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Placenta-Derived Exosomes and Their Role in the Immune Protection of the Fetus

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1. Introduction

The mammalian pregnancy is an immunologic challenge to the maternal immune system. Considering the fact that transplant rejection is a well-defined immunologic phenomenon, the peaceful feto-maternal coexistence during mammalian pregnancy has been defined as "a paradox of nature" and puzzled immunologists for ages. In 1953, the immunologist and Nobel Prize laureate Sir Peter Medawar proposed that the maternal immune system is ignoring the fetus and defined his three well-known mechanisms for achievement of this: anatomical separation of the fetus and the mother, fetal antigenic immaturity and maternal inertness or indolence to the fetus (1). Although Medawar's proposal is still recognized and cited, it is only partly true and has been rightly revised in recent years. Today, it is well-proven that instead of being ignored, pregnancy is indeed recognized but tolerated by the maternal immune system, however, the responsible mechanisms for that remain unknown. The maternal-fetal interactions are highly complex and cannot be explained by, or subordinated to a single uniting theory of maternal tolerance to the fetus. In stead, multiple mechanisms, operating in concert at the systemic- as well as the local level are moulding the framework of successful pregnancy (reviewed in 2). Several of these mechanisms are mediated by the placenta, the key organ for successful mammalian reproduction (2).

Apart of its function as a hormonal, nutritional and oxygen provider of the fetus, placenta stands out as an important immunomodulatory organ actively secreting signal substances and factors that alter the maternal immune responses during pregnancy. Recently, several reports have shown that the human placenta participates in the feto-maternal cross-talk by secretion of nanometer-sized endosomally produced membrane-bound microvesicles (MV) called exosomes that act as fetal messengers and transfer packages of information to the maternal organism for adaptation to the ongoing pregnancy (3-8). The main focus of this chapter is on placenta-derived exosomes and their role in reproduction. A comparison to the placenta-released exosomes, larger placental microvesicles/microparticles, which are shed from the apical cell membrane of syncytiotrophoblast, will be presented and discussed. Initially, as a background, a short description of cell-cell communication and various microvesicles and their generation and roles is given.

2. Cell-cell communication – A basic necessity for all living organisms

Communication between individual cells is imperative for all living organisms. For a long time, cell communication was considered to be effectuated by three different ways: 1) by direct adhesion contacts between cells, such as receptor-ligand signalling and trogocytosis; 2) by soluble mediators, such as hormones, cytokines, chemokines and other signalling substances, bioactive ions and lipids, released in an autocrine and paracrine manner; and 3) by shuttling of information through intercellular channels called nanotubules (9, 10).

Recently, however, attention was focused on the fourth way of intercellular communication built on release and uptake of membrane-bound MVs. Communication by MV combines secretion of molecules with preservation of their membrane attachment and three-dimensional structure thus preserving the biologic activity of these molecules. Secreted or shed MVs execute cell-cell contact "by proxy", delivering information from a donor to a recipient/target cell in the near vicinity or at a distance. The exosomes are the smallest members of the MV family and the only ones that are produced in the endosomal compartment in multivesicular bodies (MVB) and secreted by exocytosis in the intercellular space, blood and various bodily fluids. The function of exosomes is highly diverse and dependent on the cells from which they originate. One very prominent feature is their immunomodulatory potency (reviewed in 9, 10). The rest of the MVs are larger in size and are produced and released from the plasma membrane by shedding or blebbing.

2.1 Microvesicles are everywhere

The existence of various membrane-bound MV in the intercellular space is easily observed by electron microscopy but for a long time was considered to be inert debris from cellular damage and of no importance. Thus, the first descriptions of exosome-like microvesicles during the 1980s by Heine et al. and Jonstone et al., who also named them exosomes and pointed out a biological role for these vesicles, were completely ignored (11-13). Only recently, less than 10 years ago were they rediscovered and identified as tools for intercellular communication. The realization that there are MVs produced by various cells and found in the blood and all bodily effusions in both health and disease has opened new perspectives in biology, understanding cell-cell communication and various biological mechanisms and their regulation. The MVs are a heterogeneous group, released both in health and disease. Their composition depends on and reflects the state of the cells that produce them and their physiological and/or pathogenetic roles are diverse depending on the donor cells, the recipient cells and in what environmental context they act. MVs are divided by size, morphology and mode of generation into (i) large MV, produced by budding of the cellular membrane, 0.1-2 μm in size with various roundish, oval or elongated shapes and (ii) small, nanosized (30-100 nm) MVs of endosomal origin called exosomes that will be separately discussed. The large MVs comprise two main types: those, produced by budding from the plasma membrane of living cells, including shed microvilli, called microvesicles or sometimes microparticles (14, 15); and those, produced by blebbing/fragmentation of the plasma membrane during the programmed dying of the cells, called apoptotic bodies/apoptotic blebs/apoptotic vesicles (16).

MVs, including exosomes, are produced by a vast variety of cells. Their "rediscovery" and upgrading in importance have caused a huge, exponential interest among scientists and

literally an explosion of reports in the literature describing the MVs under different names, which can create confusion. In Table 1 a glossary with the names and definitions of various MV, described in the literature are given. The physical and morphological characteristics of the main MV types are presented in Table 2.

Microvesicle designation	**Definition**
Apoptotic bodies/blebs/ microvesicles/ microparticles	Microvesicles produced by fragmentation of the plasma membrane and the soma of dying cells. Carry membranal and cytosolic proteins and nucleic components like DNA.
Cardiosomes	Term used to designate exosomes produced by cardiomyocytes
Ectosomes	Vesicles shed from the plasma membrane of neutrophils and fibroblasts.
Endosomes	Nanovesicles (<100 nm in size) present in the multivesicular bodies (MVB), produced by inward budding of MVB's limiting membrane and secreted by exocytosis; called exosomes in secreted form.
Exovesicles	Microvesicles shed from the plasma membrane of dendritic cells.
Exosomes	30-100 nm-sized vesicles of endosomal origin secreted by fusion of MVB with the plasma membrane. Produced by a great variety of healthy and tumor cells and by the syncytiotrophoblast of the placenta.
Microparticles	Microvesicles shed by platelets, monocytes, and by the apical part of the syncytiotrophoblast plasma membrane, should not be confused with the syncytiotrophoblast-derived exosomes that are secreted through the exosomal compartment.
Microvesicles	The term is used in two ways: to designate all types of membrane-bound microvesicles, including exosomes and/or to designate larger microvesicles (>100 nm) produced and shed by the plasma membrane of normal and abnormal cells.
Prostasomes	Microvesicles of around 600 nm to 1μm in size, produced by shedding from the plasma membrane of normal prostate gland epithelium. Presence of prostasomes in prostate secretion is associated with fertility. However, the term has been used in a substantial amount of publications to designate exosomes.
Prominosomes	Microvesicles produced from the plasma membrane of the stem cells in the neural tube and the brain
STBM	Syncytiotrophoblast microvesicles or microparticles produced by the apical part of the plasma membrane, see microparticle definition above.
Tolerosomes	Exosomes, produced by the epithelial cells in the gastro-intestinal tract, involved in oral tolerance
Vexosomes	Microvesicles/exosomes engineered in vitro to carry viral vectors for gene therapy

