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Exosomes mediate embryo and maternal interactions at implantation and during pregnancy

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TABLE OF CONTENTS

1. ABSTRACT

Shedding of exosomes and microvesicles is now a well-recognized, important method of cell-cell communication in a number of different cell types. However, their importance in the female reproductive tract and in mediating embryo-maternal interactions during pregnancy has only recently been recognized. Here we review the current literature as to release of extracellular vesicles by uterine cells, the embryo, and placental trophoblast cells; how release is regulated; and the different types of signaling molecules and genetic information contained within such vesicles. We also discuss the role of these exosomes and microvesicles in regulating critical processes during implantation and pregnancy such as angiogenesis, matrix remodeling, alterations in immune function and pathological effects in gestational diseases. A better understanding of the role of exosomes and microvesicles in reproduction may lead to the development of new therapeutic approaches for treatment of infertility and pregnancy complications.

2. INTRODUCTION

Extracellular vesicles (EVs) have been well-studied in many systems and play critical roles in angiogenesis, immunomodulation, cell survival, and inflammation; however, their role in implantation, placental physiology, and pregnancy are only beginning to be understood and appreciated. EVs have been shown to regulate many processes including angiogenesis, adhesion, proliferation, cell survival, inflammation, and immune response. Although less is known about the role of EVs in reproductive physiology and pathophysiology many of the processes known to involve EVs are critical to implantation, placental development, and placental function. The role of these vesicles in reproductive tissues is only beginning to be elucidated. Here we review current knowledge of EV formation, content, and function with an emphasis on the emerging role of EVs in reproductive physiology.

2.1. Extracellular vesicles mediate intercellular communication

EVs, although initially thought to be artifacts, have been shown to be a critical method of intercellular communication necessary for cell survival and maintenance in almost every multicellular system identified. EVs are shed from many cell types and are present in all human body fluids that have been examined to date including blood, urine, saliva, milk, uterine fluid, cerebrospinal fluid and synovial fluid (1-3). The quantity, type, composition,

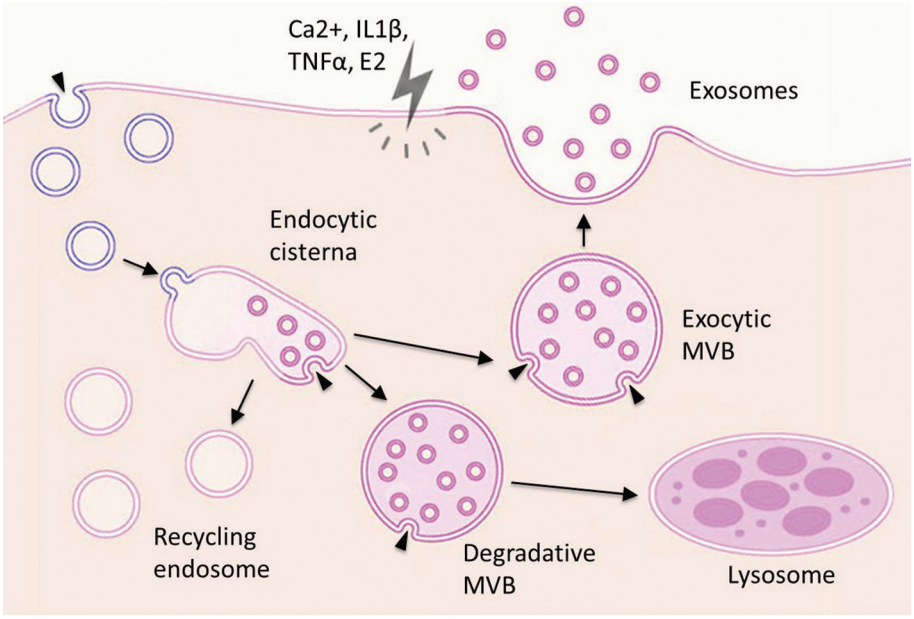

Figure 1. Generation of exosomes oocurs via formation of multivesicular bodies within the cytoplasm of the cell that then undergo exocytosis (Adapted from 8).

and functional properties of EVs are associated with the cell type of origin, physiologic or pathophysiologic state. EVs have been shown to play critical roles in a number of physiologic as well as pathophysiologic processes including coagulation, angiogenesis, tumor progression, cell survival, immunoregulation, and inflammation.

EVs provide a unique form of intercellular communication. These vesicles are an efficient mechanism of transport for soluble and membrane-bound proteins (4-6), second messengers, genetic information including mRNAs and miRNAs (4,5,7-11), and lipids with the potential for specific targeting via cell surface receptor interactions (12). These vesicles may also serve to eliminate harmful intracellular or membrane-bound components in a way that reduces exposure to other cells (13, 14). Four different types of EVs have been reported that have differing methods of generation and release: exosomes, microvesicles, membrane particles and apoptotic vesicles (1,2,4,8,15-22). It is currently unclear whether each represents an independent type of vesicle and in many cases more than one type of vesicle is present in a fluid population suggesting some overlap in vesicle type and/or release. Three vesicle types have been identified unambiguously: microvesicles, exosomes, and apoptotic bodies.

