding the pEXsF amount, incubation periods, and membrane staining. Furthermore, the origin of pEXs and the investigation of pEXs coming from venous cord blood should be considered.

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# Abbreviations

| | |
|---|---|
| AB | antibody |
| ALIX | ALG-2 interacting protein X |
| ApoBds | apoptotic bodies |
| ARF6 | ADP-ribosylation factor 6, ADP-ribosylation factor 6 |
| ARRDC1 | arrestin 1 domain-containing protein 1 |
| A-SMase | activated acid sphingomyelinase |
| BCA | bicinchoninic acid |
| BSA | bovine serum albumin |
| C19MC miRNAs | miRNAs from the chromosome 19 miRNA cluster |
| CD | cluster of differentiation |
| CHMP | charged multivesicular body proteins |
| CHMP6 | charged multivesicular body protein 6 |
| CMV | cytomegalovirus |
| DISC | death-inducing signalling complex |
| DMEM++ | Dulbecco's Modified Eagle Medium ++ |
| EAP20 | ELL-associated protein 20 |
| ERK | extracellular-signal-regulated kinase, extracellular signal-regulated kinase |
| ESCRTs | endosomal sorting complexes required for transport |
| EVs | extracellular vesicles |
| EXs | exosomes, exosomes |
| FADD | Fas-associated death domain |
| FasL | fas ligand |
| FCS | foetal calf serum |
| FGR | fetal growth restriction |
| fpAECs | Fetoplacental arterial endothelial cells |
| GADPH | glyceraldehyde 3-phosphate dehydrogenase |
| GDM | gestational diabetes mellitus |
| GRB2 | growth factor receptor-bound protein 2 |
| HBSS | Hanks' Balanced Salt Solution |
| HD-PTP | His domain protein tyrosine phosphatase |
| HLA-G | human leukocyte antigen G |
| HRS | hepatocyte growth factor-regulated tyrosine kinase substrate |

| | |
|---|---|
| HSP | heat shock protein |
| ICAMs | intercellular adhesion molecules |
| ILVs | intraluminal vesicles |
| ITIMs | immune receptor tyrosine-based inhibitory motifs |
| MHC | major histocompatibility complex |
| MLCK | myosin light chain kinase |
| MT1-MMP | membrane type-1 matrix metalloproteinase |
| MVB12 | multivesicular body subunit 12 |
| MVBs | multivesicular bodies |
| MVs | microvesicles |
| Neu5Ac | N-acetyl-neuraminic acid |
| Neu5Gc | N-gycolyl-neuraminic acid |
| NK | natural killer |
| NTA | 2.5.1 Nanoparticle tracking analysis |
| PD-L1 | programmed cell death ligand-1 |
| PE | preeclampsia |
| pEVs | placental extracellular vesicles |
| pEXs | placental exosomes |
| pEXsF | placental exosomes released to the fetal side of the placenta |
| pEXsM | placental exosomes released to the maternal side of the placenta |
| PLAP | placental alkaline phosphatase |
| PLD | phospholipase D |
| PS | phosphatidylserine |
| PT | placental tissue |
| PtdIns3P | phosphatidylinositol-3-phosphate |
| RAB | ras-related in brain |
| RABPs | RAS-associated binding proteins |
| RBPs | RNA binding proteins |
| ROCK | RhoA/Rho-associated kinase |
| sABs | secondary antibodies |
| Siglec | sialic acid binding immunoglobulin-like lectin |
| SLE | systemic lupus erythematosus |
| SNA | Sambucus nigra agglutinin |
| SNARE | soluble N-ethylmaleimide-sensitive factor attachment protein receptor |

| | |
|---|---|
| SSA | Sambucus sieboldiana agglutinin |
| STAM | signal transducing adaptor molecule |
| tfR | transferrin receptor |
| TGN | trans-Golgi network |
| TJA-I | Trichosanthes japonica agglutinin-I |
| TNF | tumour necrosis factor |
| TRADD | TNF receptor-associated death domain |
| TRAIL | tumor necrosis factor-related apoptosis-inducing ligand |
| TSG101 | tumour susceptibility gene 101 |
| Tspan | tetraspanin |
| UBAP | ubiquitin-associated protein |
| VAMP | vesicle-associated membrane protein |
| VPS | vacuolar protein sorting-associated protein |
| VTA | vacuolar protein sorting-associated protein |

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# 1 Introduction

During pregnancy, the mother and foetus are inextricably linked, which is crucial for the development of the fetus. In spite of this, the fetus is a foreign body to the woman's organism and pregnancy is therefore associated with a number of physiological changes. The placenta plays a key role in this process, as it is central to the separation of the maternal and fetal circulations, as well as being the hub of communication between them. Among other factors, placental extracellular vesicles (pEVs), and in particular placental exosomes (pEXs), appear to be involved in healthy adaptations, such as maternal systemic immune tolerance, embryo development, implantation, decidualisation, placentation and timing of parturition. In addition, an increasing body of evidence indicates that pEVs are involved in pathophysiological processes during pregnancy such as preeclampsia, gestational diabetes, and fetal growth restriction (1,2).

As an introduction to this thesis, extracellular vesicles (EVs) in general, with the main emphasis on exosomes (EXs), will be presented first, before in-depth information on the placenta, pEXs and sialic acid binding immunoglobulin-like lectin (Siglec) receptors is given.

## *1.1 Extracellular vesicles (EVs)*

EVs are nano-sized particles released by the majority of cell types into the extracellular space and can be found in all body fluids, e.g. blood, urine, sweat, cerebrospinal fluid, saliva, tears, breast milk, amniotic fluid and cord blood (3–10). For several years after the discovery of EVs, more than 70 years ago, they were considered to be nothing more than a cellular waste-disposal system, until their involvement in multiple physiological and pathological processes has been discovered and stated by various studies (11–16). Today, they are acknowledged as mediators of intercellular communication and their diagnostic, prognostic and therapeutic potential, is of great interest (17).

### 1.1.1 General composition

EVs are surrounded by a phospholipid bilayer membrane that provides a protected lumen for the transport of biomolecules through the human body (18). They contain different types of lipids, proteins, metabolites and nucleic acids, and through the process of selective cargo sorting, their purpose and function result in increased or decreased levels of certain components (18,19). Figure 1-1 schematically shows an exemplary EV and some of its potential components. It is important to note, that not all of them are present in every EV and that the specific content depends on multiple factors, e.g. the EV subtype, the cell of origin, the environment, the purpose and the function (19–21). The list of components is immense, and ongoing research still discovers new biomolecules, making the online databases EVpedia (http://evpedia.info) and Vesiclepedia (http://microvesicles.org/) very useful in understanding the complexity of EVs (22,23).

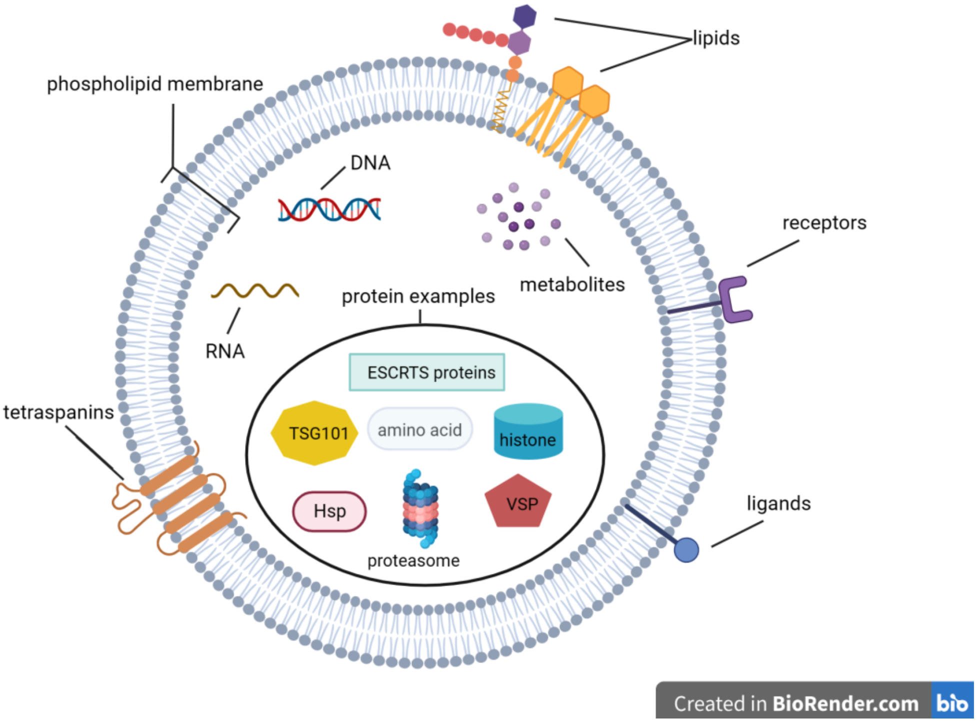

**Figure 1-1. Scheme of an EV and its composition.** Embedded in the EVs’ membrane are surface components, which are often involved in the interaction with their surroundings, e.g. other EVs or target cells: **[1] tetraspanins**: e.g. CD9, CD63, CD81, CD82, Tspan6, Tspan8, **[2] lipids**: e.g. lipid rafts, cholesterol, phosphatidylserine, sphingolipids, **[3] receptors:** e.g. TfR, MHC molecules **[4] others**: e.g. flotillin, VAMP3, glycans, cell adhesion molecules (e.g. integrins, cadherins, ICAMs), ligands ( PD-L1, WNT, FasL) (18,19,21,24). Intraluminal EVs contain: **[a] nucleic acids**: various DNAs and RNAs, **[b] metabolites:** e.g. amino acids, carbohydrates, vitamins, lipids, **[c] proteins:** e.g. cytoskeletal proteins, histones, ribosomal proteins, proteasomes, HSP 70 and 90, RABPs, GAPDH, annexins, RBPs, ARF6, ubiquitin, syntenin, ERK, clathrin, ESCRTs proteins (e.g. ALIX, TSG101, PLD, CHMP, VPS) (18,19,21,24)**.** Several of the described components can be involved in the EVs’ biogenesis (e.g. tetraspanins, lipid rafts, RBPs, ARF6, ubiquitin, syntenin, ERK, clathrin,

ESCRTs proteins**)** (18,19,24). This image was created with BioRender.com. *Abbreviations: CD, cluster of differentiation; Tspan, tetraspanin; tfR, transferrin receptor; MHC, major histocompatibility complex; ICAMs, intercellular adhesion molecules; VAMP, vesicle-associated membrane protein; PD-L1, programmed cell death ligand-1; FasL, fas ligand; HSP, heat shock protein; RABPs, RAS-associated binding proteins; GADPH, glyceraldehyde 3-phosphate dehydrogenase; RBPs, RNA binding proteins; ARF6, ADP-ribosylation factor 6; ALIX, ALG-2 interacting protein X; ERK, extracellular-signal-regulated kinase; VPS, vacuolar protein sorting-associated protein; ESCRTs, endosomal sorting complexes required for transport; TSG101, tumour susceptibility gene 101; PLD, phospholipase D; CHMP, charged multivesicular body proteins.*

### 1.1.2 Isolation of EVs

In the last couple of years, isolation protocols for EVs have been published, enabling a verifiable and in-depth study of the biological role of EVs (25). However, there is still a lack of standardised reproducible isolation and characterisation protocols available, resulting in differing and difficult-to-interpret results (26). Additionally, it is still hard to separate the different EV sub-groups from each other (27). The different approaches applied, with divergent readouts, heavily determine characteristics of EVs. (25). The currently used isolation methods are based either on size (e.g. ultrafiltration, size exclusion chromatography, hydrostatic filtration dialysis), on composition (immunoaffinity-based capture techniques like ELISA or immunomagnetic methods) or on density and size (e.g. ultracentrifugation, density gradient centrifugation) (25). Ultracentrifugation is the most commonly used approach (28). This approach uses the different sedimentation rates of particles with different sizes and densities to separate EV sub-groups from each other (25). Now, several new methods are under development to mitigate some of the challenges of current separation methods (28). They could, for example, enhance the purity of isolates, lower the EV damage, which may occur during isolation, and expedite the isolation process, making EVs as a diagnostic tool more and more attractive (13,28). In particular, microfluidics-based technologies, which combine several different processes based on size, density and immunoaffinity, seem to be very promising (29).

### 1.1.3 Sub-groups of EVs

EVs are a population of membrane vesicles with a large variety that can be divided into sub-groups depending on their origin, biogenesis, composition, size, and function (19). Nonetheless, there is an overlap between the different sub-groups.

The lack of standardised isolation and characterisation protocols, the lack of validated biomarkers, contradictory definitions and the fact that research continuously reveals new information, lead to misconceptions and confusion regarding the nomenclature of EV subgroups (16,30,31). The amount of research data keeps increasing, and it keeps getting harder to maintain the overview. In an attempt to counteract the unmanageable abundance of information on EVs, the EV-TRACK platform (https://evtrack.org) was launched to centralise research data and enhance the standardisation, reproducibility, and transparency of EV research (32). Figure 1-2 illustrates the main EV subpopulations for better comprehension.

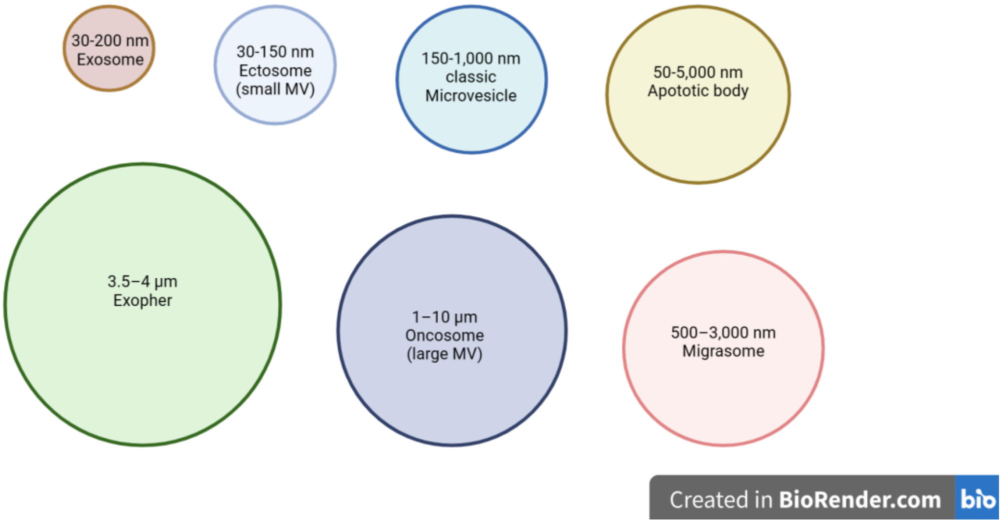

**Figure 1-2. Overview of the EV subpopulations.** Subgroups that can be distinguished from each other are exosomes, microvesicles, apototic bodies, migrasomes and exophers (30,33). So far, oncosomes have been classified as large microvesicles and ectosomes as small microvesicles because they share the same biogenesis. However, their sizes do not align with the classic microvesicle group. (150-1,000 nm) (30). Another form of classification is the division of EVs simply into large/ medium EVs (>200nm) and small EVs (<200nm) (31). This image was created with BioRender.com.

Apoptotic bodies (ApoBds), microvesicles (MVs) and exosomes (EXs) are currently considered as the three main subgroups. There are given similarities between the groups, but their different biological roles and distinct features may serve as distinguishing characteristics. Nevertheless, the International Society for Extracellular Vesicles suggests a restrained use of specific terms, such as apoptotic bodies (ApoBds), microvesicles (MVs) and exosomes (EXs) (31).

## *1.2 Apoptotic bodies (ApoBds)*

ApoBds are the largest group of EVs. Depending on the source, their size ranges from 50 to 5,000 nm (30,34). They are produced by apoptotic cells, following programmed cell death and are, therefore, part of an important biological fate of cells in normal physiology, particularly in immune homeostasis, and in pathogenic processes (35).

### 1.2.1 Biogenesis and composition of ApoBds

In the human body, approximately a million cells undergo programmed cell death every second and need to be removed efficiently (35). Therefore, they recruit phagocytes, which engulf either the whole apoptotic cell or small parts of the cell, better known as ApoBds (36). There are two ways to induce the production of ApoBds, either by an extrinsic or an intrinsic pathway (37). Following the intrinsic or mitochondrial pathway, stimuli such as drugs, hypoxia, radiation, or the absence of growth factors cause increased permeability of the mitochondrial outer membrane by inducing apoptosis regulatory proteins of the BCL-2 protein family (34,37). That eventually leads to the activation of caspase-9 (34). The extrinsic or death receptor pathway involves transmembrane receptors (36). The binding of the FasL and tumour necrosis factor (TNF) ligand leads to the binding of the Fas-associated death domain (FADD) protein and the TNF receptor-associated death domain (TRADD) protein, resulting in the formation of the death-inducing signalling complex (DISC), which activates caspase-8 (34,37).