Table 1. Glossary for terms and definitions used in scientific publication for description of microvesicles

Characteristics	Exosomes	Microvesicles/ Microparticles	Shed microvilli	Apoptotic bodies/vesicles
Size	30-100 nm	0.1-2 μm	> 400 nm	100–600to700 nm
Density in sucrose	1.13 – 1.19 g/ml	Undetermined	Undetermined	1.16-1.28 g/ml
Sedimentation (g)	100,000 -110,000	10,000 -100,000	10,000	1,500 – 100,000
Morphological shape	Cup shaped, electron translucent	Various shapes, electron-dense and/or electron translucent	Various shapes, round, elongated and cylinder-like	Irregular and heterogeneous in shape
Lipid membrane composition	Cholesterol-, sphingomyelin-, and ceramide-rich lipid rafts, expose phosphatidylserine	Expose phosphatidylserine, some enriched in cholesterol and diacylglycerol, some undetermined	Undetermined	Undetermined
Specific marker(s) for identification	Tetraspanins (CD63, CD9, CD83), ESCRT complex members (Alix, TSG101)	Integrins, selectins, CD40 and others, depending on the cell type	Various, depending on the cell type	Histones, DNA
Origin in the cell	Endosomal compartment - multivesicular bodies (MVB)	Plasma membrane	Plasma membrane	Fragments of dying cells, undetermined
Mechanism of sorting	Ceramide and ubiquitin dependent	Unknown	Unknown	Fragments of dying cells, undetermined
Intracellular storage	Yes	No	No	No
Mode of release/secretion	Exocytosis by fusion of MVB with the plasma membrane	Plasma membrane blebbing	Plasma membrane blebbing	Plasma membrane blebbing and cellular fragmentation

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Table 2. Some of the main characteristics of different types of microvesicles.*

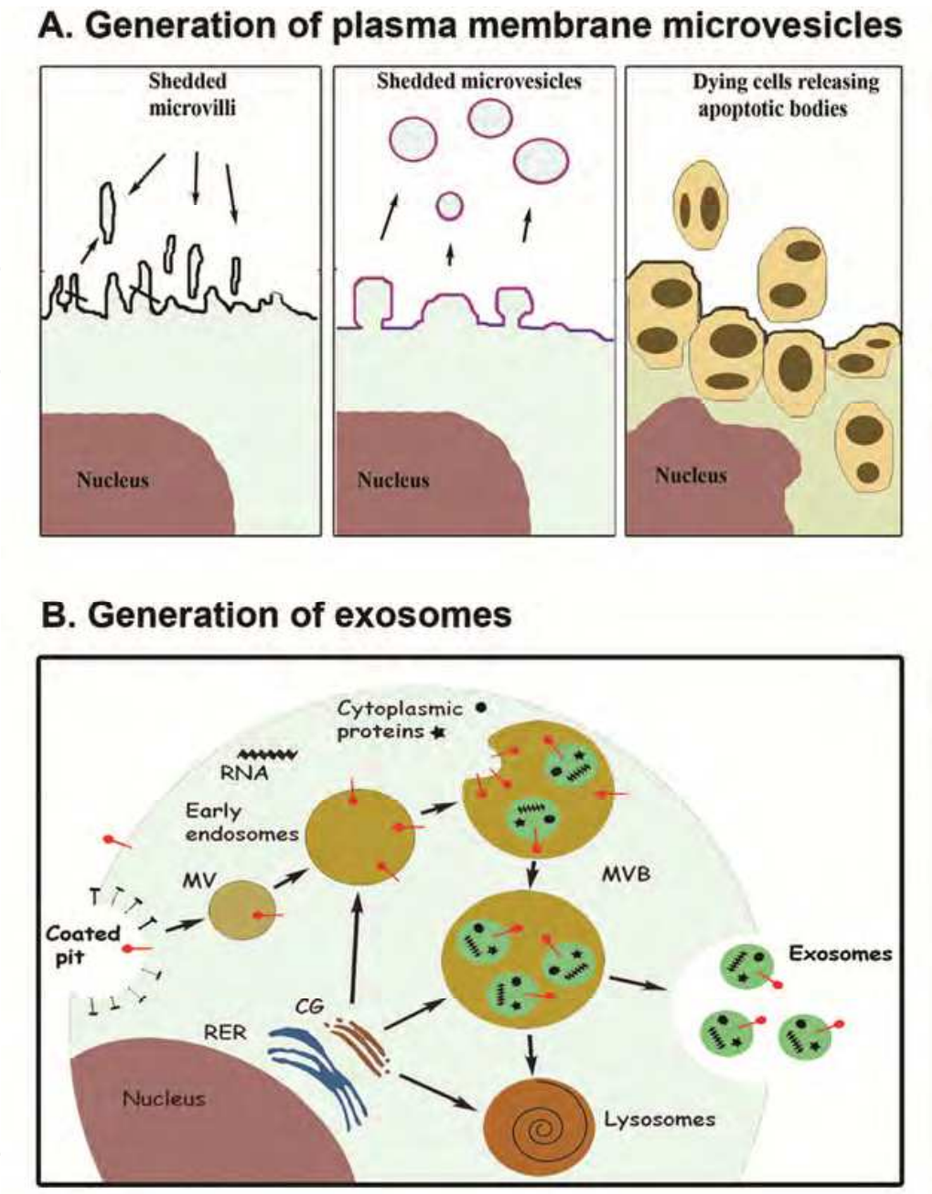
2.2 Exosomes: definition and biogenesis

The exosomes are the smallest MVs that are produced in the multivesicular bodies (MVB) in the endosomal compartments of individual cells and secreted into the extracellular space by

exocytosis. The exosomes are membrane bound nanometer-sized vesicles that carry a variety of proteins on their surface as well as inside, mRNA and microRNA and can be compared to parcels sent as "mail" between cells. The late endosomal compartment, where they are generated, can be viewed as "the post office". Each exosome carry proteins on its surface that serve as addresses of the sender and the recipient cell. Recent accumulating evidence shows that this "mail" can be powerful and transforming, determining the fate of the recipient cell.

