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Minireview

Extracellular Vesicles in the Intrauterine Environment: Challenges and Potential Functions[1]

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ABSTRACT

Extracellular vesicles (EVs), including exosomes (30–150 nm) and microvesicles (100–1500 nm), play important roles in mediating cell-cell communication. Such particles package distinct cargo elements, including lipids, proteins, mRNAs, microRNAs, and DNA, that vary depending on the cell of origin and its phenotype. This cargo can be horizontally transferred to target cells where its components can reprogram the recipient cell to modify its function. EVs have been identified within the uterine cavity of women, sheep, and mice, where they contribute to the microenvironment of sperm transport, and of blastocyst and endometrial preparation for implantation. It is likely that exosomes and microvesicles carry different cargo and coordinate different roles in this intrauterine environment. Understanding and defining these subtypes of EVs is important for future functional studies and clinical translation. Here we critically review the various purification and validation procedures for extracellular vesicle analysis and discuss what is known of endometrial-derived exosome cargo and of their hormonal regulation. The current knowledge of the functions of uterine exosomes, with respect to sperm transport and function, and of their actions on trophectodermal cells to promote implantation are summarized and evaluated in their physiological context. Given the potential importance of this form of cell-cell interactions within the reproductive tract, the critical issues discussed will guide new insights in this rapidly expanding field.

blastocyst, embryo implantation, endometrium, exosomes, extracellular vesicles, microparticles, microvesicles, receptivity, sperm, trophoblast, uterine microenvironment

ESTABLISHMENT OF PREGNANCY: THE IMPORTANCE OF THE UTERINE MICROENVIRONMENT

The uterine cavity is a unique site through which spermatozoa (sperm) migrate from the vagina to the oviducts where fertilization occurs and in which final pre-implantation development of the conceptus and the early steps of its implantation into the endometrium take place. The contents of the uterine cavity must therefore provide an appropriate milieu for these events. Uterine fluid in all species contains a rich abundance of nutrients, along with regulatory proteins, lipids, and RNA moieties. While many of these are selectively derived from blood or from tubal fluid, a large number of the proteins are derived from the endometrium and their concentrations vary with the time of the menstrual cycle (summarized in [1]).

Sperm require a series of membrane and metabolic alterations for capacitation and fertilization: such changes occur in both the male and female reproductive tracts [2]. Indeed, proteins of oviductal origin, including oviductin (human) and Annexin A2 and S110A (human and a range of species), are transferred to the sperm cell surface rendering them competent for fertilization [3–6]. It is equally likely that proteins or other mediators, are transferred to sperm during their passage through the uterine cavity.

In placental mammals, synchronous dialog between an appropriately developed embryo and hormonally primed endometrium is a prerequisite for the establishment of pregnancy, regardless of the form of placenta that subsequently develops [7]. The final stages of pre-implantation embryo development (generally from morula to blastocyst, but to filamentous forms in pigs and ruminants) occur within the nutrient and soluble mediator-rich uterine microenvironment. A large number of the proteins are derived from receptive endometrium, particularly from the highly secretory glands or luminal epithelium during the luteal or secretory phase of the female cycle [1]. It has been known since the 1950s that implantation can only occur if the embryo and the endometrium are in developmental synchrony [8, 9], and more recently, failure of implantation was demonstrated in the absence of glands in mice and sheep in which uterine gland development was inhibited [10, 11]. In women, endometrial receptivity to implantation occurs only during a brief ~3-day window during the midsecretory phase of the menstrual cycle when the glands have undergone adequate secretory transfor-

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mation [12, 13], driven by the dual actions of estrogen (E) and progesterone (P).

Significant changes in both the endometrial epithelial cells and the trophectodermal cells of the blastocyst are essential for implantation because both cell types present as polarized epithelial cell layers that are normally mutually repulsive. Thie et al. [14] termed this a cell biological paradox and postulated that the uterine cells must partially modulate their epithelial phenotype, including loss of apical-basal polarity and changes in their adhesiveness. Murphy [15] also described the epithelial cell transformation in terms of alterations of cellular junctions and intracellular organization. Since then, many molecular and functional changes in both trophoblast and endometrial epithelium have been defined as driven by soluble factors released into the microenvironment within the uterine cavity from both trophectodermal and endometrial epithelial cells [1, 16, 17]. These enable the blastocyst to undergo apposition as well as adherence (in all species) and invasion (in species with hemochorial placentation and specifically between the epithelial cells in primates). While these are the earliest steps of implantation, the early invasion, when it occurs, precedes further invasion of the constantly differentiating trophoblast cells through the decidual compartment to establish the placenta.

A new paradigm has recently been introduced for embryo-maternal interactions within the uterine cavity of humans, sheep, and mice. Extracellular vesicles (EVs) have been identified in uterine fluid from nonpregnant women [18, 19], sheep [20, 21], and mice [22]. These originate from the uterine epithelium, and in sheep conception cycles (and presumably in other ungulates), where the trophectoderm is highly elongated pre-implantation, vesicles are also of conceptus origin [21]. Given the potential importance of such EVs in the establishment of pregnancy, current knowledge of EV heterogeneity and function and their potential influence within the microenvironment of implantation is reviewed below. It is also likely that these EVs identified and characterized in the uterine cavity are the same as the uterosomes that have been studied for their uptake and effects on sperm (in mice) (summarized in [23]).

EXTRACELLULAR VESICLES

Extracellular vesicles released by cells have emerged as a distinct form of intercellular communication with important roles not only in cancer and other pathologies, but also in certain physiological situations, including reproductive and developmental processes. These membrane-enclosed packets are released from various cell types and can transfer specific proteins, lipids, RNA transcripts, microRNAs (miRNAs), and DNA to target cells, thereby reprogramming and altering their function [24]. Extracellular vesicles have been well-studied in many systems and play critical roles in angiogenesis [25–31], immunomodulation [32, 33], and inflammation [34, 35]. However, their roles in implantation, placental physiology, and pregnancy are only beginning to be characterized and understood.