3. TYPES OF EXTRACELLULAR VESICLES

3.1. Exosomes

Exosomes are the best characterized of EVs. They are cup- shaped phospholipid bilayer vesicles that range in size from 40-100 nM and are formed in the late endosomal compartment by inward budding of the membrane of late multivesicular bodies (MVBs) (1,23,24) (Figure 1). Formation of intraluminal vesicles in multivesicular bodies has been shown to involve the endosomal sorting complex required for transport (ESCRT); however, additional studies indicate that these vesicles may be formed independently of this complex (25). ESCRT has been shown to be involved in inward budding of intraluminal vesicles of MVBs and cleavage of the necks of these vesicles. Once the vesicles are present in MVBs they can be secreted as exosomes by fusion of MVBs with the plasma membrane or alternatively degraded via lysosomal fusion (26,27). Secretion of exosomes from the endosomal compartment of MVBs by fusion with the plasma membrane has been shown to be dependent on intracellular calcium (28). Release may be constitutive as seen in epithelial and dendritic cells or tightly regulated as seen in mast and T cells (29-32). The exosomal membrane is detergent resistant and high in cholesterol, sphingolipids and tetraspanins. Inhibition of sphingomyelinase decreases exosome release suggesting stabilization of lipid rafts by ceramide may be required for exosome release. Exosomes appear to be enriched in specific lipids and proteins; studies have shown protein-specific elimination of the transferrin receptor in exosomes, however, the mechanism of this enrichment remains unclear (25, 33-36). Exosomes contain proteins, mRNAs, and miRNAs based on the tissue of origin. Many cytoplasmic proteins are found in exosomes including cytostructural proteins such as tubulin, actin, annexins and actin-binding proteins as well

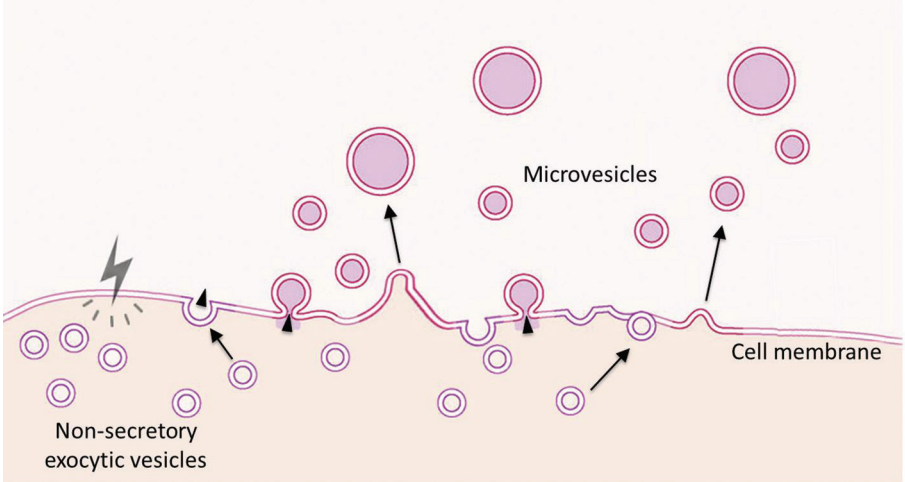

Figure 2. Generation of microvesicles occurs through reverse budding which is the result of alterations in the cytoskeletal structure underlying the plasma membrane of the cell. (Adapted from 8).

as signaling proteins such as signal transduction kinases, cytokines, and heterotrimeric G-proteins. Adhesion molecules such as β integrins and ICAM-1 are found on the exosomal surface as are the tetraspanins CD9, CD63, CD81, and CD82 which have become exosomal markers (16,21,22,37-40).

3.2. Microvesicles

In contrast to exosomes, microvesicles or shedding vesicles are thought to be formed by outward budding of the plasma membrane (Figure 2). They are heterogeneous in size and can be as large as 1000 nM. Similarly to exosomes they exhibit enrichment in specific proteins and lipids (8,41). Lipid rafts rich in cholesterol may be significant for establishing microdomains for shedding as depletion of cholesterol from the plasma membrane decreases microvesicle shedding (42). Microvesicle release has been shown to be initiated by rises in intracellular calcium which leads to subsequent activation of scramblase and calpain resulting in microvesicle formation (1, 8, 24, 43-45). Release of microvesicles is thought to be tightly regulated in contrast to exosomal release which can also be constitutive. Cytoplasmic proteins in microvesicles include annexins, caspases, FGF, and IL-1β. Typical membrane-bound proteins include integrins and matrix metalloproteinases.

3.3. Apoptotic bodies

Apoptotic bodies are formed during apoptosis. Cells shrink in size and fragment during apoptosis, forming apoptotic bodies. These vesicles are heterogenous in size ranging from 50-5000 nm Intracelullar calcium is increased during apoptosis serving as an initiating event for apoptotic body formation (8, 11).