The current definition of exosomes is: secreted membrane-bound nanovesicles that carry the following characteristics: 1) cup-shaped form, 2) 30-100 nm size, 3) tetraspanin presence in their lipid rich membrane, 4) buoyant density of 1.13-1,19 g/ml on sucrose gradient and 5) endosomal origin (9, 10).

The suggested pathway of biogenesis of exosomes separates them from all other known MVs. The exosomes are produced in the late endosomal compartment by inward budding of the limiting tetraspanin- and lipid-rich membrane of MVB and contain surface-bound and cytosolic proteins and RNA molecules. They are released when the MVB membrane fuses with the cellular plasma membrane and the MVB content of exosomes is emptied into the extracellular space. A detail schematic presentation of exosome biogenesis is shown in Figure 1B. There are two major pathways by which proteins are sorted to the MVB membrane that by inward budding becomes exosomal membrane: (i) protein recycling by endocytosis and transport of plasma membrane-expressed proteins to the early recycling endosomal compartment and from there to the late endosomal compartment and eventually to the MVB ending up on exosomes and (ii) direct transportation of proteins from the Golgi complex to the MVB where they are inserted into the MVB limiting membrane and further become expressed on the exosomal membrane as exosomes are produced in the MVB by inward budding. Previously, MVB were solely considered as an intermediate stage in the maturation of endosomes to lysosomes and were ascribed to be a "garbage station" of the cell – a dustbin for proteins aimed for destruction. Today it is known that instead of the lysosomal protein-destruction route, MVB can take an alternative route, moving to the plasma membrane and by fusion with it releasing their nanovesicle cargo as exosomes in the extracellular space. Thus, MVB are endosomal cellular organelles situated at a cross road in the cell where the fate of the proteins sorted to the MVB is decided – secretion by exosomes or degradation in lysosomes. Accordingly, two types of MVB have been suggested – degradative MVB taking the lysosomal pathway and exocytotic MVB involved in the secretion of exosomes. The process of sorting proteins to degradative MVB involves a multiprotein network called endosomal-sorting complexes required for transport (ESCRT) and ubiquitinylation process that tags with ubiquitin both cell surface and cytosolic proteins targeted for lysosomal degradation (17, 18). At present, the mechanism underlying the exocytotic MVB trafficking to the plasma membrane is less clear. The transmembrane protein TSAP6 is suggested to regulate exosome formation and Rab11, a member of the small GTPase family and calcium are considered to participate in the docking and fusion of exocytotic MVB with the plasma membrane (19, 20). Two pathways for protein sorting for exosome secretion have been suggested – the ESCRT multiprotein complex and an alternative ubiquitin-independent pathway based on sphingomyelin metabolites such as ceramide (18, 21). More studies are needed to establish the link between ubiquitin, phospholipids and ESCRT proteins and their role in the biogenesis of exosomes.



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Fig. 1. Generation of different microvesicles and their main characteristics. A) Shed microvilli, microvesicles and apoptotic bodies are all generated by the plasma membrane. B) Exosomes are generated in the endosomal compartment and carry both recycled proteins and proteins produced in the rough endoplasmic reticulum (RER) and directly sorted to the multivesicular bodies (MVB) from the Complex Golgi (CG). Note how the proteins are internalised in coated pits or inserted in the limiting membrane of the MVB and how exosomes are produced by inward budding of the limiting membrane – a way that ensures the same orientation of the membrane bound proteins on the exosomal membrane as that on the plasma membrane of the cell. The exosome-filled MVB are either fused with the plasma membrane to release exosomes or sent to lysosomes for degradation. The mechanisms deciding excretion or degradation of MVB are at present not known.

2.3 Exosomes: morphology, general biochemical composition and suggested roles

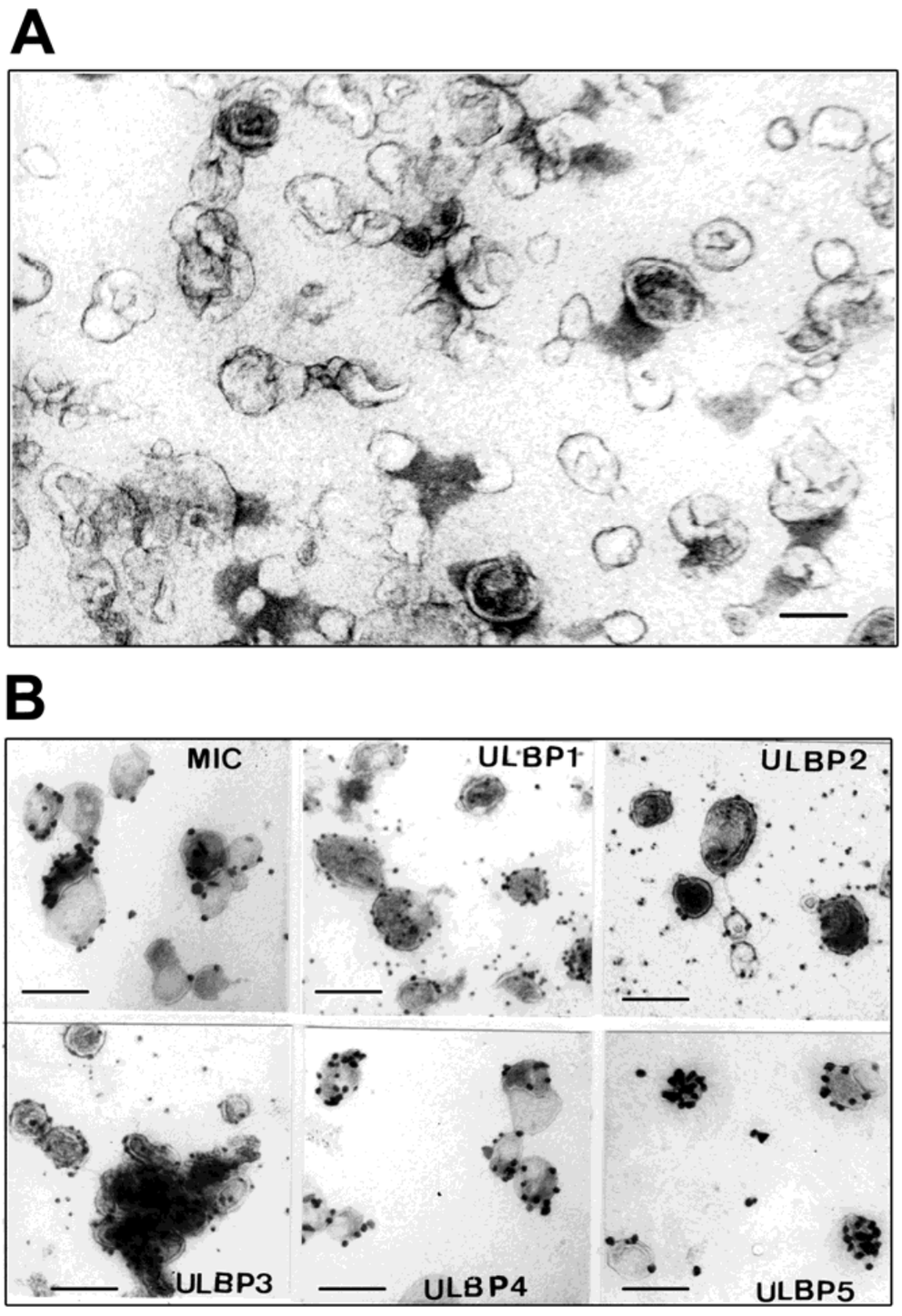
Electron microscopy is the method of choice for studies of exosomes biogenesis and morphology. Visualized *in situ* the exosomes are uniform spherical vesicles of 50-90 nm in size situated within the lumen of MVB. The electron microscopy image of isolated exosomes is different- the size varies between 30 to 100 nm, which is the upper size limit of exosomes and they have a typical cup-shaped form that is their hallmark. An illustration of isolated exosomes showing the typical heterogeneity in size and the cup shape is given in Figure 2A. The reason for the cap shape appearance is not known, but might be a consequence of the isolation procedure. The exosomal membrane is detergent and low temperature resistant and consists of lipid-rich bilayer of cholesterol, sphingolipids and tetraspanins where proteins with transmembrane or glycosylphosphinositol linkage are inserted.