Classification and Biogenesis

Most cell types release EVs including hematopoietic cells, reticulocytes, B- and T-lymphocytes, endometrial cells, dendritic cells, mast cells, platelets, intestinal epithelial cells, astrocytes, placenta, neurons, and tumor cells [18, 36–42]. Extracellular vesicles have been identified in vivo in body fluids, including amniotic fluid, urine, and blood, and isolated from uterine fluid in sheep and women [18, 20, 21, 43, 44]. Furthermore, recent publications have demonstrated that EVs are released from human endometrial epithelial cells [18, 37, 44–46], extravillous and villous trophoblast cells [47], and primary trophoblast from term placenta [44, 48].

Extracellular vesicles include exosomes, microvesicles, and apoptotic bodies (Table 1) and while the heterogeneous microvesicles (100–1500 nm) are generated by release from the plasma membrane, exosomes (30–150 nm) are derived from the endosomal pathway [49, 50] (Figs. 1 and 2). Microvesicles are formed by the outward budding and fission of plasma membrane lipid rafts or microdomains (Fig. 1A). They are enriched in phosphatidylserine, cell lineage markers, cell surface receptors, and cholesterol-rich or specialized cell membrane microdomains, and thus the membrane composition of microvesicles reflects that of the parent cell more closely than those of the membrane composition of exosomes (see below) [24, 51]. The biogenesis of microvesicles is regulated by a distinct set of molecular events, including activation of AKT and acidic sphingomyelinase, intracellular calcium flux variations, and enzymes involved in the maintenance of membrane phospholipid asymmetry [52–55]. In contrast, exosomes are smaller, have a buoyant density of 1.09–1.19 g/ml, and form by the inward budding of the luminal membrane of endosomal multivesicular bodies (Fig. 1B). Exosomes are abundant in tetraspanins (CD63, CD81, CD82) [56–59], and their biogenesis is governed by several regulatory mechanisms, including elements of the endosomal sorting complex required for transport (ESCRT), RabGTPase proteins (Rab11, Rab27, Rab35), syndecan-syntenin-Alix, p53/TSAP6 pathway, phospholipase D, ceramide, oligomerization, and neutral sphingomyelinase 2 [49, 60–65].

Extracellular vesicles package distinct lipids, proteins, mRNAs, miRNAs, and DNA contents [66] that vary depending on the cell of origin and its phenotype [67, 68]. These contents can be horizontally transferred to other cells, where they are functional [69–76]. Microvesicles and exosomes are often thought to be functionally similar; a problematic issue in the field of EV biology is the interchangeable nomenclature used in the literature. While the two predominant classes of EVs appear to share some common cargo, there have been clear demonstrations of cargo specificity and of certain proteins being found exclusively packaged in one subtype of vesicle only [24, 77]. Indeed, the use of selective membrane filtration for isolating both microvesicles and exosomes has enabled a definitive biological, proteomic, and functional characterization of these distinct EV subtypes [77]. Extensive quantitative proteomic analyses between low- and high-density exosomes have enabled their comparison and identified several classical exosome markers, including flotillin-1 and HSP70, that are present in all EVs [58]. Other identifiable proteins like CD63, CD9, and CD81 tetraspanins, syntenin-1, and TSG101 were specifically enriched in small EVs, thus defining a set of protein with different relative abundance in distinct EVs. Given that the biogenesis of microvesicles and exosomes is based on selective cellular mechanisms and cargo, it seems highly likely that these two classes of EVs have distinct biological functions.

Isolation and Purification

A significant challenge in the field of EV biology is to improve and standardize methods for EV isolation and analysis [24, 58, 78–83]. While much has been reported about the purported unique chemical and biological properties of exosomes and microvesicles [36], methods for their rigorous isolation and characterization are still largely empirical. A key issue hindering progress in understanding the underlying mechanisms of EV biogenesis and cargo selectivity has been

TABLE 1. Classification and characteristics of extracellular vesicles (EVs).

	Characteristics						
EV type	Origin	Size (nm)	Density (g/ml)	Protein markers	Cargo/contents	Isolation methods	Examples of key functions
Exosomes	Derived through maturation of the endosomal pathway; intraluminal budding of multivesicular bodies to produce intraluminal vesicles, and fusion of multivesicular bodies with cell plasma membrane to release exosomes	30–150	Sucrose: 1.13–1.21 iodixanol: 1.10–1.12	Tetraspanins (CD63, CD81, CD82), ESCRT components, PDCD6IP, TSG101, HSP70, flotillins, integrin's	mRNA, microRNA (miRNA),other noncoding RNAs, single and double stranded DNAs (dsDNAs), mitochondrial DNA, and oncogene amplifications; cytoplasmic and membrane proteins, including receptors and major histocompatibility complex molecules	Ultracentrifugation, differential centrifugation, density gradient centrifugation, sucrose cushion centrifugation, gel permeation chromatography, size-exclusion chromatography, affinity capture, synthetic polymer-based precipitation, membrane filtration	Antigen presentation, immunostimulatory and inhibitory activities, cancer progression, metastasis, cell transformation, embryo-maternal cross talk, horizontal transfer of oncogenes, premetastatic niche formation, lipid/RNA/DNA transfer
Shed microvesicles, microvesicles, microparticles	Regulated release by budding/blebbing of the plasma membrane. cell surface; direct outward budding of cell membrane	100–1500	Sucrose: 1.16 iodixanol: 1.18–1.20	KIF23, RACGAP, CSE1L, ARF6, EMMPRIN, flotillins, HSP70	mRNA, miRNA, noncoding RNAs, dsDNAs, cytoplasmic proteins and membrane proteins, including receptors	Ultracentrifugation, differential centrifugation, density gradient centrifugation, sucrose cushion centrifugation, size-exclusion chromatography, membrane filtration	Procoagulant activity, rheumatoid arthritis tumor progression, protein/RNA transfer
Apoptotic bodies	Plasma membrane/cell surface; direct outward blebbing of apoptotic cell membrane	500–2000	Sucrose: 1.16–1.28	Phosphatidylserine, histones, calnexin, cytochrome C	Nuclear fractions (dsDNAs), cell organelles	Flow cytometry, ultracentrifugation	Horizontal transfer of oncogenes, horizontal transfer of DNA, immunosuppression