4. PHYSIOLOGY AND PATHOPHYSIOLOGY OF EXTRACELLULAR VESICLE RELEASE

4.1. Angiogenesis

Angiogenesis is a critical process for many physiologic and pathologic processes. EVs have been shown to both promote and restrict angiogenesis (46-53) resulting in appropriate formation of new blood vessels to provide oxygen and nutrients to growing tissues and organs and allowing for elimination of metabolic waste (54). Endothelial cells release microvesicles containing the matrix metalloproteinases MMP-2 and MMP-9 allowing for degradation of extracellular matrix and formation of new capillaries (50). Microvesicles from platelets have been implicated in induction of angiogenesis by VEGF signaling and have also been shown to promote cell proliferation, survival, and migration in addition to capillary formation *in vitro*. Platelet microvesicles have been shown to promote revascularization via VEGF signaling in ischemic heart muscle of rats (51). VEGF is secreted upon platelet activation and associated with platelet-derived microvesicles. Platelet microvesicles have also been shown to regulate adhesion of cells. Platelet microvesicles are also able to transfer integrins and matrix metalloproteinases to cancer cells resulting in increased adherence to endothelial cells and subsequent metastasis and angiogenesis (55, 56). *In vivo* studies showed increased rates of metastasis with concomitant injection of lung cancer cells with platelet-derived microvesicles. Platelet-derived microvesicles induce expression of metalloproteinases, VEGF, hepatocyte growth factor, and interleukin-8 in lung cancer cells (56). EVs have also been shown to increase invasiveness of breast cancer cells (55). In addition, EVs derived from cancer cells have been shown to stimulate proliferation of epithelial cells. Exosomes from glioblastoma cells

contain mRNAs and miRNAs that increase tumor growth and permit stromal remodeling (46). These exosomes are enriched in markers of glioma angiogenesis and increased malignancy including angiogenin, IL-6 and IL-8. In melanoma derived EVs, increased levels of the phosphorylated tyrosine kinase receptor MET are commonly seen (57). Transfer of phosphorylated MET to bone marrow cells via melanoma-derived exosomes has been documented in a mouse melanoma model (57). Overall EVs appear to play a critical role in stimulation of angiogenesis, cell proliferation, and cell survival.

4.2. Immunomodulation

EVs, particularly exosomes, have been shown to have immunomodulatory effects. Two classes of exosomal immune modulation are seen: immunostimulatory and immunosuppressive. Antigen presenting cells including dendritic cells, macrophages and B cells typically release immunostimulatory exosomes (29,58). These exosomes activate immune effector mechanisms such as cytotoxicity, T cell stimulation, and cytokine and antibody production (21). In activated T and NK cells endosomal vesicles of MVB carry cytotoxic molecules including perforin, granzyme, FasL, and TRAIL (59-61). In contrast, exosomes released from epithelial cells typically are immunosuppressive (62,63). Many tumor cells also downregulate immune response by release of immune receptor ligands and signaling molecules (59,64-67) on exosomes resulting in inhibition of the host immune system and promotion of tumor formation, progression, and metastasis (63,68-71). In mammalian pregnancy immunologic tolerance to the fetal allograft must be established to permit fetal development and growth (72). The placenta secretes immunomodulators to modify maternal immune responses and allow fetal survival during pregnancy. The predominant and unique cell type of the placenta, the syncytiotrophoblast, has been shown to release microvesicles and exosomes during pregnancy (73-76).

5. EXTRACELLULAR VESICLES IN IMPLANTATION AND PREGNANCY

5.1. Endometrial extracellular vesicles

The uterine epithelium is a dynamic tissue that undergoes characteristic changes in morphology and function during the estrous or menstrual cycle in response to changing ovarian steroid hormones. Recent studies have confirmed that uterine epithelial cells also shed exosomes and microvesicles. The first report of this was by Habiba *et al.* (77). These authors reported the presence of a large glycoprotein, of approximately 200,000 MW in the endometrium of women. This protein was expressed in a cycle- specific fashion and showed changes in localization within the glandular and luminal epithelial cells depending on the stage of the cycle. The protein was shown to be contained in intracellular vesicles that moved to the apical edge of the cells during the midsecretory phase of the cycle. During the late secretory phase the protein was no longer intracytoplasmic but was expressed on the luminal border of the glandular epithelial cells and within the lumen. The authors hypothesized that this protein might be a member of the MUC-1 family and may be involved in uterine receptivity.

More recent studies have reported the isolation and characterization of exosomes and microvesicles from uterine luminal fluid of both women and sheep. Ng *et al.* (78) identified exosomes shed from uterine epithelial cells *in vivo* in women as well as from a cultured uterine epithethial cell line, ECC1. Uterine flushings and mucus were collected from patients and the exosomes and microvesicles isolated by differential ultracentrifugation. Immunostaining for tetraspanins, which are cell surface markers for exosomes, confirmed their presence on the apical surfaces of endometrial epithelial cells throughout the menstrual cycle though, interestingly, expression of specific proteins did vary across the cycle. The authors hypothesized that these EVs are released from uterine epithelial cells into the uterine lumen, and that they contain specific microRNAs that could be transferred to either neighboring uterine epithelial cells or to the trophectodermal cells of the blastocyst to promote implantation. MiRNA analysis of cultured uterine epithelial cells and exosomes was carried out and revealed that miRNA sorting into exosomes and microvesicles occurs. Several of the miRNAs identified were specific to EVs, while others were not present in these at all (Figure 3). The most abundant miRNAs in EVs were hsa-miR-200c, hsa-miR-17 and hsa-miR-106a. Bioinformatic analysis showed that these exosome-specific miRNAs have a number of potential targets in biological pathways highly relevant for embryo implantation such as inflammation, cell remodeling, proliferation and angiogenesis. These findings strongly support a role for EVs as a method of communication between the implanting embryo and the recipient endometrium.