Exosomes consist of proteins and RNA. The composition of the exosomes depends on the cells that have produced them and can vary even within the same cells, depending on their current differentiation and activation status. Thus exosomes reflect their donors and can influence their recipients. There is a conserved set of proteins common to all exosomes – cytosolic proteins such as tubulin, actin, actin-binding proteins, annexins, Rab proteins, heat shock proteins, signal transduction kinases, heterodimeric G proteins and the ESCRT members ALIX and TSG101. Common proteins expressed on the exosomal surface are adhesion molecules such as integrins and ICAM-1, MHC class I, and the tetraspanins like CD9, CD63, CD81 and CD82 (22).

There are many advantages of exosome-mediated cell-cell communication that can be listed as follows: (i) preservation of the 3D structure of the transported proteins and thus their biological activity; (ii) independence of cell-cell contact for signal delivery; (iii) independence of de novo synthesis; (iv) packages of carried molecules with a lower mobility and a higher concentration of the carried molecules; (v) biological effect at a distance.

Exosomes have been ascribed a variety of biological functions such as intracellular signalling, antigen presentation, immune regulation, receptor-ligand interactions, pro- and anti-apoptotic effects, delivery of proteins to plasma membrane of recipient cells thus changing their adhesive properties, transport of bioactive mRNA and microRNA, thus regulating gene expression and reprogramming recipient cells.

Exosomes can be divided into two major groups in relation to the immune defence system – exosomes with immunoactivating properties and those that are tolerogenic or immunosuppressive. In general, exosomes produced by immune cells such as antigen presenting cells (macrophages, dendritic cells and B cells) are immunoactivating. They can activate in various ways such as (i) directly by functioning as antigen presenters by proxy or indirectly by initiating immune response via dendritic cells that take up the exosomes and process the antigens carried by them; (ii) by activation T helper cells to cytokine production; (iii) by boostering cytotoxicity, antibody and cytokine production or priming of T cells (reviewed in 9, 10, 23). Interestingly, the cytolytic molecules perforin, granzyme and granulysin as well as FasL that are components of the cytotoxic machinery of cytotoxic T- and NK cells, are carried by exosome-like specialised vesicles in secretory lysosomes (24). The exosomes produced by epithelial cells and by the great majority of tumors are immune inhibitory. Normal epithelium secretes low amounts of exosomes that exert a mild immunosuppressive effect promoting homeostasis and immune tolerance. In contrast,



Bars represent 100 nm.
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Fig. 2. Isolated exosomes from supernatants of placental explant cultures. A) Negative contrast staining showing their size and the typical cup-shaped form. B) Isolated exosomes stained with antibodies against the NKG2D ligands MIC and ULBP1-2 and immunogold.

epithelialy derived tumors such as various carcinomas secrete high amounts of immunosuppressive exosomes that carry immunoinhibitory molecules, proapoptotic molecules and receptor ligands that serve as decoys, dysregulating normal immune responses and impairing the immune system of the host. Thus the net effect of tumor exosomes originating from mammary, lung, colon, prostate and ovarian cancers is a powerful immune inhibition promoting the primary tumor to establish itself and spread metastases (25). Similar to tumors, placenta-derived exosomes, produced by the syncytiotrophoblast are suppressive and able to modulate the maternal immune system.

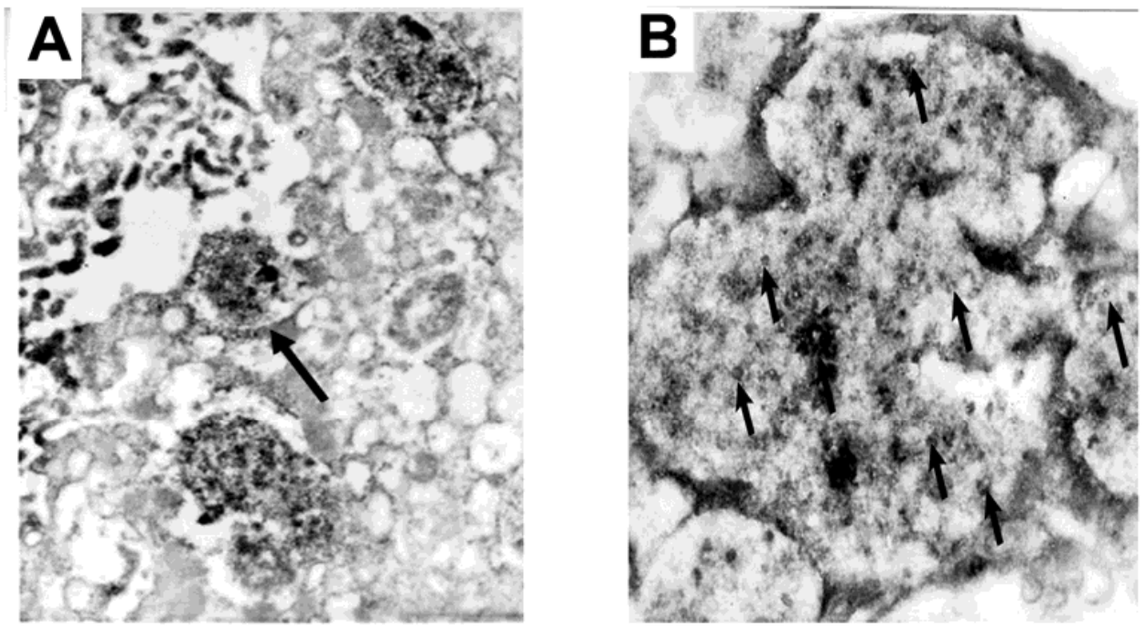
3. Human syncytiotrophoblast-a source of plasma membrane-derived shed microvesicles and endosomally secreted exosomes