The execution phase of the extrinsic and intrinsic pathways is similar, as caspase-8 and caspase-9, both, activate the “execution” or “effector” caspases 3, 6 and 7 (38). As a result, the cell begins to disintegrate, and its components are cleaved and degraded. This is followed by the formation of ApoBds through membrane blebbing, the formation of membrane protrusions (microtubule spikes, apoptopodia and beaded apoptopodia) and finally the fragmentation of the apoptotic cell (34,36,37).

ApoBds are composed of remnants of the apoptotic cells, like cytoplasmatic components, nuclear contents and residual organelles, which are a characteristic feature of ApoBds (34,36). Several surface markers to identify ApoBds have already been discussed, above all, thrombospondin, C3b and phosphatidylserine (PS) are the most prominent ones. However, PS has also been found on MVs and EXs and their uniqueness to ApoBds remains controversial (18,31,34,38).

### 1.2.2 Functions and clinical applications of ApoBds

ApoBds contribute to the efficient removal of dead cells, by ensuring controlled cell fragmentation and by influencing their environment and the immune response (34,38). When released into the extracellular space, PS-binding Annexin V, thrombospondin and complement protein C3b act as “eat me” signals, leading to the recognition and non-inflammatory clearance of ApoBds by tissue-resident and non-professional phagocytes (35,36,39). In addition, the uptake of ApoBds delivers useful components to target cells and can affect them, for example by the horizontal transfer of genetic material, such as DNA, oncogenes or miRNA (36,38).

It has already been demonstrated that ApoBds could be used therapeutically since studies showed that they could enhance angiogenesis and tissue regeneration and inhibit tumour growth and inflammation (34). Furthermore, they could be used in diagnostics (e.g. for cancer and graft-versus-host disease) and to develop anti-tumour vaccines (e.g. for myeloid leukaemia) (34). Additionally, their “eat me” signals have been successfully applied in studies investigating macrophage-targeting drug delivery (34). ApoBds and defective apoptosis seem to be involved in multiple diseases, e.g. infectious diseases (e.g. influenza A and HIV), autoimmune diseases (e.g. systemic lupus erythematosus (SLE), or rheumatoid arthritis), tumour diseases (e.g. lung cancer and prostate cancer), cardiovascular diseases (e.g. myocardial infarction and atherosclerosis) and neurodegenerative diseases (e.g. Alzheimer’s disease, Parkinson’s disease and prion disease) (34,36). In conclusion, the clinical potential of ApoBds in diagnostics, prognostics and therapeutic intervention is not yet fully developed and with that, there is a need for additional research to confirm and explore their application possibilities further.

## *1.3 Microvesicles (MVs)*

MVs originate directly from the plasma membrane and are synonymously known as microparticles, blebbing vesicles, shedding vesicles, oncosomes, ectosomes, ARRMs, migrasomes, neurospheres and sometimes even as apoptotic bodies (24,40). To prevent further confusion, only the term MV should be used henceforth

(40). The MVs' size ranges from 150-1,000 nm, and they are overall larger than EXs and smaller than ApoBds. However, despite advancements in the isolation and purification procedures of MVs, no protocol has yet been published to separate these different particles (30,31,41).

### 1.3.1 Biogenesis and composition of MVs

Cellular growth, apoptosis, or external factors, such as hypoxia can induce the formation of MVs by a process called “plasma membrane outward budding”. The mechanism is similar to the formation of ApoBds, followed by the actin-myosin-driven fission of the plasma membrane (18,41,42). However, most of the molecular mechanisms behind the outward budding are still unclear (19). Several factors have been described that could be involved in the formation and release of MVs. For example, hypercalcaemia, hypoxia, activated acid sphingomyelinase (A-SMase), ADP-ribosylation factor 6 (ARF6), ALIX, PLD, several kinases (e.g. RhoA/Rho-associated kinase (ROCK), extracellular signal-regulated kinase (ERK) and myosin light chain kinase (MLCK) and ESCRTs components such as TSG101, and VPS 4, which are also involved in the EXs biogenesis (19,43).

Besides the general EV composition described in 1.1.1, certain molecules become enriched during the formation of MVs. (18). Examples of these molecules are ARF6, major MHC-I, β1-integrin, VAMP3, PS, membrane type-1 matrix metalloproteinase (MT1-MMP), TSG101, arrestin 1 domain-containing protein 1 (ARRDC1), selectins, flotillin-2, CD40 and cholesterol (44–46). Currently, ARF6, selectins, flotillin-2, CD40 and VAMP3 are the most promising candidates as MV-specific markers (18,46).

### 1.3.2 Functions and clinical applications of MVs

MVs have been reported to be produced by a variety of different cell types, but it has been shown that mainly platelets, red blood-, endothelial- and tumour cells secrete MVs (47,48). As an important part of the intercellular communication machinery, MVs get recognised by their target cells and can affect these cells by membrane and intracellular components (41). MVs are used as transport vehicles to transfer biological information, and biomolecules, such as receptors or nucleic acids, (41). In addition, MVs have the capability to release their components into the extracellular space, thereby altering the extravesicular milieu (48).

MVs are involved in physiological and pathological cellular processes (41). Nevertheless, the specific functions of MVs are not distinctly attributed to them. Almost all pathologies linked to ApoBds, as described in 1.2.2, can be found to be linked to MVs as well (41). Additionally, MVs have been associated with apoptosis, the regulation of immunological responses and homeostasis, the unconventional export of cellular proteins, epigenetic reprogramming, angiogenesis, coagulation, feto-maternal communication, cellular repair, and cellular waste disposal (41,47,48). Notably, several studies looked at MVs and their role in different tumours (e.g. breast cancer, lung cancer and prostate cancer). To conclude, MVs are tumorigenic vesicles that support tumour development and progression, e.g. by increasing the cells' adhesion ability, by releasing metalloproteinases thereby favouring invasion, or by supporting the cells to escape the immune surveillance (40,41,48). Due to MVs' wide range of involvement in health and disease, the diagnostic and therapeutic potential of MVs is immense (e.g. as biomarkers, drug delivery vehicles and therapeutic targets in personalized patient care) (46,48). However, further research is needed to establish a universal EV classification, thereby avoiding confusion and ensuring reliable results.

## *1.4 Exosomes (EXs)*

EXs are the best-described group of EVs, characterized by their smaller size (between 30-200 nm), compared to MVs and ApoBds (30,33). However, a lack of standardization exists, and the term EXs is sometimes used generically to describe EVs, leading to inconclusive interpretations of results (49).

### 1.4.1 Biogenesis of EXs

The formation of EXs is a complex process, which is illustrated and described in Figure 1-3. Two different pathways classify the biogenesis of EXs, although there are still discussions about their coexistence and their contribution in the formation of different subpopulations of EXs (50). One of the pathways is ESCRT machinery-driven, and the other one is ESCRT-independent (50).

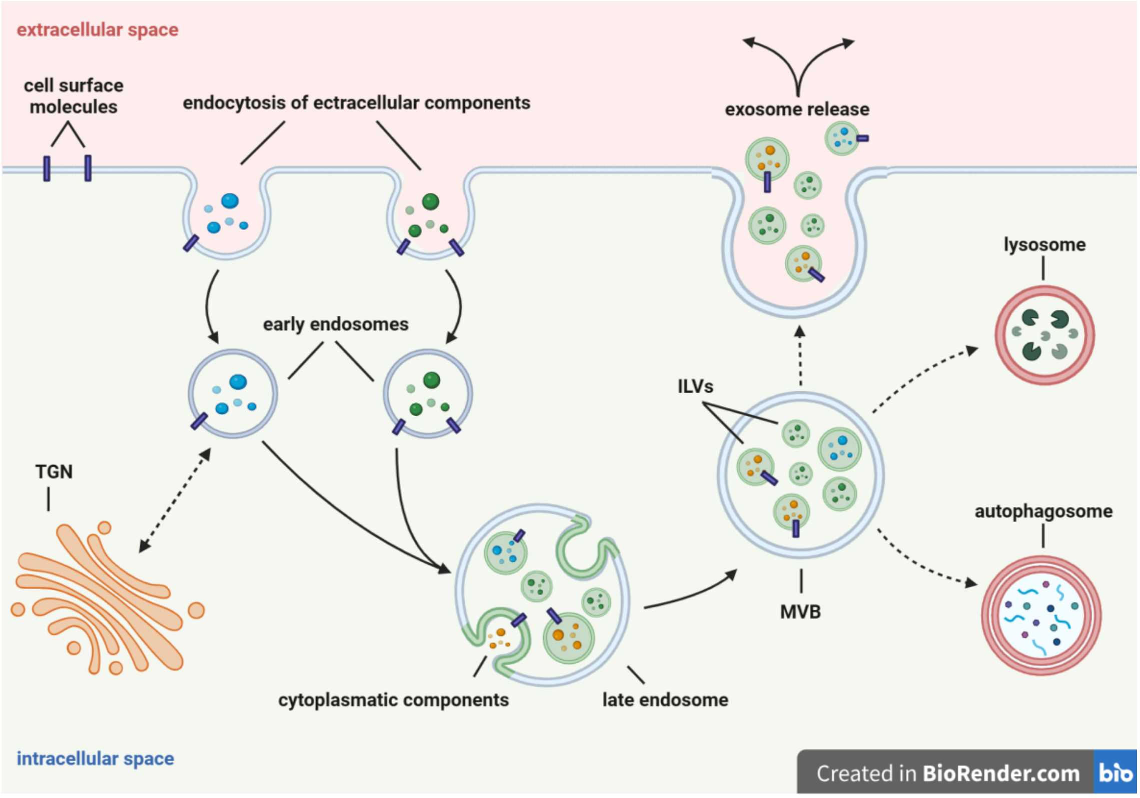

**Figure 1-3. General biogenesis of the EX.** After endocytosis of extracellular components early endosomes are formed (19). Next, their maturation process involves material exchange with the Golgi apparatus, the fusion of early endosomes and the formation of ILVs, leading to the formation of late endosomes and, eventually, MVBs (50). MVBs fuse with either autophagosomes or lysosomes for recycling or degradation of included material, respectively. Alternatively, MVBs may fuse with the cell plasma membrane to release ILVs as EXs into the extracellular space (51). This image was created with BioRender.com.

EXs generally originate from early endosomes (19). Formed from endocytic vesicles, early endosomes are formed when extracellular components are transported into the cell by invagination of the plasma membrane (19). Their composition can vary depending on whether they are destined for recycling, degradation, or exocytosis, and they can exchange components with the trans-Golgi network (TGN) (19). Subsequently, late endosomes and, later on, multivesicular endosomes/ multivesicular bodies (MVBs) emerge through a stepwise process, which is characterised by the fusion of early endosomes, the formation of intraluminal vesicles (ILVs) and their loading with specific components (proteins, lipids, nucleic acids and other cytoplasmic components) (39). Finally, MVBs either fuse with the plasma membrane, releasing ILVs into the extracellular space as EXs, or they fuse with lysosomes or autophagosomes for degradation (51). For the extracellular release, the MVBs are transported to the plasma membrane through interactions with the microtubule cytoskeleton and the binding of cortactin to actin (50). Docking of MVBs to membrane components and the subsequent fusion of MVBs with the plasma membrane is mediated by ras-related in brain (RAB) GTPases and the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex (19,50).

### ESCRT-dependent MVB formation

Following the ESCRTs-dependent pathway, the maturation process from early endosomes to late endosomes and MVBs involves the endosomal sorting complexes required for transport (ESCRTs) described in Table 1-1. This pathway is highly dependent on the ubiquitination of proteins, which are then recognized by the ESCRTs machinery through their ubiquitin moieties and sorted inside the vesicles (19). The ESCRT machinery is initiated by the binding of the hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) to endosomal phosphatidylinositol-3-phosphate (PtdIns3P) and through the recognition of the ubiquitinated endosomal cargo (19,52). As part of ESCRT-0 HRS recruits clathrin to form microdomains in the endosomal membrane, where ubiquitinated proteins are sorted (19,51). Subsequently, HRS interacts with TSG101, a subunit of ESCRT-I (19). ESCRT-I and -II are, together with ESCRT-0, responsible for cargo sorting, and serve as the major driving forces in promoting endosomal membrane budding around clusters of ubiquitinated proteins (52). ESCRT-II initiates the assembly of ESCRT-III through the interaction of the subunits ELL-associated protein 20 (EAP20) and charged multivesicular body protein 6 (CHMP6) (52). ESCRT-III is essential for cargo deubiquitination and endosomal membrane rupture, enabling the release of ILVs into the MVB lumen. Additionally, it plays a role in MVB trafficking to the plasma membrane (52,53). The interaction between ESCRT-III and the AAA-ATPase VPS4 completes the MVB formation, and ESCRT-III is degraded and recycled for another round of MVB formation, terminating the ESCRT machinery (52). However, the stepwise manner described above is not the only way in which ESCRTs-dependent MVB formation occurs (19). Some molecules interact with ESCRTs and are considered to be important accessory components (19). For example, the His domain protein tyrosine phosphatase (HD-PTP) can recruit ESCRT-0, -I, and -III while substituting ESCRT-II (19). Another example is ALIX, which can directly recruit

ESCRT-III and is closely involved in ILV biogenesis and cargo sorting, in combination with syndecan and syntenin-1 (19).

**Table 1-1. Summary of the five ESCRT subunits and their function.** ESCRTs have been identified not only in mammals but also in yeast (52). Therefore, several subunits are known by different names depending on their origin, for example, TSG101 is known as VPS23 in yeast (52). It should also be noted that there are several accessory components, such as PtdIns3P, ALIX, VTA1 or HD-PP, that are also crucial for the ESCRT system but are not listed below (19,52,53). *Abbreviations: VTA, vacuolar protein sorting-associated protein; STAM, signal transducing adaptor molecule; MVB12, multivesicular body subunit 12; UBAP, ubiquitin-associated protein.*

| ESCRTs | Subunits (human) | Primary functions |
|---|---|---|
| ESCRT-0 | HRS, STAM1 and 2 | - recognition of the ubiquitinated endosomal cargo<br>- clathrin recruitment<br>- ESCRT-I recruitment |
| ESCRT-I | TSG101, VPS28, VPS37, MVB12/UBAP1 | - ubiquitinated cargo sorting<br>- membrane budding for ILV formation<br>- ESCRT-II recruitment |
| ESCRT-II | EAP20, EAP30, EAP45 | - ubiquitinated cargo sorting<br>- membrane budding for ILV formation<br>- initiation of ESCRT-III assembly |
| ESCRT-III | CHMP1, CHMP2, CHMP3 CHMP4, CHMP5, CHMP6, CHMP7 | - cargo deubiquitination<br>- membrane budding and scission<br>- interaction with VPS-4 |
| VPS-4 | | - marking the end of the MVB formation by triggering the disassembly and recycling of ESCRT-III |

### ESCRT-independent MVB formation

It has been observed that MVB and, hence, EX formation still occurs in the absence of ESCRTs, and that cargo sorting is not solely dependent on ubiquitin recognition (50,54). Two major alternative routes have been identified. On the one hand, transmembrane proteins of the tetraspanin family cluster proteins and are, therefore, involved in the cargo sorting and ILV maturation, and they have been shown to enhance the release of EXs (50). On the other hand, lipid rafts may be involved in cargo packing, while other lipids, mainly ceramides produced by sphingomyelinases, may influence membrane quality, as well as membrane budding, fission and fusion (50,55).

### 1.4.2 Composition and markers of EXs

The general composition of EXs is the same as described in 1.1.1. In addition, the content of EXs can be highly heterogeneous, and even EXs released from one cell line may contain different components (50,55). Proteins that may be enriched in EXs include cell adhesion molecules (e.g. integrins, lactadherin, ICAMs), HSP 70 and 90, cytoskeletal components (e.g. actin), metabolic enzymes (e.g. peroxidases) and signalling receptors and ligands (e.g. TfR, TNF receptor, MHC I and II, FasL and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)) (56,57). Lipids that have been identified are cholesterol, phosphatidylcholine, PS, phosphatidylethanolamine phosphatidylinositol and sphingolipids (e.g. ceramides, sphingomyelins, glycosphingolipids) (56). They are important for the membrane structure, and besides, some may also be involved in EX biogenesis, signalling and uptake (24,55). Certain proteins, as well as lipids, can be glycosylated with N-linked and O-linked glycans, which are currently investigated for cargo sorting and EX uptake (58–60). Other biomolecules involved in the biogenesis, release and uptake of EXs include flotillins, ubiquitin, annexin, enzymes (e.g. RAB GTPases), tetraspanins (e.g. CD9, CD37, CD63, CD81, CD82), ALIX, syntenin and ESCRTs components, particularly TSG101 and VPS4 (24,56). Other components include metabolites, nucleic acids that can either transfer protein-coding information or alter the genetics of the target cell, and last but not least, cell type-specific components, such as placental alkaline phosphatase (PLAP) for placental EXs or the glutamate receptor subunit GluR3/2 for neuronal exosomes (2,56,61–63).