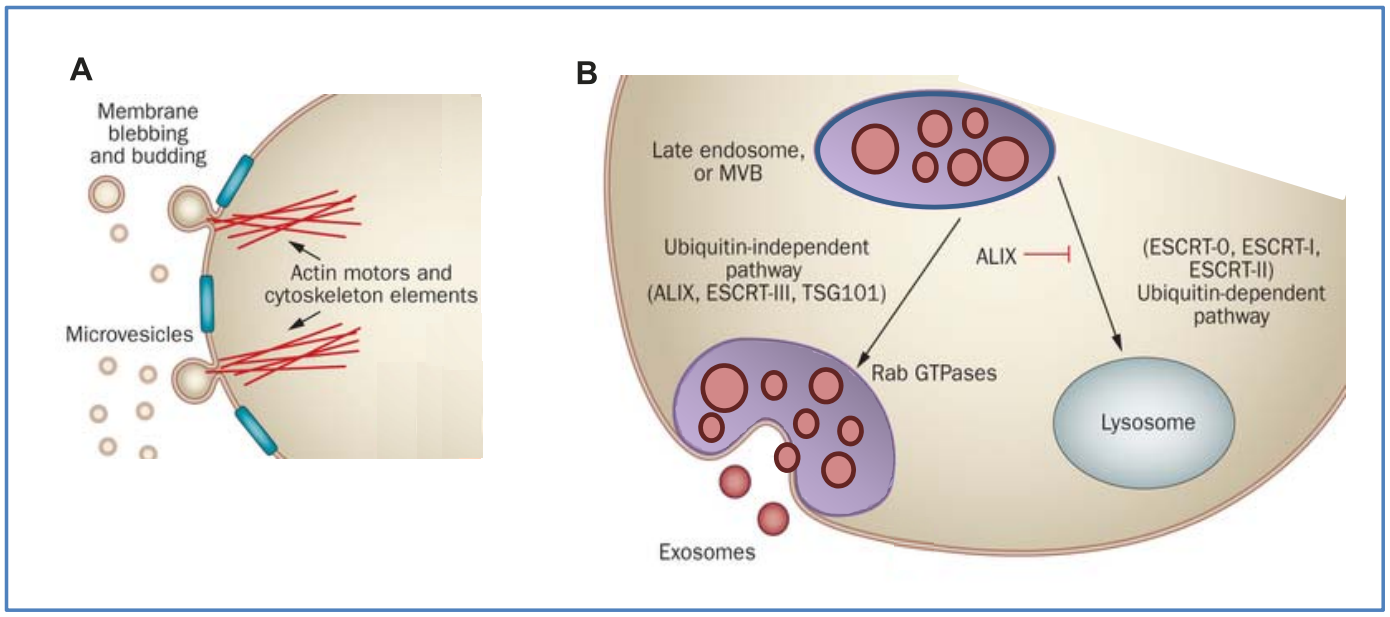

FIG. 1. Extracellular vesicles (EVs) originate through different mechanisms. **A**) Microvesicles (100–1500 nm) are formed by the outward budding and fission of plasma membrane lipid microdomains (highlighted blue), controlled by regulatory proteins and cytoskeletal elements (highlighted red) that coordinate membrane curvature at ceramide-enriched domains, resulting in vesicular protrusion and budding. Not all plasma-membrane proteins are incorporated into the shed vesicles, although the topology of membrane proteins remains intact. Microvesicles are enriched in some lipids such as cholesterol, whereas phosphatidylserine is relocated to the outer membrane leaflet specifically at sites of microvesicle shedding. Mechanisms involved in the formation and release of microvesicles is still being investigated. **B**) Exosomes (30–150 nm) initiate as intraluminal vesicles during endosomal maturation from the late endosome (multivesicular bodies [MVBs]). Following the ubiquitin-dependent interactions with ESCRT complexes, MVBs are sorted for lysosomal degradation. Alternatively, ALIX interacts with MVB cargo, preventing lysosomal degradation and facilitating an ubiquitin-independent pathway. Rab GTPases and various other components regulate MVB sorting to the plasma membrane with which the MVB fuse and are released as exosomes. Adapted from Nawaz et al. [50] with permission.

the technical challenge of isolating homogeneous EV subpopulations suitable for molecular analysis (reviewed [24]). The lack of biochemical and biophysical validation [84], along with disparate isolation schemes and annotations, has blurred the boundaries of defining and characterizing EVs. There is a large body of literature describing protocols for purifying EVs [28, 58, 77, 79, 80, 83, 85–88] and assessing their purity and concentration [89]. Various recommendations on discovery research, characterization, and diagnostic research are discussed and continually defined and updated by the research community [80, 90–93]. Accordingly, there is not sufficient experimental evidence identifying the distinct biological functions of the different pure forms of EVs; thus, further investigation on this issue is required.

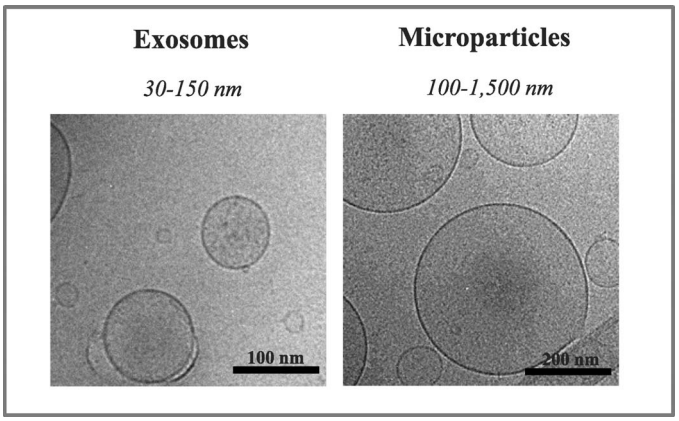

FIG. 2. Characterization of distinct EV subtypes by cryo-electron microscopy. Electron micrographs of EV subtypes released from human endometrial epithelial cells (ECC1) into cell culture media. Extracellular vesicles were harvested from cell culture media by sequential centrifugal ultracentrifugation and density-based purification, as described previously [79]. Extracellular vesicles range in diameter from small exosomes (30–150 nm) to microvesicles (100–1500 nm).