The human syncytiotrophoblast, covering the placental villi, is the most important cell type of the human placenta with several functions such as being (i) the chief regulator of oxygen and nutritional and waste transport between the fetus and the mother necessary for fetal survival and growth; (ii) the site of synthesis of a variety of proteins – such as steroid and placental protein hormones, adhesion molecules and matrix metalloproteases, signal substances, and immunomodulatory molecules of crucial importance for the pregnancy success (26, 27). The syncytiotrophoblast is responsible for and carries out the "cross talk" between the mother and the fetus during pregnancy.

The human syncytiotrophoblast comprise a continuous layer of multinucleated finally differentiated trophoblast that makes a syncytium covering completely the multitudinous chorionic villi. The free apical part of the syncytium that is in direct contact with the maternal blood, is richly covered with numerous highly pleomorphic microvilli and branched surface projections suggesting a high mobility of the apical part of the syncytioplasm (27, 28). Comprehensive electron microscopic studies have shown that there is a constitutive shedding activity of plasma membrane-bound microvesicles and even whole microvilli from the apical syncytiotrophoblast surface membrane to the maternal blood. This normal shedding activity becomes forcefully enhanced by stress enforced by various pathologic pregnancy conditions, the most well-known and studied being preeclampsia (29). Besides the intensive shedding activity from the apical membrane surface the syncytiotrophoblast has an elaborate endosomal compartment and a high protein turnover activity. Scattered between the villi are numerous bristle-coated pits or caveolae, lipid-rich spots on the plasma membrane, associated with membrane molecule uptake and recycling. The syncytioplasm is very rich in free ribosomes and rough endoplasmic reticulum with dilated cisterns, Golgi complexes distributed at intervals in the syncytium and numerous mitochondria and tubular and numerous endosomal membranes and multivesicular bodies, all characteristic of vigorous protein synthesis and a well-developed endosomal compartment. The features of active protein synthesis, protein uptake and recycling and the elaborate MVB-rich endosomal compartment warrant for biogenesis and release of exosomes. Numerous nanometer-sized vesicles are seen in the MVB that reach the apical plasma membrane and open up to release their content. Thus, in addition to MV shedding from the plasma membrane, the syncytiotrophoblast of the human placenta simultaneously and constitutively releases exosomes.

The first provided ultrastructural evidence of biogenesis of exosomes in human placenta was shown for FasL and reported by Frängsmyr et al. (4). Using immunoelectron

microscopy (IEM), it was shown that expression of FasL was completely devoid from the syncytioplasm and in stead directed to the MVB in the syncytiotrophoblast and secreted by exosomes of 60-100 nm in size (4). Furthermore, the NKG2D receptor ligands MHC class I chain related antigens A and B (MICA/B) and the human retinoic acid early transcript 1 (RAET1) proteins, also known as UL-16 binding protein (ULBP) 1-5, were used as marker molecules of the endosomal compartment in studies of the biogenesis of exosomes in the human syncytiotrophoblast (7, 8). Representative micrographs shown in Figure 3, illustrate MICA/B molecule expression in human syncytiotrophoblast. As can be seen in Figure 3A, besides surface expression these molecules are sorted to numerous MVB at different levels in the cytoplasm. Frequently, fusion of MVB with the apical microvillous membrane of the syncytiotrophoblast and release of microvesicles was seen (Figure 3B).



MICA/B: MHC class.I chain-related molecules A and B; MVB: multivesicular body.
Magnification: A) X 18,000; B) X 25,000.

Fig. 3. Biogenesis of MICA/B-expressing exosomes in the syncytiotrophoblast of human early normal placenta. A) Electron micrograph showing the apical microvillous surface and MVB stained with anti-MICA/B-antibodies. One of the MVB (arrow) is opening and releasing exosomes in the intervillous space. B) A magnification of MVB filled with exosomes and stained for MICA/B. Note also the staining of the limiting membrane. Arrows point at exosomes.

A logical question is how is it decided if a protein will be sorted to the endosomal compartment for exosome secretion? From our studies of exosome biogenesis in the syncytiotrophoblast (4, 7, 8) we propose two possible ways: (i) Proteins with lysine residues in their cytoplasmic tail such as MICB, FasL, ULBP4 and 5 are controlled by ubiquitinylation and will be sorted preferentially to MVB (30, 31). In support of this suggestion, we have reported that members of the ESCRT complex localize in the syncytiotrophoblastic MVB (8). (ii)By contrast, glycosylphosphoinositol (GPI)-linked proteins, such as ULBP1-3 and the

transmembrane MICA are preferentially expressed in lipid rafts at the cell surface and by recycling can be sorted to the MVB. Such lipid raft domains have been proposed to support sorting of proteins to MVB and formation of exosomes (32, 33). In our studies (4, 7, 8) we found frequently lipid rafts and caveolae in the apical surface of the syncytiotrophoblast. In summary, we can conclude that both plasma membranal and exosomal expression of proteins is occurring in the human syncytiotrophoblast. The rich endosomal compartment with numerous MVB and the frequent signs of exosome release from the apical surface suggests that exosomal secretion is a constitutive feature of the villous syncytiotrophoblast.