Several of these components are used to identify EXs. Common markers are TSG101, ALIX, HSP70, CD81, CD63, and CD9, as well as tissue-specific markers (55,64). However, it is important to note that some of these markers are not unique to EXs, e.g. TSG101 or ALIX can also be found in MVs (39,46,64). The International Society for Extracellular Vesicles recommends quantification of at least one protein from each of the following groups in all EX-isolates: transmembrane proteins (e.g. CD9, CD63 or CD81), cytosolic proteins with membrane binding capacity (e.g.

TSG101), and an intracellular protein that is expected to be absent in EXs (e.g. HSP90B1) as negative control (49).

### 1.4.3 Uptake and interaction with target cells

Once EXs are released into the extracellular space, they circulate in body fluids until they reach their destination and migrate through several tissue layers, probably by transcytosis, to get to their target cells (56,65). However, it is unknown whether and how they are actively and specifically transported to their target organs, or whether they reach them stochastically (65). Once they get there, EXs can affect their target cells in a number of different ways, as shown in Figure 1-4 - either by receptor-ligand interaction, by membrane fusion, or by one of the many forms of endocytosis (56). The first possibility is the fusion of the EX with the plasma membrane of the cell, releasing the contents of the EX inside the cell, which is particularly important for the delivery of proteins and nucleic acids to target cells (57). The second possibility is the recognition of EX-bound ligands by their receptors on the cell surface, which induces a cellular response through the activation of intracellular signalling pathways (65). This is critical for specific interactions and can only occur when the right combination of receptor and ligand is present (19). Additionally, a second type of receptor-ligand interaction has been described, where the ligands are cleaved from the exosomal surface and then act as free soluble signals (57). The third possibility is the endocytosis of whole exosomes, with receptor-mediated endocytosis being the most common (19,65). It is also known as clathrin-mediated endocytosis, implying that clathrin is essential for this process (56). Other forms of endocytosis include phagocytosis, non-selective macropinocytosis, and lipid raft-mediated endocytosis, together with caveolin-mediated endocytosis (57,66). Once endocytosed, exosomes have different fates, such as being targeted for lysosomal degradation or for re-secretion via the MVB pathway (56). Recently, another possibility has been described in which their cargo is delivered to the cell surface thereby triggering cellular responses, though the underlying mechanisms remain unknown (19,57).

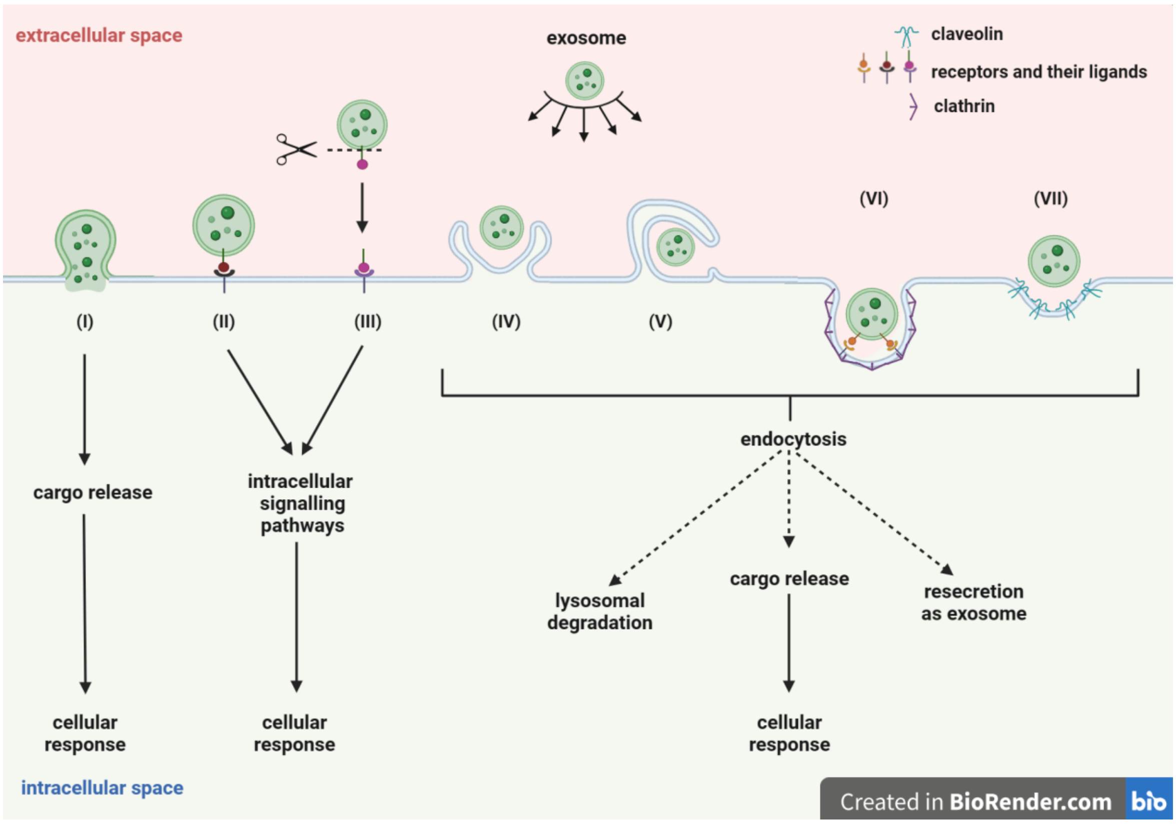

**Figure 1-4. Possible interactions of EXs with their target cells.** There can be either fusion of EXs with the plasma membrane (I), receptor-ligand interaction (II) and (III), or uptake through various forms of endocytosis: phagocytosis (IV), macropinocytosis (V), receptor-mediated endocytosis (VI) and lipid raft-mediated endocytosis (VII) (19,56,57). This image was created with BioRender.com.

### 1.4.4 The role of EXs in physiological and pathophysiological processes

EXs are produced by almost all cell types and can be found in all body fluids, including blood, saliva, urine, semen, synovial fluid, breast milk and amniotic fluid (13,67). For many years, it was thought that their sole purpose was to remove cellular waste, but although this is important, it is now known that EXs do much more (13). Depending on their origin, they are involved in the removal of cellular debris as well as in the maintenance of cellular homeostasis, and they have been identified as an important mechanism of cell-to-cell communication by having an autocrine, paracrine, or endocrine messaging function (65). In recent years, their role in immunological and metabolic processes, coagulation, apoptosis, and angiogenesis has been discovered (67). In immunology, for example, they have been identified as antigen presenters, activators of T cells, up- and down regulators of the cellular response of immune cells, and communicators between the maternal and fetal immune systems during pregnancy (51,68–70). However, many of their other specific functions remain unclear as research is still in its infancy (67).

In addition, EXs appear to be involved in various pathophysiological processes, either as promotors or inhibitors (67). Like MVs, they have been shown to assist cancer cells in local invasion, environmental protection, drug resistance and immune evasion, as well as playing a role in angiogenesis and metastasis (73,74). EXs have also been implicated in immune dysfunction (e.g. autoimmune diseases), inflammation and the spread of infectious diseases (e.g. SARS-CoV-2, prion diseases) (75-78). As part of the Trojan exosome hypothesis, HIV-carrying EXs are thought to infect dendritic cells and thereby maintain HIV infection (79). They have also been implicated in cardiovascular diseases (e.g. atherosclerosis, heart failure), renal diseases (e.g. acute kidney injury), neurological diseases (e.g. Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, autism spectrum disorder) and pathophysiological processes during pregnancy, which will be discussed in more detail in Chapter 1.5.5 (13,69,80).

### 1.4.5 Clinical applications of EXs

As exosomes are involved in several diseases, their potential applications are currently under investigation. It has already been shown that the content of EXs produced under physiological and pathophysiological conditions differs, which could be used diagnostically to infer the cause of certain diseases (13). Exosomal contents, such as specific RNAs, could be used as biomarkers in cancer (e.g. prostate cancer, oesophageal squamous cell cancer, pancreatobiliary tract cancer, hepatocellular carcinoma), renal diseases (e.g. acute kidney injury) and autoimmune diseases (e.g. rheumatoid arthritis, SLE, autoimmune thyroiditis), to assess treatment response and to monitor the progression of certain diseases like Alzheimer’s (71–80). While being far less invasive than a conventional biopsy, EXs may help to establish liquid biopsy as a diagnostic and prognostic tool in the near future (78). Additionally, their ability to target specific tissues and alter the cellular response of their target cells offers opportunities for gene therapy or targeted drug delivery, even to sites that are not yet accessible (13). The emerging field of research around bioengineered EXs could be of great importance for cancer therapy, anti-cancer vaccines, immunomodulation, and the promotion of tissue regeneration, e.g. in wound repair, type I diabetes or after myocardial infarction (13,79,81–83). However, although promising in vitro study results have been obtained, translation to in vivo applications remains a challenge for the coming years of research (13).

## *1.5 Exosomes in pregnancy*

In recent years, the role of EVs in the spatial-temporal process of pregnancy has been discovered. The level of EVs, especially of EXs, in the systemic circulation increases significantly during pregnancy and is, on average, 3.14 times higher in healthy pregnant women than in non-pregnant women (84). This suggests that EVs have pregnancy-specific functions, which are currently under investigation. It has been shown that EVs are heavily involved in the genesis of sperms and ova, the fertilisation and implantation process, and continue to play a role throughout pregnancy until the birth of the child (85,86). Various tissues of the reproductive system, including amniotic membranes and uterine tissue, secrete EXs, which are relevant in the context of pregnancy (85–90). However, the placenta secretes approximately 15-20% of total circulating EXs, suggesting that they have a distinct impact on both the mother and the fetus (91).

### 1.5.1 The human placenta

The placenta has been considered as waste material after birth in the past, and even if this point of view has already changed for a long time in the scientific context, researchers face challenges regarding ethical boundaries and the accessibility of the placenta in vivo (92). Placental research is often limited to post-delivery examinations, which is not a completely accurate representation of the organ in vivo, even if methods like the dually ex-vivo perfusion of the placenta are already advanced technologies (92,93). However, it is known that the placenta is not only the first and largest fetal organ to develop, but also influences both mother and child during pregnancy, potentially affecting them for the rest of their lives (94). The placenta is crucial for many physiological processes and its dysfunction may cause maternal and/or fetal pathologies (95).

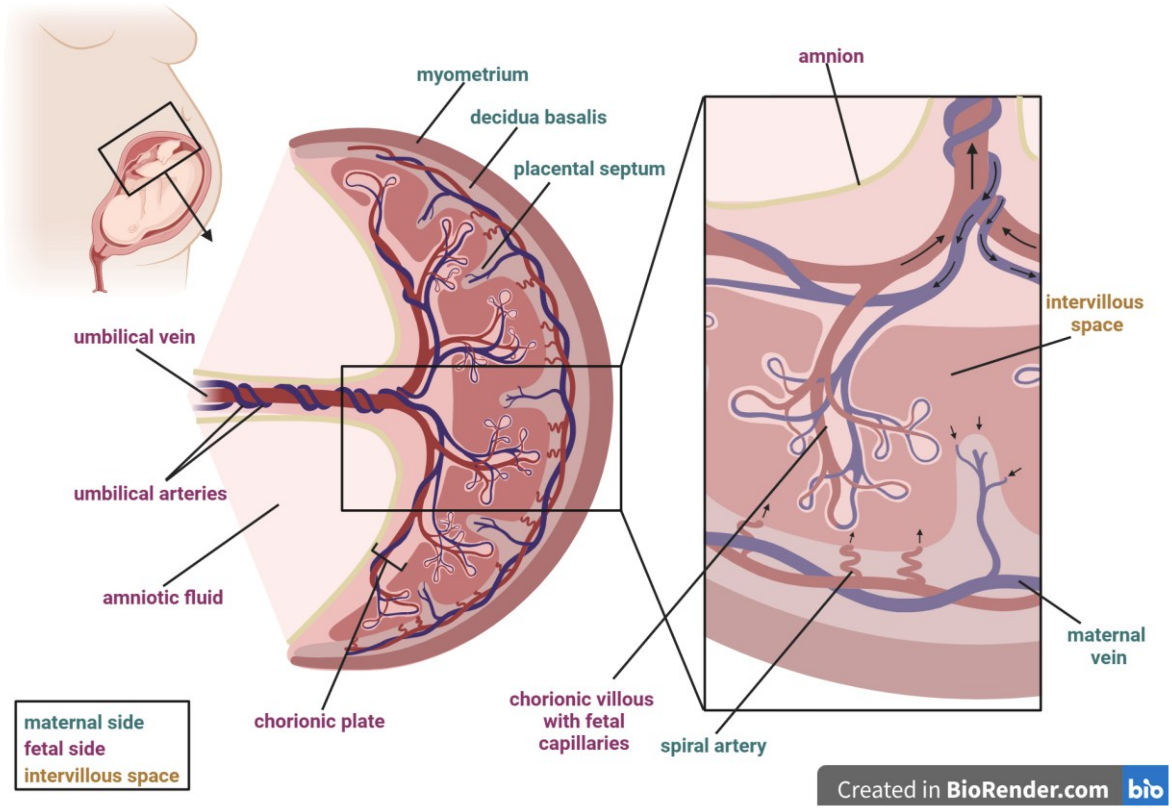

**Figure 1-5. Structure of the placenta at term.** The placenta is of fetal origin and is inseparable from uterine tissue during pregnancy. In the first trimester of pregnancy trophoblasts invade the maternal endometrium, which is then called decidua basalis, as a functional part of the placenta (94,96). This invasion results in the opening of uterine vessels and the filling of the intervillous space with maternal blood (94,96,97). The chorionic villi, which contain fetal capillaries, float freely in the intervillous space, and bidirectional molecule exchange takes place. The two circulations are completely separated by the placental tissue, consisting of fetal endothelial cells, a basal lamina and trophoblastic tissue (97,98). Within the chorionic plate, the fetal veins converge to become the umbilical vein (97). This vein transports oxygen enriched blood, nutrients and metabolites to nourish the fetus (97). Simultaneously, the umbilical arteries transport oxygen-deprived blood and metabolic waste back to the mother's circulation (97). Towards the fetal side of the placenta, the vessels are covered by the chorioamniotic membranes, consisting of the chorion and amnion (99). As the innermost layer, the amnion is in direct contact with the amniotic fluid and the fetus (99). This image was created with BioRender.com.

The placenta builds up the barrier between mother and fetus and is simultaneously the hub for exchange between both circulations. The organ's manifold functions, like gas exchange, secretion of hormones and other particles (e.g. EVs), molecule transfer (e.g. of nutrients, waste and immunoglobulins), being a fetal organ substitute, as well as the changing requirements during pregnancy are the reason of placental dynamic complexity, as illustrated in Figure 1-5 (94,97,100).

### 1.5.2 Placental exosomes (pEXs)

The secretion of pEXs is one essential placental feature (2). The secreted pEXs increase significantly six weeks after conception and peak at term in the maternal circulation (91,101). PEXs contain placenta-specific components, including PLAP, syncytin-1 and -2, miRNAs from the chromosome 19 miRNA cluster (C19MC miRNAs), CD276 and human leukocyte antigen G (HLA-G) (102–105). However, other tissues, particularly in tumours, express some of these molecules, such as PLAP and HLA-G, as well (106,107). Apart from the general EX markers described in 1.4.2, PLAP is commonly used as a placental tissue-specific marker to identify EXs of placental origin (108). Until now, different cell types of the placenta, such as mesenchymal cells, trophoblastic cells (syncytiotrophoblast and cytotrophoblast) and fetoplacental endothelial cells, have been documented to secrete pEXs (100,109). Depending on their origin, they can be secreted to the maternal or the fetal side of the placenta (110).

### 1.5.3 PEXs released to the fetal side of the placenta (pEXsF)

Most of the studies with pEXs have been in the context of release and function to the maternal side of the placenta. PEXs released to the maternal side of the placenta (pEXsM) are isolated from maternal blood after a simple blood draw, whereas the accessibility of placental exosomes released to the fetal side (pEXsF) and their function to fetal target cells is poor (108,111–113). Nevertheless, the limited number of studies conducted with cord blood-derived EXs have revealed intriguing results. Since the isolated EXs were not tested for placental markers, it is however unclear if these EXs are pEXs. It has been shown that the composition of cord blood EXs between preeclamptic and healthy pregnant women differs (114). Moreover, cord blood EXs might be involved in angiogenesis and in the function of fibroblasts (3). Interestingly, EXs isolated from fetal blood express the trophoblast specific marker PLAP, raising the question of whether or not pEXs may cross the placental barrier (115). Of note, recent studies suggest that PLAP is probably a fetoplacental-specific marker protein, rather than being solely trophoblast specific (104). Furthermore, the glycan profile of pEXsF isolated from cord blood and compared to pEXs from venous and arterial fetoplacental endothelial cells, indicates an affinity of pEXsF to bind to the α2-6 sialic acid-binding lectins Sambucus nigra agglutinin (SNA), Sambucus sieboldiana agglutinin (SSA) and Trichosanthes japonica agglutinin-I (TJA-I) (116).