Studies by Burns *et al.* (79) compared microvesicles and exosomes isolated from uterine luminal fluid of sheep at day 14 of the estrous cycle with those shed during pregnancy. These EVs were found to contain a number of mature miRNAs as well as endogenous retrovirus enJSRVs and gag RNAs that could be delivered to heterologous cells *in vitro*. Of note, the authors also found that the contents of the EVs in uterine luminal fluid of nonpregnant sheep differed significantly from those of uterine shedding vesicles of pregnant sheep (Figure 4). These results support a role for EVs from uterine epithelial cells as a vehicle to deliver enJSRVs RNA to the conceptus, which is important as enJSRVs regulate conceptus trophectoderm development. Moreover, these studies further confirm that uterine epithelial cells shed EVs containing specific miRNAs, RNAs and proteins into the uterine lumen and that these vesicles are taken up by the embryo.

Figure 3. Venn diagram comparing the miRNA profiles of exosomes versus the parent uterine epithelial cell line showing the number of shared and specific miRNAs (78).

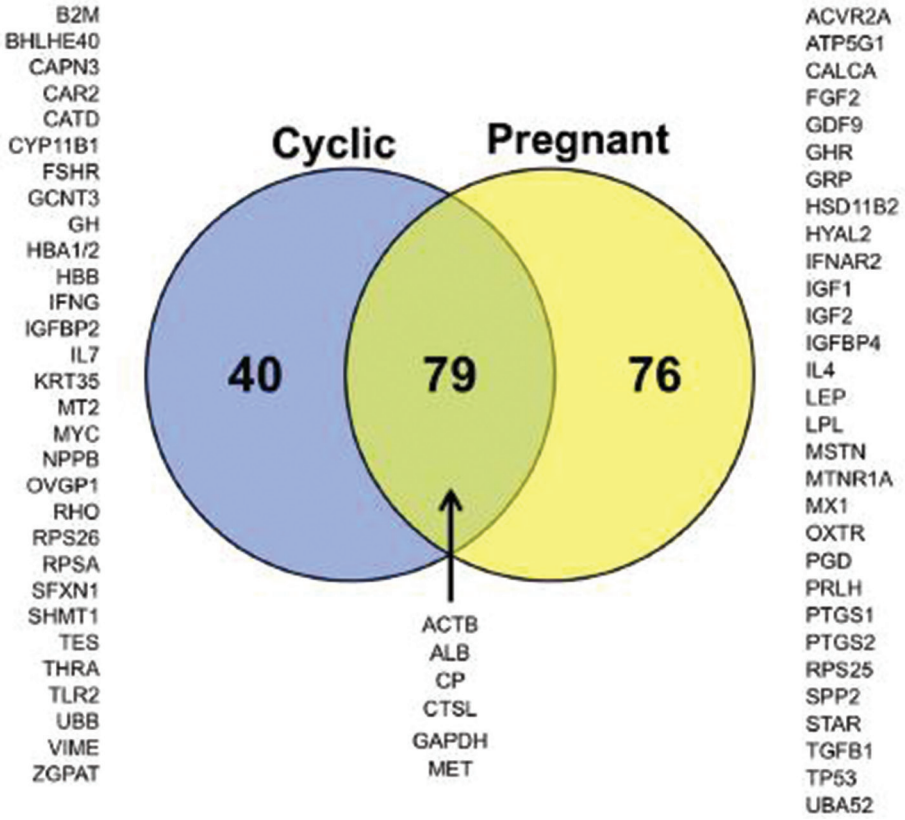

Figure 4. Proteomic analysis of extracellular vesicles from uterine luminal fluid of cyclic and pregnant ewes reveals proteins that are unique to each reproductive state (79).

5.2. Regulation of extracellular vesicle release in uterine cells

The shedding of microvesicles and exosomes can be regulated thereby allowing for direct, carefully timed communication between the conceptus and maternal endometrium and also for paracrine interactions between the different cell types within the endometrium. However, the mechanisms by which this occurs in the uterus have only recently begun to be investigated using human uterine cell lines *in vitro*. A study by Braundmeier *et al.* (80) investigated some of the factors that might regulate shedding of EVs containing the protein EMMPRIN (Extracellular Matrix Metalloproteinase Inducer), a protein that stimulates production of metalloproteinases and coordinates extracellular matrix remodeling. These investigators

found that the ovarian steroid hormone estradiol and the cytokine interleukin-1β both stimulated the shedding of EVs from uterine epithelial cells. In addition they found that, similar to cancer cell lines, activation of intracellular calcium pathways with the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) also markedly increased the release of these vesicles. They further showed that shedding of EMMPRIN-containing microvesicles by uterine epithelial cells led to stimulation of uterine stromal cell MMP production.