The next question is if there is an advantage of exosomal secretion in pregnancy? Our studies of apoptosis-inducing molecules and the stress-inducible NKG2D ligands in human placenta clearly show that exosomal secretion is chosen over plasma membranal expression of these molecules and this choice is of crucial importance for the protection of the fetal allograft. If the apoptosis-inducing FasL was expressed on the cell surface, it would be immediately cleaved by the richly expressed placental matrix metalloproteases. The resulting soluble FasL would be easily involved in induction and promotion of unwanted inflammatory responses at the fetomaternal interface in a similar way as it promotes autoimmune inflammation and hypergammaglobulinemia in a systemic lupus erythematosus-like syndrome (34). In stead, exosomal FasL expression provides a membrane-bound form of the molecule that induced apoptosis of activated immune cells and thus promotes immunotolerance (4-6, 34). Furthermore, a strategy of releasing NKG2D ligands by placental exosomes would be a decoy mechanism downregulating the activating NK cell receptor NKG2D; by contrast a membranal expression of these molecules would make the villous syncytiotrophoblast a target for attack by NKG2D receptor-bearing maternal cytotoxic T and NK cells (7, 8). In conclusion, (i) the syncytiotrophoblast of the human placenta is a site of exosomal biogenesis and release; (ii) the exosomal secretion of important immunomodulatory molecules promotes the survival of the fetal allograft and is thus preferentially used by the human placenta as a fetal immune escape mechanism. In addition, the human syncytiotrophoblast is a vigorous protein produced with a MVB-rich endosomal compartment and thus an excellent model for studies of exosome biogenesis *per se*.

4. Placenta-derived exosomes: composition, structure and function

4.1 Methodological considerations in experimental work with exosomes

The difficulties in characterizing placental exosomes so far lie in the fact that the villous syncytiotrophoblast, which is the main exosome producer, simultaneously sheds microvesicles, sometimes called STBM or microparticles, from the apical plasma membrane. In several studies, a crude bulk of ultracentrifuged pellets are analysed as exosomes. It is important to realize that such pellets contain all kinds of large MVs, apoptotic bodies, other particles and protein precipitates together with exosomes. Thus, it is of extreme importance to pay attention to the isolation procedure when separating placental exosomes for characterization of their biochemical composition and functions. Firstly, it is absolutely necessary to apply a continuous sucrose gradient (floating density 1.13-1.19) or a sucrose cushion to ensure enrichment and collection of "maximally pure" exosomes. Secondly, when immunoflow cytometry is used for phenotypic characterization of molecules on the exosomal surface, an indirect approach by loading of the exosomes on beads should be

applied, since most of the fluorescence-activated cell sorters have a discriminative capacity of around 300 nm and thus will exclude free unbound exosomes run directly in the cell sorter. Thirdly, the isolated fraction that is believed to be exosomes must be confirmed by analysing exosome specific markers, such as CD63, CD81, TSG101 and Alix.

4.2 Exosomal structure and composition

So far, studies of isolated placental exosomes have been performed on populations separated from placental explant cultures (7, 8) and peripheral blood of pregnant women (4, 5). Figure 2 illustrates syncytiotrophoblast-derived placental exosomes visualized by negative contrast staining and immunoelectron microscopy. The morphology is the typical cup shape and the size varies between 40 and 90 nm (Figure 2A). IEM reveals that they express the tetraspanins marker CD63 and the placenta-specific marker placental alkaline phosphatase, indicating their placental origin. In contrast to other exosomes, the placental exosomes are devoid of classical MHC molecules. Instead, they carry on their surface the stress inducible non-classical MHC class I chain-related molecules MICA/B and RAET1/ULBP1-5, ligands of the major cytotoxic receptor NKG2D (7, 8 Figure 2B). Besides the NKG2D ligands, placental exosomes express proapoptotic molecules such as FasL, TRAIL, and PD-L1 (3-6). In addition, they display a membranal expression of the regulatory cytokine TGFβ1. Recently, we performed proteomic analysis on placental explant culture-derived exosomes. An illustration of the major protein components are presented in Figure 4. In principal, the protein content can be divided into two compartments: proteins present on the exosomal membrane and those, entrapped in the exosomal lumen. The proteomic analysis revealed cytosolic proteins involved in biosynthesis and degradation, intracellular transport, fusion and signal transduction, heat-shock proteins and chaperons, and enzymes and ESCRT-associated proteins associated with exosome formation such as TSG101, Alix, vascular sorting protein 29 and charged MVB protein 1B and 4B (9, 10).

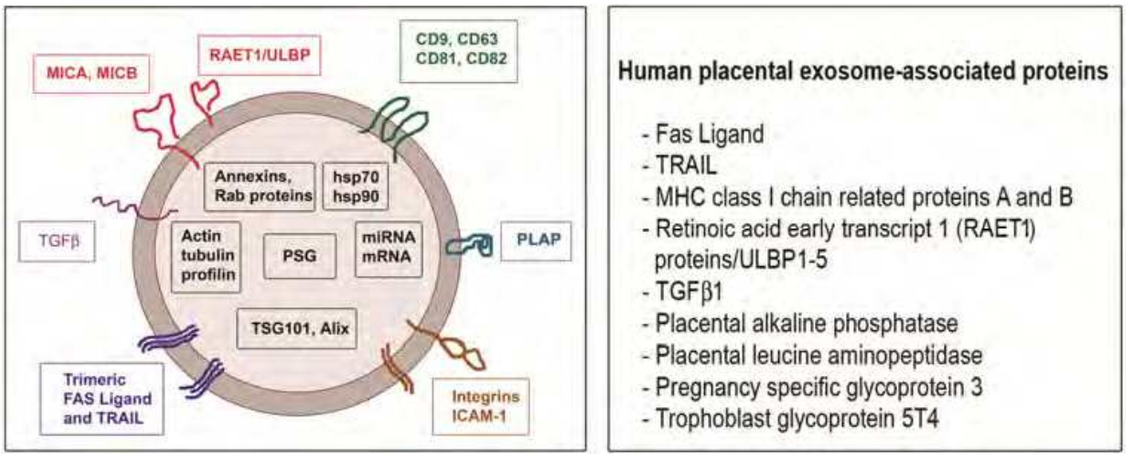


Fig. 4. Schematic presentation of a placental exosome and a list of some proteins, specific for placental exosomes.

Exosomes can transport selected mRNA and miRNA molecules from donor to recipient cells and thus influence and genetically reprogram recipient cells and regulate their intracellular metabolic pathways. So far, there are few studies of RNA content in placenta and placental

exosomes. The first detailed miRNA study of placenta revealed that most placenta specific miRNAs were localized at a miRNA cluster on chromosome 19 (35) and were up- regulated during placental development. Six novel miRNA were identified and 4 of them were placenta-specific. Placenta-specific miRNA was also identified in plasma from pregnant women (36). Urged by these reports, exploration of miRNA content in placenta exosomes was undertaken. The trophoblast-derived cell line BeWo was used as a source of exosomes. Two placenta-specific miRNAs, MIR517A and MIR21 were found in the exosome-enriched supernatant fraction from BeWo cultures. A possible involvement of MIR17A in the regulation of TNF signal transduction has been suggested. This is the first and so far only mi RNA investigation of trophoblast-derived exosomes. Thus, these results need to be repeated, confirmed and extended to exosomes from placental cultures and normal and pathologic pregnancies at different times of gestation and of placenta differentiation. Thus, placental exosome-derived mRNAs and miRNAs and their capacity to enter and reprogram maternal cells awaits to be elucidated in future studies.