PEXsF present a promising area for further research due to their different cargos and functions and with the advancement of new analytical technologies, progress in the understanding of their exact role is expected soon (111,112).

### 1.5.4 Cellular uptake of pEXs

Studies on the uptake of pEXs into cells are rare (117). It has been shown that BeWo cells, a well described choriocarcinoma cell line, internalise pEXs syncytin dependently (118). In mice, integrins are essential for the specific transport of pEVs to lung macrophages, liver endothelial cells and Kupffer cells (119). Additionally, integrin α3β1 could be involved in the cellular internalisation of pEVs in lung macrophages (119). It is also worth noting that endothelial cells take up pEVs primarily by receptor-mediated, clathrin-associated, endocytosis (120). Another study showed that chlorpromazine or LOX-1 receptor antibodies could inhibit the uptake of pEVs (121). However, it is important to acknowledge that many of these findings have only been observed in rodents and require further investigation in humans (110). It is also unclear whether the obtained findings are transferable to pEXsF. The above-described affinity of pEXsF to bind to the α2-6 sialic acid-binding lectins SNA, SSA and TJA-I indicates that pEXsF carry α2-6-linked sialic acid (116). Since sialic acid-containing glycans are already being investigated for the internalisation of EXs, they could possibly act as ligands for the uptake of pEXsF (60). However, this is merely a hypothesis that requires further investigation.

### 1.5.5 Functions and potential role of (placental) EXs in pregnancy

As already known, EXs can significantly affect the human immune system. A successful pregnancy relies on a series of immune adaptations that preferentially promote continued fetal growth, and pEXs may contribute to the communication between the maternal immune system and the rejection process against the semi-allogenic fetus (91). PEXs carry immunomodulatory molecules, like HLA-G, glycodelin-A, PDL-1, FAS-L, TRAIL, NKG2D ligands and syncytin (91,122–126). When internalised by natural killer (NK) cells, pEXs downregulate the activating receptor NKG2D, induce apoptosis of NK cells and promote the transformation of peripheral NK cells to a decidual NK cell-like phenotype (91,122,127). In addition, pEXs may have a direct effect on T-cells by affecting cell apoptosis, inhibit cell activation and downregulate cell proliferation and cytotoxicity (91). Additionally, pEXs can induce the formation of the immunosuppressive M2 phenotype of monocytes, which includes reducing effector T-cell proliferation and upregulating regulatory T-cell differentiation (128). In vitro studies have demonstrated that trophoblast cells, which are resistant to various viruses (e.g. cytomegalovirus (CMV)), can transfer their immunity to target cells through C19MC miRNAs, carried by pEXs (129,130). PEXs have angiogenic and vasculogenic potential in addition to immunomodulatory capabilities (131). Particularly under hypoxic conditions, the secretion of pEXs is upregulated and they can promote uterine spiral artery remodelling, extravillous cytotrophoblast migration, endothelial cell proliferation and cell migration (131–134). Finally, as mentioned above, pEXs contain syncytin proteins and have been implicated in syncytin-mediated fusion of cytotrophoblastic cells to form the syncytiotrophoblast (135).

In addition to their physiological roles, pEXs are thought to contribute to the development of several disorders in pregnancy, including preeclampsia (PE), gestational diabetes mellitus (GDM), prematurity and fetal growth restriction (FGR) (100). PE occurs only during pregnancy and is characterised by high blood pressure and additional symptoms, ranging from proteinuria and oedema to multi-organ failure, HELLP syndrome and eclampsia (136). As the disease is associated with a permanent state of hypoxia in the placental tissue and an angiogenic and endothelial dysfunction, the level of pEXs is significantly increased (137). Additionally, their composition appears to be different, and some of their components may contribute to vascular dysfunction and endothelial injury (138–141). As well as in PE, the amount of pEXs is higher in women with GDM, which is not surprising since glucose triggers the secretion of EXs (142,143). Furthermore, studies have shown an altered expression profile that affects insulin secretion and signalling, as well as glucose metabolism, and contributes to the release of pro-inflammatory cytokines (142,144–146). In contrast to PE and GDM, the total level of pEXs is decreased in women with spontaneous preterm birth and the ratio of pEXs to total exosomes is lower in FGR (115,147). Of note, altered exosomal cargos are found in both conditions. In preterm birth pregnancies, the miRNA profile is significantly different from that in term pregnancies and in FGR miR-520a-5p is downregulated (148,149).

Because of their apparent involvement in physiology and pathophysiology, pEXs have multiple clinical applications. For example, in PE, EVs could be used as early biomarkers for yet asymptomatic patients and to differentiate PE from other hypertensive disorders (137). A decreased ratio of pEXs to total EXs measured in maternal blood may indicate FGR and could therefore be used for monitoring and predicting FGR (115). Therapeutically, the specific inhibition of pathological EXs and the engineering of EXs, could potentially be used to treat and prevent certain pregnancy disorders, for example viral diseases such as CMV infection, which may lead to not yet preventable fetal complications (130).

## *1.6 Sialic acid binding immunoglobulin-like lectins (Siglecs)*

Siglecs belong to the I-type lectin family and are similar to immunoglobulins because of their extracellular domains (150). In humans, 15 different subtypes have been identified, which can be divided into two subgroups (150). The first group comprises Siglecs-1, -2, -4 and -15 (151). Although their sequences are diverse, they are evolutionarily highly conserved and have orthologues in different species (151). The second group includes all the other Siglecs with 50-99% similar sequences, which, however, are constantly evolving (151). Siglecs are primarily expressed by immune system cells (152). However, some are also expressed by other cell types. For example, Siglec-6 is expressed by placental cells, Siglec-10 by the human endometrium, Siglec-4 by Schwann cells and oligodendrocytes and Siglec-12 by prostate, kidney and prostate cancer cells (153–156). Overall, Siglecs play a significant role in immune regulation and are considered potential targets for cancer therapy (151,157). During pregnancy, Siglecs may contribute to the development of immune tolerance towards the fetus and the occurrence of PE (141,158).

Siglecs recognise and bind sialic acids in sialoglycans through their V-set, sialic-acid-binding domain (152). The most common sialic acids found in humans are N-gycolyl-neuraminic acid (Neu5Gc) and N-acetyl-neuraminic acid (Neu5Ac) (152). Sialic acids can be linked to surface proteins and lipids, and to other sialic acids by α2–3, α2–6 or α2–8 linkage (159). Depending on the type of Siglec, they have different affinities for binding to α2–3, α2–6 or α2–8 linked sialic acids (159). In section 1.5.3 it was already indicated that pEXsF carry α2-6 linked sialic acid (116). Siglec-2, -3 and -6, shown in Figure 1-6, have a high affinity for this linkage (160).

### 1.6.1 Siglec-2, -3 and -6

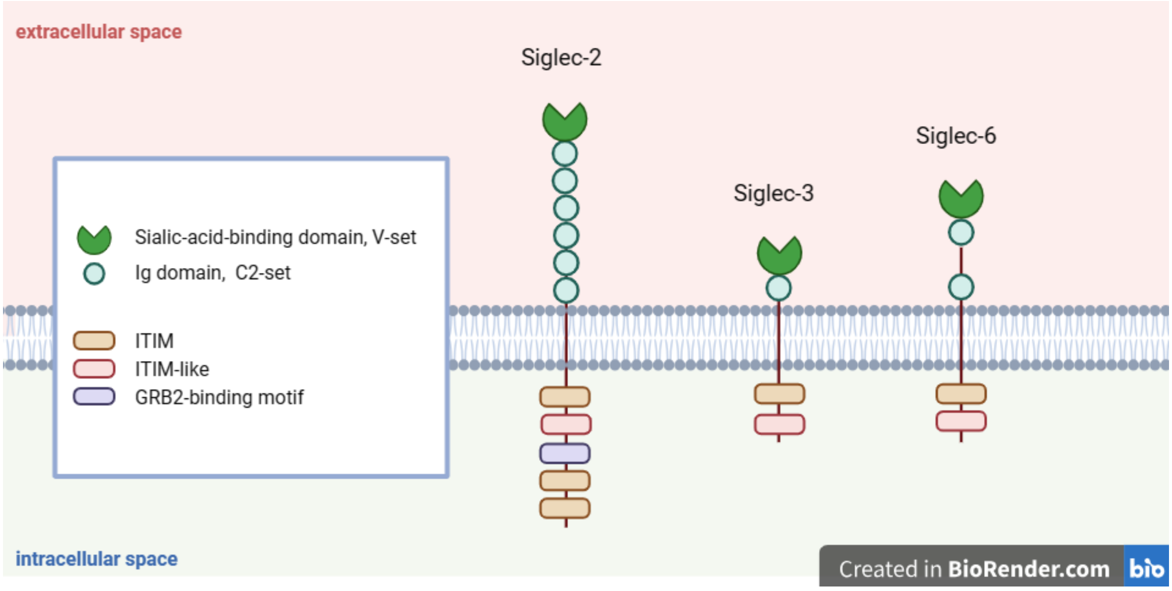

**Figure 1-6. Structure of human Siglec-2, -3 and -6.** Siglec-2, -3 and -6 are transmembrane proteins, with an extracellular domain consisting of one V-set, Ig like, domain for the binding of sialic acids and a various number of C2-set, Ig like, domains (159). Siglec-2 contains six C2-set domains, three immune receptor tyrosine-based inhibitory motifs (ITIMs), one ITIM-like and one growth factor receptor-bound protein 2 (GRB2) motif (152). Siglec-3 contains one C2-set domain, one ITIM and one ITIM-like (152). Siglec-6 contains two C2-set domains, one ITIM and one ITIM-like (152). Upon the receptors' activation, the ITIMs and ITIM-likes become phosphorylated and induce down-regulating signalling pathways (161). This image was created with BioRender.com.

Siglec-2, also known as CD22, is primarily expressed by B-cells, and can downregulate B-cell responses with its inhibitory motifs (161). Upon the binding of sialic acids, the inhibitory function is reduced (161). The receptor is specific for α2-6 linkage and binds to Neu5Ac and Neu5Gc (159). Siglec-2 can either bind to other Siglec-2 receptors in a sialic acid-dependent or -independent manner, or to IgM and CD45 in a sialic acid-dependent manner (159). The receptor has been linked to lymphoma, leukaemia and autoimmune diseases, such as SLE (160).

Siglec-3, also known as CD33, is expressed by various immune cell types, including myeloid progenitor cells, mast cells and tissue macrophages, such as microglia cells (159). The receptor binds to α2-3 and α2-6 linked sialoglycans, with a higher affinity for α2-6 linkage (161). One study showed highly specific binding to synthetic Neu5Acα2–3(6-O-sulfo)Galβ1–4GlcNAc(6′-Su-SLacNAc) (162). Siglec-3 has been associated with myeloid leukaemia, HBV infection and Alzheimer’s disease (160,163).

Siglec-6 is abundantly expressed in human placental tissue and can also be found on mast cells and B-cells (153,164,165). It selectively binds α2–6 linked sialic acid, although, one study found a preference for α2–3 linked sialic acid  (160,166). The identified sialylated ligands include glycodelin-A and Neu5Acα2-6GalNAc (sialyl-Tn), which is also the primary ligand of SNA (167–169). In early pregnancy, during placentation phase, the binding of decidual glycodelin-A inhibits the invasion of trophoblasts, and Siglec-6 may influence the duration of labour in late pregnancy (153,167). However, other physiological functions of Siglec-6 are not well understood. In non-muscle-invasive bladder cancer, higher Siglec-6 expression correlates with a lower survival, and Siglec-6 is expressed on malignant cells in acute myeloid leukaemia, and chronic lymphocytic leukaemia (170–172). The receptor is also upregulated in colorectal cancer and PE (141,173).

### 1.6.2 Siglecs and the uptake of EVs

EVs and Siglecs may interact due to their similar roles. Some studies have already shown that EVs express sialic acid-carrying surface glycans (60). For example, EVs derived from glioblastoma cells carry α2-3- and α2-6-linked sialic acid (174). Additionally, glycan profiling demonstrated that EXs derived from mesenchymal stem cells interact with sialic acid-binding lectins and pEXs specifically bind to α2-6 sialic acid-binding lectins (58,116). The receptor-mediated endocytosis of EXs is the most common form of uptake and Siglecs have an endocytotic function, possibly related to ITIM (19,151). Some studies have already shown specific interactions between Siglecs and EXs. The uptake of mesenchymal stem cell-derived EXs in HeLa cells is reduced after incubation with a Siglec-3 antibody (AB) or 11 N-acetylneuraminic acid, and they are biodistributed to and internalised by lymph node cells expressing Siglec-1, -2 and -3 (58). Another study showed that Siglec-1 is associated with the binding of B cell-derived EXs in lymph node and spleen macrophages (175). Further, Siglec-6 and Siglec-9 were also found to interact with EVs. Glioblastoma cell-derived EVs bind to Siglec-9 on dendritic cells, while Siglec-6 is involved in the binding and internalisation of EVs by binding to glycolipids

(166,174). Additionally, Siglec-6 is expressed on syncytiotrophoblast-derived placental vesicles (141).

# 2 Hypothesis and aims

The emerging role of EVs, in particular EXs, has been extensively presented in the last chapter. This young research field opens up new opportunities across multiple medical disciplines, including internal medicine, oncology, immunology as well as obstetrics (13,79,80,130,176). The role of pEXs in healthy and pathophysiological pregnancy has been discovered over the last few years, and there is a need for research in order to understand their role and to utilise this knowledge for the purposes of diagnosis, prognostics and therapeutics. However, there is hardly any knowledge, about pEXsF. Therefore, the aim of this thesis is to contribute to the exploration of their fate by analysing whether their cellular uptake could be mediated by Siglec receptors. As the fetal liver is, besides the placenta, the central fetal metabolically active organ, being the first in line to have contact with blood coming from the placenta, we hypothesize that liver cells interact with pEXsF. Hence, we use HepG2 cells, an immortalized liver cancer cell line, which have already been shown to internalise pEXsF, as our target cells for cellular uptake studies. The ultimate aim of this thesis is to answer following questions:

- Are Siglec-2, -3 or -6 receptors expressed on HepG2 cells?
- Is there a temperature-dependent difference in the uptake of pEXsF in HepG2 cells?

# 3 Material und Methods

First, the expression of Siglec-2, -3 and -6 on HepG2 cells was analysed by immunoblotting and immunofluorescence staining. In addition, pEXsF were isolated and temperature-dependent uptake studies of pEXsF were performed. The host lab provided all materials and equipment used in this thesis.

## *3.1 Cell culture*

### 3.1.1 HepG2 cells

HepG2 cells were cultured in tissue culture T75 flasks (Thermo Fisher Scientific Inc., Waltham, MA, USA), in high glucose Dulbecco's Modified Eagle Medium ++ (DMEM++; Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA), containing 10% foetal calf serum (FCS; HyClone Laboratories, GE Healthcare Life Science, Logan, US), 2 mM L-glutamine (Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA) and 1% penicillin-streptomycin (Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA). The cells were maintained under standard culture conditions in a humidified environment at a temperature of 37 °C with an atmosphere composed of 21% $O_2$ and 5% $CO_2$ until they reached 80% confluency.

### 3.1.2 Fetoplacental arterial endothelial cells (FpAECs)

Fetoplacental arterial endothelial cells (fpAECs) from healthy placentas were isolated as described by Lang et al. (177). After purity check, fpAECs were lentivirally transfected to obtain fluorescent transgene pEXsF. TSG101, a component of the ESCRT-I complex, and regulator of vesicular trafficking process in cells, was tagged with mCherry dye and used for the uptake assay. The transfected and FACS sorted fpAECs were thawed and expanded in 1% gelatine coated T75 flasks (Thermo Fisher Scientific Inc., Waltham, MA, USA) under standard culture conditions (37 °C, 12 % $O_2$, 5 % $CO_2$, humidified atmosphere). As culture medium, PromoCell++ was used, containing Endothelial Cell Basal Medium MV (PromoCell, Heidelberg, Germany) supplemented with the Endothelial Cell Growth Medium MV Supplement Pack (PromoCell, Heidelberg, Germany) and 500 µL Gentamicin (Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA). FpAECs were grown to 70-80% confluency before performing cell culture experiments.

## 3.2 Protein lysate preparation

Cell culture medium of ten tissue culture T75 flasks (Thermo Fisher Scientific Inc., Waltham, MA, USA) containing HepG2 cells was aspirated, and the cell monolayer was washed twice with Hanks' Balanced Salt Solution (HBSS; Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA). All following steps were done on ice. At first, 300 µl cold RIPA Lysis Buffer (Sigma Aldrich, Burlington, MA, USA) containing proteinase inhibitors were added per flask, and a cell scraper was used to harvest adherent cells from the surface. The cell suspension was then transferred to 1.5 ml micro reaction tubes (Eppendorf AG, Hamburg, Germany) and ultrasonicated twice for 10 seconds with an in-between cooling time on ice for about one minute, followed by centrifugation at 4°C and 13,000 rpm for 20 minutes. The supernatant containing proteins was then transferred to fresh 1.5 ml micro reaction tubes and stored at 80°C until further usage.