Estradiol is required for and modulates many normal physiologic processes in the reproductive tract. Conventionally, estradiol action involves binding to either ERα or ERβ, encoded by ESR1 and ESR2 respectively, nuclear translocation and alteration of transcription. However, estradiol can also mediate rapid, non-genomic signaling events through activation of the seven pass transmembrane receptor GPR30, encoded by the *GPER* gene. In a recent study, Burnett and colleagues (81) investigated the potential role of GPR30 in regulating release of EMMPRIN-containing microvesicles from uterine epithelial cells (EEC) *in vitro*. They found that treatment of EEC cells with estradiol stimulated release of microvesicles containing EMMPRIN. Moreover, the increase in secretion of EMMPRIN-containing microvesicles appeared to occur via the G-protein coupled receptor GPR30 as treatment of EEC cells with the specific GPR30 agonist, G1, also stimulated release of EMMPRIN-containing microvesicles. This increase was not due to an increase in EMMPRIN mRNA transcription, but rather to movement of EMMPRIN protein to the plasma membrane. Treatment with the GPR30 antagonist G15 inhibited release of EMMPRIN-containing microvesicles in response to estradiol. In addition, treatment of cells with cholera toxin, a stimulator of adenylyl cyclase and cAMP production, also resulted in increased shedding of EMMPRIN-containing microvesicles. These results reveal a mechanism by which estradiol can stimulate release of EMMPRIN-containing microvesicles, not through its classical nuclear receptors but rather, through stimulation of the cell membrane receptor GPR30 and activation of stimulatory G proteins (Figure 5). More intensive investigations into the mechanisms by which shedding of EVs from uterine cells occurs and the factors that can stimulate or inhibit their release are clearly warranted.

5.3. Extracellular vesicles from pre-implantation embryos

Communication between the uterine endometrium and implanting embryo is not a unidirectional process but involves active signaling from maternal cells to the embryo and vice versa. There is also active communication between embryos in litter bearing species such as the pig. Saadeldin *et al.* (82), using differential centrifugation and transmission electron microscopy, confirmed that porcine preimplantation embryos release exosomes and microvesicles. They also were able to demonstrate paracrine communication between different kinds of embryos such as parthenogenic and nuclear transfer embryos using a co-culture system. EVs from conditioned medium of parthenogenic embryos were taken up by nuclear transfer embryos and significantly improved their *in vitro* development. Analysis of these EVs identified a number of mRNAs including those of pluripotency genes such as Oct4, Sox2, Klf4, c-Myc and Nanog. Thus, embryos may utilize EVs as a means of communicating not only with the maternal endometrium but also with each other. This may explain the well-known phenomenon that embryos cultured in a group show improved development when compared to embryos cultured individually *in vitro*.

Two very recent studies have reported that bovine and human pre-implantation embryos secrete miRNAs into culture medium (83,84). These miRNAs are secreted within exosomes into the extracellular environment where they can be taken up by cells and act in a paracrine manner to impact gene expression. In the study by Rosenbluth et al. (83), human embryos cultured for in vitro fertilization (IVF) were found to secrete specific miRNAs that varied depending on the fertilization method used, their chromosomal state and whether they successfully implanted. The miRNA-191 was more abundant in conditioned medium from aneuploid embryos while miR-191, 372 and 645 were more highly concentrated in medium from embryos of failed IVF cycles. Kropp et al. (84) examined miRNA secretion in day 5-8 cultured bovine embryos. They observed differential miRNA expression between embryos that successfully developed to the blastocyst stage and those embryos that failed to develop from morulae to blastocysts or that degenerated. Four miRNAs- miR-25, -302c, 196a2 and -181 were found to be higher in culture medium from degenerating embryos. Human embryos also shed these miRNAs into medium. These findings demonstrate that secretion of specific miRNAs may be correlated with the developmental competence of an embryo and support the use of specific miRNAs as potential biomarkers for selecting the best quality embryos for successful pregnancy.

5.4. Placental trophoblast extracellular vesicles in normal pregnancy

Development and maturation of the placenta is a highly controlled process of trophoblast proliferation, differentiation and invasion into the maternal endometrium as well as remodeling of the spiral arteries to establish an adequate blood supply between the mother and fetus. During the first trimester of human pregnancy, the placental villi are covered by the inner mononuclear layer of cytotrophoblast. Cytotrophoblast progenitors in the placental villi can follow one of two different paths; some fuse and form multinucleated syncytiotrophoblast that overlies the cytotrophoblast layer, while others take

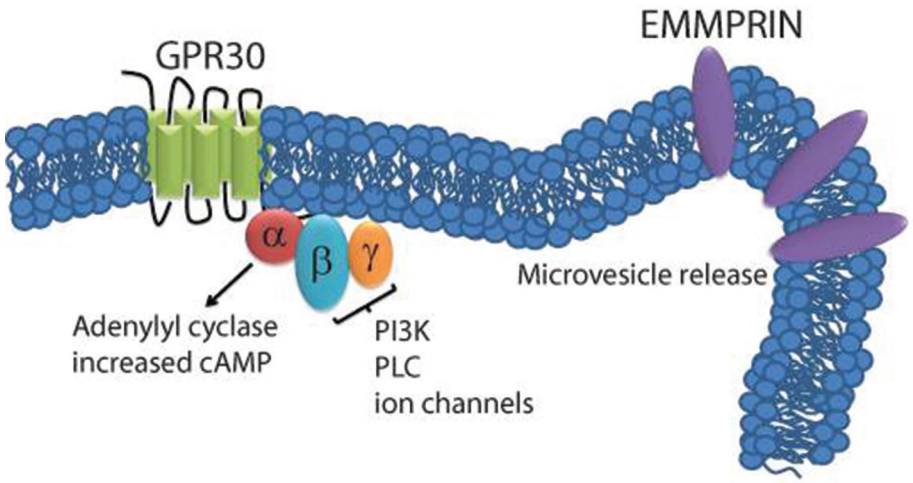

Figure 5. Estradiol stimulates release of EMMPRIN-containing microvesicles from uterine epithelial cells through interaction with the G-protein coupled receptor GPR30. Activation of the receptor leads to activation of Gαs signaling pathways and subsequent microvesicle release (Burnett LA).