4.3 What do placental exosomes do?

As mentioned previously, the importance of enriching maximally pure exosome fractions for functional studies cannot be overemphasized. In numerous studies a mixture of several microvesicle subpopulations is phenotypically and functionally analysed. Such studies are difficult to interpret and reproduce and thus must be left out when discussing the function of placental exosomes. Thus, the information, reviewed here is taken from a limited number of investigations with "maximally pure" exosome fractions isolated from peripheral blood of pregnant women (4-8) and placental explant cultures. These studies can be summarized as follows: (i) Placental exosomes are able to impair T-cell immune responses by downregulation of the intracellular signalling through the CD3-z chain of the accessory molecule CD3 and the enzyme Janus kinase 3 (JAK3). (ii) NKG2D ligand-bearing placental exosomes act as decoy and downregulate the major activating NK cell receptor NKG2D on cytotoxic T-, NK- and γδT cells with a consequent impairment of the maternal cytotoxicity and protection of the fetal allograft against maternal cytotoxic immune attack. (iii) Placental exosomes carry the apoptosis-inducing molecules FasL, TRAIL and PD-L1 in active functional form that is able to induce apoptosis in activated immune cells. Thus it is clear that placental exosomes are involved in the control of critical immune mechanisms such as cytotoxicity, T cell response and apoptosis in the local vicinity and /or at a distance from the feto-maternal interface. These functions define the placental exosomes as immunosuppressive, using in a redundant way a number of mechanisms that inhibit the function of the maternal immune system during pregnancy and promote the survival of the fetal allograft. In other words, placental exosomes are important players in the establishment of the maternal tolerance towards the fetus.

5. Exosomes in amniotic fluid

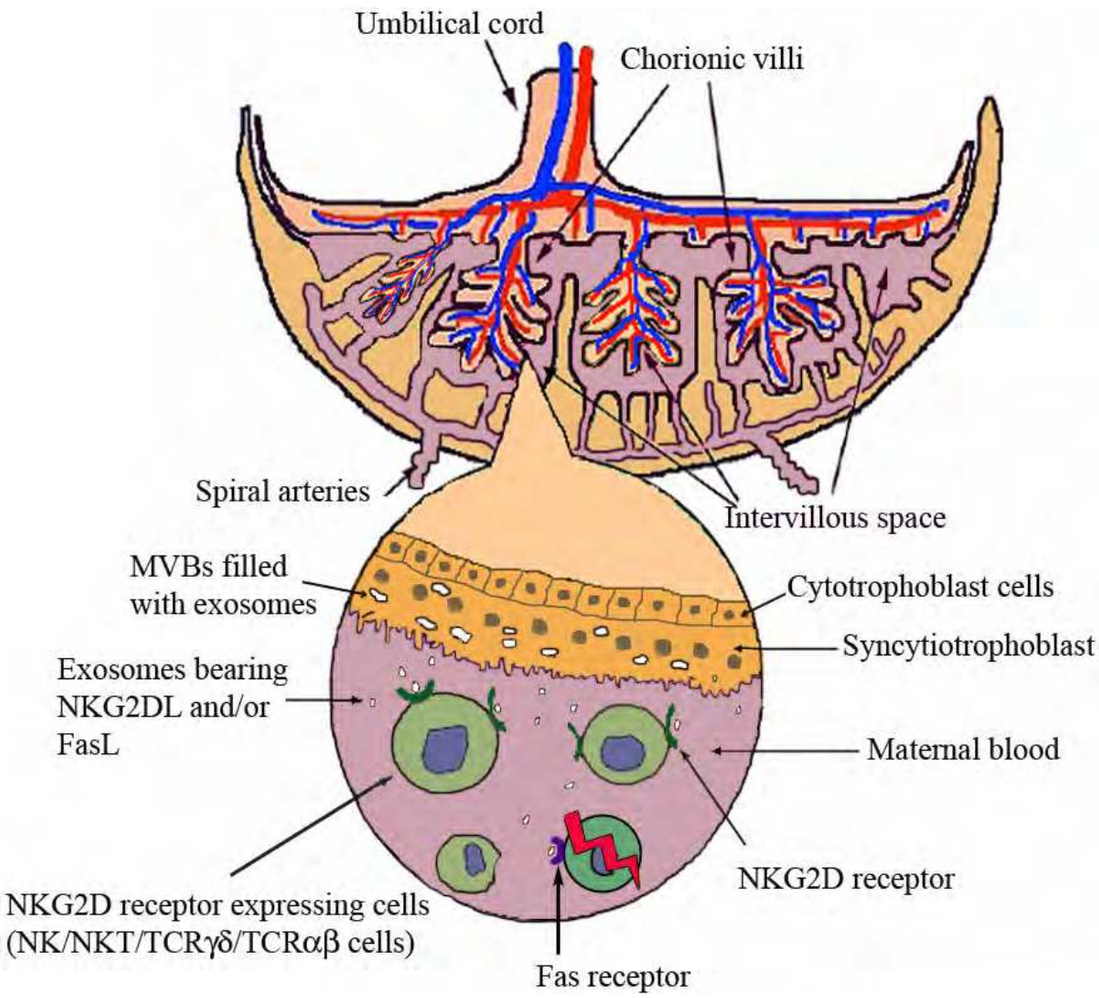
Often, when discussing placental exosomes in pregnancy, exosomes in amniotic fluid are mentioned and discussed in the same terms as placental exosomes. However, placental exosomes and amniotic exosomes differ in origin and function and are thus different entities. The placenta specific exosomes are syncytiotrophoblast-derived and are secreted in the intervillous space of the chorionic villi directly into the maternal blood and influence the

physiological adjustment of the maternal body to the ongoing pregnancy. The amniotic fluid stays in the amniotic cavity enveloped in the amniotic membranes and thus does not enter the maternal circulation. The fetus constantly produces amniotic fluid, most of which is fetal urine, and constantly swallows amniotic fluid throughout the pregnancy. At any developmental stage of pregnancy the amount of amniotic fluid is determined by the balance between these two processes. Convincing evidence has shown that the main source of amniotic fluid exosomes is the fetal kidney and the fetal urinary system. These exosomes carry on their surface CD24 as their specific address marker, annexin-1 and kidney markers such as aguaprin-2 and have a similar composition to exosomes from the urine of newborn infants (37, 39). It is logical to assume that their presence in the amniotic fluid is because of the fetal urine production rather than a special role in the immunomodulation of the maternal immune system. Amniotic exosomes have their niche and importance in monitoring the prenatal development of the renal system and in developing of prenatal diagnosis of kidney diseases and genetic malformations in the fetal kidney and urinary tract.