## 3.3 BCA protein assay

In order to determine the protein concentrations of the samples, a commercially available protein assay (Pierce® BCA, Thermo Fisher Scientific Inc., Waltham, MA, USA) was used.

The assay is based on two reaction steps. At first, $CU^{2+}$ ions bind to peptide bonds on proteins and are reduced to $CU^{1+}$ during this process, followed by the chelation of $CU^{1+}$ and two molecules of bicinchoninic acid (BCA). The so-formed purple-coloured complex can be detected through lightwave absorbance measurement at 562 nm. Since the correlation of absorbance and protein concentration follows a linear relation over a range of 20-2,000 µg/mL, the unknown sample protein concentration can be determined by reference to a standard curve based on a series of dilutions of known concentrations containing bovine serum albumin (BSA).

After preparing the protein standards, 5 µl of each standard and unknown sample were pipetted into a well of a 96-well microplate (Nunc™, Thermo Fisher Scientific Inc., Waltham, MA, USA). BCA Reagent A (50 parts), containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide, and BCA Reagent B (1 part), containing 4% cupric sulfate, were mixed to get the working reagent, which was then added to the microplate with a concentration of 200 µl per well.  The plate was placed on a plate shaker for 30 seconds and covered with a multiwell plate sealer (EASYseal™, Greiner Bio-One, Frickenhausen, Germany). After an incubation time of 30 minutes at 37°C, the light absorbance at 562 nm was measured on the SPECTROstar® Nano microplate reader (BMG Labtech, Ortenberg, Germany).

BCA was performed twice, initially to determine the protein concentration of HepG2 lysates, and subsequently to determine the protein content of isolated pEXsF.

## *3.4 Immunoblot*

The Siglec-2, -3 and -6 receptors' expression on HepG2 cells was analysed by immunoblotting. Table 3-1 depicts the targeted receptors, their supposed molecular weight and the tested corresponding primary ABs.

**Table 3-1. Characteristics of Siglec-receptor antibodies**. Differences in the molecular weight of Siglec-6 can be found in literature (from 50 to 70 kDa), with the main determinants of those size differences being different glycosylation patterns of Siglec-6 in various tissues (141,178–180).

| Receptor | Molecular weight | Primary AB |
|---|---|---|
| Siglec-6 | 50-70 kDa | Siglec-6 Thermo Fisher<br>Siglec-6 R&D Systems |
| Siglec-3 | ~55 kDa | Siglec-3 R&D Systems |
| Siglec-2 | ~140 kDa | Siglec-2 R&D Systems |

First, protein lysates of HepG2 cells were probed. The lab provided pEXsF samples isolated from fpAECs and placental tissue (PT) samples with known protein concentrations. Applied ABs are described in Table 3-4.

The first aim was to get reliable expression results of Siglec-2, -3 and -6 on HepG2 cells. Therefore, different antibody dilutions of the Siglec-2 AB (R&D Systems), Siglec-3 AB (R&D Systems) and Siglec-6 AB (Thermo Fisher) as described in Table 3-4 were tested.  PT has already shown to express Siglec-6 and was used as positive control to demonstrate the protocol's efficiency and specificity of applied ABs (153).

The second immunoblot served to verify expression results from previous blots with HepG2 cells, in which a different Siglec-6 AB (R&D Systems) was used, and which have shown that Siglec-6 is also expressed on pEXs (116). In addition, their Siglec-3 expression pattern was determined. The herein used pEXsF samples were already isolated from healthy maternal plasma samples and isolated as described in 3.5. To ensure that there was no interference with the ABs due to the transfection, only two out of four pEXsF samples were lentivirally transfected. Additionally, the Siglec-3 AB (R&D Systems) and Siglec-6 AB (Thermo Fisher) were probed again and served as controls.

HepG2 cell protein lysate was diluted with RIPA buffer (Sigma Aldrich, Burlington, MA, USA) and the other samples were diluted with PBS (Medicago AB, Uppsala Sweden). Thereafter, all of them were mixed with 2x Laemmli sample buffer (Sigma Aldrich, St. Louis, MO, USA). Protein concentrations were set to 1 µg/µl and in the case of the not transfected pEXsF samples to 0.615 µg/µl and 0.950 µg/µl. All samples were denatured with a thermomixer (Eppendorf AG, Hamburg, Germany) by heating up the samples to 95°C for 5 minutes, followed by 5 minutes of centrifugation at 3,000 rpm and vortexing. Proteins of each sample, as outlined in Table 3-2 and Table 3-3 were loaded onto a 4-20% Mini-PROTEAN® TGX™ Precast Protein Gel (BioRad laboratories Inc., CA, USA). As molecular weight marker 5 ug of the PageRuler Prestained Protein Marker (Thermo Fisher Scientific Inc., Waltham, MA, USA) were applied.

**Table 3-2. Samples used in the first immunoblot.**

| Sample | Protein content per well | Diluted sample in µl per well |
|---|---|---|
| PT | 8 µg | 8 µl |
| HepG2 protein lysate | 15 µg | 15 µl |

**Table 3-3. Samples used for immunoblotting.**

| Sample | Protein content per well | Diluted sample in µl per well |
|---|---|---|
| PT | 5 µg | 5 µl |

| | | |
|---|---|---|
| HepG2 protein lysate | 8 µg | 8 µl |
| pEXsF ECA225 transfected | 8 µg | 8 µl |
| pEXsF ECA255 transfected | 8 µg | 8 µl |
| pEXsF ECA221-3 | 8 µg | 12.9 µl |
| pEXsF ECA227-1 | 8 µg | 8.4 µl |

After sample loading, the gel was run at 110V, 400mA for 70 minutes. The separated proteins were transferred to nitrocellulose membranes (BioRad Technologies, Vienna, Austria) using the Trans-Blot® Turbo TM Transfer system (BioRad laboratories Inc., CA, USA). To check the transfer quality, they were visualized by Ponceau S reversible staining (Sigma Aldrich, St. Louis, USA). To remove the staining, several washing cycles with TBE-T, containing distilled water, 10% 10xTBE (Gatt-Koller, Absam, Austria), and 0.1% Tween 20 (Sigma Aldrich, St. Louis, USA), were performed, followed by blocking of the membranes with 5% milk blocking solution (5g of milk powder (Biorad, Hercules, CA, USA) diluted in 100ml TBE-T) for 1h on a vertical shaker at room temperature to prevent nonspecific background binding of the ABs to the membrane.

The primary ABs diluted in 1% milk-blocking solution (10 ml 5 % milk, 40 ml TBE-T) were added to the membranes in the concentrations described in Table 3-4 and then sealed and incubated overnight in a freezer at 4°C on a vertical shaker.

The next day, after thoroughly washing the membranes with TBE-T buffer for 1h, the secondary antibodies (sABs) diluted in 1% milk-blocking solution as described in Table 3-4 were added and once again incubated for 1h at room temperature on a vertical shaker. Another washing cycle for 1h, as described above, followed, before the blots were incubated for 5 minutes with the SuperSignal™ West Pico PLUS Chemiluminescent Substrate working solution (Thermo Fisher Scientific Inc., Waltham, MA, USA) for the blot development. For the visualization of the protein bands the Fusion FX imaging system (Vilber Lourmat, Marne-la-Vallee, France) was used. The exposure times were adapted to reach optimal visibility, for the Siglec-3 AB (R&D Systems) 5 minutes, for the Siglec-6 AB (Thermo Fisher) 3 minutes for samples and 30 seconds for PT and for the Siglec-6 AB (R&D Systems) 3 minutes for samples and 14 seconds for PT.

**Table 3-4. Specifications of antibodies.** To avoid unspecific binding due to too high AB concentration as well as no binding due to too low AB concentration, the first immunoblot was done as a test run with different dilutions of Siglec-2 AB (R&D Systems), Siglec-3 AB (R&D Systems) and Siglec-6 AB (Thermo Fisher) as described below. For the second immunoblot, the dilution 1:750 for the Siglec-6 AB from R&D Systems and the optimized dilutions for the other ABs, according to the first immunoblot, were probed: 1:666 for the Siglec-6 AB (Thermo Fisher) and 1:200 for the Siglec-3 AB (R&D Systems).

| Primary AB specificity | Host | Polyclonal/ monoclonal | Dilutions | Supplier | Reference number | Secondary AB specificity and dilution |
|---|---|---|---|---|---|---|
| Siglec-2 | goat | polyclonal | 1:1,500<br>1:2,000<br>1:2,500 | R&D Systems | AF2296 | Anti-Goat IgG 1:1,000 |
| Siglec-3 | mouse | monoclonal | 1:200<br>1:250<br>1:300 | R&D Systems | MAB11371 | Anti-Mouse IgG 1:1,000 |
| Siglec-6 | rabbit | polyclonal | 1:500<br>1:666<br>1:1,000<br>1:2,000 | ThermoFisher | PA5-82377 | Anti-Rabbit IgG 1:1,000 |
| Siglec-6 | sheep | polyclonal | 1:750 | R&D Systems | AF2859 | Anti-Sheep IgG 1:2,000 |

## *3.5 Isolation of placental vesicles*

Transfected fpAECs were grown to 70-80% confluency as described in 3.1.2. The cell monolayer was washed once with HBSS, after which cells were cultivated for 48h under standard culture conditions with PromoCell, containing Endothelial Cell Basal Medium MV supplemented with 5% exosome depleted foetal calf serum (FCS, Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA), human epidermal growth factor (Promocell, Heidelberg, Germany), hydrocortisone (Promocell, Heidelberg, Germany), 0.1% Gentamicin and without hypothalamic extract. Subsequently, the supernatant (70 ml) was collected, and a stepwise ultracentrifugation and filtration protocol, established in the lab, was used for exosome isolation as described below and in Figure 3-1.

All centrifugation steps were done at 4°C. First, the collected cell culture supernatant samples were transferred to two 50ml Falcon® polypropylene tubes (35ml each; Corning Inc., Corning, NY, USA) and centrifuged at 500 g for 10 min in the Allegra X12R benchtop centrifuge (Beckman Coulter Inc., Brea, USA). The pellet, containing cells and cell debris, was discarded, and the supernatant was transferred to fresh 50ml Falcon tubes and centrifuged at 2,500 g for 20 min. Once again, the pellet containing smaller cell fragments and apoptotic bodies was discarded, and the supernatants were split and pipetted into three ultracentrifuge tubes (Ultra-Clear™ Centrifuge Tubes, 25x89cm, 38.5 mL, Beckman Coulter Inc., Brea, USA), each containing approx. 23ml volume. Thereafter tubes were placed in the Optima XE-90 ultracentrifuge (Beckman Coulter Inc., Brea, USA) in a Type 70 Ti rotor and centrifuged at 12,000 g for 30 minutes. The supernatant was collected for further isolation of pEXsF, while the pellet containing mainly MVs was pooled, re-suspended in 0,2ml 1xPBS and stored at 4°C. Supernatants were pushed through a 0.2 μm Nalgene® Syringe Filter (Thermo Fisher Scientific Inc., Waltham, MA, USA), thereby all particles larger than 200 nm were filtered out. To concentrate the filtrate further, the volume was reduced to 4ml by using a Pierce™ Protein Concentrator (PES, 100K MWCO, 20-100 ml; Thermo Fisher Scientific Inc., Waltham, MA, USA) and centrifuging the samples at 1800 g in the Allegra X12R benchtop centrifuge (Beckman Coulter Inc., Brea, USA). To be sure to collect all concentrate left, the upper filter device of the concentrator was filled up with 5ml 1x PBS and all liquid from the upper device was collected and transferred to a Quick-Seal® tube (Beckman Coulter Inc., Brea, USA). The tube was filled up with 1x PBS to a total volume of 12ml, and centrifuged at 100,000 g for 22h using the Optima XE-90 ultracentrifuge (Beckman Coulter Inc., Brea, USA) in a Type 70 Ti rotor.

The final step was to carefully discard the supernatant without discarding the pellet containing the pEXsF. The pellet was resuspended in 350 ml 1x PBS and immediately after this, HepG2 cells were treated with the obtained pEXsF as described in 3.6. Protein concentration and the quality of pEXsF were determined by BCA and NTA.

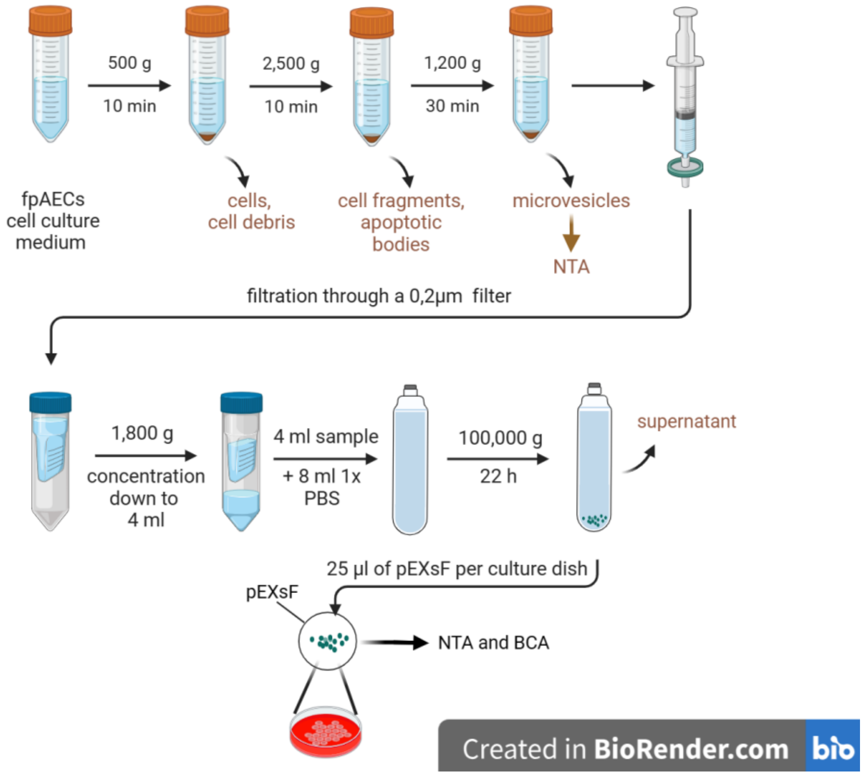

**Figure 3-1. Overview of ultracentrifugation steps performed to isolate pEXsF from fpAECs.** The figure was created adapting the template “Extracellular Vesicle Separation by Density Gradient Ultracentrifugation”, Louri Filipa, 2019. Retrieved from https://app.biorender.com/biorender-templates in 2023.

### 3.5.1 Nanoparticle tracking analysis (NTA)

Nanoparticle tracking analysis allowed to characterize isolated pEVs enriched fractions by size and particle concentration. PEXsF samples diluted in PBS (1:50) were measured, together with a negative control (PBS), and one sample containing in PBS diluted MVs (1:50).

The NTA system first excites the moving particles with a laser, to film the scattered light using a high sensitivity camera (sCMOS camera) installed on a microscope with a 20-fold objective. The captured pictures (30 per second) show the particles moving under Brownian motion and can be evaluated by a software to calculate the particle size and concentration (181).

The specific measurement was performed, according to the established protocol in the lab, with the Nanosight NS300 instrument (Malvern Panalytikal, Malvern, UK). The NTA 3.3 Dev Build 3.3.301 software was deployed to analyse the samples.

## 3.6 Immunofluorescence staining and uptake assay

Immunofluorescence staining was performed in two series of experiments. First, we aimed to detect Siglec-3 and -6 receptor expression on HepG2 cells and to test different AB dilutions, since no recommendations were provided by the manufacturer. In the second attempt, we ran a temperature dependant uptake cell culture experiment to see if there is a difference in the uptake of transfected pEXsF into HepG2 cells.

For this purpose, HepG2 cells were cultured as described in 3.1.1 and then split and counted to get $3.5 \times 10^4$ cells per Nunc™ 3.5 cm round glass bottom culture dish (Thermo Fisher Scientific Inc., Waltham, MA, USA). Thereafter, cells were incubated with CellLight™ Plasma Membrane-GFP, BacMam 2.0 (Thermo Fisher Scientific Inc., Waltham, MA, USA) for 16h under standard culture conditions. Subsequently, the medium was changed to DMEM++, and 25 µl of the isolated pEXsF suspension per glass bottom culture dish were added. For the first uptake assay, we used dishes, in which cells were incubated for 5.5h at 37 °C, 21% $O_2$ and 5% $CO_2$. For the second uptake assay, dishes were split. One of them was incubated for 24h under standard culture conditions, while the other one was put in the refrigerator at 4°C for 4h. Afterwards, the experiment was continued with the AB staining.