The isolation and purification method of choice should consider the sample source/volume, purity, integrity, yield of EVs required for subsequent analyses, and available instrumentation and processing time. A wide variety of isolation methods are described in the literature (Table 1). Typically, EVs are isolated from the supernatants of cultured cells and biofluids by differential ultracentrifugation (10 000–120 000 × *g*); microvesicles sediment at ~10 000–20 000 × *g* [58, 79, 87, 94, 95], while exosomes subsequently sediment at ~100 000 × *g* [58, 77, 83, 96, 97], although these are far from pure. Afterward, EVs can be efficiently separated from nonmembranous particles such as protein aggregates and viruses based on their relative buoyant density. Differences in floatation velocity also separate differently sized EV subtypes [58, 98]. While exosomes typically float at a buoyant density of 1.13–1.21 g/ml (sucrose gradient) [99] and 1.1–1.12 g/ml (iodixanol gradient) [58, 64, 83, 86, 88], microvesicles have been reported at ~1.16–1.19 g/ml (sucrose) [100] and 1.17–1.20 g/ml (iodixanol) (Table 1) [58, 77]. Due to the considerable overlap in the sucrose fractions, it is clear that the iodixanol gradient provides the preferred gradient for optimized EV fractionation and purification.

Purification of EVs can also be achieved following ultracentrifugation by immunoaffinity isolation [58, 83, 87, 101–103] using known protein target(s): this also selects for vesicles with an exoplasmic orientation. For the capture of exosomes from cell-derived supernatants, anti-A33 antibody-coated Dynabeads or anti-EpCAM antibody-coupled magnetic

TABLE 2. Important information required in any extracellular vesicle (EV) publication.

Nomenclature
Are the particles in the study heterogeneous or homogeneous?
Particle size characterization is required
EV isolation and preparation
Method of purification should be clearly described
Exosomal markers
Which markers do they express?
At least two or more markers should be used

TABLE 3. Current issues in extracellular vesicle (EV) research.

Do microvesicles and exosomes of the same cellular origin have similar functions?
How do EVs home to their target cells?
Is the entire EV cargo released into the cytoplasm, or does some of it target specific intracellular organelles?
Do individual components of EV have separate functions, or is it the balance of the total cargo that alters the recipient cell function?

microbeads, alone or sequentially, have been used prior to proteomic- and RNA-based analyses [79, 83, 87, 104]. For example, such sequential immunocapture revealed distinct populations of exosomes released from cancer cell organoids, which were also distinguished from microvesicles derived from the same origin [87]. Exosomal and nonexosomal subpopulations within EVs have also been immuno-isolated using anti-CD63, -CD81, or -CD9, and quantitative proteomic analysis of their respective composition was performed to reveal specific protein markers of such EVs (Table 1) [58].

Direct comparisons of methods have been helpful. Comparison of differential ultracentrifugation, density-based, and affinity-based approaches for exosome isolation and purification [79, 83] showed that immunoaffinity capture (using EpCAM) directed to the exosomal surface was superior to other methods assessed based on the selective identification and significant expression of exosome markers and proteins associated with their biogenesis, trafficking, and release (ESCRTs, RabGTPases, tetraspanins). Evaluation of single-step density-gradient against commercially available precipitation solution-based protocols, focusing on yield, purity, size, morphology, and proteome and transcriptome content, revealed that density-based purification was superior, providing the most homogenous EVs in comparison to other isolation techniques [81]. The recently available proprietary commercial kits for isolation of EVs were developed based on precipitation and rapid size exclusion chromatography, but such approaches are often limited by their abilities to distinguish differently sized EVs and membrane-free macromolecular aggregates [66], resulting in a much lower yield than some other methods. Such kits should be used with these limitations in mind. However, if the purpose of the purification of the approach is to enrich for biomarkers (protein/RNA) then such kits may be applicable. Consequently, these methods afford a rapid EV isolation/concentration step for the purpose of diagnostic assay of known EV-associated biomarkers.

Ultrafiltration devices have been suggested to provide a rapid and high yield of exosomes from conditioned media and serum/plasma when compared to ultracentrifugation [85], and when further combined with heparin-conjugated agarose beads (surface binding of EVs), superior isolation to standard ultracentrifugation and precipitation-based EV isolation [105] was established. Using sequential centrifugal ultrafiltration, we recently developed an unbiased EV-fractionation method to address the question of how many EV subtypes might be released from cells into culture media [77]. This study demonstrated the selectivity of sequential centrifugal ultrafiltration for isolating concurrently both microvesicles and exosomes and allowed a definitive biological, proteomic, and functional characterization of these distinct EV subtypes.

Characterization of EVs Populations (Subtypes)

Any publication using EVs must characterize the population used, including preparation methods. Additional requirements for characterization (covered in detail in recent reviews [50, 78, 79, 83, 93, 106–108]) should include a number of the following: single particle analyses (nanotracking, light scattering, tunable resistive pulse sensing), transmission electron microscopy, scanning electron microscopy, cryo-electron microscopy, immunoblotting for known markers, flow cytometry (immunolabeled high-resolution flow cytometry, scattering and fluorescence flow cytometry; impedance-based flow cytometry), surface charge, atomic force microscopy, mass spectrometry, and high-resolution imaging and colocalization analysis techniques. In term of stability, in our experience, exosomes derived from cancer cell lines can be stored indefinitely at −80°C without loss in recovery yield and morphology, or in biological function; the stability of microvesicles and other EV subtypes requires further investigation. For any research team entering the field, careful thought must be given to the level of homogeneity of the EVs required, yield application, and appropriate isolation methods. The EV preparations must be carefully characterized and reported in terms of size range, specific marker identification, and inherent properties of specific subtypes of EVs (Tables 1 and 2) before any claims can be made regarding their contents or functions. A number of other important issues in our understanding of EVs remain to be resolved and are listed in Table 3.