6. On the role of placental exosomes in human pregnancy: synthesis of facts and a proposed exosomal protective mechanism of action

Summarizing the current scientific data, there is no doubt that the syncytiotrophoblast of the human placenta continuously and constitutively produces and secretes exosomes. The placental exosomes are: (i) endosomally produced in the multivesicular bodies of the syncytiotrophoblast and released directly into the blood and systemic circulation of the pregnant women; (ii) pluripotent and immunosuppressive, carrying important bioactive molecules such as stress-inducible ligands, proapoptotic molecules, cytokines, other signaling molecules, mRNA and miRNA, thus being able to operate through different mechanisms and transform/reprogram recipient cells; (iii) acting by proxy to modulate the immune response of the mother locally or at a distance thus promoting maternal immune tolerance to the fetal allograft. A schematic drawing, suggesting two mechanisms that might be used by placental exosomes to alter the maternal immune response at the feto-maternal interface are presented in Figure 5. As can be seen, exosomes carrying NKG2D ligands can act as a decoy selectively downregulating cytotoxicity by internalization of the NKG2D receptor on cytotoxic T and NK cells without affecting the cytolytic machinery of the effector cells. Exosomes, bearing FasL, and/or TRAIL can induce apoptosis directed only towards activated Fas-expressing immune cells that might comprise a threat to the ongoing pregnancy. Furthermore, the exosomes, secreted by the syncytiotrophoblast to the maternal blood are most abundant in the intervillous space at the immediate vicinity of the chorion villi, where the highest risk for maternal attack and consequently the highest protection against the maternal immune cells is needed. The concentration of placental exosomes decreases with increasing distance from the placenta. Thus, the continuous secretion of exosomes by the syncytiotrophoblast creates an exosomal concentration gradient where the maternal immunosuppression and therefore immune protection of the fetus is strongest at the border between the syncytiotrophoblast and the maternal blood preventing a direct attack by the maternal immune cells. Moreover, the exosome turnover is very short, therefore the immunosuppressive influence of the placental exosomes would be "fading away" as the maternal blood leaves the placenta and enters the systemic maternal circulation. This could be one of the explanations why, although the maternal immune system during pregnancy is downregulated, it is not completely blunted. Pregnant women

are more sensitive to infections during pregnancy; however, they are still able to mount a modified immune response. The temporarily "semi- immunocompromized" maternal defence during pregnancy is the price humans have to pay for the elaborate hemochorial mode of reproduction, required to provide the huge amount of oxygen and nourishment necessary for the fetal development of the highly complicated and sophisticated human brain. Several mechanisms work in concert to meet this challenge and modify the maternal immune system during pregnancy with a minimized loss of ability to fight infections and convincing evidence suggests that secretion of placental exosomes is one of them. The syncytiotrophoblast, producing and releasing exosomes puts up a shield around the hemochorial placenta to protect it against the risk of maternal immune attack that ultimately will damage the fetus. One can imagine that the fetus, together with the placenta, is "embedded" in a cloud of exosomes that creates a beneficial and protective milieu for the fetus to grow and develop.



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Fig. 5. Schematic drawing of the human hemochorial placenta and a scenario suggesting how the pluripotent syncytiotrophoblast-released exosomes function at the feto-maternal border by down regulating the NKG2D receptor on cytotoxic lymphocytes and/or inducing apoptosis of activated Fas-expressing lymphocytes.

7. Future perspectives

The exosome research in reproduction is just at its beginning and it is logical to expect that more exosome-carried molecules and mechanisms of action will be revealed in the near future. Closing this chapter I would like to outline some issues that await elucidation. The first one is a detailed differential characterization of the placental exosomes, nanovesicles secreted through the endosomal compartment of the syncytiotrophoblast, in contrast to MV/STBM, larger vesicles shed by blebbing from the apical surface of the syncytiotrophoblast. Today, many investigations are done on mixture of exosomes and MV/STBM giving results difficult to interpret, reproduce or compare. The importance of adequate techniques for isolation of these two separate vesicle entities cannot be overestimated. Another issue is to identify and understand the exact mechanisms that govern exosome biogenesis *per se* both in general and in the placenta. Why and how do some MVB become degradative and sort to lysosomes for destruction whilst others are transported to the plasma membrane to release exosomes by exocytosis? The mRNA and miRNA content in placental exosomes has just started to shape up and needs confirmation and additional analyses to find a consensus. Finally, the role of exosomes in pathological pregnancies and related diseases, recurrent abortions, infertility and IVF failure awaits evaluation. Gaining knowledge in these areas will open possibilities for novel, exosome-based treatments of pregnancy failure and infertility. As for now, there is enough convincing evidence that the constitutively secreted immunosuppressive placental exosomes during normal pregnancy mount a protective exosomal shield around the feto-placental unit and comprise one of the keys for pregnancy success in human reproduction.

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Edited by Dr. Jing Zheng

ISBN 978-953-51-0194-9
Hard cover, 428 pages
Publisher InTech
Published online 07, March, 2012
Published in print edition March, 2012

This book contains the total of 19 chapters, each of which is written by one or several experts in the corresponding field. The objective of this book is to provide a comprehensive and most updated overview of the human placenta, including current advances and future directions in the early detection, recognition, and management of placental abnormalities as well as the most common placental structure and functions, abnormalities, toxicology, infections, and pathologies. It also includes a highly controversial topic, therapeutic applications of the human placenta. A collection of articles presented by active investigators provides a clear update in the area of placental research for medical students, nurse practitioners, practicing clinicians, and biomedical researchers in the fields of obstetrics, pediatrics, family practice, genetics, and others who may be interested in human placentas.

How to reference

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Lucia Mincheva-Nilsson and Vladimir Baranov (2012). Placenta-Derived Exosomes and Their Role in the Immune Protection of the Fetus, Recent Advances in Research on the Human Placenta, Dr. Jing Zheng (Ed.), ISBN: 978-953-51-0194-9, InTech, Available from: http://www.intechopen.com/books/recent-advances-in-research-on-the-human-placenta/placental-exosomes-in-human-pregnancy-and-their-role-in-immune-protection-of-the-fetus

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