During the incubation period NTA, as described in 3.5.1 and BCA, as described in 3.3, were performed to control the quality of the pEXsF isolation and to know the number of pEXsF applied on HepG2 cells.

After incubation, the dishes were washed with HBSS and fixated with 4% formalin (SAV, Liquid Production GmbH, Flintsbach am Inn, Germany) for 10 minutes. Cells were again washed three times for 5 minutes with PBST-T, consisting of 1x PBS, 0.1% Tween-20 (Sigma Aldrich, St. Louis, USA) and 0.1% Triton X-100 (Lactan GmbH & Co KG, Graz, Austria) and incubated for 5h with a blocking buffer containing 14 ml PBST (PBS + 0,1% Tween-20), 15 µl Triton-X100, 0.15 g BSA and 300 µl Normal Goat Serum (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA). Subsequently, HepG2s were incubated with the primary ABs, diluted in blocking buffer as described in Table 3-5, at 4°C overnight. On the next day another three-times-5-min washing cycle and 5h of blocking followed, before the dishes were incubated with the secondary ABs diluted in blocking buffer as described in Table 3-6 overnight at 4°C. After another washing cycle with PBST-T, the dishes were stored in PBS at 4°C, before nuclei of the HepG2 cells' were counterstained with DAPI (diluted 1:1,000; Sigma Aldrich, Saint Louis, USA) and cells were finally washed with PBS for 5 minutes. Subsequently, imaging on the Zeiss Axiovert 200M microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) with the ZEN 2009 software (Carl Zeiss Microscopy GmbH, Jena, Germany) was performed.

**Table 3-5. Specifications of primary antibodies**. For the first set of immunofluorescence stainings, we tested different concentrations of Siglec-6 AB (1:50, 1:100 and 1:200, Thermo Fisher) and Siglec-3 AB (1:20, 1:80 and 1:150, R&D). In the second series of experiments, we used the tested dilution for Siglec-6 (1:50). Anti-mCherry could not be used for Siglec-3-stained samples, since the host for both pABs is mouse and the sAB supposed to stain Anti-Siglec-3 would have stained the mCherry tagged exosomes as well.

| Primary AB specifity | AB | Host | Dilution | Supplier | Reference number |
|---|---|---|---|---|---|
| Siglec-6 | Anti-Siglec-6 | sheep | 1:50 | Thermo Fisher | PA5-82377 |
| Siglec-3 | Anti-Siglec-3 | mouse | 1:20 | R&D Systems | MAB11371 |
| pEXsF transfected | Anti-mCherry | mouse | 1:100 | Thermo Fisher | MA5-32977 |
| HepG2 cell membrane | Anti-GFP | chicken | 1:50 | Thermo Fisher | A10262 |

**Table 3-6. Specifications of secondary antibodies.** Siglec-3 immunofluorescence stainings were probed with anti-rabbit (DyLight™633), anti-mouse (DyLight™550) and anti-chicken (Alexa Fluor™488). Siglec-3 immunofluorescence stainings were probed with anti-chicken (Alexa Fluor™488) and anti-mouse (DyLight™633) but not with the anti-mouse (DyLight™550) since the Siglec-3 assay was not stained with anti-mCherry.

| Secondary AB specifity | AB | Host | Dilution | Supplier | Reference number | colour |
|---|---|---|---|---|---|---|
| Anti-Siglec-6 | Anti-rabbit (DyLight™633) | goat | 1:100 | Thermo Fisher | 35562 | purple |

| | | | | | | |
|---|---|---|---|---|---|---|
| Anti-Siglec-3 | Anti-mouse (DyLight™633) | goat | 1:100 | Thermo Fisher | 35512 | purple |
| Anti-mCherry | Anti-mouse (DyLight™550) | goat | 1:100 | Thermo Fisher | 84540 | red |
| Anti-GFP | Anti-chicken (Alexa Fluor™488) | goat | 1:2,000 | Thermo Fisher | A11039 | green |

# 4 Results

## 4.1 Expression of Siglec-2, -3 and -6 on HepG2 cells and pEXsF

In order to answer the question of whether HepG2 cells express Siglec- 2, -3 or -6, an immunoblot was conducted. HepG2 cells were cultured, and protein lysates were generated. All samples were diluted to obtain 15 µg of HepG2 cell protein lysate and 8 µg of PT protein lysate, serving as positive control, per well. First, we tested different AB dilutions in order to obtain traceable results for further experiments.

As illustrated in Figure 4-1, the Siglec-2 AB (R&D Systems) showed no reaction regardless of the dilution. In contrast, the binding of the Siglec-3 AB (R&D Systems) and the Siglec-6 AB (Thermo Fisher) to PT and HepG2 cells was evident, as illustrated in Figure 4-2 and Figure 4-3.

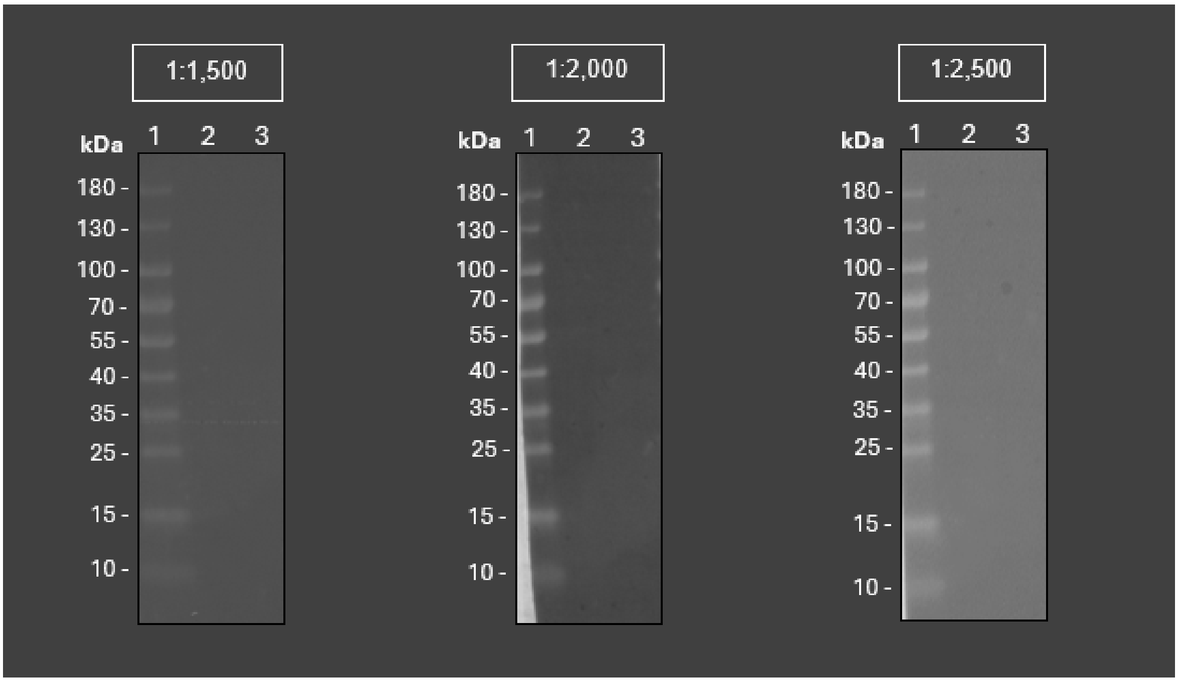

**Figure 4-1. Siglec-2 immunoblot.** We tested the dilutions 1:1,500, 1:2,000 and 1:2,500 of the Siglec-2 AB (R&D Systems). Neither PT (**2**), nor HepG2 cell (**3**) protein lysate showed a reaction. *(**1:** standard, **2:** PT, **3:** HepG2 cells)*

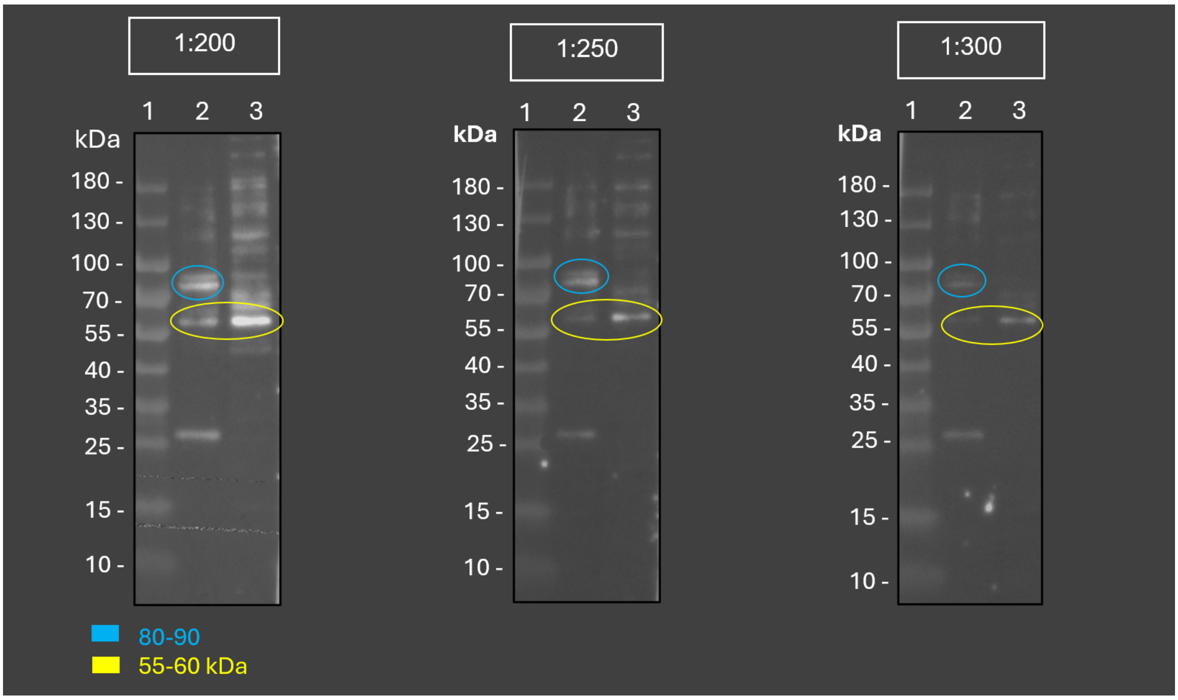

**Figure 4-2. Siglec-3, test immunoblot.** Different dilutions, 1:200, 1:250 and 1:300, of the Siglec-3 AB (R&D Systems) were tested. Siglec-3 expression was detectable in PT (**2**) and in HepG2 cells (**3**) between 55-60 kDa. However, in PT (**2**), the strongest band was between 80-90 kDa. In both PT (**2**) and HepG2 cells (**3**) there was additionally a lot of unspecific binding in the range of 70-180 kDa. *(**1:** standard, **2:** PT, **3:** HepG2 cells)*

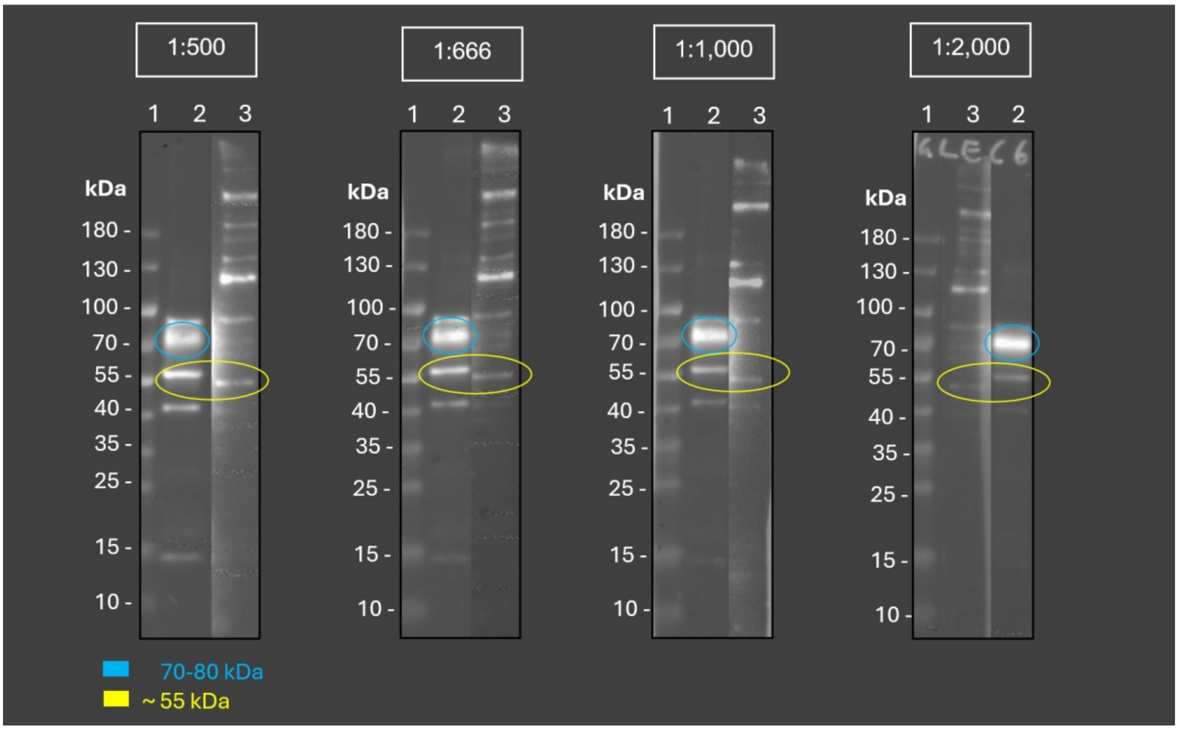

**Figure 4-3. Siglec-6, test immunoblot.** Different dilutions, 1:500, 1:666, 1:1,000 and 1:2,000, of the Siglec-6 AB (Thermo Fisher) were tested. PT (**2**) showed a strong expression at ~55 kDa and

between 70-80 kDa. In HepG2 cells (**3**) there was a band at ~ 55 kDa identifiable, but the overall binding was rather unspecific. *(**1:** standard, **2:** PT, **3:** HepG2 cells)*

Siglec-3 AB (R&D Systems) binding was detected between 55-60 kDa, as well as around 80-90 kDa in PT. In HepG2 cells, a strong band appeared between 55-60 kDa, but the Siglec-3 AB binding in HepG2 cells was overall very unspecific. The Siglec-6 expression pattern was highly specific in PT, showing the two most prominent bands at ~55 kDa and ~70-80 kDa. In HepG2 cells, the Siglec-6 expression was not restricted to one specific molecular weight, but there were several accumulations of Siglec-6 AB (Thermo Fisher) binding, as illustrated in Figure 4-3. For the Siglec-3 AB (R&D Systems), 1:200, and for the Siglec-6 AB (Thermo Fisher), 1:666, were identified as the dilutions to get reliable results.

The results with the Siglec-6 AB (Thermo Fisher) showed rather unspecific binding, prompting the decision to compare it with another Siglec-6 AB from a different supplier (R&D Systems), which has been known from previous experiments. As pEXsF are of placental origin, it was also of interest to analyse their Siglec-3 and Siglec-6 expression. In the light of these considerations, and to corroborate the findings of the initial immunoblot, we probed not only the Siglec-6 AB (R&D Systems), but once again the Siglec-3 AB (R&D Systems) and the Siglec-6 AB (Thermo Fisher).

Figure 4-4 and Figure 4-5 illustrate the results of these immunoblots. For the Siglec-3 AB (R&D Systems), the results of the initial series of immunoblots were confirmed. However, this time, both PT and HepG2 cells exhibited the two strongest bands between 55-70 kDa. Very faint Siglec-3 expression was detected in pEXsF. The Siglec-6 AB (Thermo Fisher) expression pattern resembled that observed in the first immunoblot. PEXsF showed a high degree of unspecific binding, very similar to HepG2 cells. The Siglec-6 AB (R&D Systems) demonstrated a significantly higher specificity than the Siglec-6 AB (Thermo Fisher). A band at ~55 kDa was detectable in all samples. In PT and HepG2 cells an additional band at ~70 kDa was observed.