Mechanisms of EV Uptake in Target Cells

Exosomes are released both locally and into the circulation to interact with an assortment of target cells, including tumor, stroma, and immune cells [66]. This requires considerable specificity in their uptake. Understanding EV internalization and transfer of their cargo to target cells is a key goal of the entire EV field. Mechanisms for interaction and uptake of exosomes/microvesicles to target cells appear to involve clathrin-dependent and -independent pathways that are most likely specific to a given cellular source of exosomes and a given recipient cell type [109]. Interactions between EVs and recipient/target cells can also be mediated through direct signaling interactions via surface-expressed molecules, including integrins [110, 111] and heparan-sulfate proteoglycans [112–114]. Numerous mechanisms of EV internalization are reported [24], including receptor-mediated endocytosis (LFA1, TIM1, and TIM4), phagocytosis, and direct plasma membrane fusion [55, 115].

Functions of EVs

Extracellular vesicles have distinct functions, depending on their cellular origin, and these include immune regulation, cell migration, cell differentiation, and regulation of cell and tissue polarity [36]. Given the suggested functional role of EVs in cancer and other pathophysiological processes, they emerge as potential targets of therapeutic intervention. Exosomes can transfer RNA from mast cells, resulting in a translated protein in recipient cells [103], while dendritic-derived exosomes can activate naïve T-cells upon transfer of their cargo, playing a role in antigen presentation [41]. Extracellular vesicles have

known critical roles in stimulation of angiogenesis [28, 29], lymphatic vessel formation [30], epithelial-mesenchymal transition, cell migration, and invasion [29, 31, 88]. Human breast and colorectal cancer cells release exosomes carrying EGFR-ligands (EGF, TGF-α, amphiregulin) having the capacity to modulate the cancer cell microenvironment [72]. In addition, Wnt11 exosomes activate the Wnt-planar cell-polarity signaling pathway in cancer cells, allowing the formation of protrusions necessary for cell migration [75]. A salient finding has been the role of EVs in coordinating tumorigenesis and metastasis [73, 111, 116–120]. Such intriguing studies indicate that exosomes and other EVs are agents of cross talk between cancer and stromal cells to stimulate metastasis. Furthermore, EVs, particularly exosomes, have been shown to have immunomodulatory effects, including immunostimulatory and immunosuppressive functions [33]. In the context of implantation and pregnancy, uterine, placental, and embryonic EVs are being studied to better understand their roles in uterine receptivity, placentation, and gestation. While the known functions of EVs in the uterine microenvironment are discussed below, it is anticipated that these are only the first steps in our discovery of EVs functions in this highly dynamic system.

EXTRACELLULAR VESICLES IN THE UTERINE MICROENVIRONMENT

Extracellular vesicles have been identified in uterine fluid during the estrous/menstrual cycles in a number of species, including humans, sheep, and mice [18–20, 22]. Their release from endometrial epithelial cells in culture [18, 121–123] indicates the luminal/glandular epithelium as their source. In women, EVs can also be released from the mucus contained within uterine lavage fluid [18], raising the possibility of retention or sequestration within the endometrial glycocalyx, perhaps until released by glycosidases and proteases also secreted by the luminal epithelium [124]. Extracellular vesicles derived from the endometrium contain multiple subtypes, including mixtures of small EVs, exosomes, and packaged different proteins, miRNAs, and endogenous retrovirus mRNA [18, 20–22, 37, 121, 125]. In most studies, the EVs have been shown to be of appropriate size and express a combination of transmembrane markers (CD9, CD63, CD81) and cytoplasmic markers (Alix, TSG101, HSP70). Importantly, the protein cargo contained in endometrial exosomes is regulated in a cyclical manner at least in women [37] by the ovarian steroid hormones E and P that dominate the major phases of every estrous/menstrual cycle.

RNA and miRNA Cargo in Uterine EVs

Selective packaging of RNA/miRNA profile into uterine EVs has been demonstrated using the human endometrial luminal epithelial cell line ECC1 [18]. While a total of 214 miRNAs were common to both exosomes and their cell of origin, 13 specific miRNAs were selectively packaged in EVs while the parent cells contained five miRNAs not found in the EVs. Bioinformatics analysis of the EV-specific miRNAs identified their involvement in pathways important for implantation: inflammation, cell remodeling, proliferation, and angiogenesis. The EVs also contained the noncoding small nuclear RNA NU6 involved in the spliceosome and RNU44 and RNU48, which are small RNAs that primarily guide chemical modifications of other RNAs, indicating the breadth of cellular function that may be modified following uptake of the EVs. In another human study, hsa-miR-30d, released by the endometrial epithelium into uterine fluid, was detected in both free and exosome-associated forms [45]. Treatment of mouse embryos with miR-30d mimic significantly increased embryo adhesion when compared with the action of miR-30d inhibitor. However, the study did not examine the effect of the exosomes themselves on embryos.

A large number of small RNAs and miRNAs, including 81 conserved mature miRNAs, were similarly identified in EVs from ovine uterine fluid from cyclic and pregnant ewes on Day 14, including mRNAs for a number of endogenous retroviruses [20, 122]. Importantly, the EVs' RNA profiling revealed a large number of small RNAs when compared to those in the cells of conceptus or endometrium. Of the mature miRNAs in the uterine fluid EVs, 53 were common to EVs from cyclic and pregnant ewes, with one unique to pregnancy and 27 unique to the cyclic group. Furthermore, miRNAs miR-7e, miR-7f, and miR-451 overlapped with the human endometrial exosome data [18]. A single miRNA can target many genes by inducing RNA degradation or inhibiting its translation. Because exosomes deliver a number of miRNA simultaneously, it is unlikely that examination of individual miRNA effects will be physiologically relevant with whole exosome studies likely to be more productive. However, it is quite clear that there is a high level of specificity and selectivity of the molecular cargo packaged within exosomes for delivery into the uterine microenvironment.