on an invasive path differentiating into the extravillous trophoblast (EVT). During the invasion process, EVT grows out from the villous-forming cell columns and begins to migrate from the basement membrane of anchoring villi into the decidua ultimately reaching as far as the inner layer of myometrium and into the walls of the uterine spiral arteries. The syncytiotrophoblast layer has a barrier function in the human placenta and substantially increases the surface area available for nutrient exchange between the mother and fetus. The extravillous-derived trophoblast cells play an important role in spiral artery remodeling participating in the degradation of extracellular matrix and disruption of endothelial cells/matrix/vascular smooth muscle cell interactions. Late in the first trimester of pregnancy such active EVT invasion results in conversion of low-flow, high-resistance into high-flow, low resistance spiral arteries with increased circumference and vascular compliance. Such vessel restructuring is necessary to ensure a sufficient blood supply for fetal development. Thus, the trophoblast cells of the implanting conceptus must interact and communicate with the various cell types in the uterus to ensure successful placental development, vasculogenesis and maternal tolerance of the fetal allograft.

A number of studies have confirmed that trophoblast cells shed both exosomes and microvesicles. Earlier reports described these EVs as trophoblastic debris but more recently these vesicles have been recognized as a means by which trophoblast cells communicate with other cells locally in their surrounding microenvironment as well as systemically (85-90). Proteomic studies reported the presence of a large number of proteins, including EMMPRIN, in exosomes derived from Swan71 human first trimester trophoblast cell line (85) and showed that these trophoblast-derived exosomes are able to mediate monocyte recruitment and differentiation. The authors of these studies distinguished trophoblast-derived EVs as exosomes based on the presence of exosome marker proteins and on their morphology and density (38, 85-87). However, results from other studies indicate that trophoblast cells also release microvesicles though plasma membrane blebbing (85,88-90). Atay *et al.* (85) identified 282 proteins in EVs isolated from trophoblast cells. Of these proteins, 147 had not previously been described in exosomes but instead were shown to be concentrated in the plasma membrane of microvesicles.

EMMPRIN released in microvesicles by trophoblast cells may contribute to the process of spiral artery remodeling during early placental development. Previous studies have shown that bioactive EMMPRIN is released from the surface of tumor cells into conditioned medium through microvesicle shedding (91). Other investigators reported that EMMPRIN is expressed in microvesicles derived from epithelial ovarian cancer cells and that these microvesicles induce angiogenesis and promote matrix metalloproteinase gene expression in endothelial HUVEC cells in an EMMPRIN-dependent fashion (92). Alcazar *et al.* (93) using proteomic analysis showed the presence of EMMPRIN in microvesicles derived from human retinal pigment epithelium. EMMPRIN has also been shown to increase vascular endothelial growth factor (VEGF) production and HIF1α expression in synovial fibroblasts (94) and VEGF and fibroblast growth factor (FGF) production in renal carcinoma cell lines (95). A recent report from Lee and colleagues (96) determined that EMMPRIN is also an important regulator of proliferation, invasion and MMP production in primary human cytotrophoblasts isolated from the first trimester of pregnancy. Trophoblast cells secrete a multitude of mRNAs and biologically

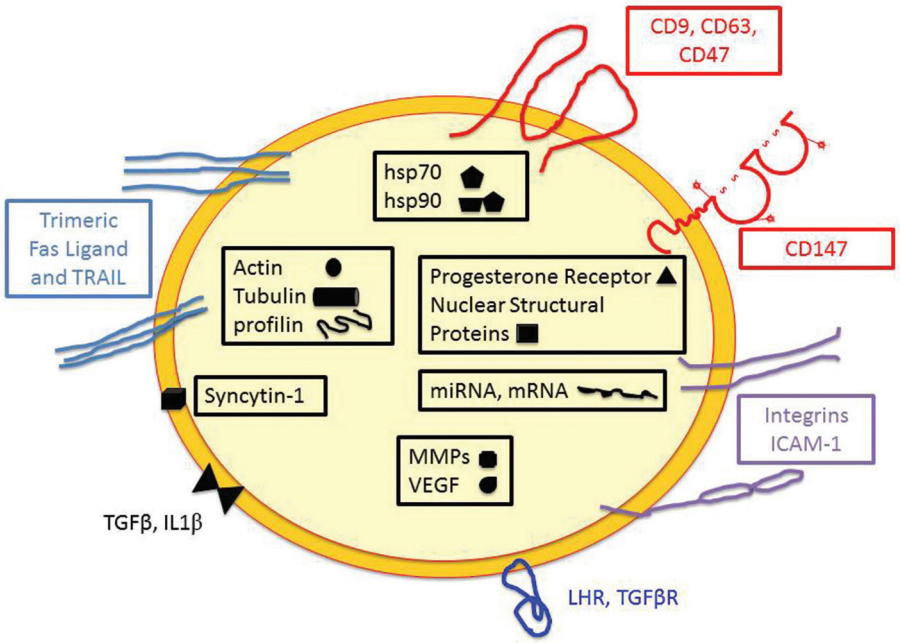

Figure 6. Examples of some of the proteins and RNAs shed in exosomes and microvesicles by placental trophoblast cells (Adapted with permission from 72).