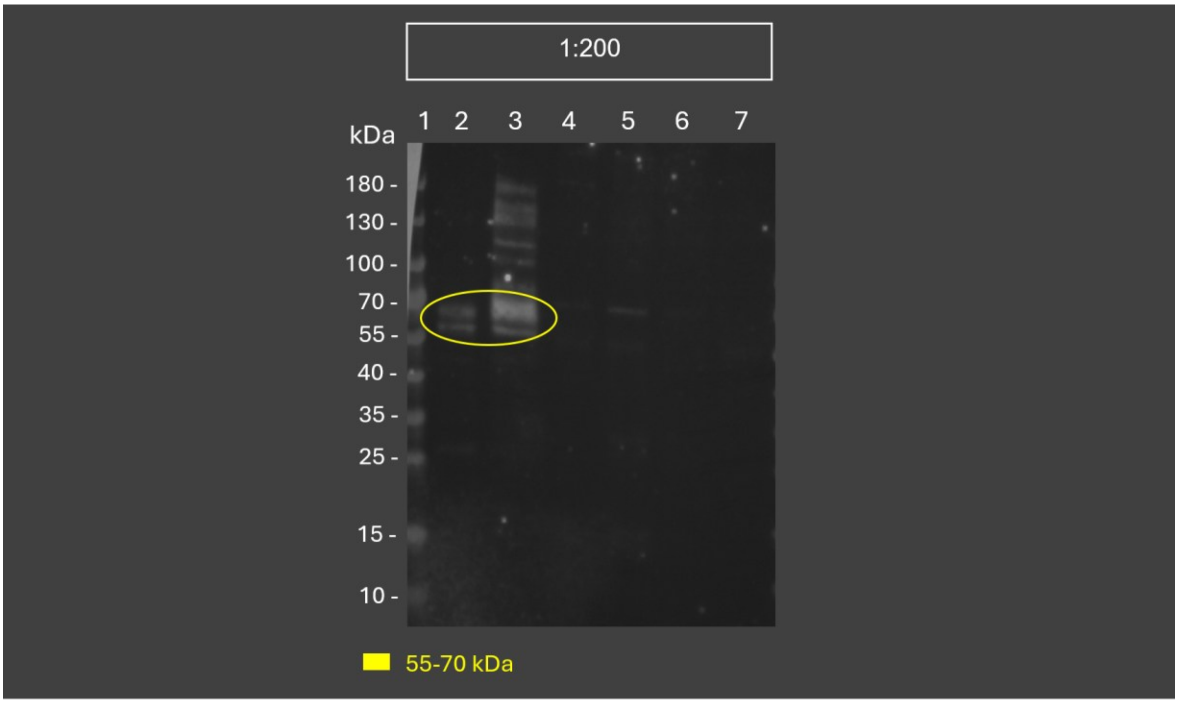

**Figure 4-4. Siglec-3, immunoblot.** Similar to the first immunoblot, there was non-specific binding, but this time it was mainly in HepG2 cells (**3**). However, in both PT (**2**) and HepG2 cells (**3**) the strongest reaction was between 55-70 kDa. Very faint binding was detected in pEXsF (**4-7**). *(**1:** standard, **2:** PT, **3:** HepG2 cells, **4:** pEXsF transfected, **5:** pEXsF transfected, **6:** pEXsF not transfected, **7:** pEXsF not transfected)*

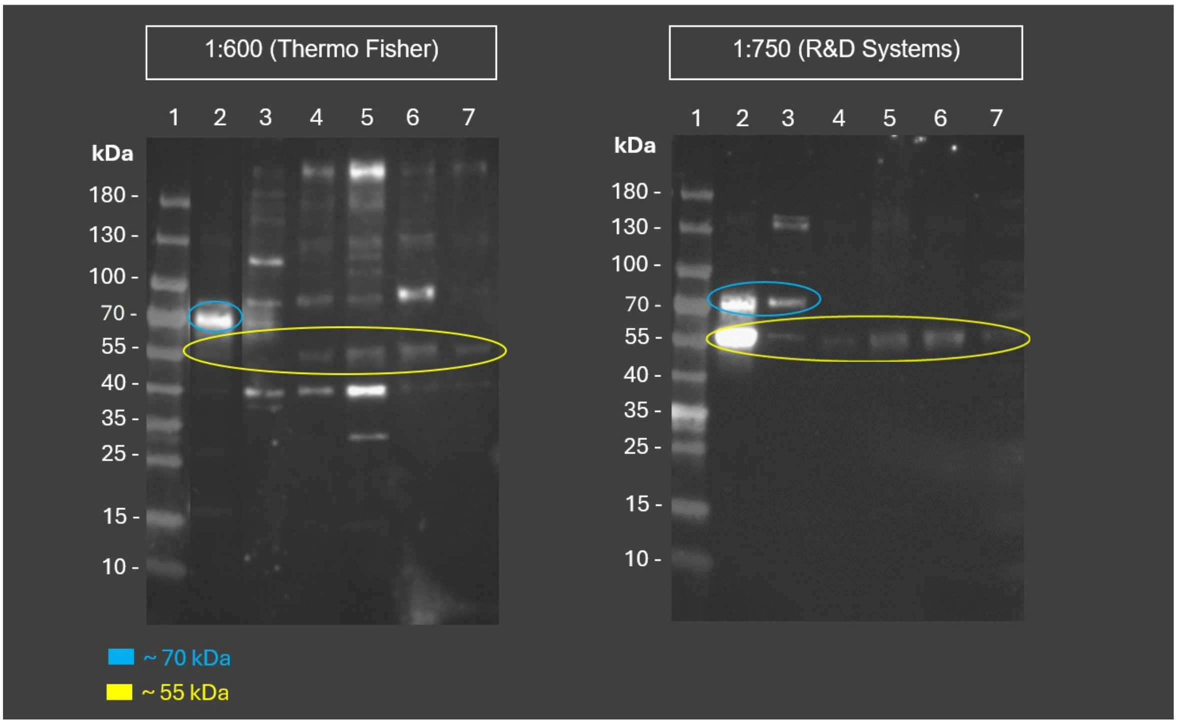

**Figure 4-5. Siglec-6, immunoblot.** Thermo Fisher AB blots are shown on the left and R&D Systems AB blots are shown on the right. The band at ~70kDA in PT (**2**) and the AB binding to all samples at ~55 kDa is evident in both blots. However, on the left, the ~55 kDa band is faint in PT (**2**) and HepG2

cells (**3**). In PT (**2**), ~70 kDA is the strongest band on the left, whereas ~55 kDA is the strongest band on the right. *(**1:** standard, **2:** PT, **3:** HepG2 cells, **4:** pEXsF transfected, **5:** pEXsF transfected, **6:** pEXsF not transfected, **7:** pEXsF not transfected)*

## *4.2 Size distribution of isolated vesicles*

For the uptake assay, pEXsF were isolated from transfected fpAECs as outlined in 3.5. NTA was employed to ascertain the size distribution and the quality of the isolation. Figure 4-6 shows that the pEXsF isolate was analysed alongside the pMV isolate and PBS, as the samples were resuspended in PBS.

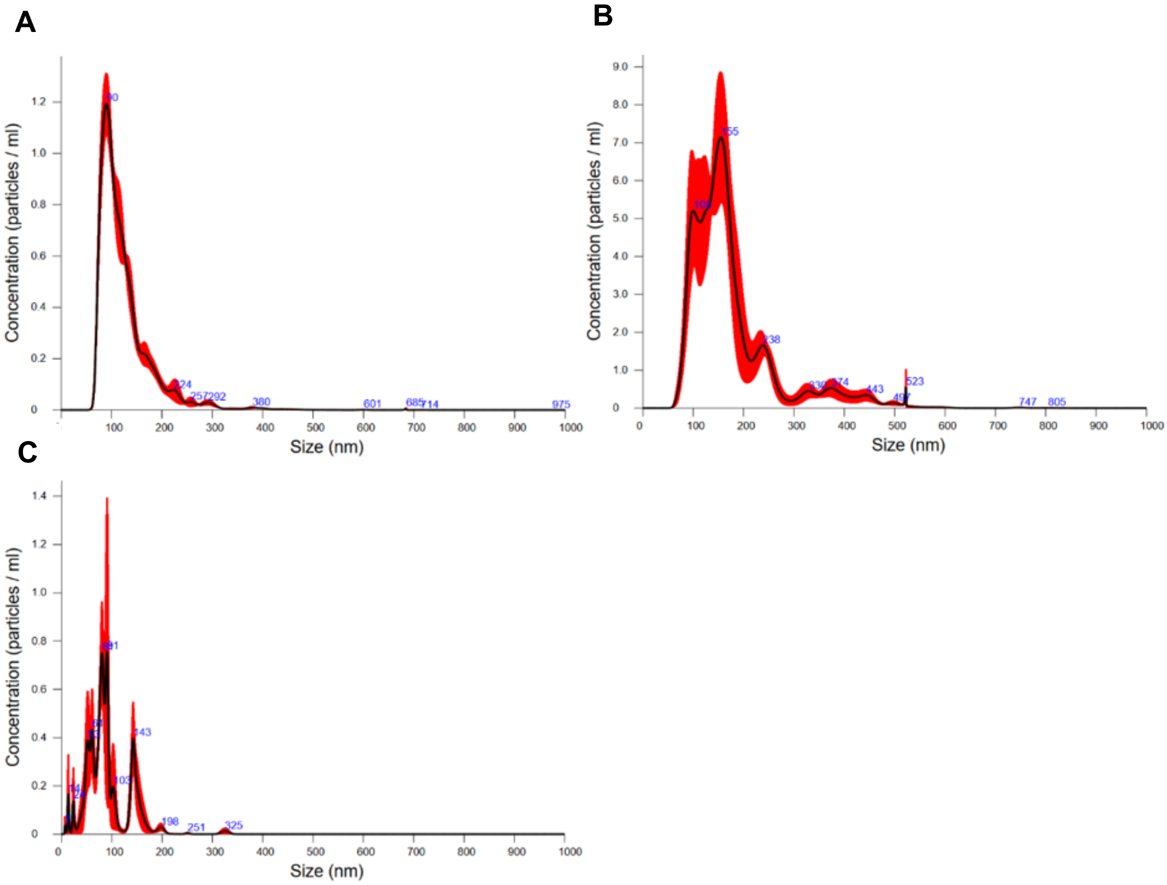

**Figure 4-6. Size distribution of isolated vesicles.** *(**A**: PEXsF. **B**: PMV, **C**: PBS)*

Depending on the source, EXs can range in size from 30-200 nm (30,33). Although there is some overlap between EXs and MVs, classic MVs are generally larger with a size ranging between 150-1,000 nm, but there are also small MVs (30-150 nm) and large MVs (1,000-10,000 nm) (30). This is consistent with our results. NTA of isolated pEXsF exhibited a right-skewed distribution with a peak at 90 nm, a mean value of 124.0 +/- 1.3 nm and a mode value of 94.2 +/- 5.9 nm. The isolated pMV showed a much broader, also right-skewed, size distribution, with three peaks at

100 nm, 155 nm and 238 nm, a mean value of 171.2 +/- 10.5 nm and a mode value of 124.7 +/- 13.2 nm. Of note, the NTA results also demonstrated that the PBS, which served as a negative control, contained particles within the size range of EXs. Nevertheless, the concentration of particles is considerably lower, at 2.9 +/- 0.2 particles/frame compared to 121.9 +/- 5.5 particles/frame in pEXsF.

## *4.3 Immunofluorescence images of Siglec-3 and Siglec-6 expression on HepG2 cells and the uptake of pEXs*

The first set of immunofluorescence stainings was conducted to assess the best dilutions of the Siglec-3 AB (R&D Systems) and Siglec-6 AB (Thermo Fisher). For this purpose, we utilised dishes from previous experiments containing HepG2 cells that had been incubated for 5.5 h at 37 °C, 21% $O_2$ and 5% $CO_2$ with 20 µg protein of transfected pEXsF per dish. HepG2 cells, Siglec receptors and pEXsF were targeted with primary ABs as described in Table 3-5. The secondary ABs, described in Table 3-6, were used to label the primary ABs fluorescently. For the Siglec-3 stainings, no ABs were used to restain the pEXsF since the primary ABs against Siglec-3 and pEXsF have the same host (mouse), and the secondary AB, intended to stain only Anti-Siglec-3, would have also stained Anti-pEXsF. However, pEXsF should have been visible in any case due to their red coloured auto-fluorescence. As shown in Figure 4-7, the HepG2 cells' membrane was fluorescently labelled with a green colour, the Siglec receptors with a purple colour and the pEXsF with a red colour. Additionally, a DAPI staining was used to make the HepG2 cells' nucleus visible in a blue colour.

The imaging revealed the presence of Siglec-3 and Siglec-6 AB binding. A dilution series of 1:20, 1:80 and 1:150, as seen in Figure 4-7, images A, B and C, was employed to test the Siglec-3 AB (R&D Systems). Siglec-3 staining was observable in the 1:20 dilution, image A, and the 1:80 dilution, image B. Interestingly, the different dilutions did not result in a difference in the staining brightness. Unfortunately, the Siglec-3 and the HepG2 cells' membrane staining did not work in the 1:150 dilution. The Siglec-6 AB (Thermo Fisher) was tested in the following series of dilutions: 1:50, 1:100 and 1:200. As visible in Figure 4-7, images D-F, all dilutions resulted in purple staining and showed the same intensity of Siglec-6 staining, but the best visibility of Siglec-6 in co localisation to the green membrane staining was obtained at the 1:50 dilution, as visible in image D. In the Siglec-3 assay, pEXsF, which were expected to be red, were not visible inside the HepG2 cells, nor in association with the cell membrane, nor outside the cells. In the Siglec-6 images D, E and F, red circles mark red particles, which were visible without a clear association with HepG2 cells. It remains unclear whether these are pEXsF or simply image noise. It must be noted, that the HepG2 cell membrane staining with Anti-GFP did not work correctly in some cases, as can be seen in Figure 4-7, images A, E and F. Instead of a distinct cell membrane staining, granulosa green spots are visible in these images and the cells' membrane is not distinctly identifiable. On the contrary, images B and D show distinct green membrane staining.

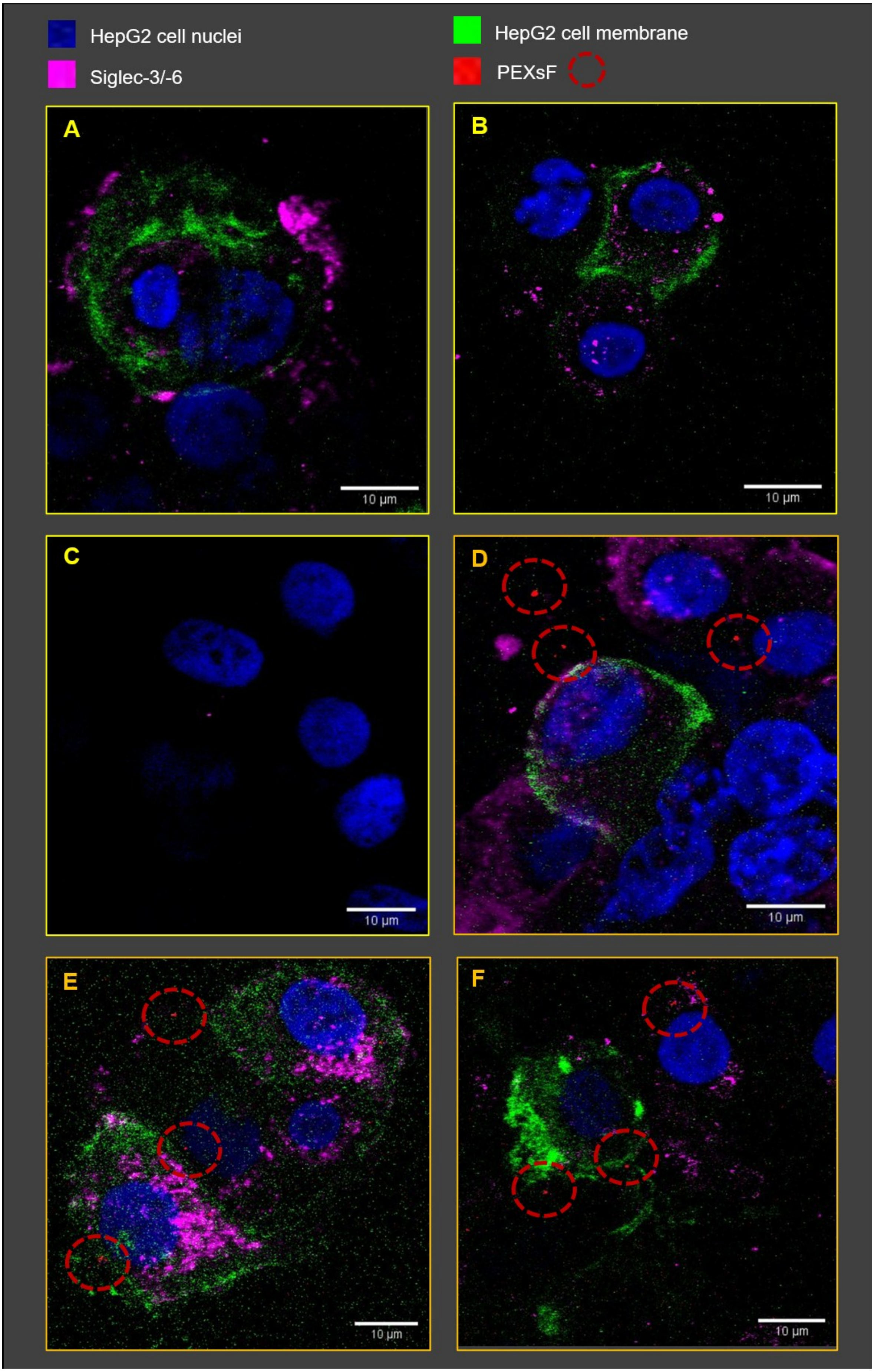
HepG2 cell nuclei
HepG2 cell membrane
Siglec-3/-6
PEXsF
A
B
C
D
E
F
10 µm

**Figure 4-7. Immunofluorescence images of different Siglec-3 and Siglec-6 AB dilutions.** The components relevant to our assays were stained with fluorescent dyes as follows: HepG2 cell nuclei blue, HepG2 cell membrane green, Siglec-3 and Siglec-6 purple and pEXsF red (only in the Siglec-6 assay). *(**A:** Siglec-3, dilution 1:20; **B:** Siglec-3, dilution 1:80; **C:** Siglec-3, dilution 1:150 (membrane and Siglec-3 staining did not work); **D:** Siglec-6, dilution 1:50; **E:** Siglec-6, dilution 1:100; **F:** Siglec-6, dilution 1:200)*

The objective of the second series of immunofluorescence stainings was to determine whether there is a difference in the temperature-dependent uptake of pEXsF. HepG2 cells were cultured under standard culture conditions as described in 3.1.1 and treated with the isolated pEXsF. BCA revealed that we applied 27 µg protein of transfected pEXsF per dish. The initial imaging demonstrated that HepG2 cells tend to remain in close proximity to one another. This makes it challenging for ABs and pEXsF to reach cells that are situated in the middle of the resulting conglomerates. Consequently, we extended the incubation period of one set of dishes from 5.5 h to 24h under standard culture conditions. The other set of dishes was placed in a refrigerator at 4°C for 4 h. We used the same ABs as in the first set of stainings, however the Siglec-3 AB (R&D Systems) was excluded and only the Siglec-6 AB (Thermo Fisher) in the 1:50 dilution was used this time. As shown in Figure 4-8, the HepG2 cells' membrane was fluorescently labelled with a green colour, the Siglec-6 receptor with a purple colour and the pEXsF with a red colour. Additionally, a DAPI staining was used to make the HepG2 cells' nucleus visible in a blue colour.