Protein Cargo of Uterine EVs

Proteomic analyses of endometrial exosomes also demonstrate considerable specificity. In the human, highly purified (buoyant density) exosomes from ECC1 cells treated with estrogen (E) or estrogen plus progesterone (EP) to mimic the hormonal profile in the proliferative and secretory phases of the menstrual cycle (E-exo and EP-exo, respectively) were profiled [37]. The data shows extensively altered programming of exosome content by hormones. Of 663 common proteins identified in the two sets of endometrial exosomes, 73% are present in all exosomes, and many of these are involved in exosomal biochemical machinery (ESCRT proteins), biogenesis, sorting, trafficking, recognition, and uptake. In addition, 254 proteins were packaged specifically within E-exos, and 126 proteins were included only in the cargo of EP-exos. Of particular interest to this field is that 35% (189) of the proteins are specific to endometrial epithelial exosomes and do not appear in any of the exosome databases or other publications [37].

Similar analysis of EVs isolated from ovine uterine fluid on Day 14 from cyclic and pregnant sheep identified that their EVs contained 40 and 76 unique proteins, respectively, from a total of 195 total proteins definitively identified [20]. Interestingly, comparison between the nonpregnant secretory (luteal phase) phase EVs from the human and sheep studies revealed eight proteins in common: peripheral plasma membrane protein CASK, myosin light chain kinase, apolipoprotein E, β actin, glycogen phosphorylase (both brain and liver forms), ATP citrate synthase, and macrophage migration inhibitory factor. The low number of common proteins is surprising because exosomes contain an abundance of proteins associated with exosome function, including ESCRT-associated proteins, tetraspanins, annexins, and Ras-related proteins. This most likely relates to differences in the EV isolation strategy and enrichment approach (precipitation vs. iodixanol density-based fractionation) and sensitivity of the proteomic profiling analysis.

Exosomes of Pre-implantation Conceptus Origin

Particularly in pigs and ruminants (in which the peri-implantation conceptus exists in filamentous form) [7], but also probably in mammals with invasive implantation, EVs derived from the pre-implantation conceptus most likely contribute to embryo-maternal communication. Indeed, analysis of proteins in EVs harvested from culture medium of Day 14 sheep conceptuses, identified at least 15 protein classes [21], and it is assumed that such conceptus EVs would have been present in the uterine fluid from pregnant ewes previously analyzed [20]. Pre-implantation (Day 14), labeled conceptus EVs were observed in the endometrial epithelium, indicating communication via exosomes in vivo [21].

It is not possible at this time to obtain sufficient primary human trophectodermal cells or human blastocysts to produce EVs for analysis or to model pre-implantation communication with the mother. The most common models used to date are the well-established, more differentiated extravillous trophoblast cell lines, including HTR8/SVneo and JEG-3. Extracellular vesicles have recently been isolated from both of these [126]. HTR8/SVneo secreted 2.6-fold more exosomes compared to JEG-3. The two sets of exosomes contained 26 common proteins, but also distinct protein sets of 59 and 58 proteins, respectively, most likely reflecting their different states of differentiation. Exosomes from HTR8/SVneo packaged more proteins associated with migration and cell movement and also induced a higher rate of migration in vascular smooth muscle cells [126], probably reflecting their original isolation from the highly migratory first-trimester trophoblast [127].

In the broader context of trophectodermal preparation for implantation, EVs have been shown to mediate communication between the inner cell mass (ICM) and the trophectoderm [128]. Uptake of EVs from mouse embryonic stem cells derived from the ICM, influenced HTR8/SVneo trophoblast cell migration via the JNK and focal adhesion kinase (FAK) pathways. Moreover, when mouse Days 3–5 blastocysts were microinjected with the microvesicles before transfer into surrogate mothers, there was a significantly enhanced likelihood of implantation. However, given the different polarity of mouse and human blastocyst in terms of the spatial relationship between the ICM and adhesive trophectoderm at implantation, it will be important to establish any species differences in such communication. It is of interest that FAK was a common pathway involved in microvesicle- and exosome-mediated communications in the endometrial-embryo dialog within the uterine microenvironment [37, 128].

Effects of Uterine Exosomes on Sperm

An impact of the uterine microenvironment on sperm would need to occur before or immediately following ovulation to allow time for the sperm to reach the newly shed ovum for fertilization in the oviduct/fallopian tube. It is clear from the literature (reviewed in [129, 130]) that EVs play an important role in sperm maturation and capacitation requiring changes in the surface membrane and in their metabolism [131]. Following release of immature sperm from the testis, they transit firstly through the epididymal fluid and are then mixed with prostatic fluid before their ejaculation into the female reproductive tract. Extracellular vesicles are present in both these fluids and are termed epididymosomes and prostasomes, respectively. Epididymosomes comprise a heterogeneous population of vesicles with diameters ranging from 50–250 nm, enabling their classification as EVs: the smaller vesicles express well-recognized exosome markers [132]. There is also considerable heterogeneity among prostasomes, and only recently two distinct populations have been characterized appropriately as exosomes [98]. These vesicles and their functions will not be further discussed here as they are outside the brief of this review. More recently, it has become apparent that EVs within the uterine cavity also influence sperm during their transit, enhancing their development. Such EVs have been termed uterosomes [22], a term inclusive of both microvesicles and exosomes, defined by their expression of CD9 and CD63. In the mouse uterus, evidence supports regulation of uterodomal content by E, in that the proteins sperm adhesion molecule 1 (Spam 1/PH-20) and plasma membrane calcium ATPase 4a (PMCA4a) are highly expressed at proestrus/estrus and only marginally present at metestrus/diestrus in the uterine and oviductal epithelium and packaged within uterosomes [133, 134] in accord with the physiological requirement. Importantly, ATPase 4a, was similarly identified in culture medium from the human endometrial epithelial cell line ECC1 but was independent of ovarian steroid hormone treatment [37]. SPAM1 was not identified in the ECC1 study, and neither protein was detected in ovine uterine fluid, perhaps reflecting methodological or species differences [20]. In the mouse, these proteins are transferred to sperm, at least in vitro, appearing on the sperm plasma membrane, [22, 135]. This indicates that vesicular docking is a likely means by which EVs are transferred to sperm within the uterine cavity and oviduct to ensure the sperm's hyperactivated motility and fertilization potential. The mechanism of fusion of oviductal EV with sperm appears to be mediated via integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ because EV binding is blocked by the appropriate antibodies and ligands. Similar mechanisms are likely for uterosomes [135].