active proteins in extracellular vesicles that regulate angiogenesis, tissue remodeling and growth of the placenta. These include miRNAs, cytokines, growth factors, and receptors such as human chorionic gonadotropin (HCG/LH) receptor, TGFβ, Flt-1, and endoglin (72, 97-105) (summarized in Figure 6).

5.5. Placental trophoblast extracellular vesicles and immune function

Another very important function of the placental trophoblast cells is to interact with the maternal immune system in early pregnancy to activate immune cells while inducing immunosuppression and tolerance for the fetal allograft at later stages (72,85-87,97). The first and early second trimesters of pregnancy resemble 'wound healing,' requiring a pro-inflammatory environment for embryo implantation, trophoblast invasion, angiogenesis and endometrial remodeling and repair. This pro-inflammatory phase of early pregnancy is characterized by the production of pro-inflammatory cytokines such as IL-1β, IL-8, and monocyte chemoattractant factor-1 (MCP1) and their appearance in the maternal circulation (86,87,106). IL-1β promotes multiple early pregnancy events, such as attraction and activation of immune cells, stimulation of endothelial cell adhesion molecules expression, and enhanced expression of extracellular matrix proteins and MMPs. Previous work (38,86,107) demonstrated that exosomes derived from first trimester extravillous trophoblast were capable of inducing the migration of monocytes and 'educating' these cells to produce pro-inflammatory cytokines including IL-1β.

While the first trimester of pregnancy is associated with an increased production of pro-inflammatory cytokines and chemokines to recruit and activate immune effector cells, the second trimester is an anti-inflammatory or Th-2 state in which the trophoblast cells must act to protect the fetus against maternal immune attack. These trophoblast cells express membrane-associated and secreted immunoregulatory factors including FasL and early pregnancy factor (EPF) that block lytic NK cell activity as well as a number of cytokines such as TGF-β and IL-10 that prevent Th1 helper type cells but enhance Th2 and regulatory T cell functions (67,72,76,108-110). Abrahams *et al.* (76) reported that isolated trophoblast cells from the first trimester of pregnancy lacked plasma membrane–associated FasL but expressed cytoplasmic FasL that was secreted in microvesicles. Studies by Abrahams *et al.* (76) and Frangsmyr *et al.* (59) also confirmed that trophoblast cells from first-trimester pregnancies lack expression of FasL in the cell membrane and exosomes released from these first-trimester trophoblast cells do not express FasL on their exterior, but do contain FasL within. This intracellular FasL, when released from microvesicles as they undergo degradation, is able to induce T-cell through apoptosis. In contrast, while membranal FasL could be detected on the plasma membrane of trophoblast cells from second- and third-trimester pregnancies, it was not contained in exosomes derived from these trophoblasts. Thus, the composition of exosomes and microvesicles shed by trophoblast cells varies depending on the

gestational age resulting in quite distinct effects on target immune cells at different stages of pregnancy.

Envelope glycoproteins of human endogenous retrovirus (HERV), such as syncytin 1 (HERV-W), are highly expressed in the placenta and are shed in trophoblast microvesicles during pregnancy (111). Treatment of peripheral blood mononuclear cells with either recombinant syncytin 1 or microvesicles from cultured BeWo trophoblast cells induced peripheral blood mononuclear cell activation and secretion of a number of cytokines including IL-2, IL-8 and tumor necrosis factor alpha (TNFα). Conversely, siRNA knockdown of syncytin 1 expression in BeWo cells inhibited such activation. These findings support a role for trophoblast-derived syncytin 1 and other HERV-W envelope glycoproteins in modulation of the maternal immune system during pregnancy.