Prior experiments have indicated that HepG2 cells are capable of internalising pEXsF, as evidenced by immunofluorescence imaging, which has demonstrated their presence near HepG2 cells' nuclei (116). However, our uptake assay could not confirm these results. Moreover, the different incubation temperatures did not result in any discernible differences, and the overall number of pEXsF was minimal. Since all images, independent of the uptake temperature, appeared very similar, only one exemplary z-stack staining image at 37° is presented in Figure 4-8, image A. The images B, C, D and E, present different sagittal cross-sectional images of A and the images F, G and H present different horizontal cross-sectional images of A. First, it must be mentioned that the green staining of the cell membrane, once again, did not work properly and the cells' membrane was not distinctly identifiable, as visible in image A. However, prominent staining of Siglec-6 in purple can be seen on the outer membrane border of the HepG2 cell conglomerate in image A. Upon examination of the vertically and horizontally cross-sectional images, it was observed that a small number of red dots, presumed to be pEXsF, were present in proximity to HepG2 cells. The red circles in the sagittal images B, C and E and the horizontal images F, G and H show pEXsF that appear to be associated with the cells' membrane and are also in close proximity to stained Siglec-6 receptors. The marked pEXF in images B and F is the same, once in the sagittal plane and once in the horizontal plane. Images C and H also show the same pEXsF, once in the sagittal plane and once in the horizontal plane. A single, potentially internalised, pEXF was observed within a HepG2 cell in close proximity to a blue stained nucleus, as visible in image D.

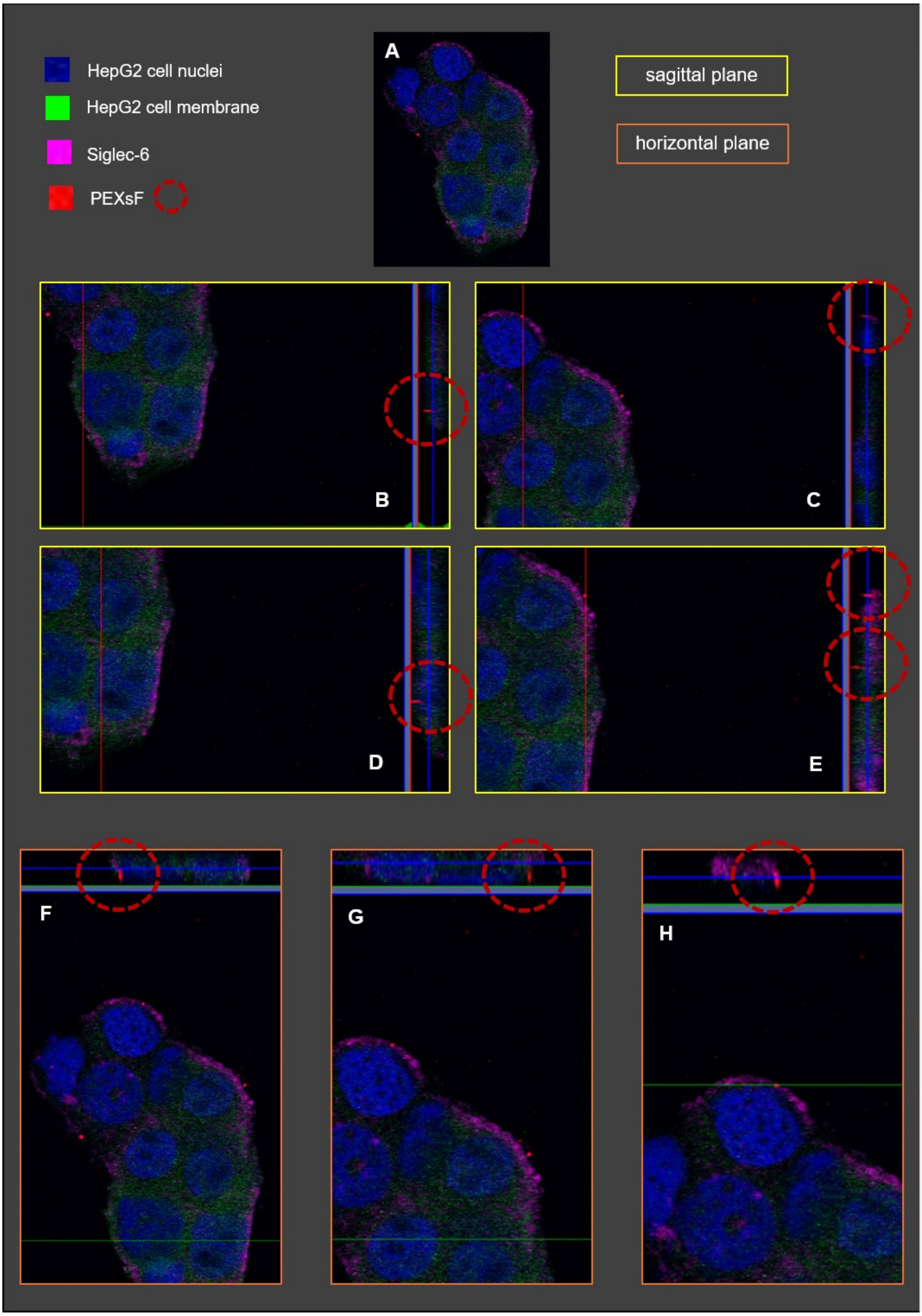

**Figure 4-8. Immunofluorescence imaging of pEXs in association with HepG2 cells. A** shows a conglomerate of stained HepG2 cells. **B**, **C**, **D** and **E** show cutouts of different cross-sectional images of **A** in the sagittal plane. **F**, **G** and **H** show cutouts of different cross-sectional images of **A** in the horizontal plane. The staining of Siglec-6 was observed to be primarily localized to the cell surface

this time. **B**, **C**, **E**, **F**, **G** and **H** show the presence of pEXsF in close proximity to the HepG2 cells' membrane and near stained Siglec-6 receptors. Only in **D** the pEXF appears to be situated within a cell, near a stained nucleus.

# 5 Discussion

EXs have been found to be involved in the complex immunoregulatory process of pregnancy (91,131,134). Given the pivotal role of the placenta in immunoregulation, the secretion of pEXs is regarded as one of the organ's fundamental functions (2). Nevertheless, previous research has primarily concentrated on pEXs released to the maternal side of the placenta (pEXsM). Since the placenta, however, also releases pEXs to the fetal side of the placenta (pEXsF), the objective of this thesis was to provide new insights into the characteristics of pEXsF, with a particular focus on their cellular uptake in liver cells. Previous experiments have indicated that pEXsF carry α2-6-linked sialic acid, a ligand for various Siglec receptors, and sialic acid-containing glycans have been shown to be involved in the cellular internalisation of EXs (58,60,116,175). Moreover, Siglec-6 has been linked to pregnancy-related functions and one study found that syncytiotrophoblast-derived placental vesicles express Siglec-6 (141,153,167). These findings are, however, poorly understood and require further investigation. Therefore, the aim of this thesis was to study the uptake of pEXsF in HepG2 cells, an immortalised liver cancer cell line, and to determine the expression of Siglec-2, -3 and -6 receptors on HepG2 cells.

The results of the immunoblotting experiments showed Siglec-3 AB (R&D Systems) binding to both PT and HepG2 cells. In pEXsF a very faint staining was detectable. The AB's data sheet describes binding at ~55 kDa, whereas our immunoblots showed binding sites between ~55-70 kDa with three dominant signals (182). According to the literature search, Siglec-3 should not be expressed on HepG2 cells, which raises questions about the specificity of the antibody used. Interestingly, we observed a similar antibody pattern in placental tissue. In addition, considerable non-specific binding sites of the AB were detectable on HepG2 cells as well as in placental tissue, even though there is no cross-reactivity described in the AB's datasheet (182). The Siglec-2 AB did not exhibit any reactivity and was therefore classified as non-human reactive and excluded from further experiments. To determine the Siglec-6 expression, we tested two different Siglec-6 ABs. However, even though both ABs did show an expression of Siglec-6 in PT, HepG2 cells and pEXsF, the expression pattern was very inhomogeneous between the two ABs. The polyclonal Siglec-6 AB from Thermo Fisher demonstrated a lot of unspecific binding, which suggests that this antibody might be less suitable for immunoblotting. Another potential explanation for the observed unspecific binding is the process of protein lysate preparation, whereby proteins may become fragmented and associate with other proteins. In contrast, this AB showed a very prominent single band around 70kDa, indicating a strong Siglec-6 expression in PT. In pEXsF the 70kDa band was not identifiable. The same samples and isolated pEXsF were used to test the polyclonal Siglec-6 AB from R&D Systems, which demonstrated a markedly higher level of specificity. Bands were observed at ~55 kDa in PT, HepG2 cells and pEXsF, and at ~ 70 kDa only in PT and HepG2 cells. This is consistent with the molecular weight of Siglec-6, described in literature ranging from 50-70 kDa, depending on differences in protein glycosylation (141,178–180). The R&D AB appears to be more suitable for immunoblotting, as fewer unspecific binding sites were identified. However, we did not use it for immunofluorescence staining, as this application possibility is not described in the AB's data sheet (182). Notably, the Siglec-6 AB (R&D Systems) data sheet describes cross-reactivity with recombinant human Siglec-3, -5. -9, -10, -2, -7 and 11 (183). For the other ABs no cross-reactivities are described (182,184).

In order to investigate the binding and uptake properties of pEXs into HepG2 cells, cell experiments, and immunofluorescence staining were performed. First, pEXsF were isolated by ultracentrifugation, and the size and respective size distribution of the isolated vesicles was analysed by NTA. In accordance with other studies, the size of pEXsF revealed a mean size of 124.0 +/- 1.3 nm, while the MVs appeared to be larger at 171.2 +/- 10.5 nm ((30,33). Moreover, in all measurements the sizes of MVs and pEXs overlapped. This result clearly indicates that isolated fractions of pEXs additionally contain larger vesicles within the range of MVs. To conclude, and as discussed in the literature, the approach to isolate exosomes by ultracentrifugation allows only the enrichment of vesicles with defined sizes from cell culture supernatants (28).

HepG2 cells, incubated with pEXsF, were used for immunofluorescence stainings. We tested the Siglec-3 AB (R&D Systems) and the Siglec-6 AB (Thermo Fisher). While immunofluorescence imaging enabled the detection of Siglec-3 and Siglec-6, a clear co-localisation of these proteins with the GFP transfected HepG2 cell membrane failed. As seen in Figures 4-7 and 4-8, the protocol for HepG2 cell membrane staining by introducing GFP constructs needs to be optimised first. We anticipated that there would be a discernible difference in the uptake of pEXsF into HepG2 cells at 4°C compared to 37°C, given that the receptor-mediated uptake should only occur at 37°C. However, the immunofluorescence imaging did not corroborate this hypothesis (data not shown). While Siglec-6 and Siglec-3 expression was detected by immunofluorescence staining, there were very few pEXsF visible in the imaging, and a direct association between a Siglec receptor and a pEXF could not be observed.

## *5.1 Conclusion and outlook*

In conclusion, our findings demonstrate that HepG2 cells express Siglec-3 and -6, which was not previously described in the literature. The expression of both receptors could be shown by immunoblotting and immunofluorescence staining. Furthermore, the expression of Siglec-6 on isolated pEXsF derived from primary fpAECs was demonstrated for the first time. The anticipated results were not achieved in the temperature-dependent uptake experiments of pEXsF into HepG2 cells. The results indicated the presence of isolated pEXsF on the surface of HepG2 cells. However, the findings were inconclusive and require further investigation.

For future experiments, several factors should be considered:

- HepG2 cells are a cancer cell line and, therefore, do not represent the same metabolic state as a primary liver cell. As a cancer cell line, there is a possibility that the expression pattern of molecules may differ from that observed in normal cells. It has been demonstrated that Siglec-6 expression is associated with several malignant diseases (170–173). The origin of HepG2 cells may thus be the reason for the Siglec-6 expression. Furthermore, the cells are of adult origin. For future experiments, it would be beneficial to consider utilising fetal liver cells, for instance, the fetal liver cell line cBAL111 (185).
- The protocol for the pEXsF uptake studies needs to be optimised. Despite previous experiments conducted in the host lab indicated an uptake of pEXsF into HepG2 cells, we could not confirm these results here. In our experiments,

only a small number of pEXsF could be identified. One potential explanation for this discrepancy is the extensive staining process, which differed from previous experiments, where only the HepG2 cells' nucleus was stained with DAPI. Additionally, we utilised transfected pEXsF, whereas the pEXsF in prior experiments were labelled with the fluorescent dyes PKH67 and Vybrant DiO Cell Labelling Solution (116). Perhaps a temperature-dependent uptake study should be performed to compare the uptake of transfected and not transfected pEXsF without membrane or Siglec staining to clarify if there is an uptake.

- The temperature-dependent uptake solely and exclusively allows to draw a conclusion about the receptor mediated binding or uptake of pEXsF in general and not specifically about a suggested Siglec-mediated uptake. Our aim was to find direct associations between stained pEXsF and stained Siglec receptors. Due to the lack of presence of pEXsF it remains unclear whether this association exists. However, if this approach is pursued, it could be worthwhile to inhibit or block Siglec receptors specifically. There is limited information in the literature about inhibitors, as this topic has not been extensively researched yet. Nevertheless, one study showed that the uptake of PKH26-labelled EXs in HeLa cells can be downregulated by incubating them with 11 N-acetylneuraminic acid or by blocking Siglec-3 with an anti-Siglec-3 AB (58).
- Immunoblotting revealed that pEXsF, derived from primary fpAECs, express Siglec-6. This prompts the question of whether the Siglec-6 receptor expression on pEXsF could be associated with the re-uptake of pEXsF in fetoplacental endothelial cells. It would also be worthwhile to determine whether fetal EXs, isolated from arterial cord blood, have an affinity for binding to Siglec-6. This could represent a mechanism by which they are internalised by fetoplacental endothelial cells and cross the placental barrier.
- PEXsF were isolated from fetoplacental endothelial arterial cells. In vivo, pEXsF coming from venous cells are the ones that reach the fetal organism. However, we did not employ those cells, as their cultivation is challenging, and they tend to be less viable. Nevertheless, this observation must be considered when formulating conclusions and planning future experiments.

Based on the current knowledge, the diagnostic and therapeutic capability of using EXs in different clinical applications is very likely. EXs have the potential to be a valuable tool, as they are minimally invasive (78). This is particularly of interest in the context of pregnancy. Studies already demonstrated the involvement of pEXsM in a healthy and disease-related context in pregnancy (91,100,134,135). It is conceivable that pEXsF may have similar functions. The potential application of such vesicles would have a profound impact on the field of obstetrics. It would represent a significant step forward in the assessment of fetal well-being and the possibility of targeted drug delivery. To ascertain whether this potential can be exploited, it is essential to gain a deeper insight into the composition and function of pEXsF and to study this in the context of their target cells during pregnancy.

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