Endometrial Exosomes Can Mediate Embryo-Maternal Interactions to Facilitate Implantation

In ruminants, the pre-implantation conceptus spends a prolonged period in the uterine cavity, and EVs play an important role in the preparation of the trophectoderm for pregnancy. Labeled EVs isolated from ovine uterine fluid, transferred to ovine trophectodermal cells in vitro within 2 h of co-incubation, resulted in both increased proliferation and release of interferon τ (the ovine pregnancy recognition signal) [125]. Interferon τ is itself regulated by endogenous jaagsiekte retroviruses (enJSRV) via activation of TLR-mediated immune pathways. Importantly, enJSRVs mRNA is contained within uterine fluid EVs and EVs derived from ovine endometrial epithelial cells [20] and is transferred as EV cargo along with other viral material such as myxovirus-resistant protein MX1 [122]. To support this finding, and because ovine trophectodermal cells will most likely themselves contain enJSRV, labeled EVs from uterine fluid were incubated with human embryonic kidney (HEK)-293 cells that do not contain these ovine viruses. The fluorescent label was clearly identified in the recipient cells, indicating EV uptake [21]. This finding, however, raises the question of cell specificity of uptake, given that both species and cell type of recipient cells were different from those of the EVs.

The bovine antiviral protein, MX1 has been hypothesized to be important for regulation of secretion from bovine uterine epithelium during early pregnancy when interferon τ levels are high, it may be responsible for the high percentages of early embryo loss seen in dairy cattle [122, 136]. To investigate this, exosomes were isolated from bovine uterine glandular epithelial cells using sequential centrifugation and a sucrose floatation gradient [122]. When the cells were pretreated with interferon τ, the MX1 gene was released into the uterine

microenvironment within exosomes (where it is protected from protease degradation) via a ceramide-dependent, ESCRT-independent pathway. Elsewhere, the human ortholog MXA appears to be important in regulating membrane homeostasis and receptor recycling independently of its role during viral infections [137, 138]. It is not yet clear whether the MXI is incorporated into exosomes because it has a role in their formation or release or whether its inclusion in the exosome cargo is important in intercellular communication [122].

In the human, endometrial exosomes also appear to have the capacity to modulate trophectodermal function as demonstrated in cell culture models in vitro [37]. Human ECC1-derived exosomes can be taken up by HTR8 trophoblast cells and enhance their adhesive capacity. Maximum altered adhesion was seen at 1.5 h of coculture and maintained for 4 h [37]. Importantly, the increased adhesion was significantly higher when the parent cells were treated with EP to mimic the receptive endometrium compared with cells treated with E alone (proliferative phase conditions) and even more so than with no steroid hormones. This stimulation of adhesion is in accord with the proteomic data on the adhesion molecule content of the exosomes. This includes fibronectin, which was increased in the HTR8 cells following exosome uptake. Furthermore, a significant increase in levels of FAK pathway members, demonstrated by a high level of expression of total FAK and phosphorylated FAK proteins (4.2- and 2-fold respectively), indicating that this pathway is likely involved in the increased adhesive capacity of the HTR8 cells following exosome uptake [37]. Interestingly, FAK is one of the pathways that has recently been identified in EV communication within the blastocyst itself and between the ICM and the trophectodermal cells [128].

Endometrial Exosomes: Interaction with Underlying Stromal Cells?

Human endometrial epithelial-derived EV (prepared by centrifugation of culture medium at 40 000 × *g*) have been examined with respect to endometrial epithelial-stromal interactions [121, 123]. Using the h-tert-EEC endometrial epithelial cell line [139], release of EVs containing the glycosylated transmembrane protein extracellular matrix metalloproteinase inducer (EMMPRIN), was stimulated by the action of estradiol-17β or cholera toxin. Importantly, this increase was not through increased secretion but rather through movement of the EMMPRIN protein to the plasma membrane; hence, its appearance in microvesicles. Unusually, the study revealed that estradiol-17β stimulates release of these EMMPRIN-containing EVs through GPR30 rather than via the classical nuclear receptors [123]. EMMPRIN mediates cell invasion [140] and can induce the release of MMP9 from human uterine fibroblast cells [121], suggesting a possible role in preparing the endometrium for embryo invasion and implantation. However, whether or not EVs of epithelial origin reach the stromal compartment during the cycle is not clear. Given the structure of epithelial layers, with their many lateral complexes providing tight barriers, release of the EVs basally and their penetration of the basal lamina would be necessary for the EVs to reach the stroma. Both basal and apical release of exosomes from intestinal epithelial cells has been demonstrated in vitro, with considerable differences in the protein content of the two cohorts [42]. Experimental work is still needed to determine if this occurs in the endometrial epithelium and whether any basally released exosomes penetrate the basal lamina. However, during the invasive phase of implantation, when the trophectodermal cells invade through a transformed epithelium, apically released EVs could readily gain entry to the stromal compartment. Recent evidence highlights the role of EVs, in particular exosomes, to stimulate invasion, migration, and matrix degradation in various cancer models [141, 142]; see the review in [31]. Oncogenic exosomes can induce the secretion of various proteases, including MMPs, particularly MMP1, generating a positive feedback mechanism between tumor and stromal cells to drive gastrointestinal tumor development [143]. Whether or not EMMPRIN of exosome origin can act on trophectoderm or epithelial cells themselves is not yet known: it has been detected on the surface of mouse blastocysts [144], but this is most likely synthesized by the blastocyst itself. Importantly, EMMPRIN was also identified in pure exosomes from a different human endometrial cell line, EEC1, under the influence of estradiol-17β, both without and with P [37] but was not identified in ovine uterine fluid [20].