5.6. Identification of RNAs in trophoblast-derived extracellular vesicles

One of the most intriguing aspects of trophoblast-derived exosomes is that they contain mRNAs and miRNAs (112-115). This allows for exchange of genetic information between trophoblast cells and specific maternal target cells in the uterus providing more direct control of maternal gene expression and cell function by trophoblast cells (96,107,116,117). Several recent studies have characterized the miRNA profiles for human placenta at different stages of pregnancy (113,115,118). Donker *et al.* (118) and Luo *et al.* (115) analyzed human first trimester and term placental tissues and demonstrated that most placenta-specific miRNAs, including MIR517A, were linked to a miRNA cluster on chromosome 19. These miRNA cluster genes were upregulated during placental development. They identified four novel miRNAs that were only expressed in the placenta. Several placenta specific miRNAs have been identified in maternal plasma confirming that the syncytiotrophoblast cells of the placenta secrete miRNAs into the maternal circulation via exosomes. *In vitro* studies using the BeWo trophoblast cell line as a model for villous trophoblast demonstrated that these cells released exosomes containing two placenta-specific miRNAs, MIR517A and MIR21, into the culture medium (115). Proteome analysis suggested that MIR17A might be involved in the regulation of TNF signal transduction. Esplin *et al.* (103) characterized and compared trophoblast-derived exosomes in the peripheral blood of pregnant women that completed normal-term pregnancies with those of women who had a preterm delivery. They found that the concentration of these exosomes was elevated in the blood of women with normal pregnancies delivering at term in comparison with preterm delivery and non-pregnant women. It is important to better characterize the placental miRNA profiles of trophoblast-derived exosomes in women with normal and pathologic pregnancies such as preeclampsia, as well as at different stages of gestation. Such studies will likely identify characteristic miRNA signatures that can be used to predict pregnancy outcomes or perhaps for prenatal testing. More functional studies are also needed to determine the mechanisms by which trophoblast-specific mRNAs and miRNAs direct the functions of maternal cells to favor survival of the fetal allograft.

6. EXTRACELLULAR VESICLES AND GESTATIONAL VASCULAR DISEASE

Vascular complications of pregnancy including gestational hypertension and preeclampsia/toxemia are a significant cause of maternal morbidity and fetal mortality. Preeclampsia is characterized by poor placentation and endothelial damage (119, 120) resulting in multi-organ dysfunction via arterial hypertension including glomerular pathology and hepatic failure (121). Key features of this disease include disruption of angiogenesis and increase in inflammatory cytokines resulting in impaired maternal endothelial function and disturbance *in utero*placental circulation (122). This increase in maternal inflammatory response and vascular dysfunction alters the progression of placentation, trophoblast proliferation, invasion, and apoptosis.

Trophoblast cells comprise the maternal-fetal interface. Proper migration, invasion, and differentiation of these cells is critical for placentation, embryo implantation, and hemostasis. Differentiation and fusion of trophoblasts to form multinucleate syncytiotrophoblasts require activation of caspases and subsequent upregulation of Bcl-2 after fusion to prevent apoptosis (123). EVs present in blood have been shown to increase in vascular disease states (124). In preeclampsia, microvesicles released from red blood cells and endothelial cells are increased consistent with other vascular pathologies (125).

Although the total number of circulating microvesicles remains similar in normal healthy pregnancies compared to those with gestational trophoblastic disease, those released in gestational vascular disease contain more proinflammatory cytokines and more pro- and antiangiogenic proteins (119,122,126,127). Shomer *et al.* (119) showed differential effects when endothelial, early stage trophoblast, and term trophoblast cells are exposed to circulating microvesicles from normal healthy pregnant women compared to those with gestational vascular disease. In human umbilical vein endothelial cells exposure to microvesicles from normal healthy pregnancies results in stable endothelial tube formation -- this does not occur in response to microvesicles from women with gestational vascular disease suggesting a role for microvesicles in endothelial cell migration and angiogenesis during pregnancy. Survival of early stage trophoblast cells increases with exposure to normal healthy pregnancy microvesicles suggesting a role for microvesicles in maintenance of endothelial and trophoblast function. Increased

migration via the erk2 pathway is also seen in response to normal healthy pregnancy microvesicles compared to microvesicles released from women with gestational vascular disease. This impairment in endothelial and early stage placental cell function may be related to failure of placental implantation seen in gestational vascular disease. In term trophoblast cells exposure to microvesicles from women with gestational vascular disease results in significantly higher rates of apoptosis compared to that seen with microvesicles from normal healthy pregnancies suggesting a role for microvesicles in trophoblast apoptosis and placental aging.

In a normal healthy pregnancy a low level of trophoblastic apoptosis is observed early in pregnancy which increases as a function of gestational age. In contrast, preeclampsia is characterized by higher rates of trophoblastic apoptosis that may result in abnormal placental invasion and potentially lead to pregnancy loss and/or early delivery. In preterm labor prostaglandins and proinflammatory cytokines are known to be increased (128). Increased trophoblastic apoptosis stimulated by microvesicles released in gestational vascular disease states may increase inflammatory cytokines and serve as putative mechanism for initiation of preterm labor. EVs clearly play an important role in the angiogenesis, inflammation, and apoptosis that determines the endothelial and trophoblast function during pregnancy.

7. SUMMARY AND FUTURE DIRECTIONS

Knowledge of intercellular communication via EVs has resulted in a more robust understanding of the physiology and pathophysiology of many processes including angiogenesis, immunomodulation, and cell survival. Many of these processes are critical to development and delivery of a successful pregnancy. Uterine, placental, and embryonic EVs are being studied to better understand the role of these vesicles in uterine receptivity, placentation, and gestation. Further elucidation of the precise mechanisms regulating EV creation, release, and actions in reproductive tissues is needed to understand the complex physiology and pathophysiology of pregnancy.

A more robust understanding of angiogenesis, immunomodulation, and cell survival in pregnancy as regulated by EVs will likely lead to better understanding of pathophysiologic conditions in pregnancy including implantation failure, abnormal placentation, and gestational vascular disease. Understanding of these processes is critical to developing novel treatment modalities to modify EV release and function.

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