Untapped Data on EV Content: The Secretome

While the secretomes (RNA, protein, and/or lipid content) of many biological fluids and cultured cells, including those of uterine fluid and of endometrial or conceptus/trophoblast culture, have been examined, the contribution of the EVs contained in the fluids was not studied. Preparation of fluid or culture medium for analysis of the soluble secreted component requires centrifugation to 100 000 × g to isolate all EVs, including exosomes. Comparison of the soluble secretome [145] and exosomal protein content [37] from ECC1 cells provides valuable insight. When ECC1 cells were treated with E alone, 917 proteins were identified in the exosomes compared with 1119 in the soluble secretome, and of these only 350 proteins were common. Likewise, following cell treatment with EP, the numbers were 789 and 976 proteins in exosomes and soluble secreted fractions, respectively, with only 270 of these being common. By contrast, miR-661, which is secreted by human blastocysts, is predominantly present in the soluble secreted medium where it is bound to Argonaute proteins; very little miRNA was detected in the 120 000 × g pellet [146]. Thus, secretome analyses need to take both soluble-secreted and EV content into account because these will act by quite different mechanisms on recipient cells.

PERSPECTIVE AND FUTURE DIRECTIONS

Extracellular vesicles represent a direct and dynamic means of communication between the endometrium and sperm as they pass through the uterine cavity as well as between the embryo and the maternal environment during blastocyst development (Fig. 3). A better understanding of the molecular mechanisms mediating such cross talk should lead to the development of new regulating agents, with novel diagnostic, biological, and therapeutic potential for supporting normal reproductive functions. Anticipating the potential implications of the contribution and select functions of embryonic- and maternal-derived EVs and their subtypes is essential. Changes in the release of placenta- and embryo-derived EVs as well as their concentration in plasma, composition, and function have been reported in association with perturbed pregnancy [38]. The data, however, are confounded by the use of different isolation methodologies and heterogeneous EV subpopulations. The application of specific and well-characterized isolation methodologies is requisite to resolving the precise role of distinct subtypes of EVs, namely exosomes and microvesicles, in establishing pregnancy, their contribution to associated complications, and their clinical utility. Therefore, advancements in definition of methods for the isolation and characterization of

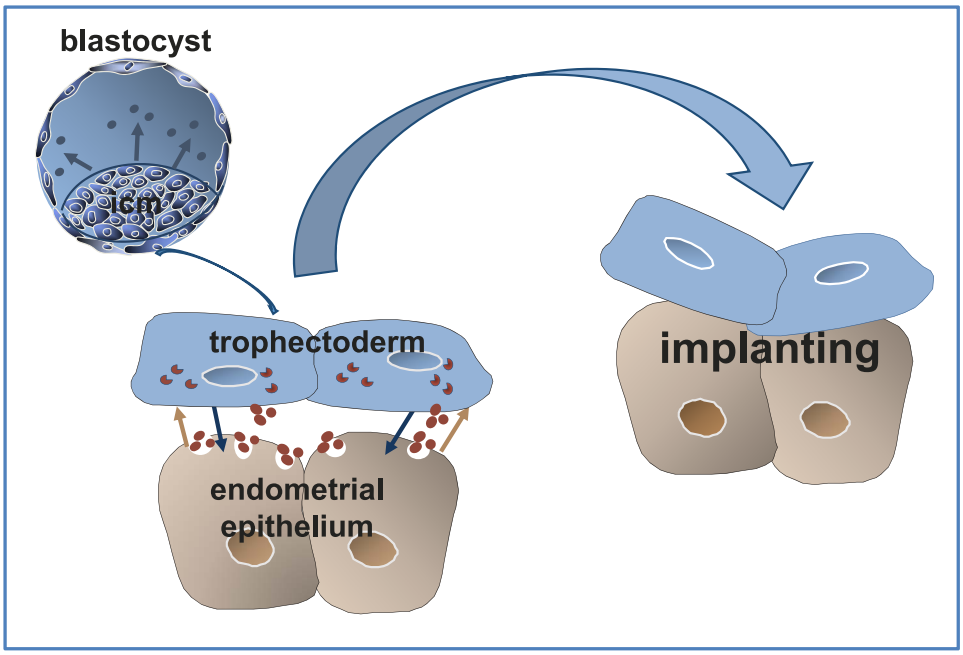

FIG. 3. Extracellular vesicles provide intercellular communication both between the inner cell mass (ICM) of the blastocyst and trophectoderm and within the uterine cavity, between endometrial epithelium and the trophectodermal cells, to favor implantation potential.

distinct EV subtypes, their physico- and biochemical properties, and analysis of their contents of protein, lipid, mRNA, miRNA, and DNA is crucial to substantiate the targets.

Further, EV biology and its development require integrated proteomic and genomic investigations to determine novel, functional transcripts and specific transcriptional activities. Further understanding of the precise mechanisms that underpin cell type-specific EV biogenesis, recognition, and entry into specific target cells is required. Because EVs contain a variety of proteins, nucleic acids, and lipids contents, it will be challenging to confirm whether EV-mediated phenotypic changes in recipient cells is a result from transfer of one or multiple EV-cargoes. Overall, EVs transfer of molecular regulators from the mother to the peri-implantation blastocyst may have a major impact on implantation and optimal placentation. Such targeted studies will enable the development of novel nanodiagnostics and nanotherapeutics to increase the success of pregnancy rates during assisted reproductive technology or in vitro fertilization treatment. These targets could also improve infertility, pregnancy loss, and pre-eclampsia and possibly provide new methods and adjuvants for contraception.

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